Pediatr. Res. 16: 227–231 (1982)

Human Erythrocyte Prolidase and Prolidase Deficiency

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Summary

Biochemical studies on human prolidase (EC 3.4.13.9) and prolidase deficiency are described. The urine sample from a 32-year-old female with prolidase deficiency was examined. Diagnosis was based on clinical features and defects of prolidase in her erythrocytes. She excreted massive amounts of iminopeptides, where three major peptides were identified; aspartyl-proline, glutamyl-proline and glycyl-proline. The prolidase was purified approximately 10,000-fold from the normal human erythrocytes through an eight step procedure. The purified enzyme consisted of two identical subunits of which the molecular weight was calculated to be 55,000. The relative cleavage rates of the enzyme for glycyl-L-proline, L-alanyl-L-proline, L-leucyl-L-proline, L-prolyl-L-proline, and glycyl-hydroxy-L-proline were 100%, 53%, 27%, 31% and 2%, respectively. The relative substrate specificity of the enzyme offers a reasonable explanation for the presence of a higher level of urinary imidodipeptides in a patient with prolidase deficiency. An attempt at erythrocyte transfusion was performed, aimed at enzyme replacement therapy. After the transfusion (erythrocytes from 800 ml of whole blood), the prolidase activity of the peripheral erythrocyte was elevated to approximately 35% of the normal values and gradually decresed (half-life, 41 days). During this period urinary peptide-bound proline was monitored, but no significant change was observed.

Speculation

The prolidase activity of transfused erythrocytes is relatively stable; however, intracellular enzyme activity has no effect on net proline loss and skin lesions. Enzyme replacement may be a possible attempt at therapy of the disease, but other forms of replacement should be tried.

Prolidase deficiency is a rare genetic disease inherited by an autosomal recessive trait and associated with chronic ulcerative dermatitis, mental retardation and massive urinary excretion of imidodipeptides (22). Since the first case was described by Powell *et al.* in 1974 (21), additional cases have been reported (3, 9, 11, 13, 20, 23). Probable cases of prolidase deficiency suspected by urinary excreted imidodipeptides and characteristic clinical features were reported (5, 10, 14, 17). The disease can be diagnosed by the assay of prolidase activity in erythrocyte (8, 13, 21, 23, 26), leukocyte (21, 20, 26) and cultured skin fibroblasts (23). Some of the properties of human prolidase derived from human erythrocytes was studied by Adams *et al.* (1). Substrate specificity of purified human prolidase has not been investigated so far. It is known, however, that substrate specificity of prolidase obtained from several animal species is not uniform (2, 7, 24). In the present report, we describe the biochemical characterization of human erythrocyte prolidase, identification of urinary excreted peptides with a female case of prolidase deficiency and the effect of erythrocyte transfusion on urinary excreted iminopeptides.

Case. A female patient with prolidase deficiency was examined. Although a detailed clinical observation will be reported elsewhere, especially on the skin lesions, the case is briefly summarized here. The patient was a 32-year-old female, who had chronic dermatitis with ulcerative lesions and mental retardation (IQ = 48) since childhood. She was the daughter of Japanese parents who were cousins. An elder brother and a first cousin of the patient were diagnosed as having the same disease (8). In addition to free amino acids, several yellow spots were found on the thin layer chromatography of her urine. Prolidase activity in her erythrocyte was undetectable (normal controls 21.5 ± 2.0 nmole/mg protein/min) (8).

MATERIALS AND METHODS

DE 52 was purchased from Whatman Ltd, (Spring Mill, England) and DEAE Sephadex and Sephadex G-200 from Pharmacia Fine Chemicals (Uppsala, Sweden). Dowex 50X8 (H^+ form) and Dowex 2X8 (Cl^- form) were obtained from Dow Chemicals (USA) and Chelex 100 from Bio Rad Laboratories (Kent, England). Glycyl-L-proline (gly-L-pro), L-leucyl-L-proline (L-leu-L-pro), glycyl-hydroxy-L-proline (gly-hydroxy-L-pro), L-prolyl-glycine (L-pro-gly), L-prolyl-L-leucine (L-pro-L-leu) and glycyl-glycine (gly-gly) were obtained from Sigma Co Ltd (St. Louis, USA). L-alanyl-L-proline (L-ala-L-pro) and L-prolyl-L-proline (L-pro-L-pro) were purchased from Serva (Heidelberg, FRG), glycyl-glycyl-glycine from Protein Research Foundation (Osaka, Japan). All other reagents were commercial products of the highest available grade of purity.

Enzyme assay. Prolidase activity was assayed by the method of Mayer and Nordwig (19) with the following modification: 0.5 ml of incubation mixture contained 50 μmole Tris-HCl, pH 8.0, 8 μmole $MnCl_2$, 5 μmole of gly-L-pro and enzyme preparation and incubated at 37°C. Hydrolyzing activity of L-ala-L-pro, L-leu-L-pro, L-pro-L-pro, L-pro-gly and L-pro-L-leu were measured by the same method. Hydrolysis of gly-hydroxy-L-pro and gly-gly was measured by the method of Josefsson and Lindberg (15). The product of hydrolysis of gly-gly-gly was measured by amino acid analyzer. Protein was measured by the method of Lowry *et al.* (18) with bovine serum albumin as a standard.

Enzyme purification. All procedures were carried out at 3–5°C unless otherwise stated. *Step 1.* Erythrocytes from 1000 ml of whole blood were washed three times with 3000 ml of cold 0.9% sodium chloride solution. Packed cells obtained after centrifugation at 1000 × *g* for 5 min were hemolysed by the addition of 8 times their volume of cold distilled water. Hemolysate was diluted to 3000 ml with 0.01 M Tris-HCl buffer pH 7.0. *Step 2.* DE 52, pre-equilibrated with the buffer, was added to the diluted hemolysate (100 g/liter of hemolysate). After stirring for 60 min, the suspension was poured through a glass column (10 x 20 cm) and the DE 52 left on the column was washed with 1000 ml of 0.05 M Tris-HCl buffer pH 7.0. The enzyme was eluted with 1200 ml of 0.25 M Tris-HCl buffer pH 7.0. *Step 3.* The enzyme solution

obtained was brought to 40% saturation by adding solid ammonium sulfate. The precipitate formed was dissolved in 50 ml of 0.01 M Tris-HCl buffer, pH 7.4 and the solution was dialysed against the buffer overnight to remove ammonium sulfate. *Step 4.* Neutralized glutathione was added to the solution obtained after step 3, to bring a final concentration of 0.001 M. The solution was heated at 58°C for 1 min, then cooled by immersion in ice cold water. Heat-denaturated protein was removed by centrifugation at 9000 × *g* for 10 min. *Step 5.* The supernatant was cooled to 0°C. Cold acetone (−20°C) was added while stirring to a concentration of 33% (V/V). The precipitate was removed by centrifugation at 9000 × *g* for 10 min. Then acetone was added again to the supernatant to reach a concentration of 60% (V/V). The precipitate was washed two times with cold acetone and dried under air. *Step 6.* The acetone powder thus obtained was dissolved in 10 ml of 0.05 M Tris-HCl buffer, pH 7.4. Undissolved material was removed by centrifugation and the supernatant was applied to a DE 52 column (2.6 x 25 cm) which had previously been equilibrated with 0.05 M Tris-HCl buffer, pH 7.4. The column was washed with the buffer, then a linear gradient elution was started with Tris-HCl buffer, pH 7.4, varying from 0.05–0.35 M in a total volume of 800 ml. The fractions containing the enzyme were pooled and concentrated through a Diaflo UM 10 membrane (Amicon Co. Ltd. Lexington, USA) under a nitrogen pressure of 3–5 atm. *Step 7.* The concentrated enzyme solution was applied to a Sephadex G-200 column (2.6 x 80 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.1 mM $MnCl_2$. The eluate was fractionated and pooled. The enzyme fraction was concentrated by ultrafiltration as described. *Step 8.* The enzyme fraction was applied to a DEAE Sephadex column (2.5 x 5 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. After washing the column with 50 ml of this buffer, the enzyme was eluted with a linear gradient varying from 0.05–0.5 M Tris-HCl buffer, pH 8.0 in a total volume of 200 ml. The fractions containing the enzyme were pooled and concentrated by ultrafiltration.

The purified enzyme was studied by analytical dis gel electrophoresis (6) and sodium dodecyl sulfate (SDS) gel electrophoresis (27).

Analysis of urinary iminopeptides: Urine samples during a 24-h period were collected under toluene and a portion of the urine was deproteinized by the addition of 1% picric acid solution. After centrifugation, the supernatant was applied to a Dowex 2X8 (Cl^- form, 1 x 2 cm) column, which was equilibrated with 0.01 N HCl. The unabsorbed fraction and eluted fraction with 0.01 N HCl were combined. One tenth volume of 1 M borate buffer, pH 11 was added to the solution and pH was brought to exactly 11 by the addition of N-NaOH. The pH adjusted solution was applied to a Chelex column (2 x 5 cm), which was treated with a saturated $CuSO_4$ solution, and subsequently equilibrated with 0.1 M borate buffer, pH 11 (4)., The eluate from the Chelex Column, with 40 ml of 0.1 M borate buffer, pH 11, was applied to a Dowex 50X8 (H^+ form, 1 x 5 cm) column. The fraction containing the peptides was eluted with 4 N NH_4OH. An aliquot was evaporated to dryness. The residue was dissolved in 6 N HCl, hydrolyzed for 24 h at 110°C and analyzed for amino acid content.

For further analysis of peptide composition, the method described above was repeated using 200 ml of urine. After the elution step through Chelex, the solution was applied to a Dowex 50X8 column (2.5 x 30 cm) and eluted in sequence with 1000 ml of 0.5 N HCl, 2000 ml of 1 N HCl, 1000 ml of water and 1000 ml of 2 N NH_4OH solutions, respectively. The eluate was fractionated and tested for Ninhydrin positive spots. The fractions containing peptides were evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of water and spotted on a sheet of paper (TOYO, NO 50). Ascending chromatography was developed with a solvent system of *n*-butanol:acetic acid:water (3:1:1). The detected peptides on filter paper were next eluted with water and hydrolyzed with 6 N HCl, as described above or digested for 1 h at 37°C with purified human prolidase in 0.1 M Tris-HCl buffer, pH 8.0. Amino acids were analyzed by an auto analyzer, Hitachi KLA 5.

Imidodipeptide catabolism by intact erythrocytes: Heparinized blood was washed three times with phosphate buffered saline (PBS, pH 7.4). The washed erythrocytes were suspended in PBS giving 1 x 10^6 cells/mm^3. A 0.45 ml of the suspension was preincubated for 5 min at 37°C and to this 0.05 ml of 0.1 M glycyl-L-proline in PBS was added. After the incubation for 1 h at 37°C, the solution was separated into supernatant and packed cells by centrifugation. The packed cells were washed twice by 20 times their volume of ice cold PBS and were disrupted by freezing and thawing method. Hemolysate was diluted with 1 ml of water and deproteinized by the addition of 1 ml of 10% trichloracetic acid. After centrifugation the supernatant was taken for proline measurement. The identical procedure, but with the addition of substrate peptide just before the separation of the reaction mixture into supernatant and packed cells, was used for zero time blank.

For the study of imidodipeptide catabolism by the hemolysate, the above described procedure was followed except that the hemolysate from 5 x 10^6 cells was used instead of the intact erythrocytes and incubated for 15 min.

Effect of erythrocyte transfusion on urinary peptides: After informed consent was obtained from the patient, erythrocytes from 800 ml of whole blood of the identical blood type were washed with saline solution and infused into the patient. The enzyme activity of the donor erythrocytes was 22.2 nmole/mg protein/min. At an appropriate interval after the blood transfusion, blood and urine samples were collected for prolidase assay and urinary peptides analysis, respectively.

RESULTS

Properties of human prolidase and the purification and yield of the enzyme at each step are shown in Table 1. The purified enzyme at the final step showed a single band on polyacrylamide gel electrophoresis and SDS acrylamide electrophoresis (Fig. 1). The molecular weight of the subunit was calculated to be 55,000 (Fig. 2).

The native enzyme appeared to have a molecular weight of 110,000. It was calculated on the basis of peptide hydrolase activity of the fractions eluted from a calibrated Sephadex G-200 column (Fig. 3). The optimal pH for enzyme activity was between pH 7.6 and 7.8 (Fig. 4). The specificity of the enzyme was investigated with a variety of peptide substrates. In this experiment, various amounts of purified enzyme were incubated with each substrate peptide at a concentration of 10 mM. The relative cleavage rates of these substrate peptides are listed in Table 2. The results indicated that the enzyme had a marked preference for dipeptides containing C-terminal proline.

Urinary peptide analysis. The results of the amino acid content test in urine and after hydrolysis of the peptide fraction are shown in Table 3. These results show that the urine contained an excess amount of peptides consisting of proline. Through ion exchange resin and paper chromatography, several peptides, including three major peptides, were isolated. The amino acid compositions of

Table 1. *Purification of prolidase from human erythrocytes*

Purification step	Volume (ml)	Total activity (unit)[1]	Specific activity (unit/mg)	Purification (fold)	Recovery (%)
Crude hemolysate	3000	4200	0.021	1	100
DEAE cellulose	1200	3612	0.589	28	84
Ammonium sulfate (40–60%)	50	2666	2.06	98	62
Heat treatment	75	2349	5.77	275	57
Acetone treatment	15	1849	13.5	640	43
DEAE cellulose (column)	4.0	687	92.1	4386	16
Sephadex G-200	10.5	361	192.5	9167	8.4
DEAE Sephadex	2.0	212	223.0	10691	5.0

[1] Unit of prolidase activity. One unit of prolidase was defined as 1 μmole of proline released from glycyl-L-proline per min.

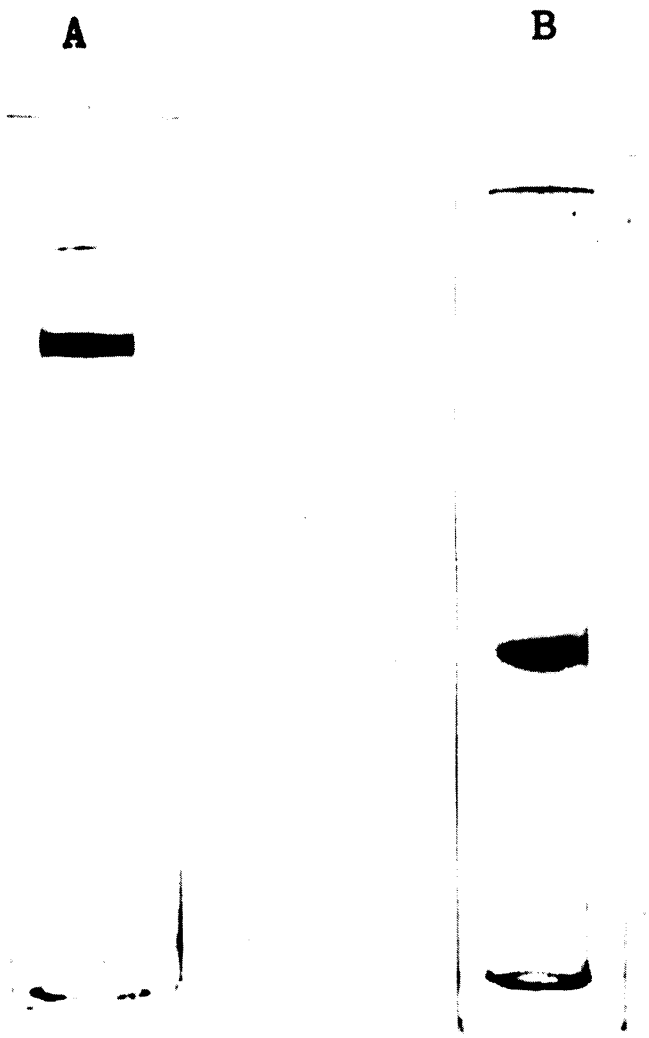

Fig. 1. *A*, Polyacrylamide gel electrophoresis; *B*, SDS gel electrophoresis in a 7.5% Gel.

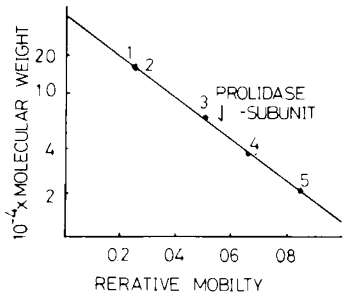

Fig. 2. Estimation of the subunit molecular weight of pure prolidase by SDS-polyacrylamide gel electrophoresis. The numbers indicate the following standards: (1) RNA polymerase β subunit (*E. coli*); (2) RNA polymerase β' subunit; (3) bovine serum albumin; (4) RNA polymerase α subunit; and (5) soy bean trypsin inhibitor.

these three peptides were analyzed by two different methods, acid hydrolysis and prolidase digestion. The results obtained were identical, showing an equimolar amount of proline associated with three amino acids; namely, aspartic acid, glutamic acid and glycine (Table 4). Thus, it is clear that these three peptides are aspartyl-proline, glutamyl-proline and glycyl-proline. In addition, results of amino acid analysis of peptides suggested that a considerable amount of prolyl-proline was excreted. Hydroxyproline content in the minor peptides was not analyzed.

Imidodipeptide metabolism by intact erythrocytes. After the imidodipeptide solution was incubated in the presence of normal intact erythrocytes, the intracellular content of free proline was significantly elevated, whereas the amount of proline release was negligible (Table 5). When imidodipeptide was incubated with the hemolysate of normal erythrocytes, the rate of hydrolysis was approximately one thousand times that of the intact cells (Table 5). The patient's intact erythrocytes, as well as the hemolysate, could not hydrolyse imidodipeptide.

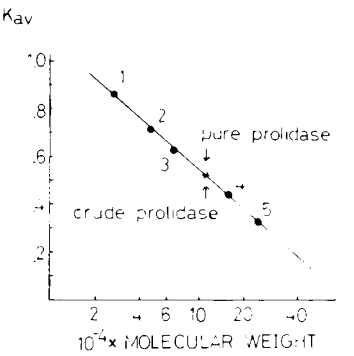

Fig. 3. Estimation of the molecular weight of native prolidase on Sephadex G-200 column. (1) chymotrypsinogen A; (2) ovo-albumin; (3) bovine serum albumin; (4) aldolase (rabbit muscle); and (5) catalase (beef liver).

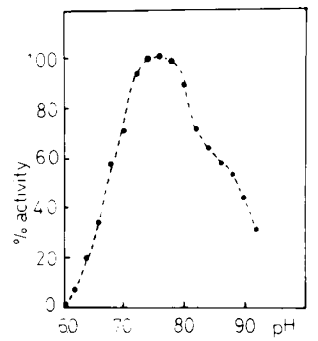

Fig. 4. Dependence of prolidase activity on pH of medium. Tris buffers adjusted to appropriate pH with maleic acid were used.

Table 2. *Substrate specificity of human erythrocyte prolidase*

Substrate (L-form)	Manufacturer	Method of assay	Relative cleavage rate (%)
gly-pro	Sigma, St. Louis, USA	Mayer and Nordwig (19)	100
ala-pro	Serva, Heidelberg, FRG	Mayer and Nordwig (19)	53
leu-pro	Sigma, St. Louis, USA	Mayer and Nordwig (19)	27
pro-pro	Serva, Heidelberg FRG	Mayer and Nordwig (19)	31
gly-hydroxy-pro	Sigma, St. Louis, USA	Josefson and Lindberg (15)	2
pro-gly	Sigma, St. Louis, USA	Mayer and Nordwig (19)	0
pro-leu	Sigma, St. Louis, USA	Mayer and Nordwig (19)	0
gly-gly	Sigma, St. Louis, USA	Josefson and Lindberg (15)	0
gly-gly-gly	Protein Research Foundation, Osaka, Japan	Amino acid analyzer	0

Table 3. *Free and peptide-bound amino acid levels in urine of the patient (nmoles per 1000 ml)*

Amino acid	Urine-free amino acid	After hydrolysis of oligo peptide fraction
Aspartic acid	232	2300
Threonine	421	596
Serine	421	344
Glutamic acid	144	1940
Proline	216	9416
Glycine	1673	1416
Alanine	207	596
Valine	[1]	532
Methionine	[1]	[2]
Isoleucine	[1]	708
Leucine	[1]	600
Tryrosine	[1]	[2]
Phenylalamine	[1]	388
Lysine	444	800
Histidine	173	26
Arginine	42	47

[1] Obscured by peptides.
[2] Undetected.

Table 4. *Identification of peptides isolated from patient's urine*

	Amino acid liberated after acid hydrolysis	Amino acid liberated by prolidase digestion	Definition
Peptide 1	aspartic acid proline	aspartic acid proline	aspartyl-proline
Peptide 2	glutamic acid proline	glutamic acid proline	glutamyl-proline
Peptide 3	glycine proline	glycine proline	glycyl-proline

Table 5. *Hydrolysis of gly-pro by intact erythrocytes and hemolysate*[1]

	Increase of free-proline content in erythrocytes incubated with gly-pro[2] (μmole/h/10^{10} cells)	Proline released from gly-pro by hemolysate[3] (μmole/h/10^{7} cells)
Control		
1	0.174	0.164
2	0.166	0.140
3	0.155	0.137
Patient	UD[4]	UD[4]

[1] The values were the means of two experiments.
[2] The substrate concentration was 10 mM and incubated for 60 min.
[3] The substrate concentration was 10 mM and incubated for 15 min.
[4] UD, undetectable.

Effect of blood transfusion on urine peptides. As shown in Figure 5, prolidase activity of peripheral erythrocytes was elevated to as high as 7.8 nmole/mg/min, corresponding to 30–40% of normal controls and 75% of heterozygotes (8). The elevated prolidase activity declined gradually as the days passed. The sixth wk after the infusion only one-third of initial activity remained. The estimated half-life of prolidase was 41 days in the patient. The amount of proline content in the urinary peptide fraction remained essentially unchanged during the observed period (Fig. 5).

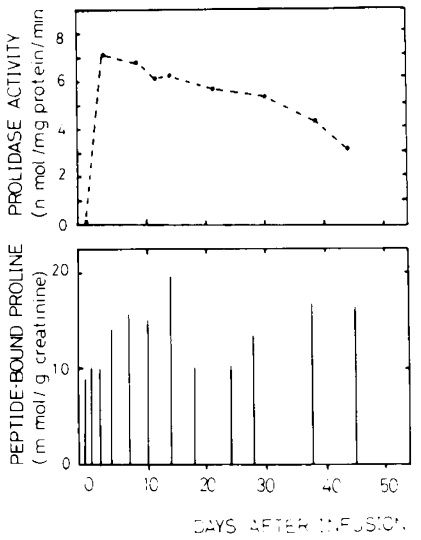

Fig. 5. Effect of erythrocyte infusion on prolidase activity in erythrocytes and peptide-bound proline in urine.

DISCUSSION

The highest purification previously achieved for prolidase (imidodipeptidase) from erythrocyte was 260-fold (2). Our own results far exceed this purification factor. The substrate specificity of human prolidase indicated that the enzyme had a marked preference for dipeptides containing C-terminal proline. The specificity of the enzyme is in accord with the definition of prolidase, as discussed by Smith and coworkers (2, 7, 25).

The enzyme requires Mn^{++} for its activity, as described by Adams *et al.* (2), for both crude and purified preparation, and is stabilized to some extent in the presence of glutathion (data not shown). The optimal pH for enzyme activity is between pH 7.6 and 7.8 (Fig. 4). These results are similar to those of Smith and coworkers, who had studied the prolidase obtained from swine kidney (7) and horse erythrocytes (2). As shown in Table 2, there is a considerable difference in the cleavage rate between the substrates, and substitution of hydroxyproline in the position of proline reduces the cleavage rate. The present enzyme preparation showed a 31% activity of hydrolyse L-pro-L-pro compared to gly-L-pro. On the other hand, prolinase (EC 3.4.13.8) obtained from porcine kidney, which hydrolyses dipeptides containing N-terminal proline, could hydrolyse L-pro-L-pro to only 0.7% of L-pro-gly (19). Prolidase from porcine intestine was unable to hydrolyse this peptide altogether (24). Prolidase from horse erythrocytes could hydrolyse L-pro-L-pro in a similar fashion as human erythrocyte prolidase (2). The present patient excreted a large amount of three peptides defined as aspartyl-proline, glutamyl-proline and glycyl-proline plus a considerable amount of prolyl-proline, as were observed in other patients (5, 11, 16, 20). It is worthy to note that the substrate specificity of purified erythrocyte prolidase reflects the excreted pattern of urinary iminopeptides and these three major peptides were hydrolyzed by the purified enzyme.

The pathologic role of prolidase deficiency is still uncertain. It was demonstrated by an experimental study that more than 90% of proline derived from collagen was reused for collagen synthesis (12). Based on this fact, Jackson *et al.* (13) surmised that the pathophysiology of this disease is a defect of the recycling use of proline in collagen synthesis. We then decided to repair this defect of the normal recycling of proline in the patient by infusing washed normal erythrocytes containing reasonable amounts of prolidase. After the injection of erythrocytes, prolidase activity in the patient's peripheral blood was elevated to as high as 35% of the control levels, and lasted the 41 days of half-life. The apparent half-life of the intracellular prolidase activity may represent the half-life of the infused cells, rather than the half-life of the enzyme in the erythrocytes. Under this condition, the amount of urinary iminopeptides was not reduced. It is shown in the present study that intact erythrocytes could metabolize imidodipeptide, but that the rate of degradation was much less than that of hemolysate (Table 5). This suggests that the transfer of imidodipeptides

through erythrocyte membrane is a limiting factor in intact cell infusion. Clinically, the patient felt better following erythrocyte transfusion, and the ulcer seemed to be slightly improved. This observation is compatible with that of Sheffield *et al.* (23) whose patient "claimed to feel better" after the administration of a large amount of proline (10 g/day). However, it is difficult to establish whether these effects are distinct from placebo effects.

The intravenous infusion of partly purified prolidase from hemolysates might give better results for imidodipeptide degradation. But on the other hand, the possibility of an antibody formation to prolidase, when there is a lack of normal enzyme, must be taken into account in such occasions. Further attempts at enzyme replacement therapy for this disorder should be considered in the future.

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28. This investigation was supported by the grant from the IBM company and a Research Grant from the Ministry of Education. (Matsuda)

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30. Received for publication May 26, 1981.
31. Accepted for publication October 1, 1981.

0031-3998/82/1603-0227$02.00/0