Irreversible Inactivation of Red Cell Chloride Exchange with Phenylglyoxal, an Arginine-specific Reagent

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ABSTRACT Chloride exchange in resealed human erythrocyte ghosts can be irreversibly inhibited with phenylglyoxal, a reagent specific for the modification of arginyl residues in proteins. Phenylglyoxal inhibits anion transport in two distinct ways. At 0°C, inhibition is instantaneous and fully reversible, whereas at higher temperature in an alkaline extracellular medium, covalent binding of phenylglyoxal leads to an irreversible inhibition of the transport system. Indiscriminate modification of membrane arginyl residues was prevented by reacting the membranes with phenylglyoxal in an alkaline extracellular medium while maintaining intracellular pH near neutrality. The rate of modification of anion transport depends on phenylglyoxal concentration, pH, temperature, and the presence of anions and reversible inhibitors of the anion transport system in fashions that are fully compatible with the conclusion that phenylglyoxal modifies arginyl residues that are essential for anion binding and translocation. Phenylglyoxal reacts rapidly with the deprotonated form of the reactive groups. It is proposed that the effects of anions and of negatively charged transport inhibitors on the rate of irreversible binding of phenylglyoxal are related to the effects of the anions on a positive interfacial potential. This potential determines the local pH, and thereby the concentration of deprotonated groups, in an exofacial region of the anion transport protein.

INTRODUCTION

The physiologically important exchange of chloride and bicarbonate across the red cell membrane is mediated by a large transmembrane protein of ~100,000 daltons known as band 3 by the nomenclature of Fairbanks et al. (1971). Evidence that the integrity of the protein molecule is decisive for normal transport function has especially been derived from studies involving covalently binding isothiocyanostilbenedisulfonate derivatives, which react with a very high degree of specificity with lysyl residues residing in the vicinity of the anion transport site with a stoichiometry of one inhibitor molecule per band 3 molecule (Passow et al., 1975; Ship et al., 1977; Ramjeesingh et al., 1981).

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It has been proposed that an arginyl residue of band 3 may be essential for normal anion transport function (Funder and Wieth, 1976; Knauf, 1979), but this suggestion was based on indirect evidence, and previous attempts to modify transport function irreversibly with arginine specific reagents have been unsuccessful (Passow et al., 1980; Wieth et al., 1980). We now report two lines of evidence that suggest that arginyl residues of band 3 may be critical for anion recognition and transport. In the preceding article we demonstrated that chloride exchange is critically dependent on the protonated form of a titratable group with an apparent pK of ~12 at 0°C (Wieth and Bjerrum, 1982). In the present work we show that anion transport in resealed human erythrocyte ghosts can be irreversibly inhibited by treatment with phenylglyoxal, a reagent that is highly specific for the covalent modification of arginyl residues in proteins (Takahashi, 1968). Anion transport is inhibited by phenylglyoxal in a fully reversible manner at low temperatures, whereas irreversible binding and inhibition occur when membranes are exposed to phenylglyoxal in an alkaline extracellular medium at higher temperatures. The reaction kinetics of phenylglyoxal with the anion transport system are fully compatible with the hypothesis that normal transport is dependent on the integrity of one or more arginyl residues in band 3, and that these arginines can be chemically modified under conditions that favor the reaction of phenylglyoxal with groups exposed to the extracellular medium. By studying the effect of pH, temperature, anions, and reversible transport inhibitors on the rate of inactivation of the anion transport system, we have been able to characterize the reaction and to define optimum conditions for modification of the transport protein. In another report we demonstrate that the amount of phenylglyoxal bound to band 3 after maximum inhibition of anion transport suffices to modify only one arginyl residue per molecule of band 3 (Bjerrum et al., 1982).

Reagents that selectively modify arginyl residues in proteins have played a decisive role in the identification of arginyl residues as essential binding sites for negatively charged substrates and cofactors in numerous enzymes (Riordan et al., 1977; Riordan, 1979). It appears that phenylglyoxal is also a useful chemical probe for the characterization and identification of essential arginyl residues in the anion transport system.

A preliminary report of this work has been published previously (Borders et al., 1981). In addition, Zaki (1981) has recently shown in a preliminary communication that 1,2-cyclohexanedione, a different α-dicarbonyl reagent that is also selective for the modification of arginyl residues in proteins, causes inhibition of sulfate transport in human erythrocyte ghosts.

METHODS

All experiments were monitored by determining the rate of ^{36}Cl efflux from resealed human erythrocyte ghosts. First, we present a general description of the techniques for phenylglyoxalation and flux determination. Second, we state information about the chemicals used and necessary background information about the membrane permeabilities of phenylglyoxal and hydroxyl ions.

Phenylglyoxalation of Resealed Ghosts

The study was carried out with ghosts because phenylglyoxal is adsorbed strongly to hemoglobin in intact red cells. Resealed ghosts buffered with 2 mM Tris (intracellular pH 7.2 at 0°C) were prepared as described by Funder and Wieth (1976). After resealing, the ghosts were washed with an unbuffered KCl solution and packed by centrifugation to a cytocrit of 80–90% (determined by measuring the extracellular space with [^{3}H]inulin). Unless otherwise stated, the ghosts contained 165 mM KCl. One sample of ghosts was washed in the flux medium and used for measuring chloride exchange in untreated cells (control), and a second sample was treated with phenylglyoxal by injecting 4 ml of the packed cell suspension into a water-jacketed thermostated vessel containing 36 ml of the alkaline-buffered phenylglyoxalation medium. Ghosts were exposed to phenylglyoxal at various temperatures from 25 to 38°C as indicated in the figure and table legends. Samples were withdrawn at appropriate time intervals (from 0.5 s to several minutes) from the reaction vessel and diluted with 4 vol of the ice-cold, acidic stopper solution described below. We have checked that the chemical reaction of phenylglyoxal with the membranes stops immediately when the temperature and pH are lowered. No irreversible inhibition of anion transport took place when ghosts were exposed to phenylglyoxal in a mixture of reaction and stopper solution with a pH of 7.2 at 38°C, which indicates that the lowering of pH alone suffices to interrupt the chemical reaction. No irreversible inhibition was found when ghosts were incubated for 10 min at 0°C in a 165-mM KCl medium containing 15 mM phenylglyoxal at pH 10.3, which indicates that lowering of temperature to 0°C suffices to prevent the chemical reaction. Unreacted phenylglyoxal was removed from the ghosts by washing the membranes three to four times with the flux medium (cytocrit 1–2%). By functional criteria this was sufficient to remove all noncovalently bound phenylglyoxal, as indicated by the complete reversal of reversible phenylglyoxal inhibition of anion transport (see below). Subsequent studies with ^{14}C-labeled phenylglyoxal showed that it is necessary to wash the membranes with 0.5–1% albumin-containing electrolyte solution to remove every trace of noncovalently bound reagent. This extra caution is not needed for kinetic studies of the effect of phenylglyoxalation on chloride transport.

Determination of the Chloride Exchange Flux

After phenylglyoxalation and washing, the 165-mM KCl ghosts were suspended in the flux medium to a cytocrit of 25–40%, labeled with ^{36}Cl (0.3–0.6 μCi per ml of suspension), kept for a few minutes to ensure isotope equilibration, and packed in nylon tubes (3-mm inside diameter) for efflux experiments carried out at 0°C and pH 7.2 in a phosphate-buffered 165-mM KCl medium by the Millipore filtering technique of Dalmark and Wieth (1972). In experiments with ghosts having a lower KCl concentration, the concentrations of the media were lowered correspondingly. The gradual inactivation of the anion transport system by exposure to phenylglyoxal was determined by measuring the chloride exchange flux as a function of the duration of the preceding exposure as shown in Fig. 2. The rate coefficient k (s^{-1}) of chloride exchange was determined as described previously (Funder and Wieth, 1976) and the exchange flux ($J^{\mathrm{Cl}}_{\mathrm{exch}}$) was determined from the relation:

$$J^{\mathrm{Cl}}_{\mathrm{exch}} = k \cdot (V/A) \cdot C_i (\mathrm{mmol} \cdot \mathrm{cm}^{-2} \cdot \mathrm{s}^{-1}), \quad (1)$$

where V is the intracellular ghost volume (usually close to 90 μm^3), A is the average membrane area (142 μm^2 per ghost), and C_i is the intracellular chloride concentration. In the figures and tables, relative fluxes are related to the chloride exchange flux of

the untreated ghost sample. Because it was found that the mean cellular volume was not affected by the phenylglyoxal treatment, relative fluxes could be expressed by the ratio

$$J_{rel} = (k_{pg}/k_o) \cdot 100,$$

where k_{pg} is the rate coefficient of chloride exchange in a phenylglyoxal-treated sample, k_o is the rate coefficient of chloride exchange for the control sample, and J_{rel} accordingly is the flux of the treated cells as a percentage of the control value.

By varying the conditions of the chemical reaction, we were able to study the effects of phenylglyoxal concentration, pH, temperature, anions, and the presence of reversible anion transport inhibitors on the rate of functional membrane modification. A preliminary report of a parallel study of the binding of phenylglyoxal to membrane proteins and its identification in band 3, the purported anion transport protein, is presented separately (Bjerrum et al., 1982).

Phenylglyoxal Media

Electrolyte media containing 10–30 mM phenylglyoxal (EGA-Chemie, Steinheim-Albuch, Federal Republic of Germany) were used for the chemical modification of ghosts. Identical results were obtained with the crude commercial phenylglyoxal preparation and with a sample recrystallized from hot water. Chloride-containing and chloride-free electrolyte media were used for the reaction. The chloride medium contained 165 mM KCl and 5 mM cyclohexylaminoethanesulfonic acid (CHES; Calbiochem-Behring Corp., Switzerland; pK_a 9.5 at 25°C). CHES has no effect on the anion transport in the red cell membrane. Phenylglyoxal was added to make up the final reaction concentrations stated in the figure and table legends. The chloride-free medium contained 25 mM potassium citrate, 200 mM sucrose, 5 mM CHES, and phenylglyoxal as above. This medium is isotonic with and has an ionic strength similar to that of the KCl medium. In experiments where only the extracellular chloride concentration was lowered (Fig. 5), proportionate mixtures of the two media were used. In those experiments of Fig. 5 where both extra- and intracellular KCl concentrations were varied, the treatment with phenylglyoxal was carried out at the appropriate concentrations stated in the figure. Before adding the cell suspension the medium was thermostated at the temperature of the subsequent reaction and titrated to the desired alkaline pH with 1 M KOH. The pH was usually completely stable and fell 0.05 units at most during the reaction. In those experiments of Fig. 6 that were performed at pH values above 10.3, the phenylglyoxalation media were buffered with 2.8 mM cyclohexyl-aminopropane-sulfonic acid (CAPS; Calbiochem-Behring Corp., Switzerland; pK_a 10.4 at 20°C). We made sure that the rates of phenylglyoxalation in CHES- and in CAPS-buffered media were identical. The reaction temperatures and pH values in individual experiments are stated in the legends.

Stopper Medium

Phenylglyoxalation was stopped by cooling and acidifying the cell suspension. The stopper medium had the same KCl concentration as the ghosts and was buffered with 4 mM KH_2PO_4 and titrated with KOH, so that mixing 1 vol of the alkaline cell suspension with 4 vol of the stopper solution would result in a pH of 7.2 at 0°C.

Electrolyte Medium for Efflux Studies

The medium used for measurements of chloride fluxes contained the appropriate KCl concentration and 2 mM K_2HPO_4. It was titrated with small amounts of 1 M HCl to

a pH of 7.2 at 0°C. The same medium was used for the study of the reversible inhibition of chloride exchange (Fig. 1). Phenylglyoxal was added to make up the concentrations stated in the figure. The medium was titrated with 1 M KOH to the appropriate pH, and the efflux of $^{36}Cl^-$ was studied using resealed ghosts that had not been exposed to phenylglyoxal before the experiment.

Radioactive Isotopes

Phenylglyoxal, labeled with ^{14}C in the C-2 position, specific activity 32 mCi/mmol, was obtained from CEA, Gif-SurYvette, France, and was kept at −20°C under a nitrogen atmosphere in methanol solution.

^3H-labeled inulin, $Na^{36}Cl$, and [^{14}C]D-glucose were from the Radiochemical Centre, Amersham, England.

Anion Transport Inhibitors

The following chemicals were used to study their possible effects on the rate of phenylglyoxal modification of the anion transport system: phloretin (K & K Laboratories, Plainview, N. Y.), phlorizin (Fluka, Switzerland), dipyridamole (Boehringer, Ingelheim), sodium tetrathionate, $Na_2S_4O_6 \cdot 2H_2O$ (Fluka, Switzerland), niflumic acid (UPSA Laboratories, Agen, France), sodium salicylate (Merck, Darmstadt), trinitrocresolate (2,4,6,-trinitro-m-cresol) (Eastman Kodak Co., Rochester, N. Y.), and DNDS (4,4′-dinitro-2,2′-stilbene disulfonic acid; K & K Laboratories and Pfaltz & Bauer Inc., Stamford, Conn.). The diuretics furosemide and bumetanide were gifts from Leo Pharmaceutical Industries, Copenhagen. The covalently binding transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was used to characterize the fraction of anion exchange that is not inhibited after phenylglyoxal treatment (Table VI). The methods employed for preparation and purification of DIDS have been reported by Funder et al. (1978).

Membrane Permeability to Phenylglyoxal

Phenylglyoxal equilibrates very rapidly across the red cell membrane. This was shown by determining the efflux of ^{14}C-labeled phenylglyoxal from resealed ghosts, loaded with phenylglyoxal at pH 7.2 where covalent reaction does not take place. Experiments at 0 and 25°C indicated that the phenylglyoxal permeability was too high to be determined with the Millipore filtering method of Dalmark and Wieth (1972), with complete equilibration of the isotope being attained in <1–2 s at both temperatures. Further studies were kindly performed by Dr. J. Brahm with a rapid flow method (Brahm, 1977). These experiments demonstrated that the half-time of isotope equilibration was 40 ms at 25°C, corresponding to a membrane permeability of 10^{-3} cm $\cdot$ s^{-1}. Identical permeabilities were found in exchange experiments and under conditions where there was a net efflux of 15 mM phenylglyoxal from the ghosts. Phenylglyoxal transport across the membrane was not affected by 0.1 mM DIDS, causing 99.6% inhibition of chloride exchange. This demonstrates that the reagent is not transported by the anion exchange mechanism. Because of the rapid intracellular equilibration, the phenylglyoxal concentration of the medium will be reduced by 10% immediately after the addition of 4 ml packed ghosts to the 36 ml phenylglyoxylation medium. Only these corrected concentrations are used in the Results section.

Membrane Permeability to Hydroxyl Ions

Phenylglyoxalation of erythrocyte membranes was carried out as described above by exposing resealed ghosts with an intracellular pH of 7.2 for brief periods to phenyl-

glyoxal in a very alkaline medium. The reaction of arginyl residues with phenylglyoxal is highly pH dependent, with increasing reactivity at higher pH (Cheung and Fonda, 1979 *b*). Studies with radioactivy phenylglyoxal indicated that even at 38°C and pH 11 the membrane barrier to hydroxyl ion transfer was sufficient to prevent labeling of the predominant intracellular membrane protein, spectrin, which reacts readily if the intracellular pH is alkaline (Bjerrum et al., 1982). The experiments of Fig. 6 demonstrated that phenylglyoxalation of the anion transport system can be carried out at extracellular pH values below pH 11.5 at 25°C. More extensive membrane modification was observed at higher extracellular pH values, where the resealed ghosts became slightly permeable even to hemoglobin. Under such conditions leak pathways are formed that prevent examination of the specific inhibition of the anion transport system.

It should be noted that anion exchange is inhibited efficiently when the ghosts are exposed to phenylglyoxal at alkaline pH values (cf. Fig. 1), because the reversible inhibition caused by phenylglyoxal is independent of the covalent binding of phenylglyoxal to the membranes. Therefore, hydroxyl ion transfer cannot be mediated by chloride-bicarbonate exchange through the so-called Jacobs-Stewart cycle (Jacobs and Stewart, 1942). The reversible inhibition of anion exchange by, for example, 15 mM phenylglyoxal is as complete as the transport inhibition in DIDS-treated cells where the hydroxyl influx at 38°C amounts to 10^{-12} $mol \cdot cm^{-2} \cdot s^{-1}$ at pH values between 10 and 11 (Wieth et al., 1980). The corresponding influx of base per liter of ghosts (cell volume 10^{-13} liter, membrane area 1.42×10^{-6} cm^2) is ~0.9 mmol per min. The ghosts have a buffer capacity of 3.5 mmol $(pH \cdot l\ ghosts)^{-1}$, so the intracellular pH will therefore increase only ~0.3 units during the first minute of incubation. Studies with ^{14}C-labeled phenylglyoxal have confirmed that intracellular pH remains sufficiently low during the first minutes of exposure to prevent modification of the interior arginyl residues of the membrane (Bjerrum et al., 1982).

The importance of the inhibition of chloride-bicarbonate exchange became evident when we tried to phenylglyoxalate membranes at phenylglyoxal concentrations that are too low to inhibit anion exchange reversibly. Gradual membrane destruction causing partial to total hemolysis was observed when the phenylglyoxal concentration was lowered below 9 mM, where the reversible inhibition of anion exchange is insuffient to prevent the bicarbonate-mediated hydroxyl ion transfer. It should be noted that only traces of bicarbonate are needed to accelerate the hydroxyl ion transport significantly if the anion exchange is working (Jacobs and Stewart, 1942), and that no special precautions were taken to keep our electrolyte solution out of contact with the CO_2 of the atmosphere.

RESULTS

Reversible Inhibition of Chloride Exchange by Phenylglyoxal

It is essential to distingish between reversible and irreversible effects of phenylglyoxal on membrane anion transport. The rate of chloride transport in resealed ghosts at 0°C is independent of pH between 7.2 and 10.8 (Funder and Wieth, 1976). When ghosts are exposed to phenylglyoxal, anion transport is instantaneously and reversibly inhibited when exposure occurs at 0°C. The extent of inhibition depends on the phenylglyoxal concentration and pH. Fig. 1 A shows the pH dependence of the reversible inhibition of chloride exchange at 0°C by 10 mM phenylglyoxal. Anion transport is inhibited 50% at pH values up to 8.5, whereas at higher pH values the extent of inhibition increases.

At an extracellular pH of 10.4, the highest value examined, 95% inhibition is observed with 10 mM phenylglyoxal. The extent of reversible inhibition is dependent on the reagent concentration. Fig. 1 B shows the dose-response curve for phenylglyoxal at 0°C and pH 10. The relationship between reversible inhibition and phenylglyoxal concentration is sigmoidal (Fig. 1 B), with a Hill coefficient of 1.7 (Fig. 1 C). Inhibition was found to be reversible when resealed ghosts were incubated for 5 min at pH 10.3 in the 165-mM KCl medium containing 15 mM phenylglyoxal. After four washes in a phenylglyoxal-free medium (pH 7.2), chloride exchange fluxes reverted to control values.

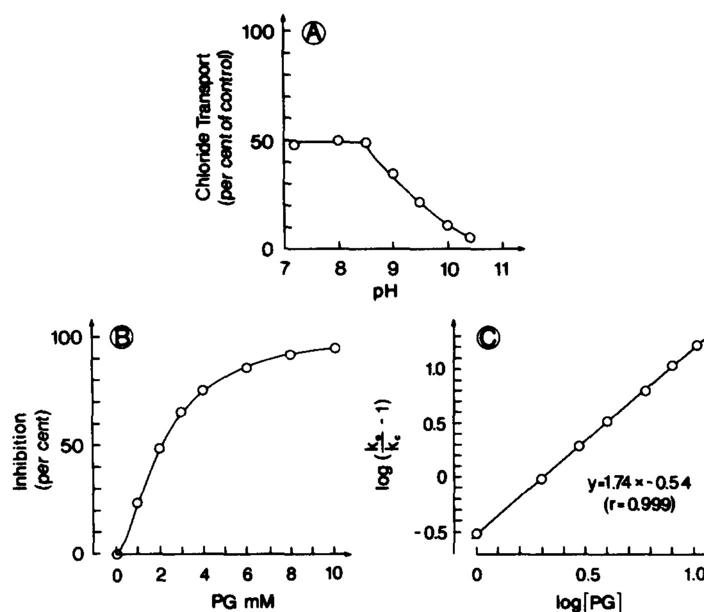

FIGURE 1. Reversible inhibiton of chloride self-exchange by phenylglyoxal at 0°C. Resealed ghosts, intracellular KCl 165 mM, pH 7.2. (A) Chloride self-exchange as a function of extracellular pH in the presence of 10 mM phenylglyoxal. Transport is expressed as a percentage of a control subjected to the same conditions, but in the absence of phenylglyoxal. (B) Chloride self-exchange at 0°C and pH 10 as a function of phenylglyoxal (PG) concentration. The relationship of chloride flux to phenylglyoxal concentration is sigmoidal. (C) Hill plot of data from part B indicates a positive cooperativity for phenylglyoxal inhibition with a Hill coefficient of 1.7. k_o is the rate coefficient for chloride efflux in the absence of phenylglyoxal and k_c is the corresponding inhibited rate at a given concentration of phenylglyoxal.

The reversible effect on facilitated membrane transport processes is not limited to anion exchange. Glucose self-exchange in resealed ghosts was examined at 40 mM D-glucose. 50% inhibition was found at a phenylglyoxal concentration of 4 mM in experiments carried out at 0°C, pH 10. The curve relating inhibition to inhibitor concentration was sigmoidal with a Hill coefficient of 1.5 (experiments not shown).

Irreversible Inhibition of Chloride Exchange with Phenylglyoxal

The anion transport system can be irreversibly inhibited by phenylglyoxal under conditions that favor the covalent binding of phenylglyoxal to groups that are exposed to the extracellular medium. Phenylglyoxal permeates the membrane rapidly with a permeability coefficient of 10^3 cm·s^{-1} at 25°C (cf. Methods section). To achieve a reproducible irreversible inactivation of ion transport, it was necessary to prevent phenylglyoxal from reacting with the arginyl residues exposed to the inside of the membrane. Because the rate of reaction increases rapidly with increasing pH (cf. Fig. 4), optimum conditions were procured by exposing resealed ghosts with a neutral intracellular pH only briefly to the alkaline phenylglyoxal-containing media.

Determination of the Rate of Inactivation

The dependence of irreversible inhibition of chloride exchange on the duration of exposure to phenylglyoxal is illustrated in Fig. 2. The resealed erythrocyte ghosts were exposed to 18 mM phenylglyoxal at pH 10 and 38°C for periods ranging from 3 to 90 s (Fig. 2 A). The reaction of the membranes with phenylglyoxal was stopped at various intervals by diluting and cooling the sample with a cold acidic phenylglyoxal-free electrolyte medium as described in the Methods section. Next the cells were washed repeatedly with 165 mM KCl medium (pH 7.2) to remove all unreacted phenylglyoxal. It was verified that the phenylglyoxalation was truly irreversible under the conditions used, for inactivation was constant independent of the number of washings with albumin-containing medium. It was also found that the degree of inhibition was unchanged after phenylglyoxal-treated ghosts were washed and incubated at 0°C for 24 h at a low hematocrit (<10%) in the presence of 1% albumin.

Fig. 2 A shows that the exchange flux of the resealed ghosts gradually approaches a residual value that amounts to about 10% of the control flux of the unexposed cells. This value was found after both 90 and 150 s exposure (the 150-s value is not shown on the graph). The residual flux (J_∞) after prolonged exposure to phenylglyoxal was independent of the extracellular conditions of phenylglyoxalation. The mean value from a large number of experiments was 9.1% (SEM 0.3, n = 20).

Fig. 2 B shows that the inactivation of anion transport proceeded as an apparent first-order reaction. The logarithmic ordinate shows the relative fluxes (after subtraction of J_∞) as a function of the duration of phenylglyoxal exposure. The pseudo first-order rate coefficient (s^{-1}) is given by the slope of the function in Fig. 2 B. In the experiment shown in Fig. 2 this rate coefficient was 0.10 s^{-1}, which indicates that inactivation of 50% of the inhibitable anion flux was achieved in ~7 s.

The kinetics of inactivation of anion transport under various conditions was studied by the method illustrated in Fig. 2. It was found that the rate of inactivation of the anion exchange depends on phenylglyoxal concentration, pH, temperature, anion composition of the medium, and the presence of some reversibly binding inhibitors of anion transport.

Phenylglyoxal Concentration

At a given pH and temperature, the rate of inactivation of the anion transport system was a linear function of the phenylglyoxal concentration. Fig. 3 A shows the rate of inactivation of chloride exchange in a 165-mM KCl medium

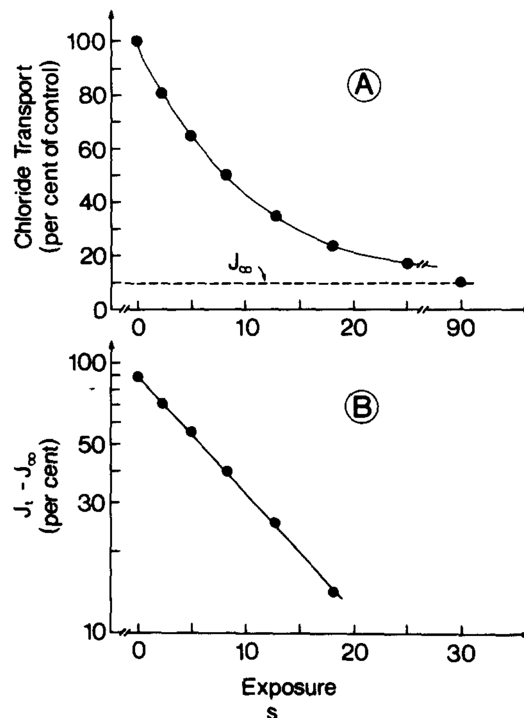

FIGURE 2. Rate of irreversible inactivation of the chloride exchange system. (A) The fractional chloride exchange flux as a function of duration of exposure of resealed ghosts to 18 mM phenylglyoxal, 38°C, pH 10 in the KCl medium. Phenylglyoxalation was interrupted by diluting, cooling, and acidifying samples at the time indicated on the abscissa. The chloride self-exchange fluxes were determined at 0°C, pH 7.2, as described by Funder and Wieth (1976) and expressed in percent of the exchange flux of an untreated control specimen. The residual flux after extensive treatment (J_∞) was 10% of the control flux (J_o) and the inhibition was not increased after 150 s exposure. The inhibitable flux ($J_o - J_\infty$) is therefore 100 − 10 = 90%. (B) The irreversible inactivation was an apparent first-order reaction at constant phenylglyoxal concentration. The results are from the same experiment as shown in A. The scale of the ordinate is logarithmic. Inactivation proceeded with a rate coefficient of 0.1 s^{-1}, as determined by linear regression analysis of the slope of ln ($J_t - J_\infty$) vs. duration of phenylglyoxalation. The chloride self-exchange flux of the control cells was 280 $pmol \cdot cm^{-2} \cdot s^{-1}$.

as a function of phenylglyoxal concentration at 38°C and an extracellular pH of 10. There was no indication of "saturation" of the reaction at the highest concentrations. The reaction could not be studied at phenylglyoxal concentrations below 9 mM because the membranes were wrecked by the reaction of

intracellular membrane proteins with phenylglyoxal that takes place when the pH gradient across the membrane is dissipated during prolonged treatment at low phenylglyoxal concentrations. Fig. 3 B shows that the inactivation of anion exchange by phenylglyoxal is a second-order reaction with a rate coefficient of 5.2 $l \cdot mol^{-1} \cdot s^{-1}$ (SE 0.11, $n = 8$) at pH 10 and 38°C.

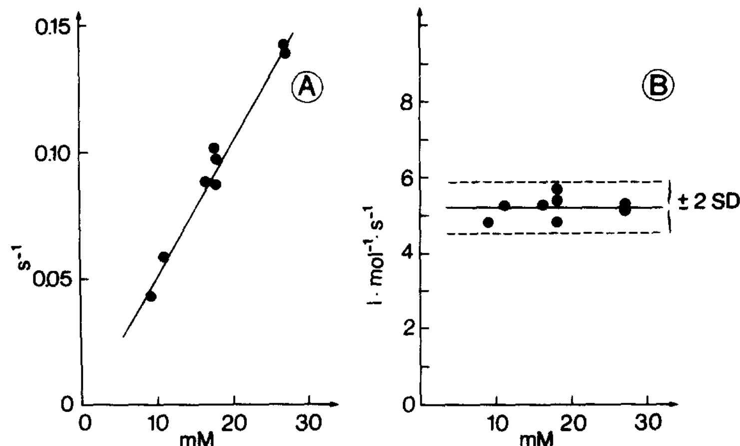

FIGURE 3. Phenylglyoxal concentration dependence of the rate of inactivation of anion transport (38°C, pH 10, KCl 165 mM, CHES 5 mM). The phenylglyoxal concentrations indicated on the abscissa are the concentrations after addition of ghosts to the reaction medium. (A) The apparent first-order rate coefficient of phenylglyoxalation (s^{-1}) as a function of phenylglyoxal concentration. The rate coefficients were determined in experiments like that illustrated in Fig. 2. The relation between the rate coefficient of phenylglyoxalation (k, s^{-1}) and phenylglyoxal concentration (C, mM) was $k = 0.0052\ C - 0.0006$ ($r = 0.991$). (B) The second-order rate coefficient (k/C liter $\cdot mol^{-1} \cdot s^{-1}$) did not vary with phenylglyoxal concentration.

TABLE I

TEMPERATURE DEPENDENCE OF ANION TRANSPORT INACTIVATION BY PHENYLGLYOXALATION

Phenylglyoxal	pH	°C	Rate of inactivation	Second-order rate coefficient
mM			s^{-1}	$l \cdot mol^{-1} \cdot s^{-1}$
13.5	10.3	38	0.150 (0.005)	11.1
13.5	10.3	30	0.037 (0.001)	2.7
13.5	10.3	25	0.0165 (0.0003)	1.2

Phenylglyoxalation was carried out in the 165-mM KCl medium. The apparent first-order rate coefficient of inactivation (s^{-1}) was determined by linear regression analysis (viz. Fig. 2). Standard deviations of the slopes are stated in parentheses.

Temperature Dependence

The rate of inactivation of chloride transport by phenylglyoxal decreased 10-fold when the temperature was lowered from 38 to 25°C (Table I), which corresponds to an apparent Arrhenius activation energy of 33 $kcal \cdot mol^{-1}$.

However, as dealt with in the Discussion section, the marked temperature dependence of the reaction partly reflects the change of ionization of the reacting guanidino group with decreasing temperature.

Dependence of Extracellular pH

The second-order rate coefficient of inactivation increases with increasing pH (Fig. 4 A). In a 165-mM KCl medium, the rate of transport modification at 38°C increased almost 10-fold for a one unit increase of pH below 10, but the slope decreased somewhat at higher pH values (Fig. 4 B). As discussed below, we believe that phenylglyoxal reacts preferentially with the electrically neutral form of a guanidino group. The fraction of uncharged residues increases proportionally with the hydrogen ion concentration at low pH values, but decreases when the p*K* of the group is approached. The data of the figure are in agreement with the assumption that the reacting group has an apparent p*K* ~11.5 at 38°C in the presence of 165 mM chloride.

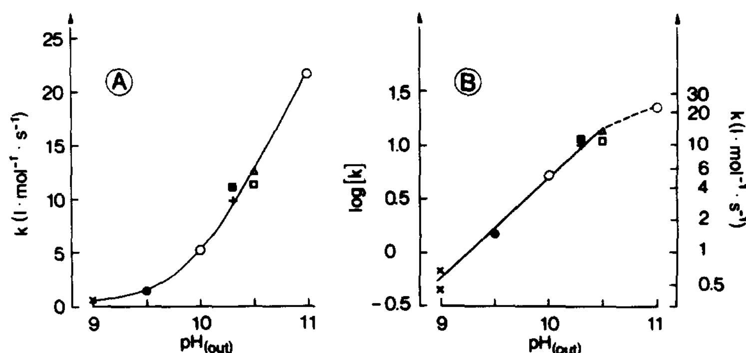

FIGURE 4. Dependence of the rate of phenylglyoxal inactivation of anion exchange on extracellular pH, 38°C, (KCl 165 mM, phenylglyoxal 9–27 mM). (A) The second-order rate coefficient, *k* (liter·mol^{-1}·s^{-1}) as a function of extracellular pH. The open circle at pH 10 represents the mean value of the eight experiments shown in Fig. 3. (B) log *k* was a linear function of the extracellular pH below 10.3.

Dependence on Extracellular Anions

The rate of inactivation of the anion transport system was found to be influenced by the chloride concentration of the extracellular medium. Fig. 5 A shows that the rate increases with decreasing extracellular chloride concentration. In one set of experiments, extracellular chloride was gradually substituted with the isotonic sucrose-citrate medium, maintaining the intracellular KCl concentration at 165 mM. In other experiments, the KCl concentrations were varied symmetrically on both sides of the membrane. Identical rates of transport inactivation were found at similar extracellular chloride concentrations under both sets of conditions, which shows that the rate of reaction is not affected by the presence of an anion gradient across the membrane. These experiments were performed at 25°C, because the rate of

inactivation at 38°C and low chloride concentrations was too rapid to permit a precise determination. The rate constant at 5 mM chloride in Fig. 5 A corresponds to a second-order rate coefficient of 10.4 $1 \cdot mol^{-1} \cdot s^{-1}$. The corresponding value at 38°C was in two experiments found to be ~80 $liters \cdot mol^{-1} \cdot s^{-1}$, inactivating 50% of the anion transport in ~0.6 s at a phenylglyoxal concentration of 13.5 mM. The dependence of the rate of inactivation on extracellular chloride is depicted in the Dixon diagram shown in Fig. 5 B. The experimental results were well described by the empirical equation:

$$k^{-1} = 0.328\ Cl_o^- + 5.418\ (r = 0.99),$$

where k^{-1} is the reciprocal first-order rate coefficient of transport inactivation at the phenylglyoxal concentration of 13.5 mM, Cl_o^- is the extracellular

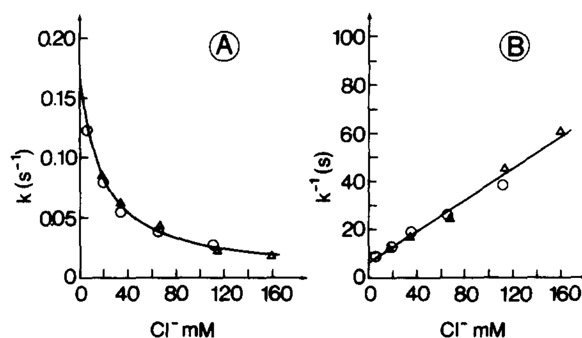

FIGURE 5. Dependence of the rate of phenylglyoxal inactivation of anion exchange on extracellular chloride concentration. All reactions were carried out at 25°C, pH 10.3, phenylglyoxal 13.5 mM. Two sets of experiments are shown; 165 mM KCl ghosts reacted in mixtures of KCl and sucrose-citrate media (○); ghosts and media with identical KCl concentrations (△). (A) The apparent first-order rate coefficient (k, s^{-1}) as a function of the extracellular chloride concentration during phenylglyoxalation. (B) Dixon diagram of the reciprocal rate coefficient (k^{-1}, s) vs. extracellular chloride concentration during phenylglyoxalation. The relation found by linear regression analysis is stated in the text.

chloride concentration (mM), and r is the correlation coefficient of linear regression. It is apparent that the rate of phenylglyoxalation is very sensitive to the chloride concentration, half-maximum rate being found in the presence of 16.5 mM chloride. In experiments with 165-mM KCl ghosts we found that the rates of phenylglyoxalation at extracellular iodide concentrations of 16.5 and 33 mM were the same as those found at the corresponding chloride concentrations (experiments not shown). We performed these experiments because iodide has been reported to have a sevenfold higher apparent affinity for transport than chloride (Dalmark, 1976). However, Milanick and Gunn (1981 *a*) have recently reported in a preliminary communication that the transport system shows no selectivity towards extracellular halides in the presence of a steep anion gradient across the membrane. It will therefore be necessary to study the protective effect of iodide in experiments with identical

iodide concentrations on the two sides of the membrane before it can be concluded that the halides exert identical effects on the rate of phenylglyoxalation.

At first sight one might think that the pronounced chloride effect reflects the affinity for the site that is modified by phenylglyoxal, the site being protected by its normal "transport substrate." However, our results do not show any increased protective effect of chloride when the apparent affinity of chloride for transport is increased 15-fold at asymmetrical chloride distribution (Gunn and Fröhlich, 1979). We have recently shown that chloride exchange depends on the protonation of a titratable group with an apparent pK of 12 at 0°C (Wieth and Bjerrum, 1982). The apparent pK decreased proportionally to the logarithm of the extracellular chloride concentration, and the effect of extracellular chloride was independent of the intracellular chloride concentration and of the substitution of chloride with iodide. By analysis of the titration data we found that the change of apparent pK with anion concentration was not likely to be caused by chloride binding to the titratable group (Wieth and Bjerrum, 1982). The pK shifts were alternatively interpreted to be caused by the effect of the halides on a positive interfacial surface potential, determining the hydrogen and hydroxyl ion concentrations at the titratable site. In view of the many similarities it is worth considering whether the titratable group is identical with the group that causes inactivation of chloride transport after binding phenylglyoxal. If we are dealing with a side group of an arginyl residue with a pK of 12 at 0°C, the pK should decrease by ~0.8 pH units when temperature is increased to 25°C, considering the ionization enthalpy of 12–13 $kcal \cdot mol^{-1}$ of a guanidinium group (Cohn and Edsall, 1943). The pH dependence shown in Fig. 4 supports the idea that phenylglyoxal reacts readily with the deprotonated form of a titratable group with a pK of ~11.5 in a 165-mM chloride medium at 38°C. Therefore, we have investigated whether the data of Fig. 5 fit the alternative interpretation, namely that the variation of the rate of reaction with chloride concentration is due to the effect of the small inorganic anions on the apparent pK of a titratable group. Fig. 6 shows that the pH dependence of the rate of phenylglyoxal inactivation of anion transport at 2 mM extracellular chloride resembles a titration curve reaching a maximum rate of ~31 $l \cdot mol^{-1} \cdot s^{-1}$ at pH values above 11. The apparent pK can be estimated if it is assumed that phenylglyoxal reacts with deprotonated groups only, because the rate of reaction will be half-maximal when 50% of the groups are dissociated. Thus determined, the apparent pK was 10.2 at an extracellular chloride concentration of 2 mM, and increased to 10.7 and 11.8 at 8 and 165 mM extracellular chloride, respectively. The increase of apparent pK with extracellular chloride concentration resembles that found by direct titration of the transport system with hydroxyl ions (Wieth and Bjerrum, 1982). According to our previous interpretation, the apparent pK is changed because a positive surface potential in an interfacial region of the transport protein is lowered when the concentration of chloride is raised. There is no reason to believe that inorganic anions bind to the electrically neutral form that reacts with phenylglyoxal, so chloride ions do not directly

"shield" the titratable group towards phenylglyoxal modification. The variation of apparent pK with extracellular chloride concentration in the experiments of Fig. 5 has been calculated in Table II. The calculation rests on the assumption that the relative rate of phenylglyoxalation is a measure of the degree of dissociation of the titratable group. Thus calculated, the apparent pK was found to increase from 10.6 at 5 mM chloride to 11.7 at an extracellular chloride concentration of 160 mM.

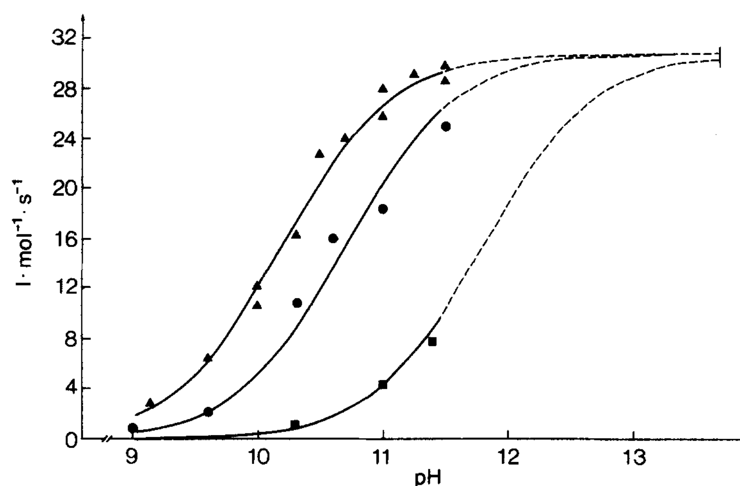

FIGURE 6. The pH dependence of the rate of irreversible inactivation of the anion transport system by phenylglyoxal at extracellular chloride concentrations of 2 (▲), 8 (●), and 165 mM (■). Resealed ghosts were treated with phenylglyoxal at 25°C in the appropriate sucrose-citrate and KCl media, and the rate of inactivation was determined by following the time-course of transport inhibition (cf. Fig. 2). At the lowest chloride concentration the rate of reaction approached a maximal rate coefficient of 31 liter·mol^{-1}·s^{-1} above pH 11. The experimental values were fitted to simple titration curves with pK values of 10.2, 10.7, and 11.8, and the maximum rate stated above.

TABLE II

CHLORIDE DEPENDENCE OF THE APPARENT pK VALUES

Extracellular chloride	Rate of phenylglyoxalation (k)	Relative rate of phenylglyoxalation	Apparent pK
mM	$l \cdot mol^{-1} \cdot s^{-1}$	k/k_{max}	
5	10.5	0.34	10.6
20	6.2	0.20	10.9
35	4.4	0.14	11.1
65	2.8	0.09	11.3
112	1.8	0.06	11.5
160	1.3	0.04	11.7

Dependence of apparent pK values on extracellular chloride concentration. The apparent pK values were calculated from the experiments of Fig. 5 (pH 10.3, 25°C). The relative rate of phenylglyoxalation (k/k_{max}) was calculated using a maximum rate coefficient for the reaction (k_{max}) of 31 l·mol^{-1}·s^{-1}, cf. Fig. 6. The apparent pK values were calculated with the assumption that the relative rate of phenylglyoxalation represents the fraction of deprotonated titratable groups at pH 10.3.

The rate of inactivation of the anion transport system was not affected by the presence of sulphate. Fig. 7 illustrates the inactivation of chloride transport in resealed ghosts as a function of the duration of the exposure to phenylglyoxal in a 110 mM K_2SO_4 medium containing 13.5 mM phenylglyoxal at pH 10.3. The reaction was carried out at 25°C, i.e., under the same conditions as the experiments shown in Fig. 5. The pseudo first-order rate coefficient of inactivation in the sulphate medium was 0.17 s^{-1}, identical with that found in a citrate sucrose medium at a low chloride concentration (viz. Fig. 5). Because the rate coefficient of transport inactivation in a 300-mM sucrose solution under otherwise identical conditions of reaction was 0.16 s^{-1} (data not shown), it appears that neither sulphate nor citrate affects the rate of reaction.

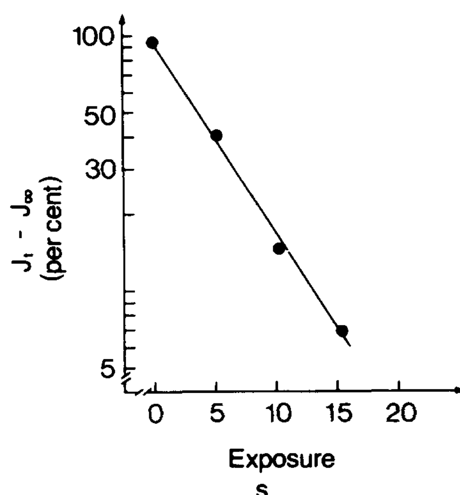

FIGURE 7. Rate of inactivation of the anion transport system by exposure of resealed ghosts to 13.5 mM phenylglyoxal in the presence of extracellular sulfate. Phenylglyoxalation was carried out at 25°C, pH 10.3, in a chloride-free medium containing 110 mM K_2SO_4, 5 mM CHES. The apparent first-order rate coefficient of transport inactivation was 0.17 s^{-1}, identical with the value predicted in the absence of chloride from the results of Fig. 5.

Interactions with Reversible Inhibitors of Anion Transport

To determine whether various reversible inhibitors of anion transport interfere with the phenylglyoxal reaction, we examined the rate of transport inactivation by phenylglyoxal in the sucrose-citrate medium in the presence of the compounds listed in Table III. Whenever possible, the inhibitor concentrations were more than 20 times the value of the apparent inhibitor constants (K_i) reported in the literature. The qualitative results listed in Table III showed that the rate of phenylglyoxalation was only significantly affected by DNDS, salicylate, and trinitrocresolate. The results of Table IV show that the rate of inactivation decreased 10-fold at a salicylate concentration of 2.5 mM, whereas in the presence of 10 mM salicylate the rate of inactivation was only 3% of the rate in the absence of inhibitor.

The effect of DNDS was subjected to closer examination. Fig. 8 illustrates the rate of inactivation of anion exchange at DNDS concentrations between 0 and 500 μM. The rate of transport inactivation by phenylglyoxal was halved at a DNDS concentration of 150 μM. The results compiled in Fig. 9 show that

TABLE III

RETARDATION OF PHENYLGLYOXALATION BY REVERSIBLE INHIBITORS OF ANION EXCHANGE

Inhibitor	Inhibitor concentration during phenylglyoxalation	K_i^{app}	Protection towards phenylglyoxalation
	M	*M*	
Phloretin	5×10^{-5}	2×10^{-6}	0
Phlorizin	5×10^{-3}	2×10^{-4}	0
Dipyridamole	5×10^{-5}	1×10^{-6}	0
Tetrathionate	1×10^{-2}	5×10^{-4}	0
Niflumic acid	1×10^{-5}	6×10^{-7}	0
Furosemide	1×10^{-3}	2×10^{-4}	0
Bumetanide	1×10^{-3}	4×10^{-4}	0
Salicylate	2.5×10^{-3}	1×10^{-3}	+
Trinitrocresolate	1×10^{-4}	5×10^{-6}	+
DNDS	1×10^{-3}	5×10^{-6}	+

Ghosts were exposed to 13.5 mM phenylglyoxal for periods sufficient to inactivate the anion exchange by 75% in a sucrose-citrate medium at 25°C, pH 10.3, in the presence of the inhibitors listed in the table. After removal of excess phenylglyoxal and inhibitor by washing, chloride self-exchange was determined. Three of the inhibitors offered partial or complete protection against inactivation of the anion transport by phenylglyoxylation. The K_i^{app} values were taken from the compilation by Knauf (1979), except the values for bumetanide and DNDS, which are our own unpublished values determined at 0°C, pH 7.2, in the 165-mM KCl medium.

TABLE IV

EFFECT OF SALICYLATE ON THE RATE OF TRANSPORT INACTIVATION BY PHENYLGLYOXAL

Salicylate	Phenylglyoxal	Rate of inactivation	Second-order rate coefficient
mM	*mM*	s^{-1}	$l \cdot mol^{-1} \cdot s^{-1}$
0	13.5	0.121 (0.004)	9.0
2.5	13.5	0.011 (0.0005)	0.8
10	13.5	0.0039 (0.00002)	0.29

Resealed ghosts (intracellular KCl 165 mM, pH 7.2) were phenylglyoxalated in sucrose-citrate media (pH 10.3, 25°C) containing 5 mM chloride and salicylate as shown. The apparent first-order rate coefficient of transport inactivation was determined with the technique illustrated in Fig. 2. Standard deviations of the rate coefficients (determined by linear regression analysis) are stated in parentheses.

a 20-fold reduction of the rate of inactivation was found at a DNDS concentration of 2 mM. It should be noted that these results were obtained in the chloride-free sucrose-citrate medium. In the presence of 165 mM chloride it was not possible to demonstrate any effect of DNDS on the rate of the

inactivation by 13.5 mM phenylglyoxal, as shown in Table V. In another experiment, ghosts were treated with 27 mM phenylglyoxal in the presence of 165 mM KCl at pH 10.3 and 38°C. The second-order rate coefficients of inactivation were 9.9 and 10.7 liter·mol^{-1}·s^{-1} in the presence and absence,

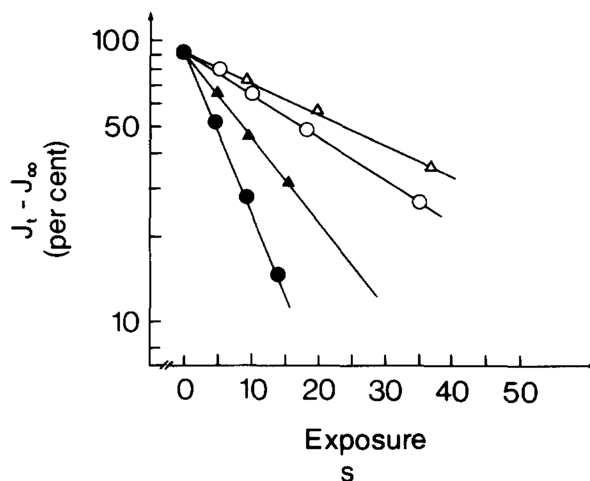

FIGURE 8. Effect of DNDS on the rate of inactivation of the anion transport system by exposure of resealed ghosts to phenylglyoxal in a sucrose-citrate medium. Phenylglyoxalation was carried out at 25°C, pH 10.3, in the chloride-free sucrose-citrate medium containing 9 mM phenylglyoxal and the following DNDS concentrations: 0 mM (●), 0.15 mM (▲), 0.3 mM (○), and 0.5 mM (△).

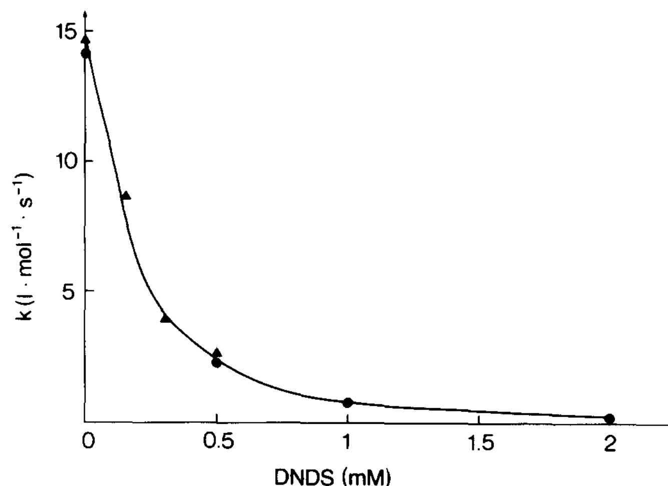

FIGURE 9. Effect of DNDS on the rate of inactivation of the anion transport system by exposure of resealed ghosts to phenylglyoxal in a sucrose-citrate medium. The figure shows the second order rate coefficients (liter·mol^{-1}·s^{-1}) from two experiments performed in media containing 9 mM phenylglyoxal (▲), and 13.5 mM phenylglyoxal (○). Phenylglyoxalation was carried out at 25°C, pH 10.3.

respectively, of 1 mM DNDS, underlining that DNDS has no major protective effect against phenylglyoxalation in the presence of high concentrations of chloride.

It seems likely that the similarities of the effects of chloride and DNDS concentrations on the reaction shown in Fig. 5 and 9 have a common cause. The results of the following section show that covalent binding of DIDS, a diisothiocyano-derivate closely related to DNDS, is delayed when the ghosts have been pretreated with phenylglyoxal, which indicates that at least the covalent reaction of DIDS with the transport protein in a 165-mM KCl medium is affected by previous phenylglyoxal modification.

TABLE V

LACK OF EFFECT OF DNDS ON THE RATE OF TRANSPORT INACTIVATION BY PHENYLGLYOXAL IN THE PRESENCE OF 165 mM CHLORIDE

DNDS	Phenylglyoxal	Rate of inactivation	Second-order rate coefficient
mM	*mM*	s^{-1}	$l \cdot mol^{-1} \cdot s^{-1}$
0	13.5	0.150 (0.008)	11.1
1	13.5	0.131 (0.004)	9.7
2	13.5	0.144 (0.006)	10.7

Resealed ghosts (intracellular KCl 165 mM, pH 7.2) were treated with phenylglyoxal in the 165-mM KCl medium (pH 10.3, 38°C) containing DNDS as shown. The apparent first-order rate coefficient of transport inactivation was determined with the technique illustrated in Fig. 2. Standard deviations of the rate coefficients (determined by linear regression analysis) are stated in parentheses.

The Nature of the Residual Flux

Maximum inhibition of anion transport after phenylglyoxal treatment never exceeds 93–94%, even when the duration of phenylglyoxal exposure of the ghosts is extended to 10 or 20 times the period necessary to inactivate 50% of the transport function. Similar observations have been made with intact erythrocytes. There are at least two possible explanations. One is that a small fraction of transport sites are not chemically modified and continue to function after the phenylglyoxal treatment. Another possibility is that the modified transport system continues to transport anions, but at a rate that is only 5–10% of that of the intact system.

Studies of the effect of DIDS binding on the residual transport in phenylglyoxal-treated membranes showed that the first explanation seems to be the right one. Phenylglyoxal-treated resealed ghosts with a transport capacity that was 12% of the control value were treated with graded amounts of DIDS. Table VI compares the effect of DIDS binding on chloride exchange in phenylglyoxal-treated and untreated red cells ghosts. Complete inhibition of anion exchange in intact membranes is achieved by the binding of $\sim 1 \times 10^6$ DIDS molecules per cell, and it can be seen in Table VI that the binding of 1.95×10^5 molecules per cell in fact caused an inhibition of 18% in the normal

ghosts. In contrast, 50% of the residual flux in the phenylglyoxal-treated membranes was eliminated after the binding of only 6.5×10^4 DIDS molecules per cell. Only ~1–2% of the control flux was retained in phenylglyoxal-treated ghosts that had bound 1.3×10^5 DIDS molecules. The results indicate that there is a small fraction of anion transport sites (1×10^5–1.5×10^5 per cell) that are still functioning after extensive phenylglyoxal treatment, and that these remaining sites are inhibited by treatment with DIDS.

The results of Table VI also show that the residual DIDS-binding sites react much more readily with DIDS than sites that have previously reacted with phenylglyoxal. If all sites, functioning or not, bound DIDS at the same rate,

TABLE VI

EFFECT OF IRREVERSIBLE DIDS BINDING ON CHLORIDE EXCHANGE IN NORMAL AND PHENYLGLYOXAL-TREATED ERYTHROCYTE GHOSTS

Number of DIDS molecules bound per ghost	Chloride self-exchanged flux	
	Untreated membranes	Phenylglyoxal-treated membranes
	$pmol \cdot cm^{-2} \cdot s^{-1}$	
0	338	40.5
6.5×10^4	306	20.3
1.3×10^5	285	5.6
1.95×10^5	277	1.0

Untreated and phenylglyoxal-treated ghosts were exposed to the number of DIDS molecules shown in the table for 30 min at 38°C, pH 7.4. Binding of 1.3×10^5 DIDS molecules eliminates almost 90% of the residual flux in phenylglyoxal treated membranes, whereas binding of 1.95×10^5 DIDS molecules eliminates only 18% of the chloride exchange flux in the ghosts that have not been pretreated with phenylglyoxal. A mean volume of 92 μm^3 was used for the calculation of the exchange fluxes.

it would not have been possible to achieve almost complete inhibition with only 10–15% of the number of DIDS molecules that can react with band 3 in the intact membrane. Therefore, our results show that covalent binding of phenylglyoxal delays or prevents the subsequent covalent binding of DIDS, permitting DIDS to react preferentially with the small number of anion transport sites that are still functioning after extensive phenylglyoxal treatment. Studies are in progress to characterize the kinetics of the binding of DIDS to phenylglyoxylated membranes (Bjerrum et al., 1982).

Specificity of Phenylglyoxal Inactivation of Anion Transport

It has already been mentioned that the facilitated diffusion processes of anions and of glucose are both reversibly inhibited by phenylglyoxal at low temperatures (*vide supra*). It has been reported that the glucose transport system of the red cell membrane can be irreversibly inactivated by reaction with butanedione in a borate-containing medium, a reaction that is very specific

for arginyl residues (Krupka and Deves, 1980). We have therefore examined whether irreversible inactivation of the chloride transport system by phenylgloxal is accompanied by irreversible inhibition of the glucose transport system. As shown in Table VII, this was found not to be the case. Resealed erythrocyte ghosts were loaded with 40 mM D-glucose. The presence of glucose did not affect the rate of inactivation of the anion transport system. While the anion transport system was rapidly inactivated under these conditions, significant inhibition of hexose transport was found only after prolonged exposure to phenylglyoxal. In the experiment shown in Table VII, exposure to 22 mM phenylglyoxal for 90 s caused a 90% reduction of chloride exchange and only a 16% decrease in glucose transport.

TABLE VII

EFFECT OF PHENYLGLYOXALATION ON CHLORIDE AND GLUCOSE EXCHANGE FLUXES

Duration of exposure to phenylglyoxal	Chloride exchange flux		Glucose exchange flux	
	$10^{12}\cdot mol\cdot cm^{-2}\cdot s^{-1}$			
s		*% of control*		*% of control*
0	294	100	105	100
5.9	166	57	107	102
9.6	128	44	99	95
13.6	86	29	95	90
90	31	10	88	84

Resealed erythrocyte ghosts (intracellular KCl 165 mM, D-glucose 40 mM, pH 7.2) were exposed to 22 mM phenylglyoxal in the 165-mM KCl medium at 38°C, pH 10. After phenylglyoxalation each sample was divided in half. One half was loaded with ^{36}Cl and used for determination of chloride exchange at 0°C, pH 7.2; the other half was labeled with [^{14}C]glucose and used for the determination of glucose exchange at 20°C, pH 7.2. Both flux media contained 40 mM D-glucose.

DISCUSSION

Our results show that the red cell anion transport system can be irreversibly modified by phenylglyoxal in a time-dependent, irreversible chemical reaction. Inactivation of transport without destruction of the membranes is attained when resealed erythrocyte ghosts with a neutral intracellular pH are exposed to phenylglyoxal in an alkaline extracellular medium. We have found that the inhibition can be achieved by the binding of two phenylglyoxal molecules per molecule of band 3 (Bjerrum et al., 1982). Because the reaction with a guanidino group has been found to involve two phenylglyoxal molecules (Takahashi, 1968), it is likely that transport inactivation can be caused by the modification of a single arginine side chain in the anion transport protein.

Surprisingly, phenylglyoxal exhibits two modes of inhibition. Anion transport is inhibited in a fully reversible manner at low temperatures, whereas irreversible binding of phenylglyoxal and irreversible inhibition of transport occur only when the membranes are exposed to phenylglyoxal in an alkaline extracellular medium at higher temperatures. Two observations suggest that

the reversible inhibition and irreversible inactivation of the transport system are unrelated processes. First, at 0°C a reversible inhibition of 95% (Fig. 1) is observed with 10 mM phenylglyoxal at pH 10, which suggests that the inhibitory sites are nearly saturated, while the rate of irreversible inactivation increases linearly when phenylglyoxal concentration is raised from 10 to 30 mM (Fig. 3), making it unlikely that the irreversible inactivation is preceded by specific reversible binding. Second, the reversible inhibition at pH 10 is characterized by a positive cooperativity for phenylglyoxal, with a Hill coefficient of 1.7 (Fig. 1), whereas the irreversible inactivation is first order in phenylglyoxal (Fig. 2). Thus the reversible inhibition by phenylglyoxal at 0°C probably does not involve interaction of the reagent with arginyl residues of the transport system, but is rather a nonspecific phenomenon related to similar reversible inhibition exerted by a number of related amphiphilic organic molecules like the acetophenone derivatives studied by Motais and Cousin (1978). Here we will limit the discussion to the indirect evidence from our kinetic studies that suggests that the irreversible modification of anion transport results from the reaction of membrane arginyl residues with phenylglyoxal.

Phenylglyoxal as a "Specific" Arginine Reagent

Phenylglyoxal is highly specific for the covalent modification of arginyl residues in proteins (Takahashi, 1968). Bovine pancreatic ribonuclease A was inactivated to the extent of 80–90% when two arginines located close to the active center of the enzyme were reacted with phenylglyoxal (Takahashi, 1968). The specificity of the reaction with guanidino groups has since been confirmed in a number of enzyme studies where phenylglyoxal has been used as a tool for the identification of arginines playing an essential role for catalytic function (e.g., Borders and Riordan, 1975; Berghäuser, 1975; Armstrong et al., 1976; Cheung and Fonda, 1979*a*; Borders and Johansen, 1980). It has become increasingly clear that anion binding sites for negatively charged substrates and co-factors in most enzymes involve positively charged guanidino groups of arginyl residues (Riordan et al., 1977), and it has been proposed that the side chain of arginine plays a general biological role for anion recognition in proteins (Riordan, 1979).

The kinetics of facilitated diffusion bear many resemblances to the kinetics of an enzyme-catalyzed reaction. The transport protein catalyzes the vectorial translation of the "transport substrate" like the enzyme catalyzes the transformation of a substrate into its product. Our suspicion that an arginyl residue may be an essential functional component of the anion transport system arose from studies of the pH dependence of chloride exchange in resealed human erythrocyte ghosts (Funder and Wieth, 1976). Anion transport was unaffected by an increase of pH from 7.2 to 10.8, although most positively charged protein groups become deprotonated in this pH interval. If chloride translocation depends on the interaction with a positive charge in the transport system, a guanidino group of an arginine, with a typical pK_a of ~12, is therefore a likely candidate. In the preceding article we have demonstrated

the existence of a titratable membrane group with an apparent pK_a of ~12, which is essential for chloride transport (Wieth and Bjerrum, 1982). We found a completely reversible inhibition of anion transport in a 165-mM chloride medium when the extracellular pH was elevated from 11 to 13 at 0°C. Together with the present evidence derived from the kinetics of irreversible phenylglyoxalation of the transport system, the titration data bear testimony to the possibility that the integrity of one or more arginines is essential for normal functioning of the anion transport system.

Since the appearance of the first report on phenylglyoxalation of arginine in enzymes (Takahashi, 1968), it has been realized that phenylglyoxal can react slowly with amino acid residues other than arginine. The only documented reports of the modification of amino acids other than arginine by phenylglyoxal comprise slow reactions with α-amino groups in ribonuclease A (Takahashi, 1968) and with a highly reactive cysteine in rhodanese (Weng et al., 1978). The evidence that the protein modification studied in this work is the result of the reaction of phenylglyoxal with membrane arginines is indirect, but strong. The pH dependence for phenylglyoxal inactivation of erythrocyte anion transport (Figs. 4 and 6) makes it highly unlikely that α-amino groups are being modified. Sulfhydryl (SH) groups do not seem to play a functional role for inorganic anion transport. Typical SH reagents do not inhibit anion transport (Passow and Schnell, 1969; Knauf and Rothstein, 1971; Deuticke, 1977), nor, do they interfere with the action of other inhibitors (Knauf and Rothstein, 1971). Our main arguments for believing that we are dealing with a specific reaction with arginyl residues are based on the fact that the kinetics of membrane modification share a number of characteristic features with the kinetics of phenylglyoxalation of well-characterized biomolecules, where the location of the reacting arginine is known with certainty. The similarities apply both to the rate and order of reaction, the pH dependence, and the ability of anionic "substrates" and inhibitors to protect the target molecules against inactivation by phenylglyoxal.

Rate and Order of the Phenylglyoxal Reaction

Phenylglyoxal inactivates many anion-binding enzymes in a second-order reaction like that illustrated in Fig. 3. Although phenylglyoxal reacts by a second-order process, it is still remarkably selective for the exclusive modification of essential arginyl residues at enzyme active sites. Many enzymes can be fully inactivated by modification of a single arginyl residue per enzyme protomer or subunit (Borders and Riordan, 1975; Berghäuser, 1975; Armstrong et al., 1976; Kantrowitz and Lipscomb, 1976; Philips et al., 1979; Cheung and Fonda, 1979 *a*; Borders and Johansen, 1980). The selectivity of the reagent towards the functionally essential arginyl residue is surprising because all the argingyl residues are potentially reactive. An important general factor for the reactivity of the essential arginines may be their typical location in a hydrophobic environment, which favors the phenylglyoxal reaction at a given pH by lowering the pK of the reactive guanidino group.

At constant phenylglyoxal concentration, the irreversible inactivation of

anion transport follows apparent first-order kinetics, as shown in Figs. 2, 7, and 8. The finding that 50% inactivation can be attained in 0.5 s at 38°C in a chloride-free medium certainly places the reaction studied by us among the most rapid phenylglyoxal reactions reported in the literature.

Dependence on pH and Temperature

We found that the rate of transport inactivation by irreversible binding of phenylglyoxal increased by a factor of 60 from pH 9 to 11 (Fig. 4). This is very similar to the pH dependence of the phenylglyoxalation observed by Cheung and Fonda (1979 *b*) for the modification of L-arginine and the inactivation of glutamate apodecarboxylase (Cheung and Fonda, 1979 *a*). Our results also agree with their conclusion that the rate of reaction increases with the increased concentration of deprotonated guanidino groups, presumably because the reaction with phenylglyoxal is a nucleophilic addition reaction in which only the uncharged guanidino group reacts rapidly with phenylglyoxal. The pH dependence of the reaction adds further support to the notion that we are dealing with a titratable group with a pK >11 at 38°C in a 165-mM KCl medium (Fig. 4).

We have found no previous studies of the effect of temperature on the rate of reaction with arginine. The apparent Arrhenius activation energy calculated from the reaction rates at 25–38°C in the present study was ~33 kcal·mol^{-1}. However, it must be noted that the pK_a of a guanidinium group increases with decreasing temperature. Therefore, the rate of reaction decreases partly because the fraction of uncharged groups decreases when the temperature is lowered. The ionization enthalpy for the guanidinium group of arginine is 12–13 kcal·mol^{-1} (Cohn and Edsall, 1943), and the pK_a will accordingly increase by ~0.4 pH units when the temperature is lowered from 38 to 25°C, as in the experiments of Table I. If it is accepted that pK values can be estimated from the rates of the chemical reaction, reasonable values of about 11.4 (38°C) and 11.8 (25°C) can be deduced from the results of Figs. 4 and 6. As discussed below, the modified group may be identical with a functionally essential group, which can be titrated with hydroxyl ions at 0°C (Wieth and Bjerrum, 1982). This group had an apparent pK of 12 at 0°C in a 165-mM KCl medium. The value is ~0.6 pH unit lower than a value extrapolated from the above results, assuming a heat of ionization of 12 kcal·mol^{-1}. This may of course mean that the two sets of groups are not identical. On the other hand, it must be realized that the discrepancy can be due to the different methods used for determining pK, or to a change of the physical properties of the environment of the protonated group. If the region becomes more hydrophobic when the temperature is lowered, this will tend to lower the pK of a positively charged group.

Effects of Anions and Anion Transport Inhibitors

The protective effect of chloride on the phenylglyoxal modification of the anion transport system (Fig. 5, Table II) seems similar to the effects of anionic substrates and co-factors on the rate of phenylglyoxalation of essential argi-

nines in enzymes, as for instance the protection offered creatinine kinase by the binding of ATP or ADP (Borders and Riordan, 1975). However, it is not certain that the protection is a direct shielding effect due to anion binding. According to the titratable carrier model, anion binding may shift the equilibrium between protonated and deprotonated groups (Gunn, 1972, 1973), thereby increasing the apparent p*K* of the anion binding group, and thus decreasing the rate of phenylglyoxalation. Taken alone, this might explain the protective effect of chloride, although it would imply that the intrinsic p*K* is surprisingly low for a guanidino group. We believe it is fortuitous that the chloride concentration causing half-maximal inhibition of the rate of phenylglyoxalation (Fig. 5) corresponds rougly to the apparent half-saturation of chloride transport found in experiments where chloride is varied on both sides of the membrane (Wieth, 1972; Gunn et al., 1973; Brahm, 1977). The rate of phenylglyoxalation was reduced by 50% by 16 mM extracellular chloride independent of the intracellular chloride concentration. The establishment of a transmembrane chloride gradient, which increases the apparent affinity of chloride for the transport system 15-fold (Gunn and Fröhlich, 1979) had no effect on the rate of phenylglyoxal binding. Therefore, we must conclude that the apparent p*K* is not affected by the condition that favors recruitment of transport sites toward the external side of the membrane (Jennings, 1980; Knauf et al., 1980).

Dalmark (1976) observed that the apparent affinity of iodide for transport is seven times larger than that of chloride. Iodide protection of phenylglyoxalation was not more pronounced than that of chloride, but this finding may be inconclusive after the recent report by Milanick and Gunn (1981 *a*) that the apparent affinities of the two ions become identical in the presence of a steep transmembrane anion gradient. It will therefore be necessary to study the rate of phenylglyoxalation in ghosts exposed to identical iodide concentrations at the two sides of the membrane to decide whether the effects of chloride and iodide on the rate of phenylglyoxalation are in fact identical. In our titration study, chloride and iodide were found to exert identical effects on the apparent p*K* of a titratable group with a p*K* of ~12 (Wieth and Bjerrum, 1982). The effects of the extracellular anions on the p*K* were independent of the intracellular anion concentration. Therefore, we concluded that the halides may affect an interfacial surface potential, which determines the local hydrogen ion concentration (and accordingly the apparent p*K*) of the titratable group. The results presented in Fig. 6 are compatible with this hypothesis, which predicts that the apparent p*K* of the reacting group decreases with extracellular chloride concentration. In Fig. 6 and Table II we have analyzed our results with the assumption that the rate of the chemical reaction is determined solely by the degree of dissociation of the reacting group. This may be an oversimplification, although the qualitative results seem clear: the apparent p*K* decreased from 11.8 to 10.2 when the extracellular chloride concentration was lowered from 165 to 2 mM. The surface potential is believed to be large and positive in the absence of anions that can permeate into the charged region of the transport protein. At high chloride concentrations where

the surface potential is low it can be little affected by DNDS, explaining why DNDS has no effect on the rate of phenylglyoxalation at an extracellular chloride concentration of 165 mM (Table V). On the other hand, DNDS has a strong effect on the rate of reaction when extracellular chloride concentration is low as in the experiments of Figs. 8 and 9.

The trivalent citrate anion cannot permeate the transport protein. This is in agreement with the observation that citrate does not inhibit the transport of chloride or bicarbonate at alkaline pH values (Gunn and Fröhlich, 1979; Wieth, 1979). Provided this interpretation involving citrate is accepted, it follows that sulfate is also excluded from the transport protein at an extracellular pH of 10.3, because the rate of phenylglyoxalation in a sulfate medium (Fig. 7) is as rapid as in the presence of citrate or sucrose. This is surprising, because there is heavy evidence that chloride and sulfate ions are transported by the same transport system, and both transport processes react very similarly to inhibitors of anion transport (Ku et al., 1979). Sulfate fluxes are orders of magnitude smaller than chloride fluxes, and, moreover, it is evident that different side chains of the transport protein are involved in the translocation of mono- and divalent ions. The clearest evidence is provided by the differing pH profiles of the transport processes. Jennings (1976) has shown that sulfate exchange takes place as a co-transport with protons. The proton donating group has a pK of ~5.2 (Milanick and Gunn, 1981 *b*), and Schnell (1972) has found that sulfate exchange in ghosts becomes minimal at pH values above 8, where chloride exchange in ghosts is maximal (Funder and Wieth, 1976). Therefore, it seems possible that sulfate does not have access to the essential arginine when all potential proton donors with a low pK_a (<8) of the region are deprotonated. We have not yet studied the effect of phenylglyoxal treatment on sulfate transport. However, a recent report by Zaki (1981), showing that sulfate transport is irreversibly inhibited after membrane modification with 1,2-cyclohexanedione, certainly suggests that arginyl residues are essential for the divalent anion transport at lower pH values.

If one accepts as a working hypothesis that the rate of phenylglyoxalation is determined by the local hydrogen ion concentration, which can be manipulated by varying the chloride and iodide concentration, it remains to be explained why the protective effects of DNDS, trinitrocresolate, and salicylate are so much stronger than that of the inorganic anions. DNDS is a nonpermeating inhibitor that binds competitively with inorganic anions to the substrate site for anion translocation (Barzilay and Cabantchik, 1979). A similar tentative conclusion has been made with regard to the site for the inhibitory action of the anionic phenol derivatives (Knauf, 1979). Structure-activity studies of organic inhibitor molecules suggest that affinity for binding increases with the lipophilicity of the inhibitors and with the ability of substituent groups to act as electron acceptors (Motais and Cousin, 1978). This may explain why the effects of the organic inhibitors on the rate of phenylglyoxalation cannot be predicted from simple electrostatic considerations, as may be the case for chloride and iodide. Possible steric effects must be taken into account. The chemical properties attracting the aromatic anions

to the transport region may assist in keeping the inhibitor anions bound, even when the guanidino group is deprotonated, thus blocking the access of phenylglyoxal to the reactive group.

We have found no evidence that the reactivity of the arginyl residues is affected by conditions that favor recruitment of transporters towards the external side of the membrane (Jennings, 1980). Our findings make it unlikely that we are studying modification of arginyl residues that are alternately exposed to the extra- and intracellular aqueous phases. This does not exclude the possibility that the modified arginyl residue(s) reside in a region of the transport protein that changes its conformation when an anion is translocated. For instance, the nonconducting anion gate proposed by Passow et al. (1980) contains several positively charged residues that change their relative positions due to a conformational change without shifting their location with regard to the inside and outside mouths of the gate.

Phenylglyoxal binding interferes with the covalent binding of DIDS, which suggest that the two inhibitors may bind in the same region. The results of Table VI demonstrate that DIDS does not bind rapidly to band 3 molecules that have already been reacted with phenylglyoxal. The covalent DIDS binding site of band 3 had recently been shown to be a lysyl residue located in a small intramembrane fragment (Ramjeesingh et al., 1980, 1981). Our preliminary results indicate that DIDS and phenylglyoxal bind to different chymotryptic fragments of band 3, contributing to the evidence that the transport region is composed by more than a single strand of the band 3 polypeptide (Bjerrum et al., 1982).

Specific and Nonspecific Effects of Phenylglyoxalation

The present work represents our first step towards the development of a specific method for phenylglyoxalation of selected arginyl groups in the anion transport system. The first important discovery made was that some degree of specific labeling can be obtained when a pH gradient across the membrane prevents internal arginines from reacting. The red cell membrane proteins contain $\sim 150 \times 10^6$ arginyl residues per cell. The results of another article demonstrate that it has been possible to improve the conditions for the specific phenylglyoxalation so that maximum inhibition of anion transport can now be achieved after modification of only $\sim 10^6$ arginyl residues per cell. These improvements have been based on the observation that the various arginyl residues that can be labeled from the external side of the membrane react at differing rates. Maximum specificity towards band 3 has been obtained by means of the very rapid phenylglyoxalation occurring in the absence of external chloride (Bjerrum et al., 1982).

Our studies of the effect of phenylglyoxalation on the facilitated diffusion of glucose (Table VII) illustrate that inhibition of anion exchange is rather specific even under nonoptimum conditions. However, we have postponed a detailed study of the permeability properties of phenylglyoxal-treated membranes until the specificity of the reaction has been increased as much as possible. In preliminary experiments we have observed that treatment of the

membranes with phenylglyoxal increases the passive permeability of the membranes to sodium and potassium. This observation may mean that the positive guanidino charge plays a role both for the translocation of anions, and for the maintenance of the permeability barrier towards monovalent cations. It has previously been shown that salicylate affects the permselectivity of the red cell membrane, inhibiting anion exchange as well as promoting monovalent cation permeability (Wieth, 1970 *a* and *b*). The finding that salicylate interferes so efficiently with the rate of phenylglyoxalation of alleged arginyl residues of the anion transport protein (Table IV) and that phenylglyoxalation also affects the selective ionic permeability of the membrane invites further investigations. In previous work, (Wieth, 1970 *a* and *b*), the effects of lyotropic anions were interpreted in the light of the fixed-charge hypothesis of Passow (1969). It was assumed that the maintenance of a low cation permeability depends on the integrity of fixed positive charges that may also be involved in anion transport. The development of methods of specific chemical modification of membranes may make it possible to investigate whether one or a few arginyl residues in band 3 play such an essential role for the permselectivity of the red cell membrane.

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