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Effects of Pronase on Passive Ion Permeability of the Human Red Blood Cell

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Summary. The effects of pronase from *Streptomyces griseus* on sulfate, potassium, sodium, and erythritol permeability of human red blood cells were studied. It was found that the proteolytic enzyme reduces anion permeability, increases cation permeability and has no effect on the nonfacilitated component of the flux of the nonelectrolyte. These findings can be explained on the basis of the fixed charge hypothesis by the assumption that the enzyme exerts its effects by altering the density of positive fixed charges in the membrane.

The effects of pronase are qualitatively similar to those of the amino reactive agent, dinitrofluorobenzene (DNFB). Therefore, attempts were made to discover if this similarity is due to alterations of the same membrane sites by the enzyme and the chemical modifier. It was found that the effects of pronase and DNFB were not additive. Hence, the enzyme and the amino reactive agent do not seem to act on two independent and parallel channels. A more detailed analysis of the data suggests that DNFB and pronase affect functionally identical sites.

Proteolytic enzymes frequently exhibit some esterase activity. However, the amino-N content of lipid extracts of red cell membranes remained virtually unaltered after exposure of the cells to pronase. This finding indicates that the positive charge of the bulk of the lipid amino groups is not involved in the control of passive ion permeability. The carbohydrate amino groups of the red cell membrane are N-acylated and hence cannot contribute to the positive membrane charge. It seems reasonable to conclude that the effects of pronase on ion permeability are primarily due to alterations of the density of charged protein amino groups in the red cell membrane.

In the course of studies on the role of positive fixed charges in the control of passive ion movements across the erythrocyte membrane, measurements of the pH dependence of anion and cation permeability were performed at various ionic strengths in the medium (Passow, 1965; LaCelle & Rothstein, 1966; Passow, 1969*a*; Lepke & Passow, 1971). The results of these measurements were compatible with the assumption that, if fixed charges are in fact involved, their pK value lies around 9. This pK value suggested that the fixed dissociable groups are protonated amino groups.

The participation of amino groups in the control of passive ion permeability was also inferred from experiments with dinitrofluorobenzene (DNFB), an amino reagent which was first used in this type of permeability research by Berg, Diamond and Marfey (1965). Essentially, this agent causes an increase of cation permeability and a decrease of anion permeability. This effect can be easily understood in terms of the fixed charge concept if the assumption is made that the interactions of the agent with uncharged amino groups leads to a concomitant decrease of the number of positively charged amino groups in the membrane (Passow, 1969*a*; Poensgen & Passow, 1971).

DNFB is a rather non-specific agent. It is not only capable of reacting with amino groups but also with SH, phenolic OH, and imidazole groups (Hirs, 1967). However, experiments with more specific amino reagents, 5-methoxy-2-nitrotropone (MNT) and trinitrobenzene sulfonate (TNBS) (Passow & Schnell, 1969; Poensgen & Passow, 1971), yielded results qualitatively similar to those found when DNFB was employed. Thus, it is likely that amino groups participate in determining the kinetics of passive ion movements.

In the red cell membrane, free amino groups are present in carbohydrates, proteins, and lipids. The available analytical data suggest that the amino groups of carbohydrates exist predominantly or, possibly, exclusively in the uncharged, N-acylated form (e.g. Winzler, 1970). Studies of DNFB- and MNT-binding showed that both agents combine with the amino groups of lipids as well as proteins in the membrane. Under the conditions of our permeability measurements, about 30 to 40% of the total DNFB bound to the membrane interacted with membrane lipids, mainly phosphatidylethanolamine (Poensgen & Passow, 1971). The present paper describes experiments in which proteolytic enzymes were used for deciding whether lipid amino groups or protein amino groups are involved in controlling ion permeability. Pronase, a highly aggressive mixture of proteases, was employed. Pronase decreased anion fluxes and increased cation fluxes. Thus, the enzyme acted essentially like the amino reagents. Under our experimental conditions, pronase did not alter the concentration of lipid amino groups in the erythrocyte membrane. This finding suggests that protein amino groups rather than lipid amino groups participate in the control of passive ion permeability.

Materials and Methods

All experiments were performed with freshly drawn, citrated human blood from healthy donors. Before use, the cells were washed three times in isotonic saline. In most experiments on anion permeability, the back exchange of $^{35}SO_4$ ions from previously

labeled red blood cells was followed. For labeling with $^{35}SO_4$ the cells were usually preincubated in isotonic 'standard medium' containing 100 mmoles/liter KCl, 50 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose, and a trace of $^{35}SO_4$. The cell density was 20 vol%. After 90 min at 37 °C, the cell suspensions were diluted 1:1 with standard medium which additionally contained sufficient pronase to establish the desired final enzyme concentration. Incubation at 37 °C was continued for another period of time whose length varied according to the experimental requirements. At the end of this second incubation period, the suspension was diluted 1:1 with ice cold $^{35}SO_4$-free standard medium containing no pronase and centrifuged in a refrigerated centrifuge. The cells were washed two more times with ice cold medium and finally resuspended in the same medium prewarmed to 37 °C (=zero time of the back exchange experiment). The described procedure ensured that the $^{35}SO_4$-back exchange was measured under conditions where the system was close to the Donnan equilibrium. In the final suspension, the cell density was usually either 5 vol% or 2.5 vol%. The pertinent values are indicated in the legends of the figures.

After mixing, samples were taken at suitable time intervals and centrifuged, and the appearance of radioactivity in the supernatant was followed. Determination of radioactivity and evaluation of data was performed as described by Poensgen and Passow (1971).

In a few experiments, the forward exchange (i.e., the entry of $^{35}SO_4$ from the medium into the cells) was followed as described by Gardos, Hoffman and Passow (1969). The results agreed with those obtained in back exchange experiments.

The experimental conditions under which the effects of pronase on cation permeability were studied are described under Results. The net cation movements were determined as described in the previous communication (Poensgen & Passow, 1971).

Commercial preparations of pronase were used (Merck). The specific activity was 70,000 P.U.K. units/g. Pronase concentrations are always expressed in mg enzyme/mliter of final suspension medium.

In the concentrated cell suspensions used for pretreating the cells with the enzyme, the buffer capacity of the cells was high enough to ensure the establishment of pH values between 7.2 and 7.3. During back exchange in the diluted cell suspension, the pH dropped by about 0.1 units.

Red cell lipids were extracted from a known number of isolated cell membranes (sucrose method, *see* Poensgen & Passow, 1971) by the method of Reed, Scott, Swisher, Marinetti and Eden (1960). Amino-N was determined by the ninhydrin method described by Troll and Cannan (1953). Amino-N assay of dilutions of a red cell lipid extract with lipid solvent yielded a linear relation between lipid concentration and light extinction at 570 nm. Hence, the method is suitable for the determination of percentage changes of the total lipid amino-N in the membrane.

The total amino-N content was measured by the method of Troll and Cannan (1953) after dissolving the membranes of a known number of erythrocytes in 10% TX-100. The hemoglobin-free membranes were obtained by centrifugation of the hemolyzed cells through several layers of sucrose solutions of increasing density as described by Poensgen and Passow (1971). Again, the results were expressed in percent of light extinction at 570 nm of a standard membrane suspension. The protein content of the sedimented membranes was determined by the method of Lowry, Roseborough, Farr and Randall (1951) as modified by Maddy (1969, *personal communication*).

Uptake of ^{14}C-dinitrofluorobenzene by red cell membranes was measured as described by Poensgen and Passow (1971). ^{14}C-DNFB was obtained from Amersham, Eng.

Results

1. Effects of Pronase on Cation Permeability

(a) Flux Measurements during the Action of the Enzyme. If washed red blood cells are exposed to the action of pronase, cation permeability greatly increases. This increase becomes singnificant after a lag period whose length depends on the concentration of the enzyme in the medium (Fig. 1a). The lag period decreases with increasing enzyme concentration (Fig. 1d). In addition, the maximal rate of K^+ loss into the NaCl medium, determined from the slopes at the inflection points of the curves relating K^+ loss to time, continually increases without reaching a maximal value over the range of pronase concentrations employed in the present experiments. K^+ loss is accompanied by Na^+ uptake (Fig. 2a). This indicates a general loss of cation selectivity rather than a modification of specific K^+-channels. Thus, the effect of pronase is quite distinct from that produced by lead, triose reductone, adenosine + iodoacetate, and other agents which induce a considerable increase of K^+ efflux with only an insignificant augmentation of Na^+ influx (Passow, 1964). Nevertheless, pronase exerts a slightly stronger effect on K^+ than on Na^+ (Fig. 2a). At least initially, when the permeability change first becomes apparent, the concentration difference for sodium ions across the red cell membrane is nearly equal to that for potassium ions while the negative charge of the cell interior, with respect to the medium, favors sodium entry into the cells. Thus, the driving force may be slightly higher for sodium ions than for potassium ions; yet, K^+ efflux exceeds the rate of Na^+ uptake. The sum $Na^+ + K^+$ passes through a minimum and remains below its initial value throughout the whole period of experimental observation (Fig. 2b).

When the pronase evoked potassium loss approaches equilibrium, hemolysis begins to develop. Lysis does not seem to be of the colloid osmotic type since it starts when the cellular cation content passes through the minimum and when the cells are maximally shrunken. The addition of sucrose to the medium delays hemolysis considerably (Fig. 2c), but does not interfere with the action of pronase on the membrane and the ensuing net ion movements. However, the addition of dextrane (average mol wt 35,000) or a series of polyvinylpyrrolidon preparations of molecular weights ranging from 15,000 to 135,000 enhanced the appearance of hemolysis. Thus, although it would be tempting to attribute the protective action of sucrose to a retardation of colloid osmotic swelling, other explanations may be needed.

(b) Flux Measurements after Interruption of the Enzymatic Modification of the Cell Membrane. In the experiments described above, K^+ loss and

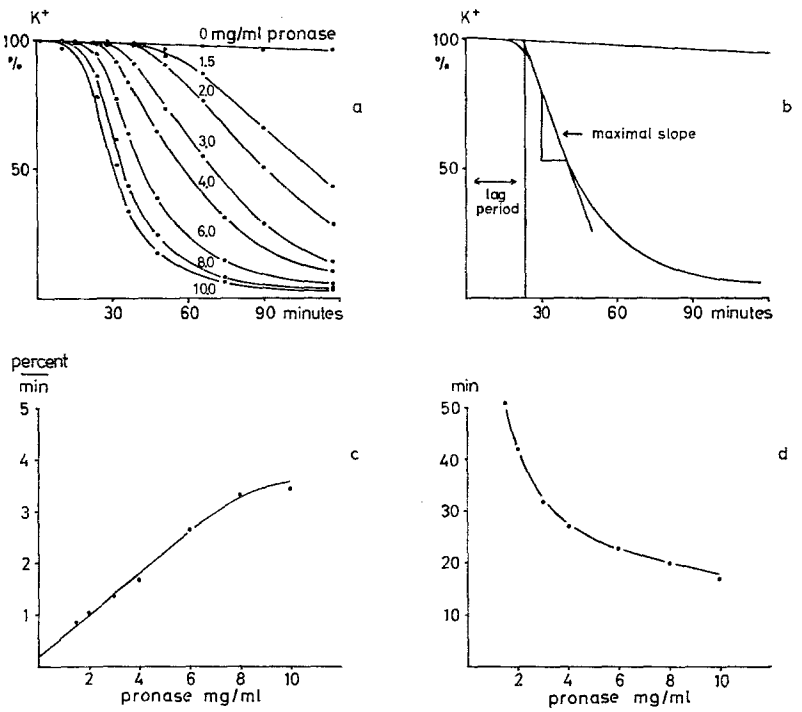

Fig. 1. (a) Time course of potassium loss from human red blood cells exposed to the action of varying concentrations of pronase in the suspension medium. All media contained 166 mmoles/liter NaCl, 20 mmoles/liter sucrose, pH 7.4. The pronase concentrations are indicated on the curves in mg/ml. At zero time, cells and incubation medium were mixed to give cell suspensions with a hematocrit of 5 vol%. Temperature: 37 °C. *Ordinate:* Potassium content of a fixed number of cells in percent of initial value. *Abscissa:* time in minutes.

(b) Schematic diagram for illustrating the method by which the length of the lag period was determined from the curves represented in (a):

1) draw a straight line with slope 2% per min (=approximately equal to the rate of potassium net loss in the absence of substrates in the NaCl medium) through the initial value for intracellular K^+ (=100%);

2) draw another straight line through the inflection point of the curve with a slope equal to the slope of the experimental curve in that point; and

3) read on the abscissa the time at which the curves intersect. This time is taken as a rough measure of the lag period preceding the onset of the change of cation permeability.

(c) Maximal rate of K^+ loss in pronase treated cells as a function of pronase concentration in the medium. The maximal rate of K^+ loss was derived from the data of (a) by determining the slopes of the curves relating K^+ loss to time at their respective inflection points. (*See* (b).) *Ordinate:* Maximal rate of K^+ loss in percent per minute. *Abscissa:* Pronase concentration in the medium in mg/ml.

(d) Lag period preceding the onset of potassium loss as a function of pronase concentration in the medium. The lag period was derived from the data of (a), as explained in (b). *Ordinate:* lag period in minutes. *Abscissa:* pronase concentration in the medium in mg/ml

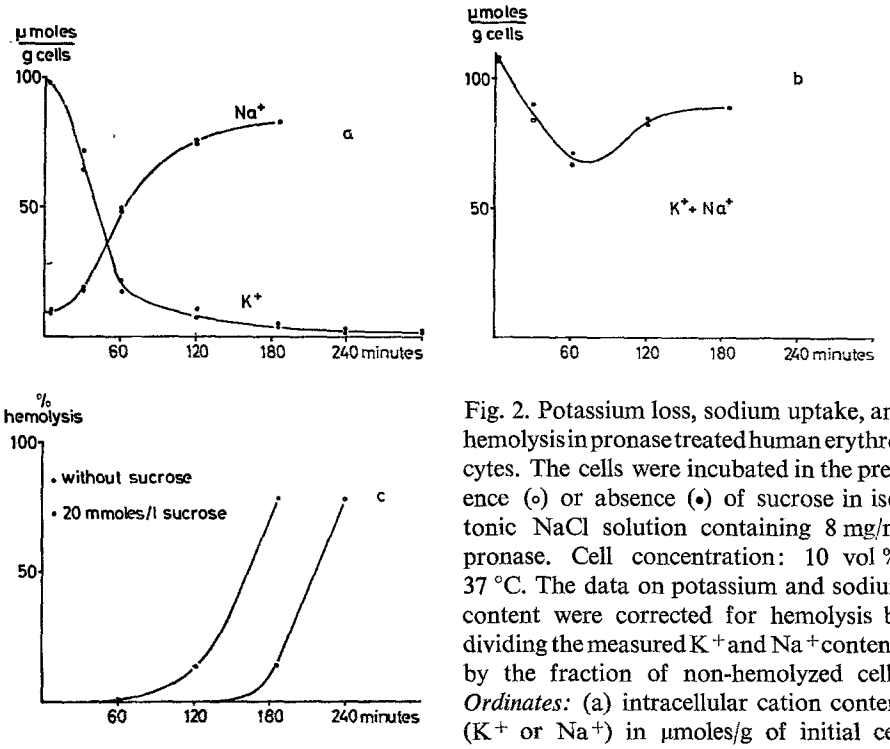

Fig. 2. Potassium loss, sodium uptake, and hemolysis in pronase treated human erythrocytes. The cells were incubated in the presence (o) or absence (•) of sucrose in isotonic NaCl solution containing 8 mg/ml pronase. Cell concentration: 10 vol %. 37 °C. The data on potassium and sodium content were corrected for hemolysis by dividing the measured K^+ and Na^+ contents by the fraction of non-hemolyzed cells. *Ordinates:* (a) intracellular cation content (K^+ or Na^+) in µmoles/g of initial cell weight. (b) sum of intracellular potassium and sodium contents in µmoles/g of initial cell weight. (c) hemolysis in percent. *Abscissa:* time in minutes

Na^+ uptake were followed while the enzyme acted on the cells. In the experiments presented below, the cells were first exposed to the action of the enzyme for a predetermined length of time. Subsequently, the enzyme was removed and flux measurements were initiated. In these experiments, the following procedure was used: The erythrocytes were first incubated in the presence of a fixed concentration of pronase at pH 7.3, 37 °C for a certain length of time, usually either one or two hr. The cell density was 20 vol %. The incubation medium contained KCl at a concentration of 100 mequiv/liter. This concentration was found to reduce the driving force for potassium exit into the medium to such an extent that no measurable K^+ loss occured[1] even if the cation permeability of the cell membrane

1 A concentration of 100 mmoles/liter is considerably lower than the intracellular potassium concentration which amounts to about 145 mmoles/liter cell water. In the figures of this paper, the cellular potassium content is expressed in µmoles/g cells. Since the cells contain about 70% cell water, the intracellular K^+ concentration is obtained by dividing the ordinates by 0.7. Nevertheless, the driving force for net K^+ movements is virtually zero, since the K^+ distribution ratio between cells and medium is close to the Donnan ratio for anions: $Cl_0/Cl_i = K_i^+/K_0^+ = 1.45$ at pH 7.3, the pH spontaneously established in our unbuffered cell suspensions.

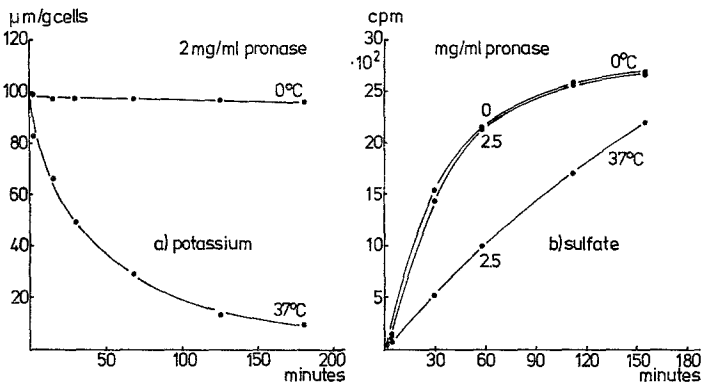

Fig. 3. Inhibition of pronase action on potassium (a) and sulfate (b) permeability by cooling to 0 °C.

(a) Prior to the flux measurements, the cells were exposed to the action of the enzyme (2 mg/ml) in 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose either at 37 °C or at 0 °C for two hr. Cell density: 10 vol%. At the end of the incubation period, both samples of cells were centrifuged, washed once in their original but pronase-free medium and then resuspended at 37 °C in isotonic NaCl solution containing 20 mmoles/liter sucrose (=zero time of the figure). *Ordinate:* potassium content in µmoles/g of initial cell weight. *Abscissa:* time in minutes.

(b) Prior to the flux measurements, the cells were equilibrated with a medium containing 151 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose and some $^{35}SO_4$ at 37 °C for two hr. Subsequently, part of the suspension was cooled to 0 °C, the rest was maintained at 37 °C. Pronase was added to both samples and incubation was continued for another two hr. Pronase concentration in the final (20 vol%) suspension: 2.5 mg/ml. A control was run in which the cells were not exposed to pronase: 0 mg/ml. At the end of the incubation period, after washing at 4 °C, the three samples of cells were resuspended at 37 °C in their original media but in the absence of pronase and of $^{35}SO_4$ and then the appearance of radioactivity in the medium was followed. *Ordinate:* $^{35}SO_4$ concentration in the supernatant in cpm. *Abscissa:* time in minutes

increased under the influence of the enzymatic attack. In addition to KCl, the medium contained 66 mmoles/liter NaCl to maintain isotonicity, and sucrose (20 mmoles/liter) to suppress hemolysis of the leaky cells. At the end of the incubation period, the enzymatic reaction was stopped by cooling the suspension to 0 °C (Fig. 3). The cells were centrifuged and washed twice in the ice cold original KCl/NaCl sucrose suspension medium containing no pronase. At zero time of the flux measurements, the packed, pretreated cells were diluted at 32 or 37 °C with isotonic NaCl solutions containing 20 mmoles/liter sucrose and sufficient HCl or NaOH to establish the desired pH values in the final cell suspensions. The final cell density was 10 or 5 vol%. In the experiments with DNFB, no acid or base was added to the isotonic saline media. Instead, the saline solutions contained the desired concentration of DNFB in addition to sucrose.

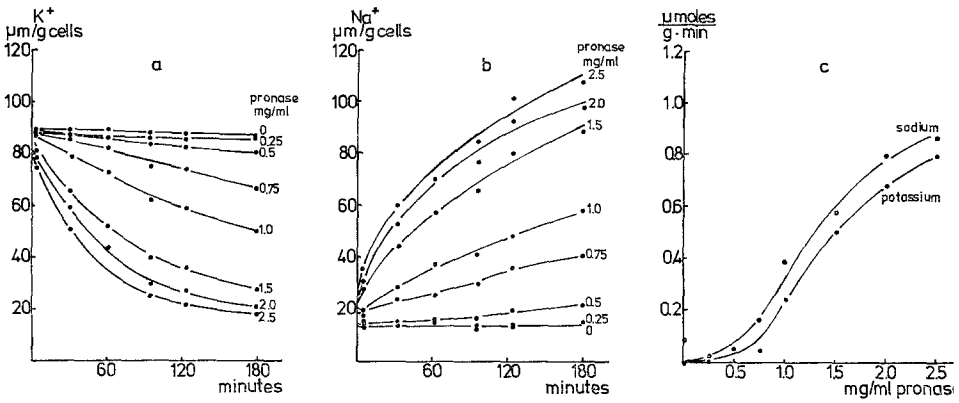

Fig. 4. Effect of pronase pretreatment on potassium and sodium permeability. The cells were incubated in the presence of the pronase concentrations indicated in the figure at 37 °C. Cell concentration: 20 vol%. The incubation medium contained 100 mmoles/liter KCl, 66 mmoles/liter NaCl and 20 mmoles/liter sucrose. After two hr, the cells were centrifuged, washed once in the described medium without pronase and then resuspended in isotonic NaCl containing 20 mmoles/liter sucrose ($t=0$ in the figure). Cell concentration: 10 vol%. 32 °C.

(a) Potassium loss after pronase pretreatment. *Ordinate:* K^+ content in µmoles/g of initial cell weight. *Abscissa:* time in minutes.

(b) Sodium uptake after pronase pretreatment. *Ordinate:* Na^+ content in µmoles/g of initial cell weight. *Abscissa:* time in minutes.

(Note: The initial sodium content increases with increasing pronase concentration in the preincubation medium. This is due to the fact that the Na^+ concentration in the preincubation medium is higher than the intracellular Na^+ concentration. Hence, some sodium enters the cells before the start of the experiment depicted in the figure.)

(c) Initial rates of K^+ loss and Na^+ uptake (*ordinate*) as a function of the pronase concentration at which the cells were pretreated prior to the flux measurements (*abscissa*)

The permeability changes which develop during exposure of the red blood cells to the enzyme in high KCl become apparent immediately after transfer of the cells to the NaCl solution. In the experiment represented in Fig. 4, no lag period precedes the net cation movements. Sodium enters the cells faster than potassium leaves. The total intracellular cation content increases and colloid osmotic hemolysis is bound to occur. This behaviour is different from that described above for erythrocytes which are exposed to pronase in isotonic NaCl, i.e., under conditions where the flux measurements are made while pronase acts on the cells. If the flux measurements are made in isotonic NaCl while the enzyme produces its effects, potassium leaves the cells faster then sodium enters them (Fig. 2b). The cells shrink. The difference between the former and the latter findings shows that the enzymatic alteration of Na^+ permeability develops more slowly than that

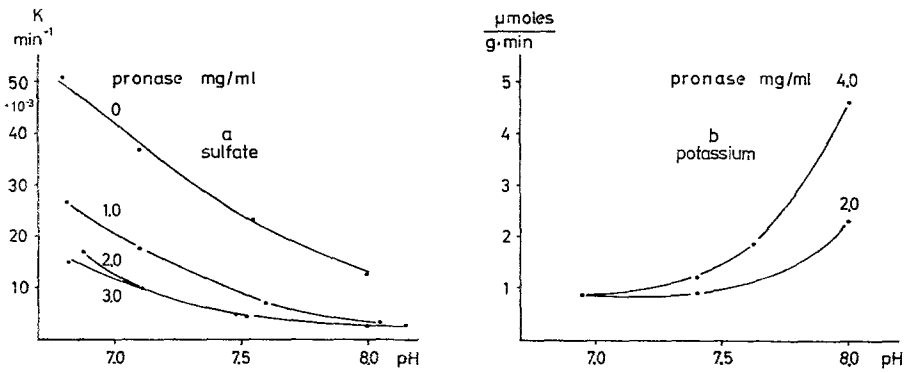

Fig. 5. (a) Effect of preincubation with pronase on pH dependence of net sulfate efflux from SO_4^{2-} preloaded red blood cells. The SO_4^{2-} loaded erythrocytes were preincubated in the presence of pronase (0, 1.0, 2.0, 3.0 mg/ml). Composition of medium: 100 mmoles/liter KCl, 51 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose and a trace of $^{35}SO_4$, pH 7.4. Cell density: 10 vol%. After 60 min at 37 °C, the cells were washed once in ice cold $^{35}SO_4$-free medium. Subsequently, they were resuspended in a medium containing 50 mmoles/liter KCl, 51 mmoles/liter Tris, 50 mmoles/liter NaCl and sufficient HCl or NaOH to yield the desired pH values in the final cell suspensions. The appearance of $^{35}SO_4$ in the medium was followed. Cell density: 2.5 vol%. 37 °C. Under the described conditions, the system is not at Donnan equilibrium and hence differs from all others described in this paper. *Ordinate:* Time constant for sulfate exit, in min^{-1}. *Abscissa:* pH in the medium.

(b) Effect of pH on potassium loss from pronase pretreated red blood cells. Prior to the experiment represented in the figure, cells were exposed to the action of the enzyme at 37 °C in a medium of the following composition: 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose, and pronase. pH 7.4. Cell density: 10 vol%. *Ordinate:* Initial rate of K^+ exit, in µmoles/g/min. *Abscissa:* pH

of K^+ permeability but continues until the membrane becomes more permeable to Na^+ than to K^+ (Fig. 4c).

Fig. 5b illustrates the pH dependence of K^+ efflux into isotonic saline from cells pretreated with pronase in a high KCl solution at pH 7.3. Potassium efflux from the pretreated cells increases as the pH of the saline solution is raised. The pH effect depends on the pronase concentration and the time of exposure to the enzyme.

(c) Effects of DNFB on Potassium Permeability of Pronase Pretreated Erythrocytes. In the context of the present paper, it was of interest to investigate the possibility that the permeability changes produced by the proteolytic enzyme pronase and by the amino reactive agent DNFB may be caused by alterations of the same sites in the cell membrane. In order to obtain some information on this question, investigations were made to find out if the combined effects of pronase and DNFB are additive.

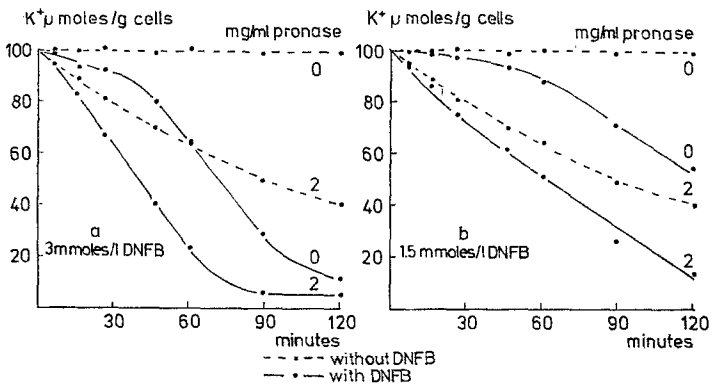

Fig. 6. Effects of dinitrofluorobenzene (DNFB) on potassium permeability of intact and pronase pretreated red blood cells. The cells were exposed to the action of the enzyme (2.0 mg/ml) in a medium of the following composition: 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose. Cell concentration: 20 vol%. Control: cells were kept under the same conditions, except that no pronase was present in the medium. After 0 and 60 min of incubation at 37 °C, samples were withdrawn and the cells were washed in cold KCl/NaCl medium without pronase. Subsequently, the pronase treated cells as well as the untreated cells were subdivided into two batches. One batch was resuspended in isotonic saline (dashed curves), the other in isotonic saline containing, in addition to sucrose, DNFB (continuous curves). The changes of intracellular K^+ were followed. 37 °C. Cell concentration: 5 vol%. (a) DNFB concentration: 3 mmoles/liter. (b) DNFB concentration: 1.5 mmoles/liter. *Ordinates:* intracellular K^+ in μmoles/g cells. *Abscissas:* time in minutes

Fig. 6 represents the effects of two concentrations of DNFB on untreated cells and on cells which were pretreated with 2 mg/ml pronase for one hr. The dashed lines indicate the extent of enzymatic modification of K^+ permeability as measured after transfer of the cells to isotonic NaCl solution. The full lines marked '0 mg/ml pronase' show that addition of DNFB to the control cells which were preincubated for one hr in the absence of pronase causes K^+ loss. This K^+ loss begins after a lag period, then attains a nearly constant rate, and finally levels out when equilibrium is approached. Increasing the DNFB concentration in the medium decreases the length of the lag period and increases the maximal rate of loss (i.e., the slopes of the linear portions of the curves become steeper). If DNFB is added to pronase pretreated cells, the lag period preceding the onset of the DNFB effect on potassium permeability is reduced. Apparently, the action of the enzyme renders the DNFB binding sites more accessible to the chemical agent. However, the maximal rate of K^+ loss remains unaltered. Thus, the maximal slopes of the continuous curves in Fig. 6b (action of 1.5 mmoles/liter DNFB) are virtually identical. The same applies to the corresponding curves in

Table 1. *Effect of dinitrofluorobenzene (DNFB) on net potassium efflux from human erythrocytes pretreated with pronase at pH 7.3* [a]

Time of pretreatment with pronase (min)	DNFB concentration (mmoles/liter)	Lag [b] period (min)	Maximal rate of net K^+ loss [b] (μmoles/g/min)
0	0		0
60	0	0	0.4
0	2.5	25	1.20
60	2.5	12	1.25
0	5.0	8	3.10
60	5.0	6	3.20
0	0		0.2
60	0	0	0.6
0	1.5	45	0.6
60	1.5	0	0.8
0	3.0	32	1.35
60	3.0	0	1.30
0	0	0	0.05
60	0	0	0.65
0	1.5	33.5	0.65
60	1.5	3.0	1.05
0	3.0	19.5	1.65
60	3.0	0	1.70
0	0	0	0.05
30	0	0	0.10
75	0	0	1.43
120	0	0	2.05
0	3.0	21	1.80
30	3.0	13	1.80
75	3.0	0	1.80
120	3.0	0	1.95
0 [c]	0	0	0.02
30	0	0	0.05
60	0	0	0.15
120	0	0	0.61
0	3.0	22	1.53
30	3.0	20	1.53
60	3.0	16	1.55
120	3.0	8	1.60

[a] Pretreatment of erythrocytes in 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose, 2 mg/ml pronase. Cell concentration: 20 vol%. Net potassium loss was measured after resuspension of the washed erythrocytes in 166 mmoles/liter NaCl, 20 mmoles/liter sucrose, and the DNFB concentrations indicated. Cell concentration: 5 vol%.

[b] For definition see Fig. 1.

[c] Pronase concentration: 1 mg/ml.

Fig. 6a (3.0 mmoles/liter DNFB), although the absolute value of the maximal slope of this pair of curves exceeds that observed with the former pair. Thus, the actions of pronase and DNFB are not additive. The time course of K^+ loss is solely determined by the DNFB concentration in the medium. This applies, provided that the DNFB concentration is high enough to produce an effect on untreated erythrocytes which exceeds that of pronase alone. If this requirement is met, then the DNFB concentration determines the extent of the permeability change, even if the agent is employed at concentrations which are far lower than necessary to produce a maximal DNFB effect on red cells which were not previously exposed to the action of pronase. It is difficult to explain in terms of a simple kinetic model, the fact that even less than maximally effective concentrations of DNFB determine the rate of K exit, regardless of the previous damage done by pronase. Nevertheless, it is evidently incompatible with the assumption that the enzyme and the chemical modifier open up separate channels. A compilation of our data about the combined effects of pronase and DNFB on maximal slope and time lag of the relationship between intracellular K^+ and time is represented in Table 1.

2. Effects of Pronase on Sulfate and Erythritol Permeability

(a) Flux Measurements after Pronase Treatment. The experimental results presented below were obtained as follows: the cells were first loaded with $^{35}SO_4$ and then treated with pronase. At the end of the incubation period, the cell suspensions were cooled to 0 °C. This reduced the action of the enzyme on sulfate permeability to negligibly small values (Fig. 3b) and decreased SO_4^{2-} penetration to such an extent that it was possible to remove the radioactivity of the intercellular fluid by repeated washings without losing radioactivity from the cells. The solutions used for washing the cells were identical with the original incubation media except that neither $^{35}SO_4$ nor pronase were present. After the last wash, the cold, packed cells were resuspended in a large excess of washing fluid at 37 °C. This initiated the $^{35}SO_4$ back exchange which is represented in the figures of this section.

Typical results of the described experiments are shown in Figs. 7 and 8. Increasing the pronase concentration at a fixed time of exposure as well as increasing the time of exposure at a fixed pronase concentration leads to reductions of sulfate flux. This is in contrast to the effects of pronase on cation permeability. Although cation permeability increases, sulfate permeability is inhibited (Fig. 8a, b). Anion permeability responds more sensitively than cation permeability. The inhibition of anion permeability

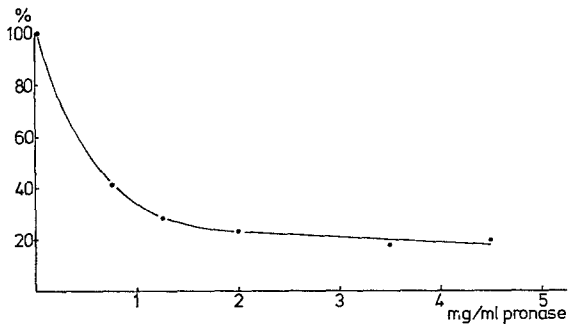

Fig. 7. Effect of pronase pretreatment of red blood cells on $^{35}SO_4$ back exchange. Cells were preincubated in a medium containing 100 mmoles/liter KCl, 21 mmoles/liter NaCl, 30 mmoles/liter Tris, 20 mmoles/liter sucrose, 10 mmoles/liter Na_2SO_4 and a trace of $^{35}SO_4$ at 37 °C for one hr. pH 7.4. Cell density: 25 vol%. Subsequently, pronase dissolved in the same medium was added to give a cell density of 20 vol% and the final pronase concentrations indicated on the abscissa. Incubation was continued for another hour. Thereafter the cells were washed once at 0 °C in a medium which was of the described composition except that no $^{35}SO_4$ was present. At zero time of the figure, the cells were resuspended in this medium at 37 °C and sulfate efflux was measured. Cell density: 2.5 vol%. *Ordinate:* rate constant for back exchange in percent of untreated control. Time constant of control $2.23 \cdot 10^{-2}$ min^{-1}. *Abscissa:* Pronase concentration at which the cells were pretreated prior to the flux measurements

develops most rapidly during the first minutes after exposure to the enzyme. The change of cation permeability commences slowly and gains increasing momentum in the course of the enzymatic attack. Moreover, the effect of pronase on anions levels out and produces maximal inhibition at a time when the alteration of cation permeability continues to increase.

In the present context, it was of obvious interest to study the effect of pronase on the penetration of a nonelectrolyte. Therefore, some experiments were performed with ^{14}C-erythritol (Fig. 8c). This polyalcohol penetrates partly by facilitated diffusion via the glucose transport system and partly via an unidentified pathway, thereby exhibiting the kinetics of a simple diffusion process (Bowyer & Widdas, 1955; LaCelle & Passow, 1971). Erythritol penetration through the glucose transport system can be blocked by phlorizin. Fig. 9 shows that pronase, at concentrations which evoke a considerable effect on passive ion permeability, does not affect the residual erythritol movements which persist when the glucose transport system is inhibited by phlorizin. Studying the effects of pronase on the penetration of one, single nonelectrolyte does not permit one to draw inferences on the bahaviour of nonelectrolytes in general, but it suggests that passive diffusion of uncharged molecules is not affected by pronase treatment.

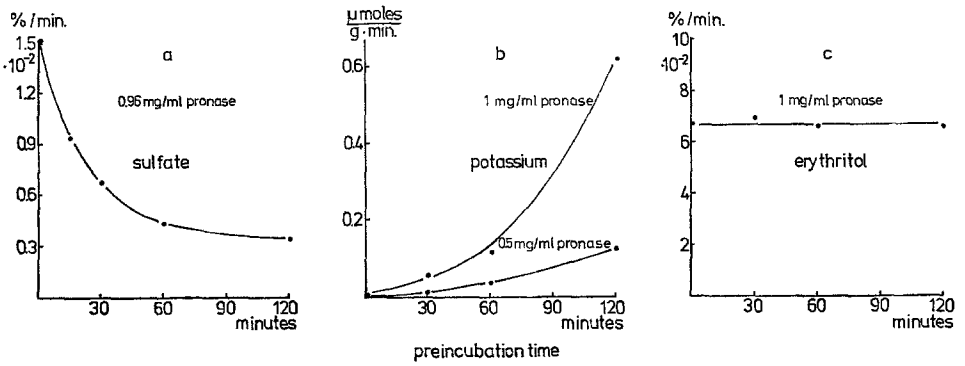

Fig. 8. Effects on sulfate (a), potassium (b), and erythritol (c) permeability of preincubating red cells prior to the flux measurements in the presence of pronase for the times indicated on the abscissas. The ordinates represent measurements of the respective penetration rates.

(a) Sulfate back exchange after pretreatment of red blood cells with a fixed concentration of pronase for varying lengths of time. Red blood cells were equilibrated in 100 mmoles/liter KCl, 50 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose, and a trace of $^{35}SO_4$ at 37 °C. Cell density: 25 vol%. After one hr, the suspension was diluted with additional medium containing sufficient pronase to establish a concentration of 0.96 mg/ml. Cell density: 20 vol%. After the times indicated on the abscissa, the enzymatic reaction was stopped by cooling to 4 °C. The cells were washed at that temperature in pronase-free medium of the described composition except that no $^{35}SO_4$ was present. At zero time of the figure, the sedimented cells were resuspended in the sulfate medium to give a final concentration of 2.5 vol%, and the $^{35}SO_4$ back exchange into the medium was followed. 37 °C. *Ordinate:* rate constant for $^{35}SO_4$ back exchange in percent per minute.

(b) Effect on potassium permeability of exposing red blood cells to the action of pronase for varying lengths of time. Cells were preincubated in the presence of either 0.5 mg/ml or 1.0 mg/ml pronase. The enzyme was dissolved in 100 mmoles/liter KCl and 66 mmoles/liter NaCl. The cell density was 20 vol%. 37 °C. At the end of the incubation periods indicated on the abscissa, the cells were centrifuged, and washed once in high KCl medium containing no pronase. At zero time of the experiment represented in the figure, the packed cells were mixed with isotonic saline containing 20 mmoles/liter sucrose, and the ensuing K^+ loss was followed. Cell density: 10 vol%. Net K^+ efflux was calculated from the initial slopes of the curves relating cellular K^+ content to time. 37 °C. *Ordinate:* net K^+ efflux in µmoles/g/min.

(c) Effect on erythritol permeability of exposing red blood cells to the action of pronase for varying lengths of time. The cells were first exposed to the action of 1 mg/ml pronase in a medium containing 100 mmoles/liter KCl, 66 mmoles/liter NaCl, and 20 mmoles/liter sucrose. Cell concentration: 20 vol%. 37 °C. At the times indicated on the curves, the cells were transferred to a pronase-free high KCl medium containing 10 mmoles/liter ^{14}C-erythritol (start of the experiment) and the entry of ^{14}C into the cells was followed. Cell concentration: 48 vol%. 37 °C. *Ordinate:* rate constant of erythritol influx in percent per minute

The kinetics of anion permeability exhibits two particularly characteristic features. (1) If the sulfate concentration in the medium is increased by replacing sodium chloride with osmotically equivalent amounts of sulfate,

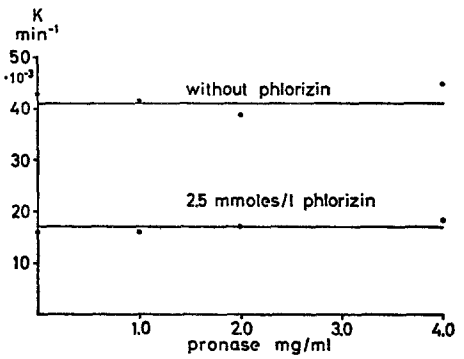

Fig. 9. Effects of pronase on erythritol permeability. Red blood cells were preloaded with ^{14}C-erythritol by equilibration in a medium containing 11.5 mmoles/liter erythritol, 166 mmoles/liter NaCl and 20 mmoles/liter sucrose. Cell density: 20 vol%. After 60 min, the suspensions were diluted 1:1 with a medium of the same composition containing pronase to give the final concentrations indicated on the abscissa. 37 °C. After 60 inin, the cells were centrifuged and resuspended in media containing 10 mmoles/liter erythritol, 166 mmoles/liter NaCl, and 20 mmoles/liter sucrose (=zero time of the experiments). One set of experiments was performed in the absence (upper curve) the other in the presence (2.5 mmoles/liter, lower curve) of phlorizin in the medium. Cell density: 2.5 vol%. The appearance of radioactivity in the medium was followed at 25 °C. *Ordinate:* rate constant for erythritol back exchange into the medium in min^{-1}. *Abscissa:* pronase concentration in the preincubation media in mg/ml

then sulfate flux increases faster than sulfate concentration in the medium. (2) Increasing the pH at constant Cl^- and SO_4^{2-} concentrations leads to a decrease of sulfate flux. It was of interest to find out if these features still persist in cells treated with pronase at a concentration which produces maximal inhibition of sulfate flux.

The execution of such experiments poses certain difficulties. The cells ought to be exposed to pronase under standard conditions before being resuspended in the various media in which sulfate efflux is measured as a function of sulfate concentration or pH. In the experiments on the relationship between sulfate flux and sulfate concentration, it was assumed that the action of the enzyme was the same regardless of the SO_4^{2-}/Cl^- ratio in the medium, and hence preincubation with pronase was performed for each SO_4^{2-}/Cl^- ratio separately. In the experiments on pH dependence, such a procedure was obviously impossible. Therefore, all cells employed in these experiments were first loaded with $^{35}SO_4$, and then – still in the presence of $^{35}SO_4$ – exposed to the action of the enzyme at pH 7.3. Subsequent to the removal of enzyme and extracellular $^{35}SO_4$ by washing at low

temperature, the sedimented cells were subdivided into several batches. They were then resuspended in isotonic media without sulfate. These media contained sufficient Tris buffer to adjust the pH in the final cell suspensions to the desired values. Under such conditions, the final system is not at Donnan equilibrium and hence the isotope flux will be accompanied by net SO_4^{2-} movements. Since the negative charge inside the cells increases with increasing pH, the driving force for SO_4^{2-} efflux also increases. This would tend to counteract the known decrease of the permeability constant with increasing pH. Hence, the absolute magnitude of the pH dependence as measured under our conditions is somewhat smaller than in previously published experiments (Lepke and Passow, 1971) where special care was taken to work at Donnan equilibrium. Thus, the present data cannot be evaluated quantitatively in terms of the fixed charge concept as represented by Eq. (1)

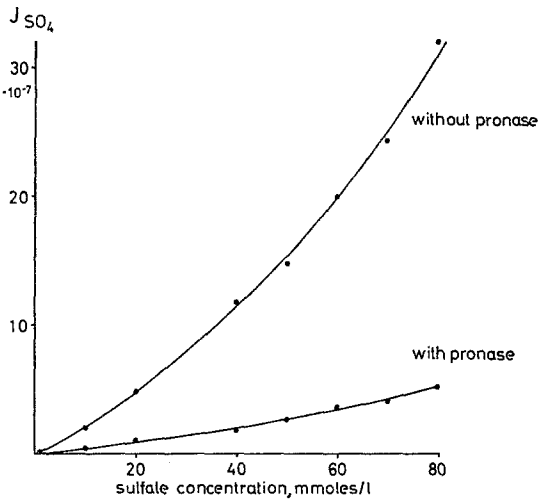

Fig. 10. Effects of pronase on sulfate flux at various sulfate concentrations in the medium. The cells were washed three times in isotonic mixtures containing varying proportions of Na_2SO_4 and NaCl and fixed concentrations of Tris (66.5 mmoles/liter, pH 7.2) and sucrose (20 mmoles/liter). For each mmole of sulfate, 1.26 mmoles of chloride were replaced in the medium. After washing, the cells were resuspended to give a hematocrit of 20 vol%. $^{35}SO_4$ was added and the suspension was incubated at 37 °C. After two hr, the cell suspensions were diluted 1:1 with additional $^{35}SO_4$ containing medium to give a final cell concentration of 10 vol%. In one set of samples, the suspension medium contained sufficient pronase to give a final concentration of 3 mg/ml. After dilution, incubation was continued for another hour. At the end of this period, the cells were washed three times at 4 °C in order to remove extracellular $^{35}SO_4$ and pronase. The sedimented cells were resuspended in media of the original composition but without $^{35}SO_4$. This initiated the back exchange of radiosulfate into the medium. Cell concentration: 2.5 vol%. 37 °C. *Ordinate:* sulfate flux in moles/g/min. *Abscissa:* sulfate concentration in the medium, in mmoles/liter

of Lepke and Passow (1971). Nevertheless, any deviation of the pH dependence of pronase-treated erythrocytes from that of a control containing untreated cells should still be easily observable.

Fig. 10 shows that the general pattern of the relationship between SO_4^{2-} concentration in the medium and sulfate fluxes is retained after pronase treatment. However, the upward bend of the curve relating flux to concentration in pronase treated cells is less pronounced and the whole curve undergoes a shift to lower flux values. Fig. 5a indicates that some pH dependence of sulfate flux persists even after inhibition of sulfate flux by pronase pretreatment.

(b) Effects of DNFB on Sulfate Permeability of Pronase-Pretreated Erythrocytes. Both enzymatic treatment with pronase and chemical modification with DNFB produce an increase of cation permeability, a decrease of anion permeability, and have little or no effect on the (nonfacilitated) movements of the nonelectrolyte, erythritol. The similarity of the effects of the two agents even applies to details. The pH dependence of anion flux persists after inhibition with DNFB or pronase. The chemical modifier as well as the enzyme produce their most pronounced effects on anion permeability immediately after addition to the cells while the change of cation permeability develops slowly and becomes faster only gradually as the time of exposure increases. Thus, the question may be asked whether the DNFB binding sites involved in the control of anion permeability are identical with those sites which are modified by the enzyme. The experiment represented in Fig. 11 was designed to provide an answer to this question.

In this experiment, washed red blood cells were first loaded with $^{35}SO_4$, then exposed to the action of pronase and finally, after removal of the enzyme and of external $^{35}SO_4$, used for measuring $^{35}SO_4$-efflux. The flux measurements were initiated by resuspending the sedimented cells in $^{35}SO_4$-free medium. Fig. 11 shows that pronase pretreatment leads to an inhibition of sulfate flux. In addition, the figure demonstrates that the presence of DNFB in the back-exchange medium produces some further inhibition. The combined effects of pronase and DNFB are independent of the degree of inhibition produced by the previous treatment with pronase. There is always the same maximal response to DNFB as in red cells which were never exposed to the enzyme. In other words, the actions of pronase and DNFB are not additive. This finding suggests that the DNFB binding sites control the same channels in the membrane which are modified by the enzyme.

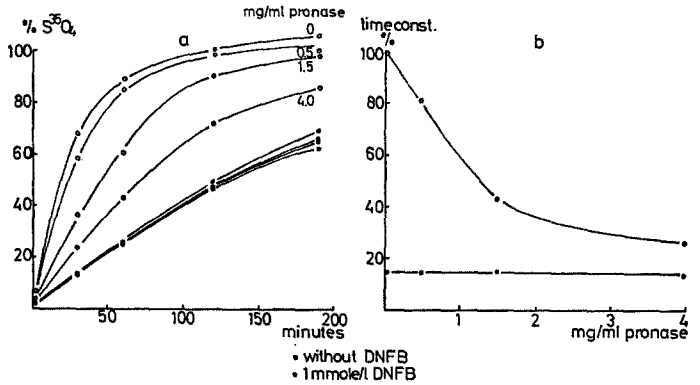

Fig. 11. Effect of dinitrofluorobenzene (DNFB) on sulfate permeability of pronase-pretreated erythrocytes. Prior to the flux measurements, the $^{35}SO_4$-loaded cells were incubated in the presence of $^{35}SO_4$ and pronase at 37 °C for 45 min. The medium ('sulfate medium') contained 151 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose and in addition the pronase concentrations indicated on the curves of Fig. (a) or on the abscissa of Fig. (b). Cell concentration: 10 vol%. At the end of the incubation period, pronase and external $^{35}SO_4$ were removed by washing the cells at 4 °C. Each batch of pretreated cells was subdivided into two samples. One sample was resuspended in pronase-free sulfate medium, the other in pronase-free sulfate medium containing 1.0 mmole/liter DNFB. Cell concentration: 2.5 vol%. The temperature of the media was 37 °C. The appearance of $^{35}SO_4$ in the supernatant was followed (a) and the calculated time constant plotted against the pronase concentration in the preincubation medium (b). Open circles: no DNFB in the resuspension medium; filled circles: 1 mmole/liter DNFB in the medium. *Ordinates:* (a) Percentage of intracellular $^{35}SO_4$ released into the medium; (b) rate constant of $^{35}SO_4$ exchange. *Abscissas:* (a) time in minutes; (b) pronase concentrations to which the cells were exposed prior to the flux measurements

3. Enzymatic Alterations of the Red Blood Cell Membrane

Pronase consists of a mixture of endopeptidases and ectopeptidases; hence, the enzyme preparation can be expected to release amino acid residues into the medium and to split peptide chains into fragments which may still remain attached to the cell membrane. If intact cells are exposed to the action of pronase (pronase concentration 3.0 mg/ml, cell density: 10 vol%) at 37 °C for three hr, about 19% of the membrane protein as determined by the method of Lowry *et al.* (1951) is removed. Under the same experimental conditions, the total amino-N content of the membrane remained virtually unaltered. Apparently, the enzymatic splitting of peptide bonds leads to the formation of about the same number of amino groups as it releases into the medium[2].

2 The membranes were prepared by the method of Poensgen and Passow (1971) from pronase-treated erythrocytes. If erythrocyte ghosts (prepared by the method of Dodge, Mitchell & Hanahan, 1962) are exposed to pronase, amino-N is released by the enzyme.

Table 2. *Effect of pronase on lipid amino-N of red blood cells*[a]

Exp. no.	Lipid amino-N		
	Control		Mean with pronase
	without pronase	with pronase	
1	101.1; 95.2; 99.8	96.1; 98.8; 98.0; 97.6	97.6%
2	98.9; 101.1	105.5; 104.3; 101.1	105.3%
3	100.3; 99.7	102.9; 99.6; 99.6	100.7%

[a] Pronase concentration: 1.0 mg/ml cell suspension. Cell concentration: 25 vol%. 37 °C. After exposure to the enzyme under the specified conditions for two hr, the lipids were extracted by the method of Reed *et al.* (1960). Amino-N was determined in the lipid extract by the method of Troll and Cannan (1953). All values are expressed in percent of the mean of the controls without pronase.

Under the described conditions, pronase removed 19% of the proteins from the cell membrane. (The membranes of pronase treated cells were isolated by the method of Poensgen and Passow (1971), and the protein content was determined by the method of Lowry as modified by Maddy (1969, *personal communication*). The electrophoretic mobility of the cells was reduced by 38% (Fuhrmann and Passow, *unpublished results*).

Proteolytic enzymes may exhibit some esterase activity. It is necessary therefore to ask whether pronase releases amino groups from phosphatidylethanolamine or from phosphatidylserine. The similarity of the total amino-N content of isolated membranes with untreated and pronase-treated red cells suggests that the lipid amino-N content also remains unaltered. Direct determinations of the amino-N content of lipids extracted from untreated and pronase treated cells showed that there was no significant difference (Table 2). This result was obtained if, in addition to the usual washings with aqueous KCl solutions, the lipid extracts were washed first with $n/10$ HCl and subsequently with $n/10$ KOH. Thus, it is unlikely that amino groups liberated by the action of pronase are retained in the lipid extract and analyzed together with the unaltered cephalins. Therefore, one may conclude that pronase does not release significant amounts of lipid amino-N.

In addition to measuring the effects of pronase on the amino-N content of the intact erythrocyte membrane, we tried to determine the influence of the enzyme on the number of DNFB binding sites. Regardless of the enzyme concentration employed, ^{14}C-DNFB binding to membranes of intact erythrocytes is not measurably affected by exposing them to the action of pronase prior to the addition of the agent (Fig. 12). Again, the failure to observe a difference may be due to the formation of a number of new binding sites equivalent to the number of released binding sites.

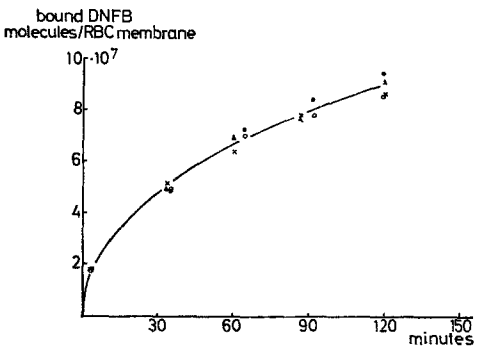

Fig. 12. Binding of dinitrofluorobenzene (DNFB) to the membranes of pronase-pretreated red blood cells. Red blood cell suspensions were incubated in the presence of 0.0 (×), 0.25 (△), 0.5 (⊙), and 1.0 (●) mg/ml pronase in isotonic saline at 37 °C for two hr. Cell density: 20 vol%. At the end of the preincubation period, the cells were washed twice in isotonic saline containing 20 mmoles/liter sucrose and resuspended in the saline-sucrose medium containing 3 mmoles/liter ^{14}C-DNFB. Cell density: 5 vol%. 37 °C. The membranes were isolated according to the method of Poensgen and Passow (1971). *Ordinate:* DNFB binding to the isolated membranes, number of bound molecules per red blood cell membrane. *Abscissa:* time in minutes

Discussion

The interpretation of the described experiments rests on the assumption that the effects of pronase are due to some enzymatic action on the membrane rather than to simple adsorption of the enzyme protein to sensitive loci in the cell surface. The enzymatic nature of the observed effects is suggested by the protracted time course of the development of inhibition of anion permeability and by the lag period preceding the onset of progressive changes of cation permeability. Adsorption is usually a rapid process which is completed within a few minutes. Periods of 30 min or more were required to produce maximal effects on anion permeability or to initiate changes of cation permeability. More direct evidence in favor of an enzymatic action consists in the finding that the development of the changes of both anion and cation permeability can be prevented if the reaction mixture containing cells and enzyme is cooled to 0 °C.

Pronase induced alterations of the cell surface can be directly demonstrated by measurements of electrophoretic mobility. The enzyme releases neuraminic acid (Uhlenbruck, Pardoe & Heggen, 1968) and reduces the mobility of the erythrocyte by about 38%. The time course of the development of the effects of pronase on mobility is also considerably temperature dependent. At 37 °C, the pronase induced changes of erythrocyte mobility are completed within a few minutes after the addition of the enzyme to the

red blood cells (Fuhrmann & Passow, *unpublished results*). Hence, they do not seem to be related directly to the effects of pronase on ion permeability which develop more slowly.

The fact that our pronase preparations possess enzymatic activity is further demonstrated by the release of some proteins from the erythrocyte membrane (*c.f.* legend to Table 2). In addition, pronase could be shown to remove a considerable fraction of previously bound ^{14}C-DNFB from the membrane *(unpublished results)*. However, as was the case with electrophoretic mobility, the proteins are released much faster than the effects on ion permeability develop. Hence, there does not seem to exist a causal relationship between the observed changes of the membrane's protein content and the permeability changes.

The most striking effects of pronase on ion permeability consist in an inhibition of anion permeability and an acceleration of passive cation movements. There are little, if any effects on the non-facilitated component of the permeability for the nonelectrolyte, erythritol. These findings are consistent with the fixed charge concept: for electrostatic reasons, the enzymatic dislocation, or removal of positive fixed ions, leads to a decrease of the concentration of counterions and an increase of the concentration of coions in the membrane and hence to correspondingly inverse changes of anion and cation permeability. The movements of uncharged molecules are not affected by alterations of the charge density in the membrane.

Cation permeability responds less sensitively to the action of pronase than anion permeability and the effect of the enzyme on anion permeability develops faster than on cation permeability. Moreover, at a given concentration of the enzyme its effect on cation permeability continues to increase without reaching a maximal level for many hours. In contrast, inhibition of anion movements approaches completion after about one hr. Correspondingly, if varying concentrations of the enzymes are applied for a fixed length of time, the effects on cations are always less fully developed than those on anions.

The same reduction of the charge density of an ion exchange membrane does not necessarily affect cation and anion movements to the same extent. In an anion exchange membrane, anion flux is reduced in proportion to the enzymatic reduction of the fixed charge density. However, a few remaining positive charges may still be capable of effectively blocking cation flux. If the fixed charges were located in narrow channels, their capacity to produce cation-anion discrimination could be very much higher than anticipated on the basis of a simple Donnan exclusion of coions in a homogeneous ion exchange membrane (Sollner, 1945; Passow, 1969*a*).

Although the assumption of an ion exchange membrane with narrow channels could qualitatively explain the difference in sensitivity of anion and cation permeability to the action of pronase, other assumptions may be equally useful. For example, it would seem possible that anion permeability is predominantly controlled by charged groups which are more easily accessible to the enzyme than those involved in the control of passive cation permeability. Such an assumption would be supported by our previous observation that the amino reagent 5-methoxy-2-nitrotropone (MNT) inhibits anion permeability without enhancing cation movements. Only if the agent is added to the cells in the presence of fairly high concentrations of ethanol does it produce the expected increase of passive cation fluxes (Passow & Schnell, 1969). This observation is in accord with the finding of Knauf, Sauda and Rothstein (1969), that the non-penetrating amino reagent 4-acetamino,4′-isothio-cyanostilbene-2,2′-disulfonic acid (SITS) strongly inhibits anion permeability without inducing net potassium loss.

The concentration of positive fixed charges within the membrane is postulated to be much higher than the cation concentration in isotonic suspension media. Therefore, maintenance of electrical balance would require that the concentration of diffusible anions within the membrane considerably exceeds that of the medium. Cl^- and SO_4^{2-} should compete for the positive fixed charges. As a consequence, an increase of the concentration of divalent sulfate ions at the expense of monovalent chloride ions in the medium should result in a more than proportional increase of sulfate concentration in the membrane. This effect is further amplified by a cooperative facilitation of sulfate penetrations at increasing sulfate concentrations in the membrane (Lepke & Passow, 1971). Enzymatic removal of the fixed charge region should largely abolish Cl^-/SO_4^{2-} competition for the fixed charges and reduce the sulfate concentration at the rate determining barrier within the membrane to the much lower concentration existing in the medium. At low sulfate concentrations within the membrane, sulfate flux is nearly proportional to sulfate concentration. One would anticipate, therefore, that pretreatment of the cells with pronase would not only diminish the absolute values of SO_4^{2-} penetration rates, but would also tend to reduce the curvature of the relationship between sulfate flux and sulfate concentration. The experiment represented in Fig. 10 seems to support this inference.

According to the fixed charge hypothesis as discussed in the present series of papers, decreasing the pH shifts the equilibrium $R{-}NH_2 + H^+ = R{-}NH_3^+$ to the right. For electrostatic reasons, this should be associated with an increase of the concentration of negatively charged, diffusible

counterions in the membrane and hence with an increase of anion flux across the membrane. If those amino groups which are allegedly involved in the control of ion permeability could be completely removed by the action of pronase, one would expect the pH dependence of anion permeability to disappear. Fig. 5a indicates that in the range of pH 7.4 to 8.0, the pH dependence is, in fact, nearly abolished. However, at lower pH values, in all our experiments, variations of pH still produce considerable effects. In one of the experiments, the curves representing the pH dependence at the two highest pronase concentrations nearly coincide. This would suggest that the pronase concentrations were sufficient to produce a maximal effect. Hence, the residual pH dependence may be due to the persistence of some unassailable, dissociable membrane constituents or to the survival of some inaccessible protein amino groups. It is not yet known whether these pronase resistent groups control sulfate flux through the original, enzymatically modifiable channels or through parallel pathways which, in the intact cell, cannot be distinguished from the pronase sensitive ones.

The pH dependence of potassium flux which is observed after pronase treatment does not seem to fit into the fixed charge concept. In this context, it may be pertinent to recall that we found neither a significant decrease of the amino-N content in membrane of pronase treated erythrocytes nor a reduction of the number of DNFB binding sites. Perhaps, after enzymatic splitting of peptide bonds, newly formed amino groups may continue to influence passive ion permeability and thereby cause some pH dependence.

The failure to observe additive effects of pronase and DNFB on ion permeability suggests that the enzyme and the amino reactive agent act on the same channels. Since DNFB reacts with protein as well as with lipid amino groups (Poensgen & Passow, 1971), it was important to observe that pronase removes little if any lipid amino-N. Our crude analytical methods are accurate to within $\pm 5\%$ of the total lipid amino groups. Hence, our inability to detect an enzymatic removal of lipid amino groups by pronase does not definitely rule out the possibility that enzymatic hydrolysis of special amino-containing lipids causes the observed permeability changes. Nevertheless, it shows quite conclusively that the positive charge of the bulk of lipid amino groups is not involved.

The nature of the defect which leads to the described permeability changes remains obscure. One possible explanation for the finding that the alteration of cation permeability produced by a less than maximally effective concentration of DNFB is independent of the extent of the damage done by previous exposure to pronase consists in the assumption that, after pronase treatment, the DNFB binding sites still remain attached to the

Table 3. *Effect of trypsin on sulfate penetration*[a]

Concentration of trypsin (mg/ml)	Time constant for SO_4 back exchange (min^{-1})
0	$12.3 \cdot 10^{-3}$
1.0	12.7
2.5	12.9
5.0	13.9
0[b]	11.9
2.5[b]	12.2
5.0[b]	13.2
0	14.0
1	14.95
2.5	14.55
9	18.42
0[b]	13.75
2.5[b]	14.6
5.0[b]	16.9

[a] Prior to the flux measurements at 32 °C, the cells were exposed to the action of the enzyme at 37 °C for two hr. Cell concentration: 20 vol%. Suspension medium: isotonic saline.

[b] The medium contained 0.1 mmoles/liter $CaCl_2$ in isotonic saline.

membrane and hence continue to be available for DNFB binding although they are incapable of exerting their effect on cation permeability. The experiments on anion permeability also leave little doubt that DNFB and pronase modify identical channels in the membrane. However, it is not clear whether DNFB binding sites are released into the medium or remain attached to the membrane.

The commercially available pronase of *Streptomyces griseus* is composed of at least 11 different proteolytic enzymes (Narahashi, Shibuya & Yanagita, 1968). Seven of them are endopeptidases, three are aminopeptidases, and one is a carboxypeptidase. Observation of the effects produced by such a complex mixture of proteolytic enzymes does not permit one to draw any conclusions about the nature of the hydrolyzed peptide bonds. We employed, therefore, in addition to pronase trypsin, an easily available, highly purified protease with better defined substrate specificities. Trypsin had only insignificant effects on the passive anion or cation permeability if applied at 37 °C, pH 7.4, at concentrations up to 2.5 mg/ml cell suspension (20 vol%) for up to two hr. Yet, the enzyme produces some hydrolysis in the outer cell surface of the erythrocyte as evidenced by a reduction of the electro-

phoretic mobility (confirmed for our experimental conditions by Fuhrmann and Passow, 1969, *unpublished*).

Trypsin catalyzes the hydrolysis of peptide bonds between the COOH group of lysine, or the COOH group of arginine, and the NH_2 group of the adjacent amino acid (Hirs, Moore & Stein, 1956). Thus, the enzyme attacks a site which is of particular interest in studies of the role of amino groups in the control of ion permeability. The conspicuous ineffectiveness of the enzyme to alter passive ion permeability may have many reasons. It would be intersting to know whether the absence of an effect is simply due to the inaccessibility of the peptide bonds adjacent to the decisive basic amino acid residues or if lysine and arginine are not present in the ion permeable regions.

Essential portions of the material described in this paper were first presented in August 1969 during an ICRO/UNESCO training course on membrane biophysics at the Dept. of Radiation Biology of the University of Rochester, Rochester, N.Y.

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