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EFFECT OF TEMPERATURE ON SULFATE MOVEMENTS ACROSS CHEMICALLY OR ENZYMATICALLY MODIFIED MEMBRANES OF HUMAN RED BLOOD CELLS*

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SUMMARY

The effect of temperature on sulfate equilibrium exchange was measured in cells which were modified by papain, pronase, salicylate, or 1-fluoro-2,4-dinitrobenzene (FDNB).

Following modification at 37 °C with the enzymes or FDNB the excess modifiers were removed and sulfate exchange was measured at 4 °C and 37 °C. The reversibly bound salicylate was also present during the exchange.

With increasing concentrations of salicylate or papain, SO_4^{2-}-permeability shows a saturation type decrease at both temperatures. The apparent activation enthalpy (E_A) does not change significantly.

After treatment with FDNB or pronase, saturation type inhibition is observed at 37 °C. If measured at 4 °C, sufficiently high concentrations of both modifiers accelerate SO_4^{2-}-transfer. At pH 7.2, E_A decreases from 33 to 10 kcal per mole per °K.

If measured at 37 °C, in FDNB or pronase treated cells the rate of anion movement decreases with increasing pH. If measured at 4 °C, the pH dependence is reversed. E_A is correspondingly lowered with increasing pH down to 6.4 kcal per mole per °K at pH 8.3. In contrast, the pH dependence of untreated cells is generally similar at both temperatures. At 37 °C a maximum is observed at pH 6.2. This is shifted to pH 5.8 at 4 °C. Variations of E_A with pH are relatively small over the range pH 5.8 to 8.0.

A study of the effects of purified pronase subfractions showed that neither aminopeptidase nor carboxypeptidase activity is responsible for the change of E_A. Among the endopeptidases those with activity towards casein and the ester *p*-nitrophenyl acetate were found to be particularly potent.

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; BAEE, benzoyl-L-arginine ethyl ester; PNPA, *p*-nitrophenyl acetate

* Part of this work was presented at a recent symposium [1].

INTRODUCTION

Chemical and enzymatic modification of cell membranes plays an important role in identifying functional groups involved in the control of penetration processes and in pinpointing those molecules in the membrane to which these functional groups are attached. The usefulness of chemical and enzymatic modifiers depends on the specificity of interaction with those sites whose modification is responsible for the observed effect. The present note is concerned with an assessment of the specificity of the effects produced by a number of typical inhibitors of sulfate permeability of the red blood cell membrane. Two proteolytic enzymes, papain and pronase, an irreversibly bound amino reactive agent, 1-fluoro-2,4-dinitrobenzene (FDNB), and a reversibly bound amphiphilic substance, salicylate were used. The results suggest that the effects of pronase and FDNB represent the net result of inhibition and facilitation of sulfate equilibrium exchange. Over the concentration range studied, papain and salicylate acted only as inhibitors.

MATERIALS AND METHODS

Blood from healthy donors was collected in acid–citrate–dextrose buffer and used within the next 4 days. Erythrocytes were separated from the plasma by centrifugation and washed 3 times in the respective incubation media.

Determination of sulfate permeability

Incubation media were usually composed of KCl (100 mM), NaCl (51 mM), Na_2SO_4 (10 mM), sucrose (20 mM). The influence of pH was studied in media containing KCl (80 mM), NaCl (16 mM), K_2SO_4 (10 mM), Tris (40 mM), sodium citrate (10 mM), sucrose (20 mM). In some of these experiments KCl and K_2SO_4 were replaced by equivalent amounts of NaCl and Na_2SO_4. The pH was adjusted to values ranging from 5.25 to 8.7 by the addition of Tris–OH or HCl. pH measurements were always made at the temperature at which the respective experiments were performed.

After equilibration of the cells in the appropriate media (30 min, 37 °C), $^{35}SO_4^{2-}$ was added. Incubation at 37 °C was continued for at least another 90 min until Donnan equilibrium was established. After equilibration the various inhibitors were added. Care was taken to avoid disturbing the previously established Donnan equilibrium. Papain was added together with 2–2.5 mM BAL. Incubation with FDNB was performed in the presence of ethanol. In each experiment dinitrophenylation was carried out at a constant concentration of ethanol. Treatment with the inhibitors took place at a cell density of 10% except in the case of pronase where the cell density was 20%. After incubation at 37 °C for 60 min, the cells were washed thrice at 4 °C to remove external $^{35}SO_4^{2-}$ and the inhibitors including the ethanol used for solubilizing FDNB. However, in case of the reversibly bound inhibitor salicylate, the inhibitor was also present in the washing media and in the final media used for flux measurements .After washing the cells, they were resuspended at 4 °C to give a final hematocrit of 2.5%, and $^{35}SO_4^{2-}$ exit was followed for about 20 h. At the end of this period the cells had lost about 10–15% of their original radioactivity. Subsequently, the suspensions were brought to 37 °C and the exchange of the remaining

intracellular radioactive sulfate was followed at 37 °C for additional 2–3 hours.

The radioactivity in the supernatant was measured in a liquid scintillation-counter by using a toluene–methanol mixture (1:1 v/v) containing 4 g/l PPO and 50 mg/l POPOP. From the time course of appearance of $^{35}SO_4^{2-}$ in the supernatant, a rate constant [2] was derived. The quantitative evaluation was done either by using a least square fit or by a graphical technique. In the latter case the initial slope of the curve was divided by the equilibrium concentration of $^{35}SO_4^{2-}$ in the supernatant. The dimension of the rate constant (k) is min^{-1}. In experiments with blood from different donors the rate constant in untreated cells may vary by a factor of two. So far it is not known whether these differences reflect biological variation or are due to other unidentified causes. In the present context it is significant that the E_A values observed

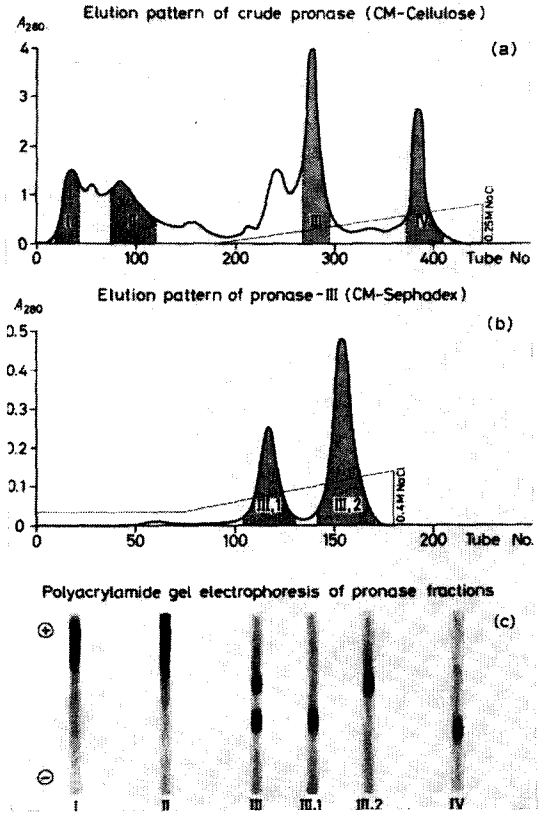

Fig. 1. (a) Separation of crude pronase on CM-cellulose (30 cm × 3 cm; Whatman CM-52, W&R Balston, Ltd). Elution was started with 500 ml of 10 mM sodium acetate, pH 5.2 containing 5 mM $CaCl_2$ and continued with a linear gradient ranging up to 0.25 M NaCl. Flow rate: 40 ml/h; fraction volume: 6.2 ml (b) Rechromatography of Fraction III (Fig. 1a) on CM-Sephadex (60 cm × 3 cm; CM-Sephadex C-50, Pharmacia AB, Uppsala). After washing the column with 250 ml of 10 mM sodium acetate, 7 mM sodium chloride, pH 4.2 a linear gradient was developed by use of 0.4 M NaCl in the starting buffer. Flow rate: 50 ml/h; fraction volume: 10 ml. (c) Analysis of individual pronase fractions by means of gel electrophoresis according to Löfqvist and Sjöberg [5]. Anodic electrophoresis (not shown) revealed no additional band for Fraction III and IV; however, anodic components from Fraction I and II were detected.

are independent of the absolute values of k. The apparent activation enthalpy was calculated by means of the Arrhenius equation. To determine the pH-dependence of the activation enthalpy the rate constants for corresponding pH values at 4 °C and 37 °C were read off smooth curves which were plotted through the data points obtained at various pH values at each of the two temperatures.

Separation of pronase components

Commercially available pronase (Merck No. 7433, LAB) was fractionated according to Narahashi et al. [3] and Narahashi [4] with some minor modifications concerning the geometry of the columns used. Eventually five fractions were recovered which by criteria of polyacrylamide gel electrophoresis [5] were found to be heterogeneous (Fractions I and II) or homogeneous (Fractions III,1; III.2 and IV), respectively (Fig. 1). No further attempts were made to resolve the complex mixtures of Fractions I and II. Pronase fractions I, II, III.1, III.2 and IV, together with the unresolved Fraction III.1 were selected for an examination of their effects on SO_4^{2-}-permeability.

For determination of enzymatic activity of the isolated pronase fractions casein, L-leucyl alanine, carbobenzoxyglycyl-L-leucine, benzoyl-L-arginine ethyl ester (BAEE) and *p*-nitrophenyl acetate (PNPA) were used as substrates. Caseinolytic, aminopeptidase and carboxypeptidase activities were measured according to Narahashi [4], esterolytic activity against BAEE according to Löfqvist and Sjöberg [5], against PNPA according to Wählby [6]. The measurements were referred to protein content as determined according to Lowry et al. [7].

RESULTS

The main result of the present investigation consists of the finding that the nature of the permeability change observed after modification of the red blood cell membrane by a number of chemical agents and enzymes depends on the temperature at which the permeability of the modified membrane is studied.

Fig. 2a shows the result of an experiment in which salicylate was used as a modifier. Increasing concentrations of the modifier produced saturation type inhibition of sulfate equilibrium exchange at 4 °C and at 37 °C .The apparent activation enthalpy calculated from these data was about 33–35 kcal per mole per °K for unexposed cells and decreased slightly and possibly insignificantly with increasing salicylate concentration. These results are in agreement with those previously reported by Schnell [8].

Salicylate is a reversible inhibitor which is present in the medium at the time of the flux measurement. Hence in experiments of the type described above it is impossible to discriminate between the effects of temperature on the interactions between the modifier and the membrane on the one hand and the temperature dependence of the sulfate equilibrium exchange across the modified membrane on the other.

This ambiguity does not exist in experiments with enzymes and chemical modifiers which react irreversibly with the membrane. Using irreversible enzymatic or chemical modification it is possible to subject the membrane to modification under standard conditions at 37 °C and to remove the modifiers prior to the flux measurements at 4 °C and 37 °C. Under these conditions, the observed changes of sulfate equilibrium exchange represent the behavior of the modified membrane and not the

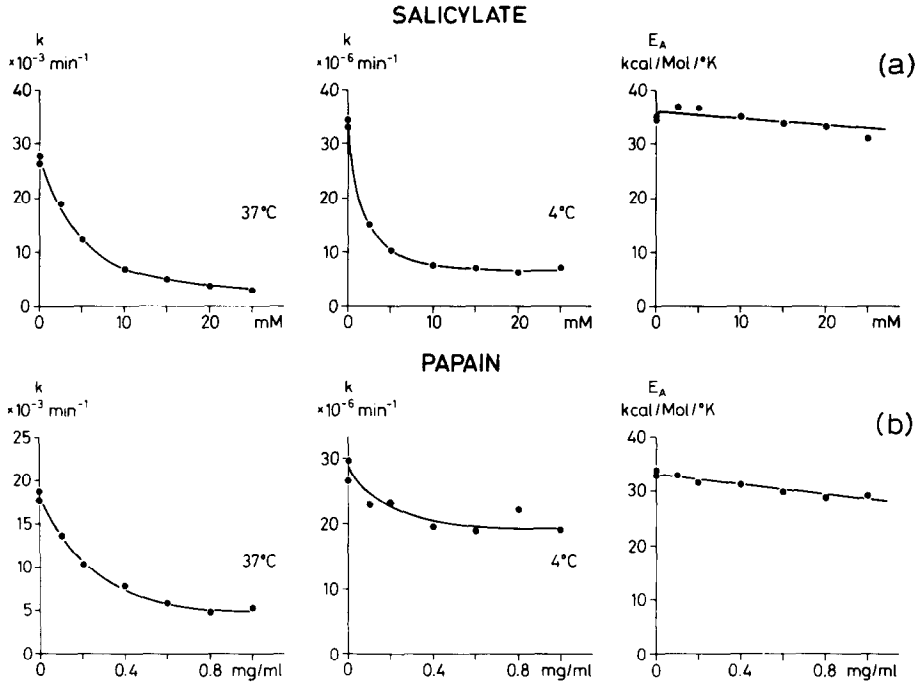

Fig. 2. Rate constants (k) for SO_4^{2-} equilibrium exchange at 37 °C and 4 °C, pH 6.9–7.2, in cells pretreated with varying concentrations of salicylate (a) or papain (b) and the corresponding apparent activation enthalpies (E_A).

effect of temperature on the process of modification. Consequently, the following experiments were done with irreversible modifiers.

Fig. 2b shows that treatment of red blood cells with papain leads to an inhibition of sulfate equilibrium exchange. This inhibition is seen regardless of whether the flux is measured at 37 °C or at 4 °C and at all papain concentrations to which the cells were exposed. The calculation of E_A from these data shows a result which is similar to that obtained with salicylate: E_A decreases only slightly and probably insignificantly with increasing degree of inhibitors.

Irreversible modification with increasing concentrations of pronase or FDNB prior to flux measurements leads to different effects (Fig. 3). If the sulfate permeability of the membrane is measured after modification with crude pronase or FDNB a saturation type reduction of sulfate permeability is also observed provided the flux measurements are performed at 37 °C. However, if the flux measurements are carried out at 4 °C sulfate permeability passes through a minimum. After exposure of the cells to sufficiently high concentrations of the modifier the rate of sulfate movements may greatly exceed the rate in untreated cells. For example, after treatment with 6 mM FDNB the penetration rate as measured at 37 °C is about 5.5% of the control value in untreated cells. If measured at 4 °C it is about 5–6 times faster than in the control with no FDNB. The acceleration beyond the control value does not indicate prehemolytic leakage. This can be inferred from the finding that, after warming up to 37 °C the

very same cells which showed an acceleration at 4 °C show the strong inhibition which is typical for the modified cells at that temperature (Fig. 4). Observations, which are essentially the same as those with FDNB have been obtained with crude pronase and certain purified components of the crude enzyme. After treatment with certain pronase subfractions (III, III.1) inhibitory effects may be completely absent at 4 °C. For example in cells which after treatment with increasing concentrations of Fraction III.1 (see Fig. 10) show up to 86% inhibition at 37 °C, there is only acceleration and no indication of inhibition at 4 °C.

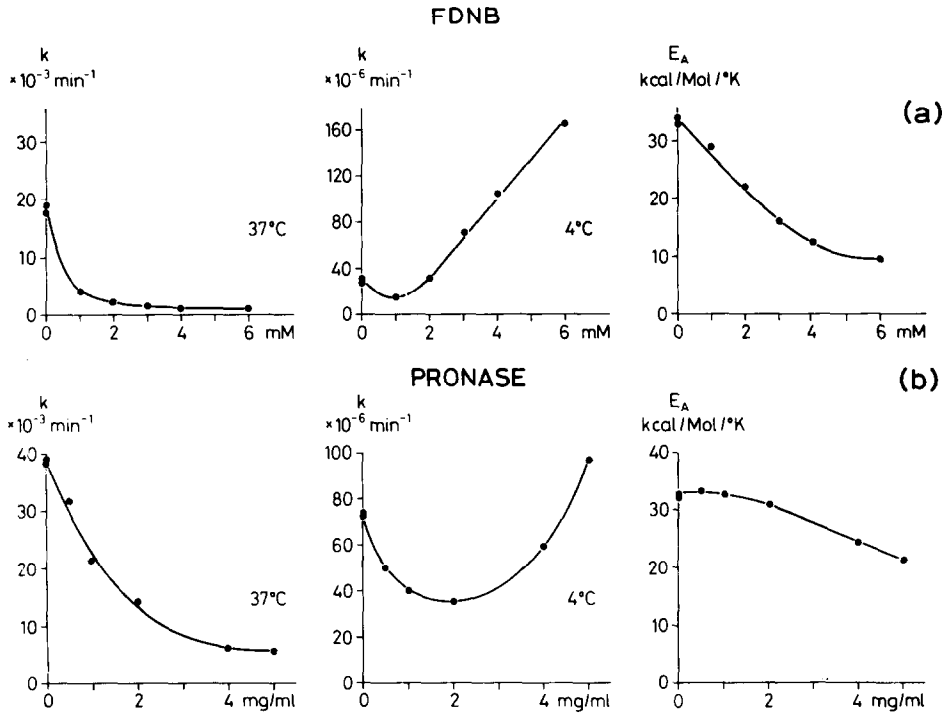

Fig. 3. Rate constants (k) for SO_4^{2-}-equilibrium exchange at 37 °C and 4 °C, pH 6.9–7.2, in cells pretreated with varying concentrations of FDNB (a) or pronase (b) and the corresponding apparent activation enthalpies (E_A).

Fig. 3 (a and b) demonstrates that the modification of the red cell membrane with FDNB or pronase is associated with the decrease of the apparent activation enthalpy for sulfate equilibrium exchange. Control experiments in which penetration rates were measured not only at 4 °C and 37 °C but also at intermediate temperatures showed that in the pretreated and untreated cells E_A was independent of temperature (Fig. 5). The effect of the modifiers on E_A suggests that the rate controlling step in the modified cells is different from that in the intact cells.

Further support for this inference comes from the finding that the pH dependence of sulfate movements in the treated cells as measured at 37 °C is inverse to that measured at 4 °C (Fig. 6). At 37 °C increasing the pH from 6.4 to 8.3 reduces the penetration rate across the dinitrophenylated membrane by about 75%. If measured

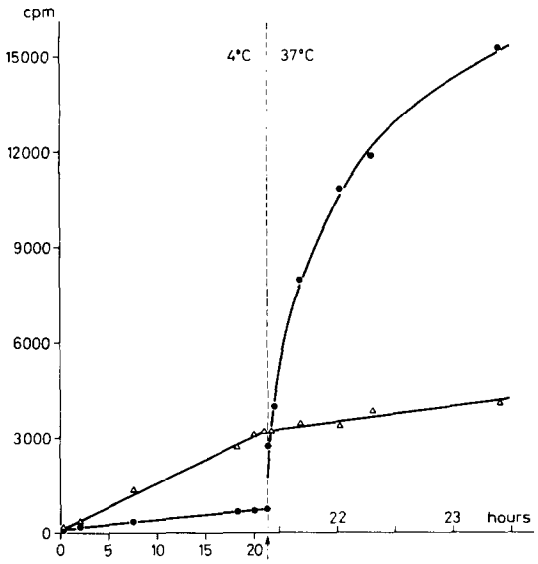

Fig. 4. Time course of appearance of radioactivity in the supernatant of $^{35}SO_4^{2-}$-loaded red cells. During the first 21.5 h the loss of radioactive sulfate was followed at 4 °C, during the subsequent 2 h at 37 °C. The transfer of the cell suspensions from 4 °C to 37 °C is indicated by an arrow. Note change of time scale. ●–●, untreated cells; △–△, cells pretreated with 6 mM FDNB.

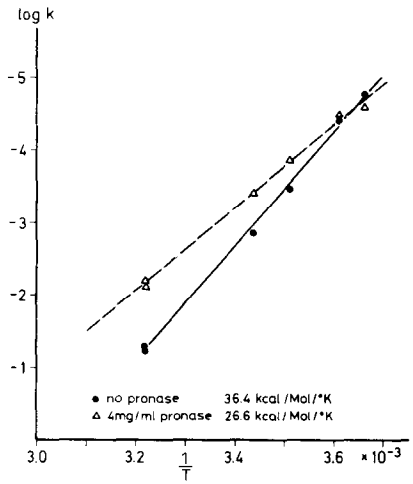

Fig. 5. Arrhenius plots of rate constants (k) for SO_4^{2-}-equilibrium exchange (pH 6.8–6.9) across the membrane of untreated or pronase pretreated erythrocytes.

at 4 °C there is a 2-fold increase in the same cells. As a consequence the E_A of the modified cells is a function of pH. At pH 6.4 E_A was 15 kcal per mole per °K, at pH 8.3, 6.4 kcal per mole per °K. This latter value is close to the activation enthalpy for unrestricted diffusion in aqueous solutions. Similar results were obtained with pronase

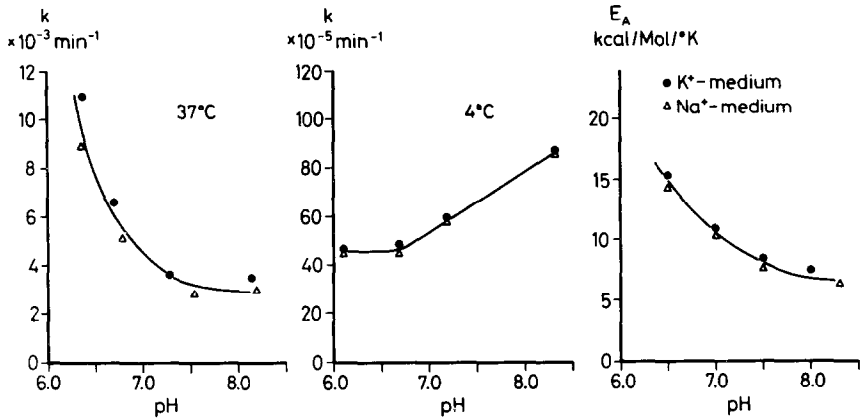

Fig. 6. Rate constants (k) for SO_4^{2-}-equilibrium exchange at 37 °C and 4 °C, respectively, of dinitrophenylated cells as measured at the pH values indicated on the abscissae, and apparent activation enthalpies (E_A) as calculated from the rate constants determined at the two temperatures at corresponding pH values. Dinitrophenylation was performed by preincubation of the cells with 5 mM FDNB (1 h, 37 °C). The flux measurements were done in media which contained either Na^+ (△) or K^+ (●) as the predominant cation species.

treated cells (not shown).

Control experiments with intact cells at 4 °C and 37 °C showed that the pH dependence of sulfate equilibrium exchange follows the same general pattern at the two temperatures (Fig. 7). At both temperatures the exchange rate passes through a maximum. At 37 °C the maximum is located at pH 6.2. At 4 °C it is shifted to pH 5.8. In the pH range covered in the experiments with the modified cells, the untreated cells show a decrease of the penetration rate with increasing pH at both temperatures. The apparent activation enthalpy is 36 kcal per mole per °K at pH 7.0 and varies relatively little over the pH range from 5.3 to 8.0. Control experiments at pH 6.8 and 7.7 show that E_A is independent of temperature. This applies also to FDNB treated cells (Fig. 8).

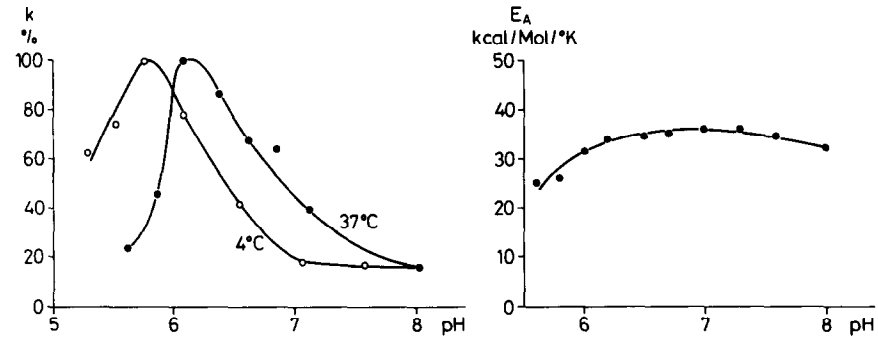

Fig. 7. pH-dependence of the rate constants (k) for SO_4^{2-}-equilibrium exchange across the erythrocyte membrane at 37 °C and 4 °C and of the apparent activation enthalpies (E_A) as calculated from the rate constants determined at the two temperatures at corresponding pH values. The rate constants are represented in percent of the values at the respective maxima ($k = 65.5 \cdot 10^{-3}$ min^{-1} at 37 °C and $k = 13.5 \cdot 10^{-5}$ min^{-1} at 4 °C).

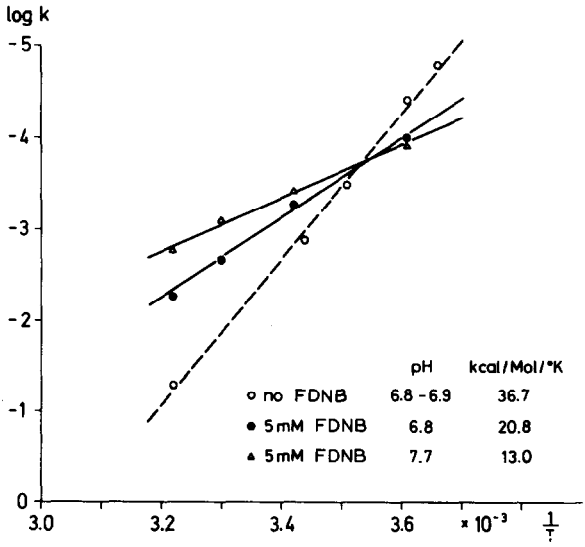

Fig. 8. Arrhenius plots of rate constants (k) for SO_4^{2-}-equilibrium exchange across the membrane of untreated (– – –) cells and (———) cells which were pretreated with 5 mM FDNB (1 h, 37 °C). SO_4^{2-}-exchange was measured at the pH values indicated.

The commercial pronase used in the experiments described above is a mixture of different proteolytic enzymes, including aminopeptidases and carboxypeptidases [9, 3]. In an attempt to obtain some information on the nature and specificity of the damage done by pronase the crude enzyme was fractionated into several subfractions (see Fig. 1) whose effects on permeability (Figs 9 and 10) and whose substrate specifities (Table I) were determined.

Fraction I has little effect on SO_4^{2-}-permeability at 4 °C and 37 °C and does not change E_A. This fraction contains aminopeptidase and carboxypeptidase activity.

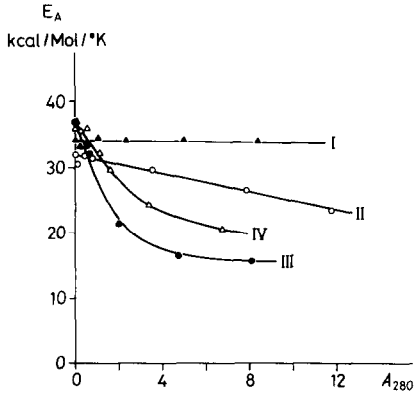

Fig. 9. Apparent activation enthalpies (E_A) for SO_4^{2-}-equilibrium exchange (pH 6.8–7.0) in red cells which were pretreated with varying concentrations of pronase subfractions I-IV (see Fig. 1). The concentration of the pronase fractions in the preincubation mixture is indicated in $A_{280\ nm}$ units.

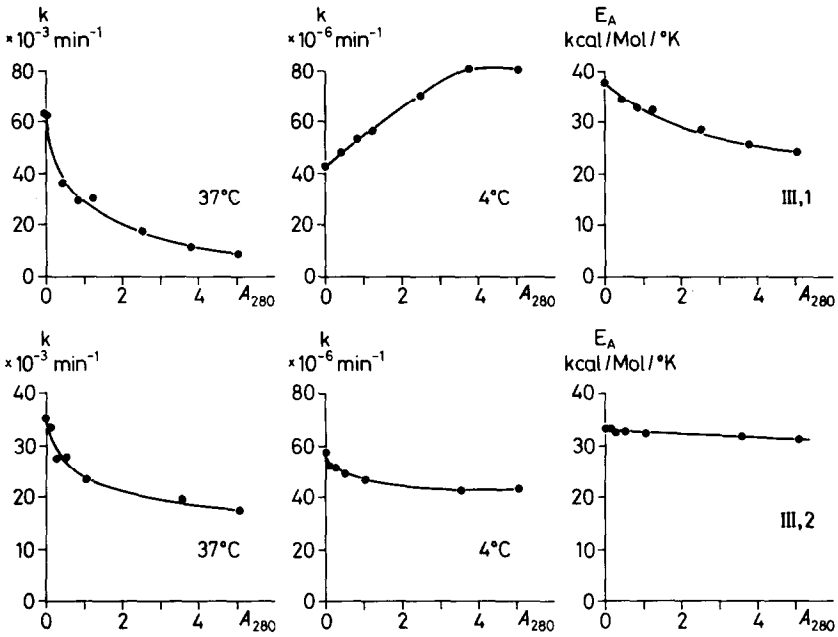

Fig. 10. Rate constants (k) for SO_4^{2-}-equilibrium exchange at 37 °C and 4 °C, pH 6.4–6.6, in cells pretreated with varying concentrations (indicated in $A_{280\ nm}$ units) of the pronase Fractions III.1 and III.2 (see Fig. 1) and the corresponding apparent activation enthalpies (E_A).

TABLE I

THE SUBSTRATE SPECIFICITY AND EFFECT ON SO_4^{2-}-PERMEABILITY OF PRONASE FRACTIONS

Activity against each tested substrate is expressed for Fractions I-IV relative to their maximal values. The data are based on mean values ($n = 6$). Substrate specificity of the purified enzymes III.1 and III.2 is shown qualitatively. On the right part of the table is shown the percentage of inhibition of SO_4^{2-}-transport at 37 °C and the percentage change of the apparent activation enthalpy after preincubation of the red blood cells (1 h, 37 °C) in media containing 5 $A_{280\ nm}$-units of the respective enzyme fractions. Abbreviations: n.s. = not significant, L-Leu-Ala = L-leucylalanine, Z-Gly-Leu = carbobenzoxy-glycyl-L-leucine, BAEE = benzoyl-L-arginine ethyl ester, PNPA = *p*-nitrophenyl acetate.

Fraction	Substrate					% Inhibition of SO_4-flux at 37 °C*	% Decrease of activation enthalpy*
	L-Leu-Ala	Z-Gly-Leu	Casein	BAEE	PNPA		
I	100	100	46	n.s.	n.s.	18	0
II	7	45	100	5	58	65	11
III	0	8	15	100	100	90	55
IV	0	n.s.	12	n.s.	55	82	38
III.1	−	−	+	−	+	86	36
III.2	−	−	+	+	−	48	6

* Enzyme concentration: 5 $A_{280\ nm}$.

Like crude pronase, Fractions III and IV are potent modifiers of sulfate transport. Both fractions mainly contain endopeptidases which differ in their activity against various esters. Fraction III contains two proteinases, one active against PNPA (III.1), the other active against BAEE (III.2). Fraction IV resembles the enzymatic properties of Fraction III.1. Pretreatment of the cells with Fraction III.1 affects SO_4^{2-}-transport in a similar way to crude pronase*, while the action of Fraction III.2 resembles that of papain. So one may assume that endopeptidases as specified by their hydrolytic activity against PNPA, are responsible for the ability of pronase to lower E_A of SO_4^{2-}-transfer. An enzyme with such characteristics is also included in pronase Fraction II. In contrast to the later eluted enzymes this enzyme is inhibitable by EDTA (see also 3). The observation that, at 37 °C, addition of EDTA reduces by 40% the inhibition of SO_4^{2-} flux by pronase does suggest a participation of the PNPA hydrolysing endopeptidase in Fraction II in the effect of crude pronase.

DISCUSSION

Inhibition of anion permeability by enzymatic or chemical modification can be easily accounted for in terms of two alternative models of anion permeability: the fixed charge model [10] and the carrier model [11]. Enzymes as well as chemical modifiers could either reduce the concentration of fixed charges or of carrier molecules and thereby reduce the rate of anion transfer across the red blood cell membrane. However, reactions of this type could only explain the simple inhibitory effect of salicylate and papain but not the dual actions of pronase and FDNB. It has been postulated that the rate-limiting barrier is in series with a layer of fixed charges [10]. The reduction of the concentration of fixed charges by FDNB or pronase would, in terms of this model, be counteracted by an additional effect on this rate determining barrier. Alternatively, in terms of the carrier model, one could assume that a reduction of the number of carrier molecules is associated with a change of the membrane structure which facilitates the penetration of the surviving carriers. Regardless of the specific properties of the model of anion transfer an explanation of the effects of pronase and FDNB requires the assumption that at least 2 effects are superimposed. Unfortunately, there is no unequivocal criterion to decide from flux measurements whether or not the sites of action involved are arranged in series in one and the same pathway of if they control two parallel and possibly independent pathways.

In this context it is worth mentioning that anion penetration across the red blood cell membrane has been postulated to proceed via two parallel pathways: an exchange pathway and a conductance pathway [12]. The question has been raised whether or not the findings represented in this paper are reconcilable with such an assumption [13]. If one assumes that in the untreated cell the rate of penetration via the exchange pathway greatly exceeds the rate across the conductance channel, then inhibition observed at 37 °C would represent the inhibition of the exchange pathway. If papain and salicylate would have no effect on the conductance channel or if the two agents would inhibit both pathways to the same extent then one has to stipulate that

* The fact that Fraction III.1 reduced E_A to a lesser extent than Fraction III is probably due to a loss of proteolytic activity (about 45%) from Fraction III.1 during its dialysis before use. This loss of activity does not take place during dialysis of Fraction III.2.

pronase and FDNB facilitate the penetration across the conductance channel.

An entirely different but equally plausible explanation would consist in the assumption that pronase and FDNB open up a parallel pathway which does not exist in the untreated cell. For this reason our experiments do not contribute evidence for or against the existence of a conductance channel in the intact erythrocyte.

Inferences about "pathways" are derived from flux measurements and are of purely operational significance for the interpretation of kinetic data. Biochemical evidence which could add a more concrete basis to the speculations about the mode of action of the modifiers used in our work is still inadequate.

Pronase is known to degrade a membrane protein of molecular weight 100 000 [14]. Cabantchik and Rothstein [15] showed that certain stilbene derivatives, which are very specific inhibitors of anion permeability, are bound to this particular protein. Moreover, Zaki and Passow [16] demonstrated that the binding of FDNB to that protein is largely prevented by pretreatment with one of these stilbene derivatives (SITS). Possibly the 100 000 mol. wt. band in sodium dodecylsulphate polyacrylamide gel electrophoresis is composed of several different proteins. Hence for the time being it cannot be decided whether those inhibitors which lower E_A for anion transport alter other proteins in the 100 000 mol. wt. region than those which do not change E_A or if the two types of inhibitors affect different sites on the same molecule.

The experiments with the different pronase enzymes lead us to the following conclusions: Exopeptidase activity seems to play no significant role in inhibition at 37 °C and produces no change of E_A. Those pronase fractions which lower the E_A most effectively (III.1 and IV) consist primarily of endopeptidases. Unfortunately, the specificity of these enzymes is low [17, 18] and hence does not provide definitive information on the nature of the bonds cleaved. However, in view of the suggested involvement of amino groups in the control of anion permeability [19, 20] it is interesting to note that the potential to cleave peptide bonds at the carboxyl end of arginine or lysine residues cannot be related to the decrease of E_A. This may be inferred from the absence of a decrease of E_A after treatment of the cells with pronase Subfraction III.2. This Fraction is believed to possess trypsin-like specificity since it cleaves the A- and B-chains of oxydized insulin only at the bonds mentioned above [21]. Moreover, the rather unspecific enzyme papain which is also capable of attacking these bonds does not affect E_A. Similar conclusions may be drawn about inhibition without concomitant change of E_A: although the trypsin-like enzyme III.2 does produce such inhibition, trypsin does not.

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