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MEMBRANE-BOUND ENZYMES

III. PROTEASE ACTIVITY IN LEUCOCYTES IN RELATION TO ERYTHROCYTE MEMBRANES

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SUMMARY

Protease activity was detected in membranes of human and bovine erythrocytes prepared by the conventional procedures which include washing and removal of the "buffy layer". The enzyme was extracted by 0.75 M KCNS or $(NH_4)_2SO_4$ and was activated by 0.4 to 0.5 M of the same salts. Colored, particulate hide powder-azure, membrane fractions and soluble proteins such as hemoglobin, casein or albumin were susceptible to hydrolysis by the membraneous protease.

Partial purification of the enzyme was accomplished through disc-gel electrophoresis on polyacrylamide in the presence of 0.25 % positively charged detergents like cetyltrimethylammonium bromide. An alkaline protease (pH 7.4) with properties similar to those of the erythrocyte enzyme was found in leucocytes. The similarity between the properties of the leucocytic and erythrocytic proteases and the correlation of the activity in erythrocyte membranes with the content of white cells in these preparations, suggest that enzymatic activities in the contaminating leucocytes are responsible for the activity of membraneous proteases in erythrocytes.

INTRODUCTION

The occurrence of enzymes catalyzing the degradation of proteins, present in suspensions or extracts of erythrocytes, leucocytes, platelets or other cells of the blood has been considered and studied over the last 30 years. Pioneering attempts to identify such enzymes in erythrocytes were made by Morrison and Neurath [1] in 1953. These investigators characterized three "erythrocyte proteinases, I, II and III" which were extracted from disrupted erythrocyte membranes by high concentrations of salts or by *n*-butanol. The distinction between the three enzymes was based on pH optima, responses to reducing agents, or to Zn^{2+} and Fe^{2+}. The substrate employed was denatured human hemoglobin. These studies were later confirmed and extended by

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Moore et al. [2] and by Bernacki et al. [3]. The mature red cells comprises the majority of the blood cell population and is accompanied by a small percent of immature red cells (less than 3 %) and leucocytes (a fraction of a percent). Morphologically, the mature red cell lacks intracellular membraneous structures, and any enzyme which has properties of being attached to particles will be considered as a plasma membrane-bound enzyme (e.g. acetylcholine esterase, $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase, K^+-activated phosphatase, etc.) [4, 5]. The leucocytes or immature and very young red cells, however, contain intracellular organelles, e.g., endoplasmic reticulum, mitochondria, lysosomes, etc. It is therefore not surprising that proteolytic activity was detected in the lysosome-containing leucocytes [6, 7]. On the other hand, it was rather unusual to have protease(s) attached to the membranes of the red cells, unless these enzymes were remnants of earlier stages of the red cell's life cycle, or of cytoplasmic origin. Indeed, several comments were recently made, which cast some doubt on the validity of earlier observations concerning the presence of protease in red cell membranes [8, 9].

In view of the similarity in the properties of erythrocyte protease(s) and those of the enzymes from leucocytes [6, 10], we re-examined the possibility of cross contamination and concluded that unless the erythrocytic enzyme was very labile or its activity extremely low, the membranes of the red cells seem to be devoid of proteolytic activity. An account of these results has been previously reported in a preliminary communication [11].

METHODS

Preparation of blood, cells, membranes and extraction with salt

Human blood was obtained by venesection, and fresh bovine blood was collected from adult cows in the municipal slaughter house. Clotting was prevented by citrated dextrose (cf. ref. 4) and the blood was used immediately or after storage of 2–3 days at 4 °C.

Separation of the blood cells from the plasma and their washing were done by established procedures [4, 12].

Erythrocyte ghosts were prepared according to Dodge et al. [12] or Weed et al. [13]. Stroma were prepared by the procedure of Morrison and Neurath [1]. Extraction of the membranes with high concentrations of salts and with Triton X-100 was accomplished virtually according to Morrison et al. [1] and Bernacki et al. [3], respectively.

Leucocytes were obtained from fresh human blood by the Dextran sedimentation procedure outlined below. The cells were disrupted by repeated freezing to −30 °C and thawing to 37 °C; or by ultrasonically irradiating for a total time of 1 min. The process was carried out in an ice-water bath using a homogenizer (Brown, model sonic 300, Quigley, Rochester Inc., N.Y.) operating at 60 % of its maximal output.

Assay of proteolytic activity

(A) In most experiments the proteolytic activity was assayed with hide powder-azure as substrate. The particulate protein (hide powder) is covalently linked to a chromogen (Remazo brilliant blue) by an ether bond [14]. The release of the chro-

mogen during hydrolysis is taken as a measure of the proteolytic cleavage of the peptide bonds located in the vicinity of the ether linkage. The reaction mixture contained 0.2 M phosphate buffer, pH 7.4; 0.45 M KCNS, and 25 mg of the substrate. The reaction was started with 0.25 to 1.00 ml enzyme suspension in a final volume of 5 ml. Following incubation at 37 °C for periods up to 4 h, the reaction was terminated with a 10 % (w/v) solution of trichloroacetic acid and filtration through Whatman No. 1 filter paper. The intensity of the blue color was determined in a Gilford spectrophotometer at 595 nm [14]. 100 % hydrolysis of the substrate was accomplished by the addition of 0.5 mg trypsin, 40 mM Tris · HCl, pH 8.1 and 0.1 M $CaCl_2$. Absorbance, following complete hydrolysis was 1.79 ± 0.03, and one unit of protease activity was thus defined as the amount of enzyme which hydrolyzes 1 % of the substrate per min (i.e., increase in the absorbance of 0.018/min).

(B) Digestion of various proteins was assayed following incubation under conditions similar to those outlined above, except that the substrate concentration ranged from 1 to 5 mg protein/ml, and the activity was followed by the release of "Lowry" positive, acid soluble products [15]. Under the conditions of the Lowry et al. procedure [15] as modified by McDonald and Chen [16] for proteinase activity, 100 μg of tyrosine yield a colored product with the absorbance of 1.060 at 700 nm. One unit of protease is thus defined as the amount of enzyme releasing 1 μg tyrosine/min.

Electrophoresis on polyacrylamide gels in the presence of detergents

Electrophoresis of the blood cell protease was carried out on gels, 10 cm long, containing 5 % acrylamide [17, 18]. The runs were done in 50 mM Tris-glycine, pH 9.2 with 2 mA per tube for the first 15 min then increasing to 5 mA per tube. The protein samples were concentrated by ultrafiltration on Diaflo membranes (Amicon, Holland), dialyzed to remove excess KCNS and applied onto the gels.

Samples of membrane proteins were subjected to electrophoresis in the presence of detergents. 0.1 % (w/v) Triton X-100 or 0.25 % (w/v) of either cetyl-trimethylammonium bromide or cetylpyridinium bromide were employed. Staining of the gels was done with Coomassie blue G-250, except when detergents were used which interfered with the staining process.

At the end of electrophoresis, the gels were sectioned and the proteolytic activity was determined following an overnight soaking at 4 °C in a solution of 0.75 M KCNS dissolved in 0.3 M phosphate, pH 7.4. The gels or the detergents did not interfere with the assay. Preparative polyacrylamide disc-gel electrophoresis on two gel combination as described by Heller et al. [18], in the presence of 0.25 % (w/v) of cetyl trimethylammonium bromide, was carried out.

Separation of cells

(A) Sedimentation of washed blood cells or whole blood in a solution of Dextran, 3 % (w/v), M_r 228 000, using a modification of the procedures of Klein et al. [19] or Skoog et al. [20]. The erythrocytes were removed by rapid hemolysis in water and bringing the tonicity back to normal (isotonic) with NaCl. Following centrifugation at low speed, the intact leucocytes sedimented, leaving the erythrocyte ghosts in solution.

(B) Density gradient centrifugation. A sample of 4 ml washed blood cell mixture was layered on top of a stepwise density gradient made of 17.9 % to 35 %

(w/v) Dextran 60–90 (M_r 60 000 to 90 000) and centrifuged in a Spinco, swinging bucket rotor SW 25.1 (Beckman) at 90 000 $\times g$ for 30 min.

(C) Selective adsorption of leucocytes on cotton wool made into a column, allowing the passage of erythrocytes [21]. The eluted blood was passed 2 to 3 times through the column for maximal efficiency. At the end of each separation the cells were washed by centrifugation and resuspended in isotonic NaCl solution to remove the medium.

The number of cells in the original blood sample and in various steps of the process of separation was monitored by counting under the light microscope using Levy hemocytometer or with a Coulter counter [22].

Proteins and acid soluble peptides were determined by the method of Lowry et al. [15] or its modifications [23].

MATERIALS

Hide powder-azure, grade B was a product of Calbiochem. (Lucerne, Switzerland); Dextrans were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), cetyl trimethylammonium bromide, cetylpyridinium bromide, sodium deoxycholate were obtained from Sigma Israel (Ramat-Gan, Israel); trypsin and denatured bovine hemoglobin ("for protease assay") were products of N. B. Co. (Cleveland, Ohio); Triton X-100 was a generous gift from Rohm and Haas (Philadelphia, Penn.), Coomassie blue G-250 from Serva Feinbiochimica (Heidelberg, G.F.R.); Cotton threads were purchased at the Bedouin market, Beersheba, Israel.

RESULTS

Properties of the membrane-bound proteolytic activity

Protease activity was assayed in high salt extracts of red blood cells membranes. Extraction with 0.75 M KCNS or $(NH_4^-)_2SO_4$ yielded an enzyme activity of about 0.5 to 1.0 units/mg membraneous protein. Both stroma [1]and ghosts [12, 13] gave similar protease activities. The presence of a similar protease activity in salt extracts of human erythrocyte membranes, degrading the chromogenic substrate hide powder-azure, was earlier described by Morrison and Neurath [1]. The activity could be preserved upon storage at 4 °C, pH 4 to 6, in presence of 0.1 % merthiolate for three days only, whereas at −20 °C, considerable activity was retained even after periods of 3 to 4 weeks.

The proteolysis was determined at pH values between 3 and 8, and the highest activity was obtained in the neutral (7.4) to alkaline (8.0) pH range. In addition to the hydrolysis of hide powder-azure, several other particulate or soluble protein preparations were used as substrates. Among these were: native or heat-denatured membrane preparations; residues of membranes remaining after salt extraction, as well as hemoglobin, casein and albumin. The hydrolysis was followed by the release of acid soluble peptides and amino acids reacting with "Lowry's" reagents [15, 16]. Table I shows that the enzyme preparations obtained from bovine or human red blood cells, exhibited high activities with the particulate substrates but had lower activities towards the soluble substrates.

In order to enable further purification of the protease on one hand, and to

TABLE I

PROTEOLYTIC ACTIVITY IN SALT EXTRACTS OF BOVINE AND HUMAN ERYTHROCYTE MEMBRANES

Stroma were prepared from bovine or human washed erythrocytes according to Morrison et al. [1], and were extracted with 3 vol. of 1 M KCNS. The salt extracts were assayed for proteolytic activity at pH 7.4 with either hide powder azure (5 mg/ml) or with the other substrates (1.5 mg/ml). Incubations were carried out at 37 °C for periods up to 3 h. For hide powder-azure as substrate, one unit of activity is defined as the hydrolysis of 1 % of the substrate/min; for the rest of the substrates: the formation of 1 μg equivalent of tyrosine/min.

Substrate	Activity (munits/mg enzymatic protein) in extracts	
	Bovine	Human
Hide powder azure	327	983
Stroma, native	198	188
Stroma, boiled	404	472
Casein	76	–
Hemoglobin	74	–
Albumin	2	–

expose the membrane-bound enzyme to the particulate substrates, we explored the effects of detergents on the enzymatic activity. Concentrations up to 0.05 % of the non-ionic detergent Triton X-100 or the cationic detergents cetyl trimethyl ammonium bromide or cetyl pyridinium bromide did not have a considerable effect on the activity of the membraneous protease. The anionic detergent deoxycholate, however, at similar concentrations, caused a 90 % inhibition of the enzyme.

Partial purification of the protease

Dialyzed salt extracts obtained from human erythrocyte membranes were submitted to polyacrylamide disc-gel electrophoresis with either 7.5 % or 3 % acrylamide, but only partial penetration into the gel matrix occurred. To facilitate penetration of the proteins, detergents were added to the applied extract and the electrophoresis buffer. With 0.1 % of Triton X-100, about 60 % of the applied activity was recovered from the gel. In spite of the relatively good recovery, the enzymatic activity was smeared over a large section of the gel. On the other hand, positively charged detergents at a concentration of 0.25 % (w/v) enabled complete penetration of the protease into 7.5 % acrylamide gels.

Table II outlines the scheme for a partial purification of the membraneous protease from human red blood cells. Highly active enzymatic preparations could be obtained using this method, which consists of extraction with salt, ultrafiltration through PM-30 Diaflo membranes and dialysis to concentrate the extract and remove the salt, followed by disc-gel electrophoresis on polyacrylamide in the presence of 0.25 % (w/v) cetyl trimethylammonium bromide.

Protease activity in leucocyte membranes

A mixed population of leucocytes containing granulocytes, lymphocytes and platelets was disrupted by repeated freezing and thawing of the cell suspension in

TABLE II

SCHEME FOR PURIFICATION OF PROTEASE FROM STROMA

Details are given in the Methods section.

	Protein (mg)	Activity (units)	Spec. act.
Stroma in 0.75 M KCNS Mix overnight, then centrifuge at 100 000 × *g*, 60 min, 4 °C; discard precipitate	74.1	19.30	0.262
Supernatant Ultrafiltration through Diaflo membrane, PM-30; residue dialyzed overnight to remove KCNS	25.4	14.60	0.570
Concentrate Add 0.25 % cetyltrimethyl-ammonium bromide; electrcphoresis on polyacrylamide disc gel, 5 %, in above detergent for 2 h			
Overnight total extraction with 0.75 M KCNS	4.80	6.40	1.33
Active band on gel	0.005	0.11	22.00

TABLE III

PROTEASE ACTIVITY IN LEUCOCYTES

Leucocytes from fresh or up to 4 days-old stored blood, were separated from the erythrocytes and plasma by sedimentation in Dextran [19, 20]. Stroma were prepared by either (A) freezing and thawing repeatedly; (B) procedure (A) combined with 1 min ultrasonic irradiation; (C) treatment with 0.1 % Triton X-100, centrifugation for 30 min at 100 000 × *g*, discarding the supernatant, retaining precipitate for assay; (D) stroma were ultrasonically irradiated for 1 min in the presence of 0.1 % Triton X-100, centrifuged as in (C) and both precipitate and supernatant assayed. Assay was carried out in pH 7.4, as outlined in "Methods" with 0.2 to 0.9 mg protein for periods up to 4 h at 37 °C with constant shaking in a water bath. The substrates were either (a) hide powder-azure; product determined at 595 nm, or (b) denatured, bovine hemoglobin; the products were determined according to Lowry [15, 16]. (For units, cf. "Methods" and Table I). The numbers in parentheses indicate the number of experiments done.

Enzyme preparation	Substrate	Activity (munits/ mg enzymatic protein)
A	Hide powder-azure	925 (2)
C	Hide powder-azure	556 (1)
B	Hemoglobin	423 (3)
D (supernatant)	Hemoglobin	38 (1)
D (precipitate)	Hemoglobin	495 (1)

isotonic solutions of NaCl. Alternatively these cells were disrupted either by ultrasonic irradiation or by addition of Triton X-100 to a final concentration of 0.1 % (w/v). Such disrupted leucocytes exhibited a high neutral-alkaline protease activity (pH 7.4) with either hide powder-azure of hemoglobin as substrates (Table III).

The possible contribution of proteolytic activity arising from leucocytes to the erythrocyte membranes was subsequently explored. The ineffectiveness of the conventional washing procedure in separating the white from the red cells was observed when after 6 washings, the number of leucocytes per 10 000 erythrocytes was 5.3 compared to 14.3 in the original, unwashed blood. This means that the repeated washings removed only 63 % of the original leucocytes, leaving a considerable percentage in the red blood cell samples.

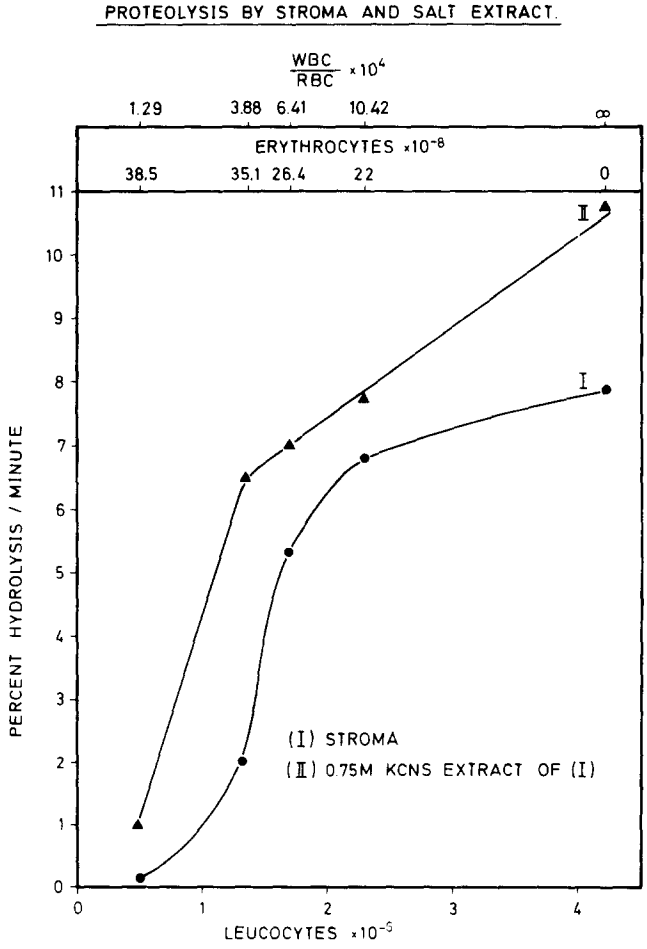

Fig. 1. Proteolytic activity of membranes and salt extracts with various preparations of erythrocytes and leucocytes. Samples of cells were counted for their erythrocytes or leucocytes content; membranes ("stroma") were prepared and extracted with KCNS. The proteolytic activity was determined with hide powder-azure; and the activity was expressed in units (= percent hydrolysis/min of substrate, in which 1 % means 1 unit). WBC, leucocytes; RBC, erythrocytes.

Fig. 1 shows a plot of the neutral-alkaline protease activity as a function of red cell content or white cell count. The enzyme was assayed either in the membraneous preparations (stroma) or their salt extracts prepared from mixtures containing known amounts of red and white cells. It is therefore possible to correlate protease activity to the cell count. It can be seen that the activity was proportional to the leucocyte content of the preparation. At low white cell content, only a very low digestive capacity was detected despite the high content of red cells. Highest protease activity was observed when the leucocyte/erythrocyte ratio approached infinity.

Triton X-100 extracts of human erythrocyte membranes were shown by Bernacki and Bosman to contain acid (pH 3.4) and neutral (pH 7.4) proteases [3]. Preparation of ghosts according to their method from samples containing a known number of erythrocytes and leucocytes showed proteolytic activity only when the white cell count exceeded $5 \cdot 10^5$ cells/sample (Table IV). Furthermore, the detergent, Triton X-100 did not extract the neutral-alkaline enzyme from the ghosts.

We have extended this study and carried out disc-gel electrophoresis of salt extracts obtained from a cell suspension of known erythrocyte and leucocyte content. The results indicated clearly the similarity in the electrophoretic pattern, based on protease activity, between the leucocytaric enzyme and the enzyme which is supposed to be in the erythrocyte membranes.

In order to clarify this point further a few methods were employed to obtain a clearer separation between the white and the red cells. Although not adequate for a large-scale preparation, we have found that density gradient centrifugation in Dextran solutions was superior to the other methods tried. Stroma were prepared from the mixture of erythrocytes and leucocytes obtained after such separation procedures were applied. It is clearly seen that with a low ratio of white to red cells, the activity of the protease decreased enormously (Table V). It seems that the proteolytic activity stems primarily from the leucocytes, since as shown in Expt. 4 of Table V, a tenfold decrease in the leucocyte content resulted in a tenfold reduction in protease activity.

From the data of Fraenkel-Conrat et al. [24], the appropriate range of leucocytes for optimal proteolytic activity is at 10^6 to 10^7 cells per assay. Consequently, fresh human blood was washed and the cells were separated by sedimentation in 3 % Dextran. The leucocytes, freed from erythrocytes by rapid hemolysis and removal of

TABLE IV

PROTEASE ACTIVITY AFTER TRITON X-100 EXTRACTION

Ghosts were treated with 0.1 % Triton X-100, and the assay of protease activity was carried out with hide powder-azure in presence of 0.45 M KCNS at pH 7.4. The measurements in "ghosts" were done 48 h after their preparation to ensure leucocyte disruption.

Expt No.	Red blood cells $\times 10^{-9}$	White blood cells $\times 10^{-5}$	White blood cells	Total activity (units)			
	Assay	Assay	10^4 red blood cells	"Ghosts"	Ghost-free hemolyzate	"Triton X-100 extract"	"Triton X-100 precipitate"
I	4.50	1.75	0.39	0	0	0	0
II	3.00	5.50	1.83	1.69	0	0	1.32
III	0	10.80	–	5.05	1.06	0	4.92

TABLE V

PROTEASE ACTIVITY IN STROMA OBTAINED FROM PURIFIED ERYTHROCYTE

For details see Methods section. Assay: Exp. 1: 1 ml stroma of the sediment was assayed with hide-powder azure for 120 min at 37 °C. Exp. 2, 3: 0.5 ml stroma or eluate was assayed for 120 min at 37 °C. Exp. 4: 0.5 ml stroma prepared from cells which were obtained after density gradient centrifugation in Dextran 60-90 and collected from the band having the density of 1.1082. The activity was assayed for 290 min at 37 °C.

Expt No.	Method of separation	Activity		
		White blood cells / 10^4 red blood cells	munits / mg protein	Total units
1	Washed red blood cells	1.26	64.4	1.718
	Dextran 200 sediment	1.73	80	2.165
2	Washed red blood cells	10.10	319	6.666
	Cotton column	1.09	16	0.660
3	Washed red blood cells	3.40	57	1.112
	Cotton column	1.56	42	0.804
4	Washed red blood cells	1.48	73	–
	Dextran gradient band ($d = 1.1082$)	0.13	7.7	–

TABLE VI

PROTEASE ACTIVITY IN DISRUPTED MEMBRANES OF ERYTHROCYTES AND LEUCOCYTES

The blood cells were separated by sedimentation in 3 % Dextran. The erythrocytes were hemolyzed according to Dodge et al. [12]. The erythrocyte membranes and the leucocytes were ultrasonically irradiated for 1 min at 4 °C. The proteolytic activity was assayed with hide powder-azure at 37 °C for 120 h at pH 7.8 with a fixed number of leucocytes in the vessel.

Preparation	White blood cells $\times 10^{-6}$ / Assay	Red blood cells $\times 10^{-8}$ / Assay	White blood cells / 10^4 red blood cells	Protein (mg) / Assay	munits / mg protein	Units $\times 10^8$ / white blood cells
Pure white blood cells	1	0	–	0.03	874	2.6
Mixture of white blood cells + red blood cells	1	8.7	11.4	0.21	171	3.6
Enriched red blood cells	1	108	1	3.9	4.5	1.8

the ghosts, were obtained as well as a fraction enriched with erythrocytes. A third fraction of erythrocytes with a higher content of leucocytes was also tested. Disrupted membranes were obtained from hemolyzed erythrocytes and from leucocytes following ultrasonic irradiation. Table VI indicates clearly that when a fixed amount of leucocytes (10^6) were present in the assay, a constant activity of approximately $3 \cdot 10^{-8}$ units/leucocyte was obtained. A considerable variation in the specific activity (expressed as munits/mg protein) was noticed. This leaves little doubt as to the major contribution of proteolytic activity by leucocytes, in blood cells.

DISCUSSION

Several studies on erythrocyte membrane-bound enzymes were carried out with red cells obtained after a partial removal of the white cells [25, 26]. The conventional procedure of washing and removal of the buffy layer usually leaves, however, relatively large proportions of the original leucocyte count. Furthermore, the activities of some enzymes in leucocytes are several orders of magnitude greater than in erythrocytes and some enzymes are only present in white cells and absent from red cells. Therefore, contamination by leucocytes can result in erroneous high enzymatic activities.

The results of the present study leave little doubt as to the contribution of the white cells to the proteolytic activity in preparations of human or bovine erythrocyte membranes. The activity was found to be in a direct correlation with the white cells content of the preparations and seems to be altogether absent in preparations of erythrocytes made deficient of leucocytes.

In a survey of hydrolases in human erythrocyte membranes, Bosmann [27] has shown that neither cathepsin nor trypsin-like activities could be detected by the method of Anson [28] with hemoglobin as substrate. On the other hand, he was able to determine the activity of a general protease, which was assayed at pH 7.5 with azocoll as substrate. This enzyme was activated by 0.1 % (w/v) of Triton X-100, but was not extracted with this detergent. In a subsequent paper, Bernacki and Bosmann [3] described a procedure for purification of an acid protease, which was extracted by the very same detergent from membranes of human erythrocytes, prepared in such a way as to retain sufficient leucocytes with the red cells. In our hands, membranes of human erythrocytes had neither the neutral nor the acid (pH 7.4 and 3.8, respectively) protease activities described by these authors. A major difference, however, should be mentioned between the present study and that of Bernacki et al. [3]. The substrate employed in our studies was primarily hide powder-azure, but also hemoglobin, whereas Bernacki et al. used [^{3}H]acetyl hemoglobin, the hydrolysis of which might reflect activity of an amidase, yielding radioactive, free acetic acid, in addition to a protease, releasing radioactive acetylated peptides.

It might be possible that with the hide powder-azure, hemoglobin or other proteins used as substrates in the present study, and with rather long incubation periods, we missed the erythrocyte membrane-bound proteases (either acid or alkaline). In view of the sensitivity of the acid cathepsin (cf. the review of Barrett [29]), and also of the alkaline enzyme of the leucocytes [6], the existence of such enzymes associated with erythrocyte membranes cannot be completely excluded, especially in younger red cells or reticulocytes, in which elimination processes of nuclei, mitochondria and endoplasmic reticulum occur during maturation.

Our results on the partial purification of the neutral-alkaline protease from the mixture of blood cells suggests that regardless of the true source of enzyme, it can be considerably purified from that mixture without having to separate the white from the red cells completely. The techniques employed here, are thus advantageous for further studies on the properties of this enzyme. A clear-cut verdict on the real source of the proteolytic activity will have to wait for a more rigorous and complete separation of the white from the red cells.

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