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Red Cell Hydrolases

II. Proteinase Activities in Human Erythrocyte Plasma Membranes

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Summary. A 0.1% Triton X-100 extract of human erythrocyte plasma membranes contained high proteolytic activity as determined by a very sensitive assay utilizing ^{3}H-acetylated hemoglobin (162 cpm/pmole) as a substrate. Two proteolytic enzymes having optimum activity at pH 3.4 and pH 7.4 were isolated from Sephadex G-100. The protease active at pH 3.4 was 75 times as active as the pH 7.4 enzyme and it was purified 182-fold over the original homogenate and characterized. A linear relationship for activity versus time and activity versus concentration of enzyme was found. The optimum temperature was 37 °C and the K_m was 1×10^{-5} M hemoglobin. No enzyme activation was observed with any cation studied and EDTA had no inhibitory effect; (10 mM Fe^{+3} and Hg^{+2} were inhibitory). The pH 3.4 protease was stable indefinitely at −20 °C in 0.1% Triton X-100. Gel electrophoresis was performed on a sodium dodecylsulfate-mercaptoethanol enzyme preparation and two protein bands (mol. wt. 33,000 and 54,000) were evident for the Sephadex G-200 eluate containing the pH 3.4 protease.

The degradation of red blood cells *in vivo*, the prevention of hemolysis *in vitro* of stored blood-banked whole human blood, and the preservation of functional red blood cells after transfusion are all very important phenomena that may be associated with degradative enzymes located on or within erythrocyte plasma membranes. These enzymes are perhaps, in part, capable of degrading portions of the erythrocyte membrane. Morrison and Neurath (1953) have reported that proteolytic enzymes were present in the erythrocyte; and, recently, Moore, Kocholaty, Cooper, Gray and Robinson (1970) have reported on the isolation of a proteinase active at pH 7.4 from human erythrocyte membranes. A membrane acid phosphatase has been identified in rabbit erythrocyte membranes (Berry & Hochstein, 1969) and a number of glycosidase and protease activities have been identified from human erythrocyte membranes (Bosmann, 1971). This laboratory has an interest in membrane associated enzymes, and with the advent of a highly sensitive,

well characterized proteolytic enzyme assay (Hille, Barrett, Dingle & Fell, 1970) the problem of human erythrocyte plasma membrane proteases was reinvestigated. This communication describes the extraction, purification and characterization of two proteolytic enzymes located on human erythrocyte plasma membranes using ^{3}H-acetylated hemoglobin (Hb) as a substrate. Gel electrophoresis was performed on sodium dodecylsulfate (SDS)-mercaptoethanol membrane extracts (Lenard, 1970) using Coomassie Blue and periodic acid-Schiff (PAS) staining for protein and glycoprotein (Bosmann, Case & Shea, 1970), to determine the nature of the enzymes found on human erythrocyte plasma membranes.

Materials and Methods

Blood

We collected 500 ml of blood in acid-citrate-dextrose from young healthy male donors. Blood was used immediately after drawing.

Preparation of Erythrocyte Membranes

Erythrocyte membranes were prepared by the osmotic lysis method of Weed, Reed and Berg (1963) and Van Stevenrick, Weed and Rothstein (1965). Further washing in 30 mosm phosphate buffer, pH 7.2, yielded white ghosts.

Fractions Tested for Enzyme Activity

Whole erythrocyte membranes, erythrocyte membranes extracted for 30 strokes with a Ten Broeck Homogenizer in 0.1% Triton X-100 (TX-100) according to Bosmann (1971), a $50{,}000 \times g$ supernatant of the 0.1% TX-100 extract and a Sephadex G-100 and G-200 column eluate were tested for enzyme activity. Routinely, erythrocyte membranes were homogenized in 3 volumes of 0.1% TX-100 for 30 strokes with a Ten Broeck homogenizer at 4 °C, centrifuged at $50{,}000 \times g$ for 20 min, and the supernatant used as the starting material for purification of the proteinases by Sephadex G-100 column chromatography. The $50{,}000 \times g$ supernatant of the 0.1% TX-100 extract was placed on a Sephadex G-100 gel column, previously equilibrated with 0.1% TX-100. The column (45.0×2.5 cm) was eluted at 4 °C with 0.1% TX-100 with a flow rate of 10 ml/hr; fractions of 3 ml were collected. Fractions were kept at 4 °C during collection and assayed for protein at 280 nm using 0.1% TX-100 as a blank, and those fractions containing protein were assayed for proteinase activity. Those fractions showing highest activity were pooled and placed on a Sephadex G-200 column. The elution method was exactly the same as that used for the Sephadex G-100 column and a flow rate of 4 ml/hr was obtained and 3 ml fractions were collected. Enzyme assays were done on those fractions showing protein content.

Protein

Total protein in any of the various enzyme preparations was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Crystalline bovine serum albumin was used as a standard.

Preparation of Substrate

^{3}H-acetylated hemoglobin was prepared by the method of Hille *et al.* (1970). ^{3}H-acetic anhydride (specific activity 400 C/mole; New England Nuclear) was reacted with Type I beef blood hemoglobin purchased from Sigma. The purified ^{3}H-acetylated hemoglobin specific activity was 162 cpm/pmole based on mol. wt. of 68,000. Routinely, 50 µliters (115 µg ^{3}H-acetylated hemoglobin) were added per assay.

Cathepsin (E.C. 3.4.4.23)

Cathepsin activity was measured by a modified method of Anson (1938) and Hille *et al.* (1970). Routinely, 50 to 100 µliters of enzyme extract (3 to 150 µg protein, depending on the source of enzyme) were added to 100 µliters of a solution of 1.35 M acetic acid and 0.02 M ammonium sulfate, pH 3.4. A portion of 50 µliters of ^{3}H-acetylated hemoglobin was added as the substrate. This mixture was incubated for 1 hr at 37 °C in a Dubnoff metabolic shaker. The reaction was terminated by placing the assay tubes in an icewater bath (0 to 4 °C) and adding 100 µliters of 2.5% hemoglobin and 50 µliters of 50% trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation at $5{,}000 \times g$ for 5 min and an aliquot of the supernatant fluid was plated on a glass fiber filter; the radioactivity was determined by counting in a liquid scintillation counter (Bernacki & Bosmann, 1971). Activity is expressed as pmoles of hemoglobin degraded per hr/mg enzyme protein. Suitable blanks consisting of 0.1% TX-100 or boiled enzyme (10 min at 100 °C) were added in place of the enzyme extract and incubated simultaneously. The blank readings never exceeded 5% of the enzyme activity. All experiments were performed in duplicate or triplicate. To insure that proteolytic activity was being measured with this assay system the method of Anson (1938), utilizing unlabelled hemoglobin substrate, was employed simultaneously on fractions containing high specific activity at both pH's according to Bosmann (1971).

Trypsin (E.C. 3.4.4.4)

Trypsin-like activity was analyzed as above except that 100 µliters of 0.1 M phosphate buffer, pH 7.4, was substituted as the buffer.

Enzyme Characterization (pH 3.4 Proteinase)

All experiments were performed with the purified enzyme (Sephadex G-200 extract); enzymes from several purification runs were pooled for the experiments.

Analytic Electrophoresis of SDS Extracts

Extraction and electrophoresis were carried out on the various fractions obtained from the erythrocyte membranes by the method described by Lenard (1970). Membrane extracts and standards were disaggregated by dialysis in 5 mM EDTA-5 mM mercaptoethanol for 16 hr. SDS, to a final concentration of 2 to 3%, was added and the extracts were then heated for 3 min at 100 °C. Gels were 10 cm long and contained 5% acrylamide 0.1 M phosphate buffer (pH 7.1), and 0.1% SDS. The samples were subjected to electro-, phoresis at 8 ma per gel for 5 hr. The electrophoresis buffer contained 0.1 M phosphate buffer (pH 7.1) and 0.1% SDS. All gels were run toward the anode.

Following electrophoresis, the gels were stained for either protein or glycoprotein. For protein, the gels were fixed in 20% sulfosalicylic acid for 16 hr, stained with 0.25% Coomassie Blue for 3 hr, and destained with several washes of 7.5% acetic acid. For glycoprotein, the gels were fixed in 20% sulfosalicylic acid for 16 hr and then stained with a modified PAS technique exactly as described by Zacharius, Zell, Morrison and Woodlock (1969).

Results

Purification of a Cathepsin Proteinase from Erythrocyte Membranes

The purification scheme described above yielded a preparation purified 182-fold over the original membrane preparation (Table 1). The scheme was based on homogenization in 0.1% TX-100 (which activated and solubilized about half of the prepared membrane), and centrifugation and chromatography on Sephadex G-100 and Sephadex G-200. Two major protein peaks containing proteolytic activity were evident from the Sephadex G-100 column chromatography. The first peak, containing most of the membrane protein, contained proteolytic activity active at pH 7.4. The second protein peak contained proteolytic activity active at pH 3.4. The specific activity evident at pH 3.4 was approximately 75 times greater than that seen at pH 7.4 (Fig. 1). The proteinase active at pH 3.4 was pooled and placed on Sephadex G-200 column chromatography. One major protein peak containing pH 3.4 proteolytic activity was recovered (Fig. 2). 60% of the total activity based

Table 1. *Purification and isolation of a proteolytic enzyme active at pH 3.4 by 0.1% TX-100 extraction, centrifugation and column chromatography*[a]

	Total protein[b] (mg)	Total activity (pmoles Hb/hr)[c]	% Recovery[d]	Specific activity (pmoles Hb/mg/hr)	Purification factor
Human erythrocyte plasma membranes	550	143,000	—	260	1
0.1% TX-100 homogenate	550	271,700	100	494	2
50,000 × *g* supernatant	177	260,832	96	1,475	6
Sephadex G-100 fraction	9.2	211,926	78	23,008	89
Sephadex G-200 fraction	3.4	163,020	60	47,325	182

[a] All procedures were carried out at 0 to 4 °C. The experiments were repeated five times and were quantitatively and qualitatively reproducible. Assays were performed under optimal conditions for the cathepsin proteinase.

[b] Yield from 500 ml of whole human blood.

[c] 1 pmole hemoglobin (Hb) yields 162 cpm.

[d] Based on the total activity of the 0.1% Triton X-100 homogenate.

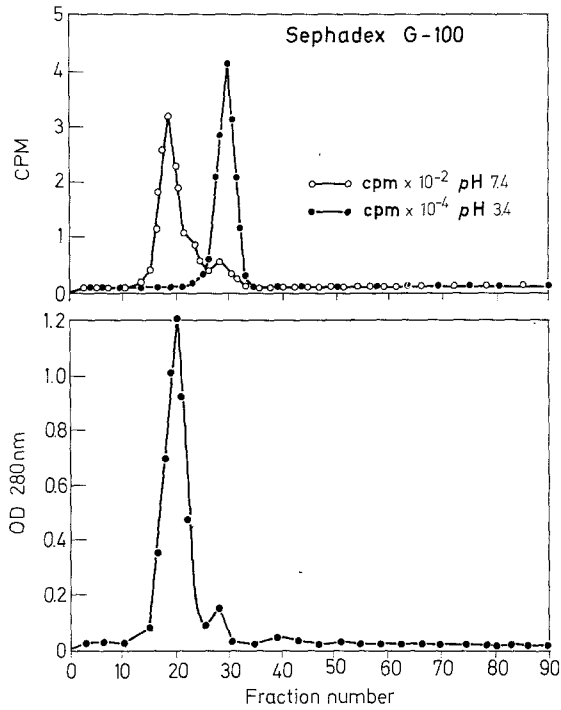

Fig. 1. Chromatography of erythrocyte membrane proteolytic activity on Sephadex G-100. 50,000 × *g* 0.1 % TX-100 supernatant (177 mg protein) in 30 ml of 0.1 % TX-100 was applied to the column (45.0 × 2.5 cm). Elution was with 0.1 % TX-100 and 3-ml fractions were collected. All procedures were carried out in a jacketed column at 4 °C

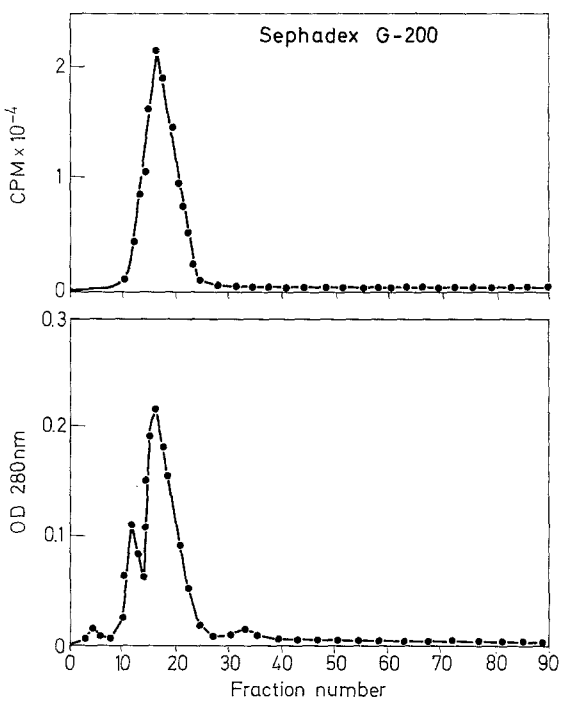

Fig. 2. Chromatography of erythrocyte membrane cathepsin activity on Sephadex G-200. Enzyme (212 mmole Hb per hr, 9.2 mg protein) in 24 ml of 0.1 % TX-100 was applied to the column (45.0 × 2.5 cm). Elution was with 0.1 % TX-100, 3-ml fractions were collected. All procedures were performed in a jacketed column at 4 °C

on the 0.1% TX-100 homogenate was recovered by these procedures (Table 1). The highest purification step was achieved with the Sephadex G-100 chromatography. Activity at pH 7.4 was not evident in the original membrane preparation but could be located in the first protein peak from the Sephadex G-100 chromatography. Possible explanations of this are that the homogenization released the previously bound enzyme or that inhibitors were present in the original preparation which were lost in the purification. The proteinase active at pH 3.4 had a much higher specific activity than the pH 7.4 enzyme using ^{3}H-acetylated hemoglobin as the substrate, and further studies were performed in order to characterize this enzyme active at pH 3.4.

pH Optimum of the Enzyme

The effect of pH on the proteolytic activity of the cathepsin like enzyme was studied using ^{3}H-acetylated hemoglobin in citrate-phosphate buffers ranging from pH 2.0 to 8.2. The pH activity profile shown in Fig. 3 indicates a pH optimum of 3.4.

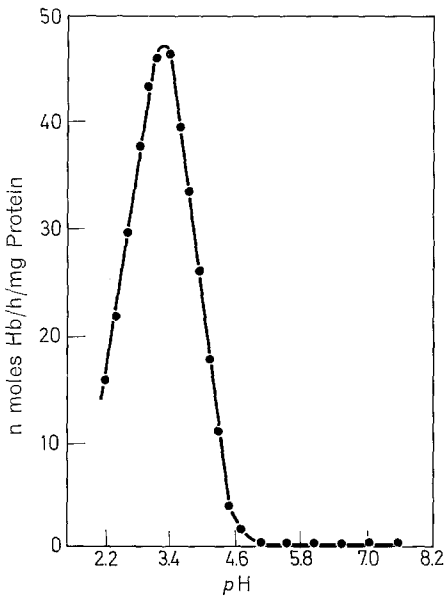

Fig. 3

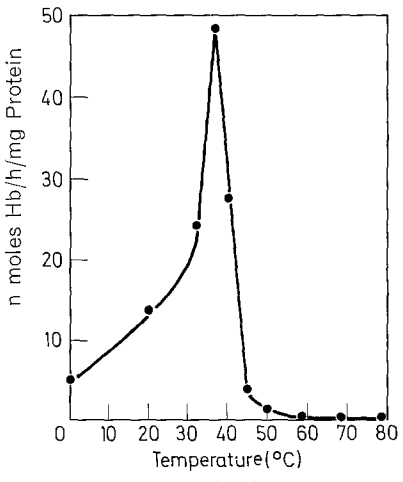

Fig. 4

Fig. 3. Influence of pH on the activity of the cathepsin proteinase. McIlvaine's 0.1 M citric acid-0.2 M sodium phosphate buffer was used. Enzyme (10 µg), substrate (50 µliters), and buffer (100 µliters), were incubated for 30 min at 37 °C and the TCA soluble fraction was analyzed for activity as described in Materials and Methods

Fig. 4. Effect of temperature on the activity of the erythrocyte membrane cathepsin activity

Effect of Temperature

An optimum temperature of 37 °C (Fig. 4) was evident for the cathepsin proteinase. Activity decreased sharply at temperatures higher than 40 °C.

Effect of Added Enzyme Protein

The activity of the erythrocyte membrane cathepsin was linear at low concentrations with respect to enzyme protein (1 to 7μg) and gradually diminished at higher concentrations (Fig. 5) although it was fairly linear up to 100 μg of enzyme protein (not shown).

Time Course

Proteolytic activity at pH 3.4 is linear up to 60-min incubation (Fig. 6), and the rate of ^{3}H-acetylated hemoglobin degradation decreases thereafter. In all the experiments performed, assays were terminated at either 30 or 60 min.

Effect of Cations on Erythrocyte Membrane Proteinases

$FeCl_3$ and $HgCl_2$ were inhibitory at 10 mM, pH 3.4 (Table 2). EDTA at 10 mM had no effect at both pH's tested, indicating a lack of endogenous divalent metal cofactor. The only cation showing an acceleratory effect was $FeCl_2$, at 10 mM, pH 7.4. Although the activity was greatly enhanced at 10 mM $FeCl_2$, the effect was anomalous; that is, the complete system minus enzyme protein or substitution of boiled enzyme protein in the complete system (pH 7.4) with the addition of 10 mM $FeCl_2$ resulted in activity that was still greatly elevated over the activity evident at pH 7.4 with the complete

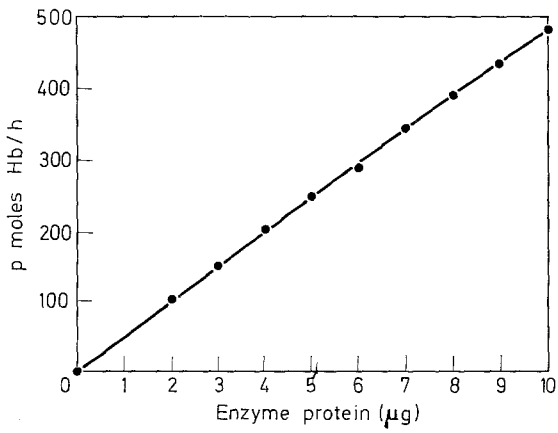

Fig. 5. Effect of enzyme concentration on the activity of the erythrocyte membrane cathepsin activity. Incubations were done for 30 min at 37 °C (pH 3.4) and the TCA soluble protein was analyzed as described in the text

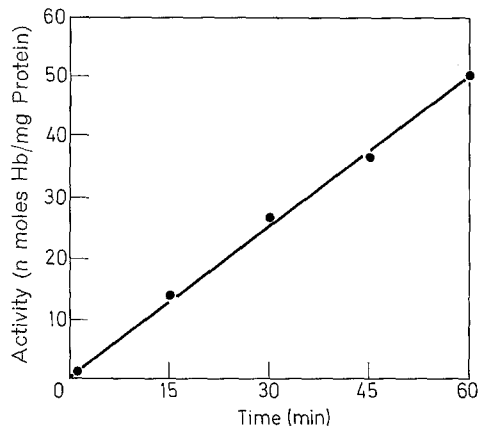

Fig. 6. Time course of ^{3}H-acetylated hemoglobin degradation by the cathepsin enzyme. Enzyme protein was kept constant

Table 2. *Effect of cations on erythrocyte membrane proteinase activity*[a]

Ion or addition	Concentration (mM)	Specific activity (% maximum of control)	
		pH 3.4	pH 7.4
Control		100	100
$FeCl_2$	1	99	140
	10	100	1,540
$FeCl_3$	1	91	100
	10	69	100
$HgCl_2$	1	70	—[b]
	10	52	—
$MgCl_2$	1	102	—
	10	103	—
$CaCl_2$	1	100	—
	10	100	—
$CdCl_2$	1	100	—
	10	100	—
$PbCl_2$	1	100	—
	10	98	—
EDTA	10	100	100
KCNS	250	10	51
$MgCl_2$	250	12	53

[a] The control values for the two different pH's are expressed as 100%. 0.1 M salt solutions were added to the assay to the final concentrations listed. Sephadex G-200 and G-100 aliquots were the enzyme source for the pH 3.4 and 7.6 enzymes, respectively.

[b] Experiment not performed.

system. Therefore, the effect of 10 mM $FeCl_2$, pH 7.4 cannot be attributed as a cofactor of the enzyme active at pH 7.4 but must be regarded as an anomalous effect causing degradation of the ^{3}H-acetylated hemoglobin substrate. Erythrocyte membranes extracted with 1 M Postassium thiocyanate (KCNS) or 1 M $MgCl_2$ (3:1 by volume) for 1 hr at 4 °C and then centrifuged at 20,000 × *g* for 20 min had little activity expressible in the supernatant fraction. Either the extraction period was not suitable or the final concentration of 250 mM KCNS or $MgCl_2$ was inhibitory. The latter seems to be true since the 0.1% TX-100 extract was also inhibited at pH 3.4 and pH 7.4 at these high concentrations of KCNS and $MgCl_2$ (Table 2). These results seem to be contradictory to those of Moore *et al.* (1970), who showed an acceleration of a pH 7.4 proteinase at high concentrations of KCNS. This may be due to the much shorter assay periods used and the fact that Moore *et al.* (1970) found a lag period of 1 to 2 hr in their assay system which lasted at least 6 hr. In any case extraction with 0.1% TX-100 seemed to be more suitable.

Control Characterization of Proteolytic Activity

Various control conditions were tested, and the results are presented in Table 3. Under optimum conditions, as previously established (37 °C, pH 3.4 and 7.4, 30 min), the Sephadex G-200 Triton extract degraded 47,325 pmoles of ^{3}H-acetylated hemoglobin per hr/mg enzyme protein at pH 3.4 and the Sephadex G-100 Triton extract degraded 305 pmoles Hb per hr/mg protein at pH 7.4 as based on the tritium cpm detected in the TCA soluble supernatant. Various control experiments were carried out and the activity found in all cases was significantly less than the control values. Trypsin, 10 μg (purchased from GIBCO), and soybean trypsin inhibitor, 100 μg (obtained from Sigma), were incubated alone or together in place of the erythrocyte membrane extract and their activities are shown in Table 3. Trypsin at pH 7.4 was very active (> 100,000 pmole Hb/hr/mg) and its activity was decreased 50% when incubated with 10 times excess (w/w) soybean trypsin inhibitor. The soybean trypsin inhibitor had no effect on the erythrocyte membrane proteinases at either pH.

Effect of Substrate Concentration on Enzyme Catalysis

By varying substrate concentrations of ^{3}H-acetylated hemoglobin, a Lineweaver-Burk plot was obtained (Fig. 7). The plot of $1/v$ versus $1/[S]$ shows a straight line relationship. The value for the apparent K_m as computed from this plot was 1×10^{-5} M hemoglobin.

Table 3. *Control characterization of erythrocyte plasma membrane proteinases*[a]

	Specific acitivity (pmoles Hb/hr/mg protein)	
	pH 3.4	pH 7.4
Complete[b]	47,325	305
Boiled enzyme[c]	37	35
Zero time[d]	35	30
Complete-enzyme	32	32
Complete-substrate	0.02	0.02
0 °C[e]	6,235	44
Trypsin (10 μg)	600	>100,000
Soybean trypsin inhibitor (100 μg)	30	32
Trypsin plus soybean trypsin inhibitor	350	55,000
0.1 % TX-100 extract plus soybean trypsin inhibitor	47,100	300

[a] Experiments were performed with ^{3}H-acetylated hemoglobin as a substrate. Assay conditions were similar throughout except as noted and any balancing of assay volumes necessary was made with 0.1 % TX-100 or distilled water.

[b] The complete assay system consisted of 50 μliters of enzyme protein (10 μg), 100 μliters buffer and 50 μliters substrate. Distilled water or 0.1 % TX-100 were substituted as needed to maintain constant volumes. Sephadex G-200 and G-100 aliquotes were the enzyme source for the pH 3.4 and 7.6 enzymes, respectively.

[c] Aliquots of enzyme protein were heated at 100 °C for 10 min and then assayed.

[d] Immediately after the substrate was added to the incubation assay, 0.1 ml of 2.5 % hemoglobin and 0.05 ml 60 % TCA were added and the TCA soluble fraction was analyzed.

[e] The incubation assay was incubated for 30 min in an ice-water bath at 0 °C.

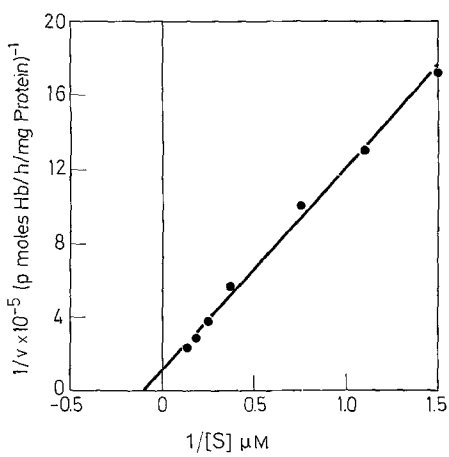

Fig. 7. Lineweaver-Burk plot of cathepsin activity from erythrocyte membranes. Assay was carried out under the conditions described in the text

Polyacrylamide Gel Electrophoresis

The distribution of protein in human erythrocyte membranes and the purification of a cathepsin-like enzyme on polyacrylamide gel electrophoresis, as visualized with Coomassie Blue, is evident as shown in Fig. 8. Red blood cell membranes as obtained by osmotic lysis are shown in gel *A*. Ten distinct protein bands are evident with their molecular weights ranging from 285,000 to 33,000. The second gel (*B*) represents red blood cell membranes after homogenization in 0.1% TX-100. It is interesting to note that the higher molecular weight protein species disappear with this treatment, leaving seven distinct protein bands with molecular weights ranging from 135,000 to 33,000. Gel *C* represents the 50,000 × *g* 0.1% TX-100 supernatant and contains three distinct protein bands with molecular weights of 84,000, 54,000, and

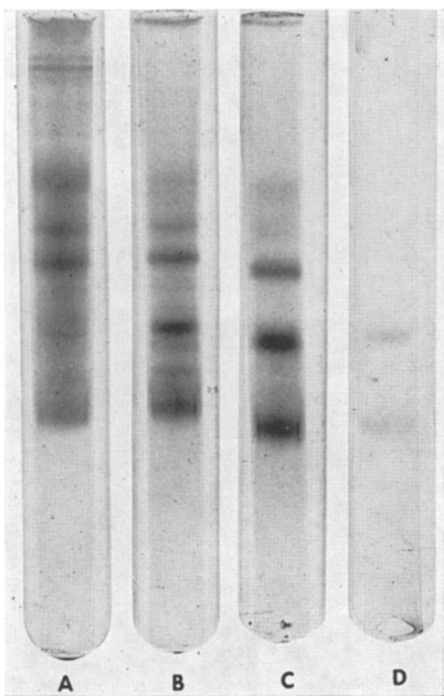

Fig. 8

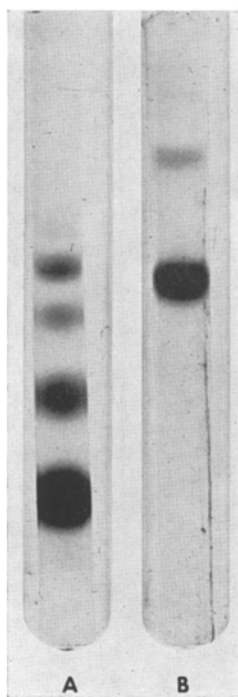

Fig. 9

Fig. 8. Gel electrophoresis of membrane proteins stained with Coomassie Blue. *A*. Red blood cell membranes. *B*. 0.1% TX-100 homogenate of red blood cell membranes. *C*. 50,000 × *g* 0.1% TX-100 supernatant. *D*. Sephadex G-200 aliquot containing cathepsin activity. All gels were run toward the anode (bottom)

Fig. 9. Gel electrophoresis of protein standards stained with Coomassie Blue. Gel *A*, hemoglobin and gel *B*, albumin

33,000. The last gel (*D*) is an extract from the Sephadex G-200 gel chromatography and contains two distinct bands of 54,000 and 33,000 molecular weight. The molecular weight approximations are based on the simultaneous gel electrophoresis of hemoglobin, bovine serum albumin, pepsin, and β-glucuronidase prepared and run in the same manner as the membrane extracts. Hemoglobin (gel *A*) contained four distinct protein bands (Fig. 9). Placing a value of 68,000 mol. wt. per hemoglobin molecule, the lowest or most prominent band is a sub-unit of hemoglobin with a 17,000 mol. wt. The other bands visualized may be multiples of the sub-unit. Bovine serum albumin (gel *B*) gives one prominent protein band. Assuming a unit molecular weight of 69,000 for bovine serum albumin, this value was given to its most prominent band. Pepsin, (mol. wt. 35,000) and β-glucuronidase (mol. wt. 280,000) were analyzed and included in Fig. 10.

Measuring the migration in cm from the origin of the gel to the midpoint of each band of the standards, an exponential relationship between molecular weight and migration was obtained. Plotting the molecular weights of the standards *vs.* migration from the origin, we obtained a straight line on a semilogarithmic chart (Fig. 10). From this chart we obtained our approximate molecular weights of the protein moieties found in the various extracts of human erythrocyte membranes.

The distribution of glycoproteins in the gels was scanned by PAS staining. Only two bands were evident in the original membrane preparation with approximate molecular weights of 135,000 and 84,000, which do not coincide with the two protein bands evident from the Sephadex G-200 cathepsin extract.

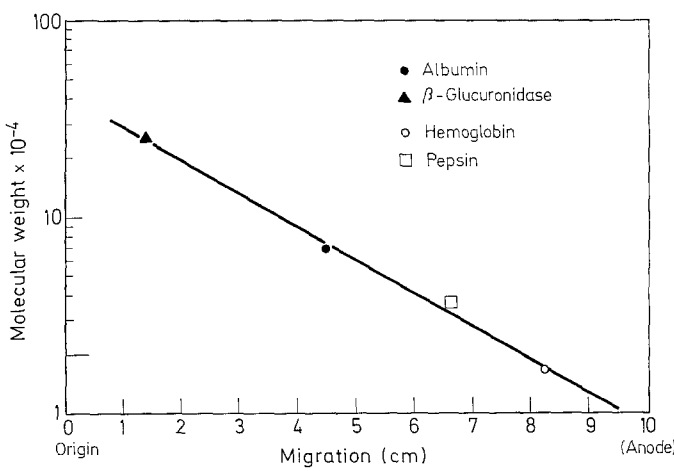

Fig. 10. Electrophoretic migration as a function of molecular weight of bovine serum albumin (•), hemoglobin (○), β-glucuronidase (▲), and pepsin (□)

Discussion

Morrison and Neurath (1953) reported the presence of proteolytic activity in red cell membranes using various extraction procedures and located proteinases active at pH 3.4 and 7.6. Moore *et al.* (1970) concentrated their efforts on isolating and characterizing a salt extracted proteinase active at pH 7.4. This present paper demonstrates that 0.1% TX-100 is a suitable agent for extracting proteinases from red blood cell membranes. The proteinase active at pH 3.4 has many properties similar to that of cathepsin *D* isolated from chick bone rudiments (Hille *et al.*, 1970) and bovine uterus (Woessner & Shamberger, 1971) and was purified and extensively characterized using a ^{3}H-acetylated hemoglobin as a substrate that proved to be much more sensitive than other substrates previously used (Bosmann, 1971).

The original membrane preparation contained a high amount of cathepsin activity but little can be ascertained as to whether the enzyme resides on the interior or exterior of the membrane since the incubation conditions employed (pH 3.4) lyse the prepared ghosts.

Column chromatography on Sephadex G-100 shows the cathepsin enzyme eluting after the major band of membrane protein containing the glycosidases (Bosmann, 1971) and pH 7.4 proteinase. The Sephadex G-200 eluate containing the cathepsin activity is shown to contain two protein bands on polyacrylamide gel electrophoresis of 33,000 and 54,000 mol. wt.

The exponential relationship of molecular weight and migration as seen with the SDS-treated proteins (Shapiro, Vinuela & Maizel, 1967; Weber & Osborn, 1969) provides a simple and accurate means of estimating molecular weights of unknown proteins. The basis of this separation according to molecular weight is still unknown but must be due to conformational changes of the proteins and masking of the protein's original charge by the SDS, leaving only size or molecular weight as a parameter that is acted upon by the exponential sieving effect of the polyacrylamide gel.

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