creased to 85 mm Hg (pH 7.1–7.4; temperature 37 °C). The measured values are compared with the saturation values calculated after THEWS[4], curve 1 standing for a plane, unlimited hemoglobin layer 1.8 μ thick, and curve 2, for a cuboid that replaces the erythrocyte (see Figure 1).

Another example is shown in Figure 6 (A and B). This concerns the hemoglobin O_2 saturation increase in monoerythrocyte blood lamellas, when the external O_2 and CO_2 partial pressures are suddenly changed. The dots, with their standard deviations, stand for the mean values obtained from 25 and 29 measurements, respectively (FRECH et al.[28]). Again the curves are calculated after THEWS[4]. In A, a change was made from a 'venous' to an 'arterial' gas mixture (P_{O_2} = 40 mm Hg, P_{CO_2} = 47 mm Hg → P_{O_2} = 100 mm Hg, P_{CO_2} = 40 mm Hg), and, in B, there is a corresponding change in the hypoxia range (P_{O_2} = 24 mm Hg, P_{CO_2} = 40 mm Hg → P_{O_2} = 47 mm Hg, P_{CO_2} = 35 mm Hg). Examinations of this kind indicate the physiological saturation processes taking place during the passage of the erythrocyte through the lung capillary. In this case, though, the retarding effect of the alveolar capillary membrane, which protracts the time by approximately the submultiple 1.3, must also be taken into account. According to the results shown in Figure 6, it is to be expected that the alveolar contact time of the erythrocyte, with the gas phase in the human lung, is about 0.2–0.3 sec.

Zusammenfassung. Es wird eine Übersicht über die mathematischen und experimentellen Aspekte des O_2-Austausches im Erythrozyten gegeben. Der Gesamtprozess kann als eine Sauerstoffdiffusion mit gekoppelter Hämoglobin-Sauerstoff-Reaktion beschrieben werden. Für die mathematische Analyse muss also die partielle Differentialgleichung der Diffusion durch ein Gleichungssystem erweitert werden, das dem chemischen Reaktionsablauf Rechnung trägt. Voraussetzung hierfür ist die Aufstellung eines Modells zur molekularen Interpretation der O_2-Bindungskurve. Die vereinfachten Differentialgleichungen für Diffusion und Reaktion lassen sich unter Beachtung der Rand- und Anfangsbedingungen nur näherungsweise integrieren.

Es werden ferner die experimentellen Methoden beschrieben, die für die Verfolgung der schnellen O_2-Austauschvorgänge im Erythrozyten geeignet sind. An einigen Beispielen werden die mit diesen Methoden gewonnenen Untersuchungsergebnisse erläutert. Es zeigt sich, dass die mathematische Analyse und die experimentellen Untersuchungen zu übereinstimmenden Ergebnissen führen.

Chemical Modifiers of Passive Ion Permeability of the Erythrocyte Membrane

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In current permeability research the use of chemical modifiers of cell membranes is part of the experimental routine, as is the application of enzyme inhibitors in the study of metabolic sequences and enzyme kinetics. Enzyme inhibitors are widely used in attempts to elucidate the nature of the enzymatic reaction sequence which accomplishes active transport. The inhibition of the K-Na-sensitive membrane ATPase by ouabain (SKOU[1]) is an indispensable tool for the identification of the transport enzyme in tissues and cells. The application of N-ethyl-maleimide facilitated the separation of the ATPase reaction into two distinct steps: A sodium activated P exchange between ATP and ADP and a potassium activated hydrolysis of some product of the reaction between ATP and membrane (FAHN et al.[2]). Finally, the effects of ethacrynic acid stimulated discussions about the possible occurrence of a specific sodium transport mechanism in addition to the K-Na-membrane ATPase (HOFFMAN and KREGENOW[3], WHITTEMBURY[4]).

Chemical modifiers of passive permeability have been employed even more extensively than inhibitors of active transport (cf. PASSOW[5]). It is the aim of the present paper to discuss a number of representative results obtained with a few selected types of modifiers of passive permeability and thus to demonstrate some of the special problems encountered in the interpretation of their actions. Obviously, such a discussion requires frequent reference to existing concepts of the molecular mechanisms which supposedly control passive permeability. Therefore, before focussing on the

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subject matter of this article, it seems worth-wile first to summarize briefly the more widely employed hypothesis about the nature of the pathways crossing the red cell membrane. Subsequently, some of the ambiguities encountered in the interpretation of experimental results obtained with chemical modifiers will be reviewed. Typical difficulties in the elucidation of the effects of metabolic poisons on passive permeability will be emphasized and illustrated. Finally, attention will be concentrated on inhibitors of anion permeability. A more detailed presentation of experimental results obtained in this laboratory will be given with special reference to the so-called 'fixed charge hypothesis' of ion selectivity of the red cell membrane.

Penetration pathways across the erythrocyte membrane

Existing evidence, which has been summarized by PASSOW[5] and, more recently, by POST et al.[6] suggests that active and passive ion movements proceed through parallel and independent pathways ('pump' and 'leak'). The essence of this evidence is briefly the following: The potassium flux across the red cell membrane can be subdivided into 2 components (GLYNN[7]); one of them exhibits saturation kinetics and can be reduced by substrate depletion; the other exhibits a linear relationship between flux and concentration up to the highest K concentrations tested and is insensitive to the presence or absence of substrates. Ouabain mainly affects active fluxes. Moreover, glycolytic poisons which reduce the energy supply to the pump reduce the uphill movements of K and Na but have little or no effect on their downhill movements. Finally, the assumption of the independence of pump and leak fluxes proved to be highly successful in TOSTESON and HOFFMAN'S[8] quantitative formulation of the interrelationships between cell volume, ion composition of cells and medium, and passive or active ion fluxes.

Small anions such as Cl^- and HCO_3^- penetrate about 10^6 times faster than cations of comparable size. They always move passively down electrochemical gradients until Donnan equilibria are established. In 1950 and the following years a number of authors (e.g. GOURLEY and GEMMILL[9]) postulated that phosphate ions constitute an exception from this rule and are actively transported. However, the authors of more recent papers on this subject (e.g. VESTERGARD-BOGIND[10]; PASSOW[5]; DEUTICKE[11]) agree that phosphate migrates downhill and uses the same pathways as sulfate and other slowly permeating anion species.

In the absence of a better hypothesis, it is usually assumed that the capacity of the red cell membrane to discriminate sharply between anions and cations is mainly due to the presence of fixed positive charges inside the membrane. These charges prevent the passage of cations without blocking the movements of anions. Systematic studies of the dependence of anion and cation permeability on pH and ionic strength of the medium lead to the assumption that the pK of the fixed charges is about 9, which is indicative of amino groups (PASSOW[12–14]). The participation of amino groups was also suggested by the finding that the decrease of the selectivity of the membrane with increasing temperature could be explained by an increase in the dissociation of the fixed charges and that the calculated heat of dissociation (about 10 kcal/mole) was within the range anticipated for the dissociation of amino groups (LACELLE and ROTHSTEIN[15]).

It is not yet clear whether the observed passive cation leakage occurs through the positively charged anion channels or via other pathways. The fixed charge density within the red cell membrane is thought to be about 2.5 moles/l membrane water. If the ion selectivity of the membrane could entirely be related to coion exclusion by a Donnan effect of the fixed charges, such a charge density would allow a cation flux which is even larger than the observed one (for a full discussion, cf. PASSOW[13]). It would seem, therefore, not impossible that passive cation leakage proceeds only via the anion channels. However, there is no direct evidence to substantiate such an assumption. Earlier work of HOFFMAN[16] suggested the occurrence of some carrier mediated exchange diffusion of Na besides diffusion through a leak pathway. Today, the situation seems to be even more complex (GARRAHAN and GLYNN[17]). There is, however, a tentative agreement (LACELLE and ROTHSTEIN[15]; PASSOW[12,13]) that the increase of cation flux observed under experimental conditions which can be expected to decharge the membrane NH_3^+ groups is due to cation flux across anion channels.

Problems encountered in investigations on the mode of action of chemical modifiers

Perplexing situations may arise if typical metabolic inhibitors evoke a change of passive permeability. It

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usually requires a detailed study to decide whether this change is an indirect consequence of metabolic inhibition, or the independent result of a direct modification of the membrane, or a combination of both effects. Fluoride poisoning of human red blood cells is a good case in point. When the effect of fluoride on potassium permeability was first discovered (WILBRANDT[18]), there was reason to believe that the potassium loss induced by the agent was causally related to the effect of fluoride on glycolysis. It was assumed that the inhibition of enolase directed glycolysis to abnormal pathways and that the resulting abnormal metabolic products increased the leakage of potassium from the cells. Such and similar actions of other metabolic inhibitors has led one of the present authors (PASSOW[19]) to extensive speculations about a possible metabolic control of passive permeability. It was argued that many of the constituents of cell membranes participate in metabolic reactions and hence are liable to catabolic breakdown after inhibition of glycolysis. Such breakdown could, it was further suggested, lead to the specific increase of passive potassium permeability (Na-permeability is little affected under these conditions) and it was thought that metabolically induced variations of K-leakage could be used by a cell with a stoichiometrically coupled K/Na pump as a means for regulating separately its K and Na content. A closer investigation of the effects of fluoride showed, however, that the inhibitor exerts a twofold action. Its effect on permeability is a consequence of the formation of a complex with an alkaline earth ion and a ligand as yet unidentified in the cell membrane. This reaction, however, can take place only if the level of an intracellular complexing agent, ATP, is reduced below a critical value. This is achieved by fluoride inhibition of glycolysis. In other words, inhibition of glycolysis by fluoride is a prerequisite for a direct action of that poison on the membrane and not in itself the immediate cause of the change of passive permeability (LINDEMANN and PASSOW[20]; LEPKE and PASSOW[21]). Thus, in the case of fluoride poisoning, the accessibility of the permeability controlling sites within the membrane to the poison depends on metabolism, but not the control of passive permeability itself. There are, however, a good many other metabolic inhibitors, which produce similar permeability changes as fluoride (cf. PASSOW[5]). In particular, iodo acetate can induce an increase of K efflux similar to that of fluoride, provided a suitable substrate, adenosine or inosine + NH_4Cl, is present (GARDOS[22]). The biochemical mechanisms of this 'Gardos effect' is not yet really understood. It still remains to be seen whether or not the action of the inhibitor, iodo acetate, reveals a physiological interrelationship between metabolism and passive permeability or represents only a complicated interplay of metabolism and a direct drug action on the membrane.

Once the membrane is established as the site of action of a poison, the further elucidation of the poison's mode of action encounters the same difficulties as the interpretation of the effects of enzyme inhibitors. Most chemical modifiers are not really specific with regard to specific groups in the membrane: Even if specific modifiers are available they may be bound not only to the rate limiting sites but also to other sites in the membrane which do not participate in the control of ion permeability. Thus, it is usually difficult to establish a meaningful relationship between binding and effect. A typical example is mercury poisoning. The Hg ions not only bind to the membrane but also penetrate into the cell where they are soaked up by hemoglobin and, perhaps, other cell constituents. Thus, it is almost hopeless to establish a clear relationship between Hg binding and its effects on passive potassium and sodium fluxes (WEED et al.[23]). An organic mercurial, parachloromercuribenzenesulfonate (PCMBS), however, does not penetrate and yet produces an increase of K efflux and Na influx similar to that of mercury. With this agent, it was possible to correlate its effect to the number of blocked SH groups within the membrane. Nevertheless, the estimate is still subject to some uncertainties, since only a small fraction of the SH groups present in the membrane is involved in the control of cation permeability (SUTHERLAND et al.[24]).

In conjunction with the interpretation of the effect of PCMBS, another typical difficulty arises: In the case of this agent, as with many others, one does not know whether a modifier affects a site which is normally involved in the control of ion movements, or if not normally existing pathways are opened. Moreover, chemical agents may produce allosteric effects which might not occur under physiological conditions. In short, the interpretation of the action of chemical modifiers is usually dubious. Nevertheless, if considered in conjunction with other evidence about the nature of penetration pathways, study of the effect of modifiers may help to decide between existing theories and to formulate better ones. Below, a few examples of the effects of chemical modifiers of passive anion permeability of the red blood cell will be briefly reviewed and the scope and limitations of their usefulness for the interpretation of penetration processes in terms of the fixed charge concept will be illustrated.

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The effects of the amino-acid reagents 1-fluoro-2,4-dinitrobenzene (DNFB) and 2-methoxy-5-nitrotropone (MNT)

DNFB, a chemical modifier of amino groups, was found to inhibit anion permeability (PASSOW[13,14]) and to induce an increase of cation permeability (BERG et al.[25]). Such an effect is to be expected if the number of fixed charges within the ion permeable pathways is reduced by reaction with the agent: the efficiency of the permeability barrier to prevent cations from diffusing down their electrochemical gradients is reduced, hence K and Na leakage is bound to occur. On the other hand, the decrease of the fixed charge density is associated with a decrease in the number of diffusible counter ions, i.e. anions, within the membrane. Hence, the anion flux is diminished.

The effects on anion permeability are already apparent at the very lowest DNFB concentrations used since any reduction of the fixed charge density leads to a more or less proportionate decrease of flux. Cation permeability, in contrast, shows a marked increase only if the charge density of the barrier is reduced to such an extent that it becomes comparable to the concentration of the diffusible ions in the medium. In a membrane with an estimated fixed charge density of about 2.5 moles/l, the DNFB dose, which must be bound to the membrane in order to achieve K loss from the cells, considerably exceeds the minimal dose which is required to affect measurably anion permeability.

DNFB arylates only uncharged amino groups. The dinitrophenylation of the membrane should therefore be favoured by alkaline reaction within the membrane. The pH within the membrane can be affected in 2 ways, (1) by raising the pH in the medium at a given ionic strength, (2) by lowering the ionic concentration in the medium at a given pH. The latter method is based on the fact that the OH concentration within the positively charged membrane is different from that of the medium. If Donnan's law applies to the anion distribution between cell membrane and medium one may write

$$\frac{Cl_m}{Cl_0} = \frac{OH_m}{OH_0}$$

where the indices m and 0 refer to membrane and medium, respectively. Any reduction of Cl_0 at a given OH in the medium and a given Cl in the membrane (the Cl concentration within the membrane is, for electrostatic reasons, about equal to the concentration of positive fixed charges) must lead therefore to an increase of the pH inside the membrane. Regardless of the method employed, any increase of the pH within the membrane should increase the efficiency of a given dose of DNFB in reducing anion permeability. This is actually observed (PASSOW[14]) and thus supports the contention that the action of DNFB can be interpreted on the basis of an interaction with amino groups. Unfortunately, closer inspection reveals a number of ambiguities.

If the cells were first exposed to a given dose of DNFB at high pH and then resuspended in a solution of neutral pH, they should show a stronger inhibition of anion permeability than cells which were reacted with DNFB at neutrality. This effect would be anticipated since the arylation of amino groups with DNFB is irreversible and hence the abolition of a larger number of amino groups at high pH should show itself after resuspension of the treated cells at neutral pH. This could not be observed. The degree of inhibition of anion permeability was entirely determined by the pH at which the anion flux was measured and independent of the pH at which the membrane was dinitrophenylated.

Difficulties with the interpretation of the action of DNFB were not unexpected. DNFB is not specific with respect to amino groups. It may also react with SH, imidazole, and tyrosine OH-groups. A reaction with SH-groups is, however, unlikely to explain the described permeability change. Other SH reagents, such as IAA or PCMBS do not affect anion fluxes (PASSOW[13]). Moreover, if the cells are dinitrophenylated after the SH groups were protected by combination with PCMBS or iodoacetate, the effect of DNFB remains unaltered; this holds true even if the PCMBS is removed after dinitrophenylation by washing the cells with cysteine (KNAUF and ROTHSTEIN 1968, personal communication, confirmed in our laboratory, 1969). Effects via modification of phenolic OH or imidazole groups could not be excluded so far.

The ambiguity of the interpretation of results obtained with DNFB becomes further apparent if one tries to correlate DNFB binding with its effects on permeability. In isotonic saline, at a concentration of 4 mmoles/l, DNFB reduces sulfate permeability about 80%. Under these conditions a single cell *membrane* binds about 5×10^7 molecules of DNFB. In a recent paper by FORSLING, REMFREY and WIDDAS[25a] on DNFB binding to human erythrocytes in relation to inhibition of glucose transfer it is shown that inhibition is fully developed when 4×10^8 molecules/cell are bound. Since we were able to demonstrate DNFB penetration into the cells, and DNFB binding to hemoglobin, it is easily understood why the figure of these authors is about one order of magnitude higher than ours which represents binding to the membrane. FORSLING et al. presented evidence which suggests that only a small fraction of the bound DNFB is involved in blocking glucose transfer. They conclude that DNFB is not particularly useful as a tool for the identification of glucose transport sites.

[25] H. C. BERG, J. M. DIAMOND and P. S. MARFEY, Science *150*, 64 (1965).

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Approximately 30% of the membrane bound DNFB molecules reacted with lipids. Preliminary studies of DNFB binding to the lipids in our laboratory (POENSGEN, unpublished results) revealed that DNFB combines with 2 different substances, one of which takes up 90% of the total amount fixed. Interestingly enough there is virtually no DNFB binding to phosphatidylserine under conditions where a dramatic permeability change is observed. It is not quite clear whether all of that fraction of the DNFB, which is not bound to the lipids, is associated with the protein. It also remains to be shown whether or not unreacted DNFB is present in the lipid phase of the membrane in addition to the 2 compounds mentioned above. DNFB is known to be highly lipid soluble and it is not certain that washing cells or membranes removes all of the unreacted DNFB. Moreover unreacted DNFB is quite volatile. It may, therefore, easily get lost during the purification of the ethanol chloroform extract of the membrane lipids which involves evaporation to near dryness. In view of the inhibitory action of dinitrophenol (DNP) on anion permeability of the red blood cell (OMACHI[26]), it seems conceivable that the structurally related DNFB exerts a similar effect. In other words, the DNFB effect could conceivably be completely unrelated to the formation of covalent bonds with the lipids and protein of the membrane.

In the red cell, DNFB is rapidly converted by the enzyme carbonic anhydrase into dinitrophenol (HENKART et al.[27]). In contrast to DNFB, DNP can be removed quantitatively from the red cell membrane. The fact that washing the cells restores the original anion permeability after exposure to DNP but not after DNFB treatment, suggests that a possible formation of DNP has little, if anything, to do with the DNFB effect on permeability.

Due to ambiguities in the interpretation of DNFB action, experiments were performed with another amino reagent, 2-methoxy-5-nitrotropone (MNT). This agent is supposed to be much more specific than DNFB. In particular, it is claimed not to react with SH, phenolic OH, or imidazole groups (TAMAOKI et al.[28]). MNT is only sparingly soluble in water. Nevertheless, if added in the solid state to a cell suspension, it reacts with the membrane, thereby lowering anion permeability to a similar extent as high concentrations of DNFB (Figure 1). There is also an acceleration of cation efflux (Figure 2), but this effect is much less pronounced than that produced by DNFB. The accelerating effect can be increased by the addition of ethanol to the medium. The action of ethanol does not seem to be related to its rather slight effect on the solubility of the agent. It appears as if the ethanol renders the binding sites involved in the control of cation permeability more accessible to the action of MNT. This is supported by the observation that ethanol has little if any effect at concentrations below 1.3 moles/l. Above this concentration range its effectiveness sharply

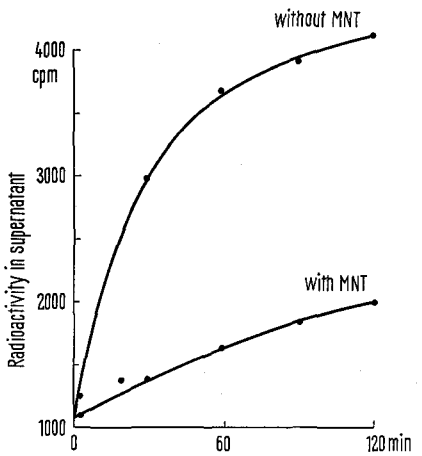

Fig. 1. Effects of 2-methoxy-5-nitrotropone on sulfate permeability of human red blood cells. The cells were equilibrated in 2 tubes with $^{35}SO_4$ at 37 °C in a medium containing 10 mmoles/l Na_2SO_4, 10 mmoles/l *Tris*, 141 mmoles/l NaCl, pH 7.4, and 37 °C for 30 min. Cell concentration: 20 Vol %, total volume of each sample 6.0 ml. After 30 min 5 mg of solid MNT was added to one of the tubes and incubation was continued for another 2.5 h. Subsequently the cells were centrifuged and resuspended in a medium of the same composition as that previously used except that neither MNT nor $^{35}SO_4$ was present. The final cell concentration was 2.5 Vol %. The appearance of $^{35}SO_4$ in the supernatant was followed. Ordinate: Radioactivity in supernatant in cpm. Abscissa: Time in min.

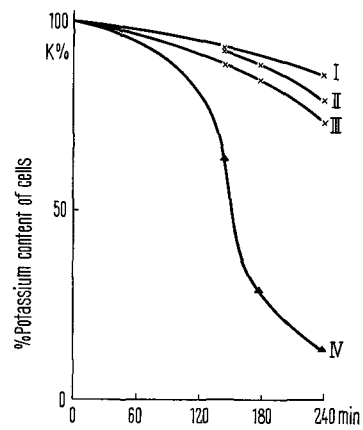

Fig. 2. Effects of 2-methoxy-5-nitrotropone on potassium permeability of human red blood cells. The cells were suspended in 50 mmoles/l *Tris*, 166 mmoles/l NaCl, and 10 mmoles/l sucrose (to prevent colloid osmotic hemolysis). Cell concentration 2.5 Vol %, pH 7.4, temperature 37 °C. To 11.0 ml of samples II and IV 2.0 mg each of solid MNT was added. Sample IV contained in addition 0.075 ml ethanol/ml suspension. Samples I and III are controls without MNT. I is without ethanol, III contains ethanol in the same concentration as sample IV. Ordinate: Potassium content of cells in percent of original value. Abscissa: Time in min.

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increases. This sudden increase of effectiveness occurs at ethanol concentrations which are just a little lower than those required to induce K loss without added MNT. At the concentrations used in the experiments on cation permeability ethanol has little if any effect on the MNT action on anion permeability. This suggests that the rate limiting barrier for anions and cations is not identical – a conclusion which had already been drawn for reasons beyond the limited scope of the present paper (cf. PASSOW[13,14], see also below).

In summary: The experiments with DNFB are far from conclusive. However, if considered in the light of other, extensive evidence on the participation of amino groups in the control of passive ion permeability, it would seem far fetched not to relate its actions to arylation of amino groups. The action of the more specific NH_2-reagent, MNT, substantiates this conclusion. The amino groups of phosphatidylserine can be excluded as possible binding sites of DNFB. Obviously, they play no role in the control of passive ion permeability. There is still a long way to go until one will be able to relate the binding of a fraction of the total of about 5×10^7 DNFB molecules/cell membrane to the observed effects on passive permeability.

Inhibitors of passive anion permeability which do not seem to interact with fixed charges

The permeability of the red cell membrane to phosphate (GERLACH, DEUTICKE and DUHM[29]) and other anions (PARPART[30], SCHNELL and PASSOW[31]) can be inhibited by a large number of unrelated compounds whose chemical structure seems to preclude the formation of covalent bonds with amino groups. This group of inhibitors includes anions, cations, and uncharged molecules. It comprises aliphatic as well as aromatic compounds. The only property which all inhibitors have in common is their polar-apolar character. They are therefore liable to accumulate at lipid water interfaces.

The effectiveness of the inhibitors varies with the ionic composition of the medium, in particular with pH and concentration of penetrating anions (Figure 5). However, for any given composition of the medium, the relationship between concentration and effect of an inhibitor can be described quantitatively in surprisingly simple terms provided the flux measurements are made with radioisotopes at Donnan equilibrium (cf. PASSOW[13]) and the ion concentrations in the medium are kept constant in spite of the addition of varying amounts of the inhibitor. For example, in experiments with a penetrating uncharged inhibitor (e.g. primary alcohols, phenol), the inhibitor has to be added to the medium in addition to the other constituents. If the inhibitor is a monovalent anion (e.g. benzoate) it has to be added in exchange for an equivalent amount of chlorid from the medium, etc.

For the specified conditions, the observed relationship between concentration and effect of the inhibitor (Figure 3) can be fitted to the equation

$$\frac{1}{P} = \frac{1}{P_0} + \frac{c}{P_0 \cdot K} \qquad (1)$$

(Figure 4) where c represents the concentration of the inhibitor in the medium, K the mass law constant for the interaction between inhibitor and membrane sites; P and P_0 indicates the permeability constant in the presence and absence of the inhibitor, respectively. An equation of this mathematical structure is well known in enzyme kinetics. In our context, it can be easily derived on the following assumptions: (1) the inhibitor reduces anion flux by interactions with a limited number of sites in the membrane, (2) the reaction between inhibitor and membrane sites can be described by the

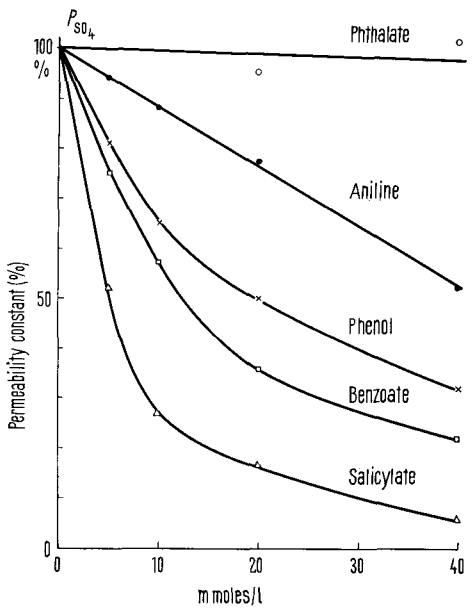

Fig. 3. Relationship between concentration and effect on SO_4 permeability of various benzene derivatives. Flux measurements were made with $^{35}SO_4$ after equilibrating cells and media until the Donnan equilibrium was established (cf. GARDOS et al.[32]). Composition of suspension medium: 166 mmoles/l sucrose, 68 mmoles/l NaCl, and 10 mmoles/l Na_2SO_4. Anionic inhibitors were added in exchange for chloride. The penetrating inhibitors phenol and aniline were added in addition to the components listed. Cell concentration: 46 Vol %, temperature 37 °C, pH 7.3–7.4. Ordinate: Permeability constant in percent of the value measured in the absence of inhibitor. Abscissa: Concentration of inhibitor in the medium prior to mixing with the cells.

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mass law or – this would be mathematically equivalent – by a Langmuir adsorption isotherm, (3) inhibition is proportional to the number of occupied sites.

The observed inhibition is non competitive. It does not seem to be associated with a change of the charge density of the membrane. This statement rests on the following considerations and experimental findings: The flux of a given anion species such as sulfate across a charged membrane depends on the concentration of that species whithin the membrane. This concentration may be quite different from that in the medium and is a function of the concentrations of all other anion species present in the medium. It is primarily determined by the competition between diffusible counterions for the fixed charges and by the pH which determines a degree of dissociation of the membrane amino groups and hence the concentration of fixed charges.

Assuming the applicability of the mass law to the dissociation of fixed charges, the existence of electroneutrality within the membrane, and the establishment of Donnan equilibria between membrane and adjacent media it is possible to calculate, from the known concentrations in the media of all diffusible on species, including OH ions, the concentration of the considered ion species within the membrane. The flux across the membrane should be a unique function of the calculated concentration of this species within the membrane, provided adequate values for the concentration of dissociable groups $\overline{A}$ and dissociation constant K of the fixed charges are chosen (PASSOW[13]). The Figure 5 shows that the flux of SO_4 across the membrane depends on the concentrations of all 3 anionic constituents of the medium: SO_4, Cl, and OH. However, after replotting the fluxes against the calculated $SO_{4\,m}$ all points tend to scatter around a single straight line, provided $\overline{A} = 2.5$ moles/l and $K = 10^{-9}$ are used for the calculation of $SO_{4\,m}$. Similar results are obtained with data which were derived from flux measurements in the presence of an inhibitor phenol (Figure 6). In other words, the dependence of the observed 1000-fold change of sulfate flux on the 3 variables, SO_4, Cl, and OH on the medium, can be described by attributing the same numerical values to the 2 constants, $\overline{A}$ and K, regardless of the presence or absence of the inhibitor.

If the data are replotted on a semilog scale, it is obvious, that, over a very wide range, the flux, j_{SO_4}, increases exponentially with SO_{4m}:

$$j_{SO_4} = j_0 \cdot e^{a \cdot SO_4 m} \qquad (2)$$

j_0 and a are constants over the range of sulfate concentrations within the membrane, SO_{4m}, covered in this experiment. This suggests that SO_4 penetration is a highly cooperative phenomenon: increasing the sulfate concentration facilitates sulfate penetration (PASSOW[13]). Obviously, the inhibitor has little effect on the slope a of the curve which represents a measure for the cooperativity of SO_4 permeation. The main effect is on j_0. In other words, the action of the inhibitor, which is highly dependent on the ion composition and pH of the medium (Figure 3) can be quantitatively

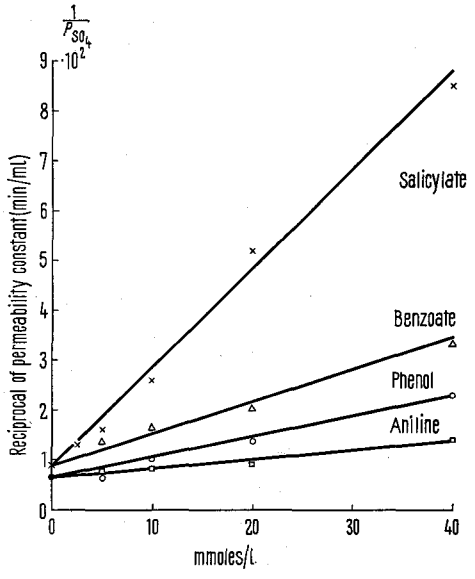

Fig. 4. Data of Figure 3 replotted according to equation (1) (see text). Ordinate: Reciprocal of permeability constant. Abscissa: Concentration of inhibitors in the medium prior to mixing with the cells.

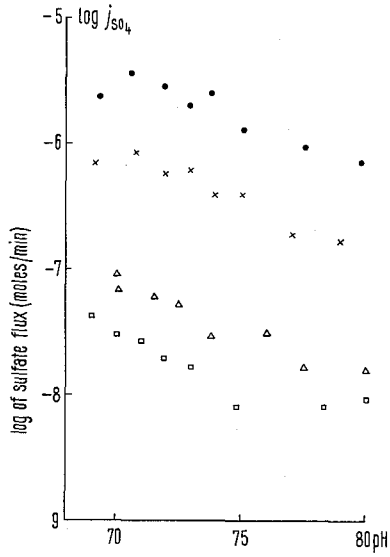

Fig. 5. pH-dependence of inhibitory action of phenol on sulfate permeability as measured at 2 different sulfate concentrations. The measurements were made with $^{35}SO_4$ at Donnan equilibrium (GARDOS et al.[32]). The concentrations of SO_4 and Cl at equilibrium amounted to 55.0 and 0 mmoles/l respectively, for the upper set of points (× and ●) and 5.5 and 150 mmoles/l respectively for the lower set of points (△ and ○). Isotonicity was maintained by sucrose. Cell concentration: 46 Vol %, temperature: 32 °C ×, □, phenol concentration: 12 mmoles/l; ●, △, controls without phenol. Ordinate: log of sulfate flux as measured in moles/m in (cf. GARDOS et al.[32]). Abscissa: pH.

described by its effect on 2 of the 4 constants necessary to represent the data. The effect of the inhibitor concentration on j_0 can be derived from dose-efficiency curves such as those represented in Figure 3. It should be mentioned in passing that j_0 is not constant over the whole range of possible SO_{4m} values. At low concentrations of SO_{4m}, j_{SO_4} should tend towards zero not towards j_0, as equation (1) suggests. Unpublished experiments show that j_0 becomes in fact concentration dependent at lower SO_{4m}'s. It would seem plausible to suspect that

$$j_0 = \text{const}_I \cdot \frac{SO_{4m}}{\text{const}_{II} + SO_{4m}} \quad (3)$$

could represent the behaviour of the curve at low SO_{4m} values. Formally, the SO_4 flux j_{SO_4} could then be considered to follow saturation kinetics, although saturation is never observed. The penetration rate j_{SO_4} continues to increase exponentially with SO_{4m} after j_0 has reached its maximal value. This formal description of our results may help to understand why the effect of the inhibitor can be described on the assumption of the inactivation of a limited number of membrane sites although even at the highest concentrations used, sulfate fluxes fail to approach a concentration independent level.

Of course, this formal 'explanation' is far from satisfactory since it tells nothing about the molecular mechanism involved. This is the main limitation of the present studies of the effects of inhibitors on the kinetics of passive permeability. Nevertheless, it seems gratifying that the effect of these inhibitors can be related to the decrease of only 2 constants among the 4 necessary to describe, phenomenologically, the kinetics of anion transfer. In particular, it is noteworthy that the results fit into the fixed charge concept: those parameters which supposedly describe the concentrations and chemical properties of the fixed charges are not affected at all. Among the 2 other constants j_0 is greatly reduced whereas the cooperativity a is only little affected by the inhibitor.

At least in the concentration range employed in the present experiments, many of the inhibitors of passive anion permeability (e.g. benzoate, aniline, dinitrophenol) exert little if any effect on passive cation movements. Others, including phenol, induce potassium loss from the cells. The effect of phenol increases with decreasing pH and thus is different from that of DNFB. These findings support the view that the mode of action of the inhibitors dealt with in this section is different from that of amino acid reagents which affect both, the movements of anions and cations.

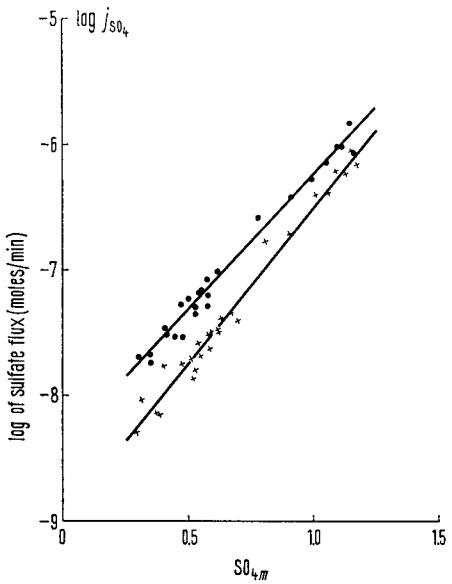

Fig. 6. Sulfate flux j_{SO_4} as function of sulfate concentration within the membrane, SO_{4m}. The values for SO_{4m} were calculated according to equation (2) from PASSOW[12] (see also PASSOW[13]) using $\overline{A} = 2.5$ moles/l and $K = 10^{-9}$. Data are taken from Figure 6 and an additional set of experiments where the sulfate and chloride concentrations in the medium amounted to 8 and 66 mmoles/l, respectively. The straight lines were calculated by the method of least squares. ● control, × with phenol. Ordinate: log of sulfate flux. Abscissa: SO_{4m} in moles/l.

Conclusions

In view of our present ignorance about the structure of the red cell membrane, the application of chemical modifiers in the study of the kinetics of passive ion permeability cannot be anticipated to yield definite information about the nature of penetration pathways. Nevertheless, results can be obtained which may be used as evidence for or against already existing hypotheses. Our experiments support the previously expressed view (PASSOW[13,14]) that, on their way across the red cell membrane, anions have to penetrate across at least 2 barriers in series. The effects of amino acid reagents indicate that one of them carries amino groups as positive fixed charges. The action of a large variety of other, completely unrelated organic compounds, suggests that the anions also pass through a region in the membrane where hydrophobic interactions may occur.

In the absence of ethanol, the amino reagent 2-methoxy-5-nitrotropone inhibits anion permeability but exerts little effect on cation permeability. Addition of ethanol increases the efficiency of the agent to provoke cation leakage but does not influence the inhibition of anion permeability. This suggests that the rate limiting barriers for anions and cations are not identical (cf. PASSOW[13,14]). This conclusion is further supported by the finding that many of those inhibitors of anion permeability which do not interact with amino groups only affect the movements of anions but not of cations[33].

[33] Acknowledgments. Our thanks are due to Miss I. GUTH, Miss S. LEPKE and Mr. J. NITSCHKI for their efficient collaboration. The work was supported by the Deutsche Forschungsgemeinschaft and the Volkswagen Foundation.

Zusammenfassung. In der vorliegenden Arbeit wird ein Überblick über die Probleme gegeben, die bei der Interpretation der Wirkung von Substanzen auftreten, welche die passive Ionenpermeabilität der Erythrozytenmembran beeinflussen. Dabei wird besonderes Gewicht auf Hemmstoffe der Anionenpermeabilität gelegt.

1-Fluoro-2,4-Dinitrobenzol (DNBF) und 5-Methoxy-2-Nitrotropon (MNT) können mit Aminogruppen kovalente Bindungen eingehen. Beide Substanzen werden von der Erythrozytenmembran irreversibel gebunden. Sie hemmen die Permeabilität für Anionen und steigern sie für Kationen. Die Wirkung des MNT auf die Kationenpermeabilität wird allerdings nur sichtbar, wenn es zusammen mit einer nahezu hämolytischen Menge an Äthanol dem Inkubationsmedium zugesetzt wird. Die Beobachtungen stehen im Einklang mit der Hypothese, dass die Erythrozytenmembran ein Anionenaustauscher ist, in dem Aminogruppen als Träger der positiven Festladungen vorhanden sind.

Schwieriger lässt sich die Hemmwirkung einer Reihe von Stoffen erklären, die nicht mit Aminogruppen reagieren können. Dinitrophenol und Benzoat hemmen den Sulfationenfluss nichtkompetitiv, ohne dabei eine Steigerung des Kaliumeffluxes herbeizuführen. Auch aliphatische Verbindungen, darunter primäre Alkohole und Amine, können die Anionenpermeabilität vermindern, wobei bereits ein deutlicher Hemmeffekt auftritt, bevor der Kaliumefflux vergrössert wird. Die einzige Gemeinsamkeit dieser chemisch sehr heterogenen Gruppe an Hemmstoffen ist der polar-apolare Charakter ihrer Moleküle, was zu einer Anreicherung dieser Stoffe an Grenzflächen zwischen Lipid und Wasser führen sollte.

Die Beziehung zwischen Sulfatpermeabilität und Hemmstoffkonzentration lässt sich mit Hilfe einer einfachen, aus der Enzymkinetik bekannten Formel beschreiben. Zwischen dem gemessenen Sulfatfluss und der aufgrund der Festladungshypothese berechneten Sulfatkonzentration in der Membran besteht ein exponentieller Zusammenhang, der durch eine empirische Gleichung wiedergegeben werden kann. Mit Hilfe dieser Gleichung lässt sich der Anionenfluss auch in Gegenwart eines Hemmstoffes (Phenol) beschreiben, wobei sich nur eine der beiden empirischen Konstanten erheblich ändert.

Die geschilderten Ergebnisse legen die Vermutung nahe, dass die Anionen bei der Penetration durch die Erythrozytenmembran mindestens zwei verschiedene, hintereinander geschaltete Permeabilitätsbarrieren überwinden müssen.

Hemoglobin

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Hemoglobin is a spheroidal molecule of 65 by 55 by 50 Å in diameter[1] and contains 2 unequal polypeptide chain pairs with a heme linked to each chain. During human life 4 different hemoglobins are formed: the embryonic Hb Gower 2, the fetal Hb F and the 2 adult hemoglobins Hb A and Hb A_2. They differ in one polypeptide chain pair: Hb A = $\alpha_2\beta_2$, Hb F = $\alpha_2\gamma_2$, Hb A_2 = $\alpha_2\delta_2$ and Hb Gower 2 = $\alpha_2\varepsilon_2$. The α-chain contains 141, each of the other chains 146 amino acids[1a]. Recently SCHROEDER et al.[2] described 2 types of Hb F in normal infants with glycine and alanine respectively in position 136 of the γ-chains. In all polypeptide chains an identical heme is situated near the surface between 2 helices of the chain[1,3]. Out of the 6 coordination bonds of the heme-Fe^{++}, 4 are bound to the porphyrin ring, the 5th is linked to the so-called proximal histidine α^{87} and β^{92} respectively and the 6th is located opposite the distal histidine α^{58} and β^{63} and represents the site of reversible combination with molecular oxygen. In deoxyhemoglobin the oxygen is replaced by a water molecule and the distance between the Fe^{++} atoms of both β-chains is increased from 33.4 to 40.3 Å[4]. The heme-heme interaction resulting in the sigmoid shape of the oxygen dissociation curve requires 2 unequal polypeptide chain pairs. During red cell aging a slight increase of the O_2-affinity of the hemoglobin and a decrease of the heme-heme interaction occur, which are attributed to an alteration of electrostatic interactions in the molecule[5].

Besides the normal hemoglobins an increasing number of abnormal human variants have been described, designated first by Latin capital letters and later by proper names. At the present time more than 70 ab-

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