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Action of 1-Fluoro-2,4-Dinitrobenzene on Passive Ion Permeability of the Human Red Blood Cell

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Summary. Dinitrofluorobenzene (DNFB) inhibits the penetration of anions such as sulfate, phosphate, succinate, and lactate, and facilitates the penetration of cations such as K^+ and Na^+. The phlorizin-glucose insensitive fraction of erythritol permeability is not affected by the agent. The effects of DNFB on ion permeability are similar to those of more specific amino reactive agents like trinitrobenzene sulfonate and 2-methoxy-5-nitrotropone.

Anion permeability reacts more sensitively to DNFB than cation permeability. At a given concentration of DNFB in the medium, the inhibition of anion permeability develops faster than the facilitation of cation permeability. At a given time of exposure, lower concentrations of DNFB are required to produce a nearly maximal response of anion permeability than are necessary for maximal effect on cation permeability.

The response of anion and cation permeability to DNFB is augmented by increasing the pH at which dinitrophenylation is allowed to take place.

DNFB binding to the cell membrane is about one order of magnitude lower than DNFB binding to the whole cell. In the cell membrane, proteins as well as lipids are dinitrophenylated. Among the lipids, only phosphatidylethanolamine binds significant amounts of DNFB. Phosphatidylserine does not seem to react with the agent under the experimental conditions under which DNFB produces its effects on ion permeability.

The experimental results are compatible with the assumption that removal of uncharged NH_2-groups by dinitrophenylation of the membrane leads to a concomitant reduction of fixed NH_3^+-groups and hence of the positive membrane charge. This leads to an acceleration of cation movements and an inhibition of anion permeability while nonelectrolyte permeability remains unaffected. However, other explanations of our observations cannot be ruled out.

Addition of dinitrofluorobenzene (DNFB) to human red blood cells induces cation leakage (Berg, Diamond & Marfey, 1965; Passow, 1969*a*) and inhibition of anion permeability (Passow, 1969*a*). DNFB can be expected to interact with a variety of functional groups: -SH, $-NH_2$, imid-

azole, and phenolic-OH. In addition, dinitrophenylation of side chains should lead to alterations of the tertiary structure of proteins. All conceivable DNFB binding sites abound in the red blood cell membrane. Nevertheless, the effect of the agent on ion permeability is attributed to interactions with amino groups. It is assumed that DNFB removes uncharged amino groups from the equilibrium $R-NH_3^+ = R-NH_2 + H^+$ by the formation of $(NO_2)_2-C_6H_3-NH-R$. This reaction would be associated with a lowering of the concentration of fixed positive charges in the membrane. For electrostatic reasons, a concomitant increase of the concentration of diffusible cations and a decrease of the concentration of diffusible anions should occur in the ion permeable regions of the cell membrane. Such concentration changes could give rise to the observed inverse changes of the penetration rates for cations and anions.

In spite of the significance which has been attributed to the effects of DNFB in recent assessments of the function of fixed charges in the control of passive ion permeability (Passow & Schnell, 1969), no detailed description has been published so far of the conditions under which this agent produces its effects on the red cell membrane. The present paper serves to supply some of this information and gives a more detailed account of the number and chemical nature of the sites of binding and the mode of action of the agent.

Materials and Methods

All experiments were performed with freshly drawn human blood obtained from healthy donors. Citrate was used as an anticoagulant. The cells were washed twice in isotonic saline. After removal of the buffy coat, the sedimented cells were used for the experiments.

Measurement of Cation Movements

If not expressly stated otherwise, the erythrocytes were dinitrophenylated by incubation in the presence of DNFB at 37 °C. The agent was added in alcoholic solution to cell suspensions in isotonic saline buffered with sodium phosphate to pH 7.4. The ethanol concentration in the final medium amounted to maximally 1.0%. The hematocrit was usually 5%. The ensuing effect on potassium and sodium permeability was followed by centrifuging measured samples at suitable time intervals, dissolving the sedimented cells in distilled water and determining their potassium and sodium content by means of flame photometry. In a number of experiments, the cells were first dinitrophenylated in media in which 100 mmoles/liter of the isotonic sodium chloride solution were replaced by 100 mmoles/liter of potassium chloride solution. In the presence of KCl in the medium, no driving force existed for a net movement of K^+ out of the cells. Hence, dinitrophenylation could be performed in the absence of significant potassium movements. At the end of the desired reaction time, the cells were centrifuged and resuspended in buffered saline. Thus, the driving force for net K^+ and Na^+ movements was reestablished and, by following the change of the amounts of K^+ and Na^+ in a constant number of red

blood cells, it was possible to determine the effect which DNFB produced prior to the flux measurements. Except in the experiments which were designed to test the influence of sucrose on DNFB induced changes, all media contained 20 mmoles/liter of sucrose.

Sodium and potassium were determined by flame photometry. Intracellular sodium was calculated after correction for extracellular sodium trapped between the sedimented cells (Passow, 1969*b*). Hemolysis was measured photometrically at 540 nm after converting hemoglobin into cyanmethemoglobin.

Measurement of Anion Movements

(a) '*Back*' *Exchange.* The cells were incubated at 37 °C in a medium ('sulfate medium') which contained 10 mmoles/liter Na_2SO_4, 151 mmoles/liter NaCl, 20 mmoles/liter sucrose, and a trace of $^{35}SO_4$. After 90 min, when the anion distribution between cells and medium had attained the Donnan equilibrium, alcoholic DNFB solution was added to the suspension and the incubation was continued for another period of time (=preincubation period). The ethanol concentration never exceeded 1.0%. In every experiment, controls were run with ethanol but without DNFB. Although ethanol is, in principle, capable of inhibiting anion permeability, the concentrations employed in the present work were found to be low enough to produce no significant effects. At the end of the preincubation period, the cell suspensions were centrifuged, washed once in ice cold sulfate medium without DNFB and without $^{35}SO_4$, but otherwise identical with the sulfate medium employed for suspending the cells during the preincubation period. Washing at the end of the preincubation period was essential for the removal of unreacted DNFB and its breakdown product, dinitrophenol. If washing was performed at a temperature of 4 °C or less, replacement of labeled sulfate medium by unlabeled sulfate medium was accompanied by little if any loss of radioactivity from the $^{35}SO_4$ loaded cells. After washing, the cells were resuspended in fresh medium which was prewarmed to 37 °C ($t=0$ of the back exchange) and the appearance of radioactivity in the supernatant was followed as described by Lepke and Passow (1971). The final cell density was 2.5%. The described procedure ensured that $^{35}SO_4$ efflux was measured in the absence of significant net sulfate movements.

(b) '*Forward*' *Exchange.* In a number of experiments, the entry of $^{35}SO_4$ from the medium into the cells was followed. The cells were first equilibrated with a sulfate medium of the composition described above at 37 °C for 90 min until the Donnan equilibrium was established. Subsequently, alcoholic DNFB solution was added and the incubation continued for another two hr. Thereafter, the flux measurement was initiated by the addition of a trace of $^{35}SO_4$. In contrast to the back exchange experiments, that fraction of the agent which did not react with the cells before the start of the experiment (i.e., before $^{35}SO_4$ was added), continued to react with the cells while the radioactivity penetrated across the membrane. However, although the binding of DNFB still proceeded, the effect of DNFB on anion permeability was already maximally developed. The maximum of the effect was reached before the radioactive sulfate was added to the medium and did not significantly increase during the flux measurements. Further details of the experimental procedure were described by Gardos, Hoffman, and Passow (1969). The hematocrit was 40%.

Erythritol Permeability

Erythritol movements were measured as described by LaCelle and Passow (1971). The method is essentially similar to that used for the measurement of sulfate back exchange in the present work.

In all experiments with $^{35}SO_4$ or ^{14}C-erythritol, the time course of appearance or disappearance of radioactivity in the supernatant followed a single exponential. The rate

constant (dimension: min^{-1}) was estimated by the method of least squares. The computer program was designed by Dr. Martin Pring of the Johnson Foundation, University of Pennsylvania, Philadelphia.

Determination of DNFB Binding to the Cell Membrane

DNFB binding to the membrane was determined by the following procedure: Small volumes of ^{14}C-DNFB in ethanol were added to phosphate buffered cell suspensions (10 vol%). In suitable time intervals, a measured volume of the suspension was diluted in the ratio 1:4 with suspension medium containing no DNFB, and was centrifuged down. The sediment was washed once in DNFB-free medium and finally resuspended to give a 25 vol% suspension in isotonic Tris solution containing 1% saponin, pH 8.0.[1] 1.0 ml of the hemolyzed suspension was transferred to a centrifuge tube (28 ml capacity) containing three layers of sucrose solution. The sucrose concentrations amounted to (from top to bottom) 0.500, 0.585, 0.750 moles/liter. All three sucrose solutions were buffered with 166 mmoles/liter Tris to pH 7.4. The hemolyzed blood suspension was carefully placed on top of the uppermost layer of sucrose. Subsequently, the tubes were centrifuged at $90{,}000 \times g$ in a swing out rotor for 12 min. Using this procedure, it was possible to collect quantitatively pure white ghosts of a measured volume of cell suspension at the bottom of the centrifuge tube. The hemoglobin is largely retained in the original hemolysate on top of the sucrose solutions. Some of the hemoglobin mixes with the first, sometimes also with the second layer of sucrose. However, the sediment contains no detectable trace of hemoglobin.

The radioactivity of the sediment was measured after dissolving the sedimented ghosts in 3.0 ml glacial acetic acid and mixing with 7.0 ml methanol-toluene (4:6, v/v) scintillation fluid containing 4.0 g/liter of 2,5-diphenyloxazole (PPO) and 0.050 g/liter of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP).

Lipids were extracted by the method of Reed, Scott, Swisher, Marinetti, and Eden (1960) from sucrose centrifuged ghosts. Care was taken to remove traces of protein from the lipid extracts by washing with KCl solutions. In some experiments, the washes with KCl of the extracts were preceeded by washes with N/10 HCl and N/10 NaOH solutions. Counting of ^{14}C was done in a liquid scintillation counter as described above after dissolving the dried lipid extract in glacial acetic acid. Quenching was determined by means of an internal standard.

We obtained ^{14}C-DNFB from Amersham, Eng. In thin layer chromatograms, nearly all of the radioactivity moved with the unlabeled DNFB, the rest with traces of 2,4-dinitrophenol, a decomposition product of DNFB. In one batch, about 1.5% of the radioactivity was neither DNFB nor dinitrophenol. The chemical nature of the contaminant is not known. Unlabeled DNFB was obtained from Sigma Corp. The product contained some 2,4-dinitrophenol. This contamination was insignificant for the present purpose.

Cell concentrations were determined by means of a Coulter counter.

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1 Saponin removes little if any ^{14}C from the membrane of ^{14}C-dinitrophenylated red blood cells. This was shown as follows: Ghosts were prepared by the osmotic lysis method of Dodge, Mitchell and Hanahan (1962) from ^{14}C-dinitrophenylated red blood cells. These ghosts were exposed to saponin and then centrifuged through sucrose. About 5% of the ^{14}C activity remained in the supernatant. This was similar to the amount left behind by ghosts which were centrifuged under the same conditions without prior exposure to saponin. The release of the small quantity of ^{14}C from intact and saponin-treated ghosts was probably caused by the dissolution of some of the water soluble proteins of the red blood cell membrane in the supernatant.

Chemical Methods

Dinitrophenylation of Erythrocytes. Red blood cells, washed twice with isotonic saline, were suspended in an equal volume of 6.0 mmoles/liter DNFB in isotonic saline (containing 1% ethanol). They were incubated at 37 °C for two hr, separated by centrifugation from the DNFB solution and washed twice with isotonic saline. The pH was 7.0.

Dinitrophenylation of Erythrocyte Ghosts. To 3 ml of red cell ghosts (Dodge, Mitchell & Hanahan, 1962) 25.5 mg NaCl and 0.75 ml potassium phosphate buffer (0.1 mole/liter, pH 7.2) were added. The suspension was mixed with 4.48 mg DNFB dissolved in 0.04 ml ethanol and incubated at 37 °C. After two hr, the lipids were extracted by the method of Reed *et al.* (1970). At the end of the incubation period, the pH of the suspension was 6.9.

Dinitrophenylation of Erythrocyte Lipids. Ten mg of red cell lipids were emulsified in 10 ml saline (containing 0.02 moles/liter potassium phosphate, pH 7.2) by sonicating the mixture for 15 min in a Branson Sonifier B-12. Then 11.2 mg DNFB, dissolved in 0.1 ml ethanol, were added and the emulsion was incubated at 37 °C for two hr. At the end of the reaction, the lipids were extracted three times with chloroform/methanol (1:1, v/v).

Dinitrophenylation of Phosphatidylethanolamine. 12 μmoles phosphatidylethanolamine, dissolved in 3 ml chloroform/ethanol (1:1, v/v) were incubated with 50 μmoles DNFB and 0.02 ml triethylamine for two hr at 37 °C. After evaporation of the solvents, DNP-phosphatidylethanolamine was purified by thin layer chromatography in chloroform/methanol/acetone and eluted with chloroform/methanol (1:1, v/v).

Lipid extraction was performed according to Reed *et al.* (1960). *Phosphatidylethanolamine* was isolated from normal erythrocytes by thin layer chromatography of the lipid extract in chloroform/methanol/water. Phosphatidylethanolamine and phosphatidylserine did not separate and were both eluted with chloroform/methanol (1:1, v/v). They were separated by thin layer chromatography in chloroform/methanol/25% NH_3. R_f of phosphatidylserine = 0.2; R_f of phosphatidylethanolamine = 0.39. *Thin layer chromatography* was performed on glass plates (20 × 20 cm) layered with Kieselgel H (Merck) in the following solvent mixtures: chloroform/methanol/water = 13:6:1 (v/v/v); chloroform/methanol/acetone = 4:2:1 (v/v/v + 2.5% water); *n*-propanol/25% NH_3 = 7:3 (v/v); chloroform/ethanol = 2:1 (v/v); ethylacetate/chloroform/methanol = 1:2:1 (v/v/v); chloroform/methanol/25% NH_3 = 12:4:1 (v/v/v).

Phosphate was determined according to Bartlett (1959). DNP-ethanolamine was estimated by measuring the extinction at 350 nm of a solution of DNP-phosphatidylethanolamine in chloroform. The amount of DNP-ethanolamine present was calculated by comparison with a standard curve prepared from pure DNP-ethanolamine. DNP-phosphatidylethanolamine was hydrolyzed in 6 N HCl for 20 hr. The acid was evaporated and DNP-ethanolamine extracted with ethylacetate.

Results

Cation Permeability

Incubation of human red blood cells in isotonic saline containing DNFB induces potassium loss, sodium uptake, and, eventually, hemolysis. The changes in cation permeability are preceeded by a lag period whose length decreases as the DNFB concentration in the medium is raised (Fig. 1 a, b, d).

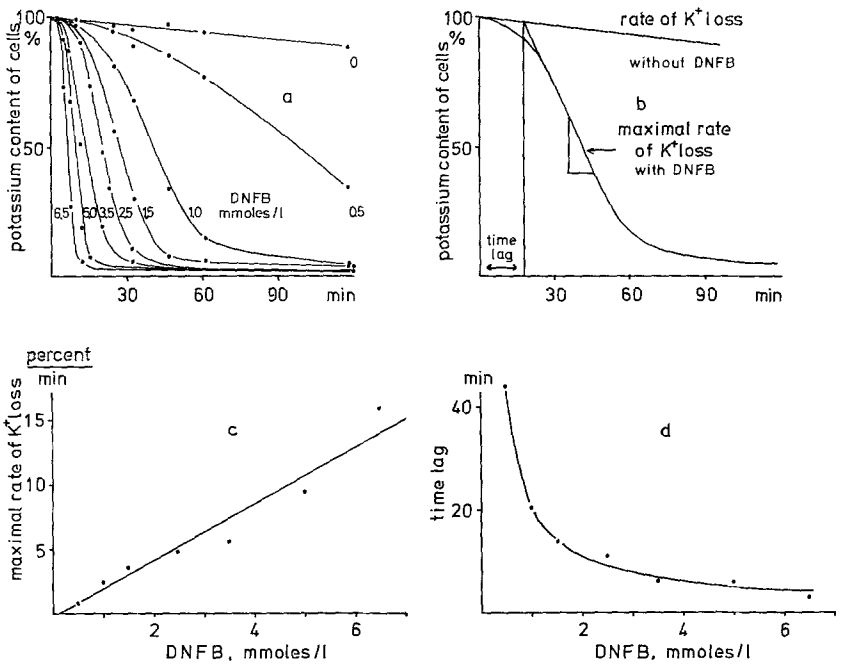

Fig. 1. (a) Effects of increasing concentrations of DNFB on K^+ content of human red blood cells. At the start of the experiment, the cells were suspended in a medium containing 50 mmoles/liter Na_2HPO_4, 91 mmoles/liter NaCl, 20 mmoles/liter sucrose, and varying concentrations of DNFB. The changes in cellular K^+ were followed. The pH in the final suspension was 7.8, except at the highest DNFB concentration where it dropped to 7.52. Hematocrit: 5%. 37 °C. *Ordinate:* Potassium content in percent of initial value. *Abscissa:* time in minutes;

(b) Illustration of the method for determining time lag and maximal rate of K^+ loss from the cells. *Ordinate:* Potassium content in percent of initial value. *Abscissa:* time in minutes;

(c) Maximal rate of K^+ loss as a function of DNFB concentration in the medium. *Ordinate:* rate of K^+ loss in percent/min. *Abscissa:* DNFB concentration in mmoles/liter; and

(d) *Ordinate:* lag period preceding the onset of K^+ loss in minutes. *Abscissa:* DNFB concentration in mmoles/liter

The maximal slopes (Fig. 1 b) of the curves which relate potassium content of the cells to time increase with increasing DNFB concentration in the medium. At high DNFB concentrations, the rate of loss becomes too high to be accurately measurable by our methods. Nevertheless, in spite of a fairly large scatter of the estimates, the available data suggests that the maximal slopes do not reach a limiting value within the range of DNFB concentrations employed in our experiments.

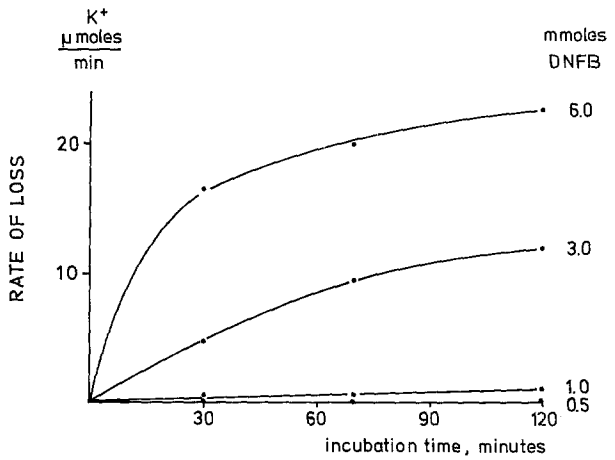

Fig. 2. Rate of potassium loss of cells which were exposed to 0.5, 1.0, 3.0 and 6.0 mmoles/liter DNFB for various lengths of time prior to the flux measurements. Cells were dinitrophenylated in media containing 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose and the DNFB concentrations indicated on the curves. Cell density: 10 vol%. 37 °C. At the times indicated on the abscissa, aliquots were transferred to 0 °C and subsequently centrifuged. After removal of the supernatant, the sedimented cells were resuspended in a medium containing 166 mmoles/liter NaCl and 20 mmoles/liter sucrose. The ensuing net potassium efflux was followed. From the curves relating K^+ efflux to time the initial slopes were taken as a measure of the action of DNFB. *Ordinate:* rate of K^+ loss as determined from the initial slopes in µmoles/g cells/min. *Abscissa:* time of preincubation at 37 °C in the presence of the DNFB concentrations indicated on the curves

Since the cation movements were measured while the agent was still reacting with the membrane, the ion distribution between cells and the medium may approach equilibrium before chemical modification of the membrane is complete. Hence, the measured rates of cation movements cannot be attributed to definite states of modification. To avoid this ambiguity, in another series of experiments, the cells were first exposed for a predetermined length of time to the action of the agent in a high potassium medium whose cation composition was at equilibrium with the cation composition in the cells. In this medium, no driving forces exist and hence chemical alterations of the permeability barrier cannot lead to changes of intracellular cation composition. The reaction between DNFB and erythrocytes was stopped by washing the cells in their original suspension medium without DNFB. Subsequently, the packed cells were resuspended in isotonic saline. This reestablished driving forces for net cation movements. The ensuing net K^+ effluxes (as estimated from the maximal slopes of the curves relating intracellular K^+ content to time), are represented in Fig. 2 as functions of DNFB concentration and time of exposure to the agent during

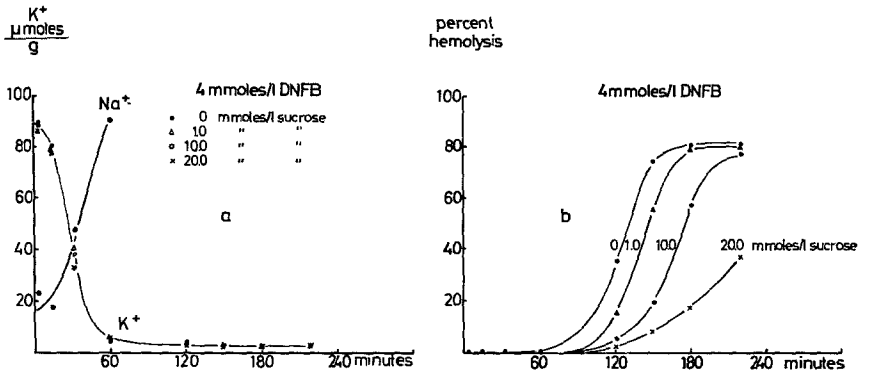

Fig. 3. Effect of sucrose on potassium loss, sodium uptake (a), and hemolysis (b) in DNFB treated red blood cells. At the start of the experiment, cells were suspended in isotonic saline containing 4.0 mmoles/liter DNFB and 0, 1.0, 10.0 and 20.0 mmoles/liter sucrose, respectively. Cell concentrations: 5 vol%. 37 °C. *Ordinate:* (a) K^+ content of the cells in µmoles/g of initial weight; (b) hemolysis in percent. *Abscissa:* time in minutes

the preincubation period. At 0.5 mmoles/liter, DNFB causes no effect at reaction times up to two hr. At 6 mmoles/liter, after about 30 min, the effect tends to approach a limiting maximal value. At intermediate concentrations, the effects continue to increase for the whole period of experimental observation. These results show that the concentration efficiency curve obtained in experiments where the cells are exposed to the agent in isotonic saline are somewhat misleading since dinitrophenylation of permeability controlling sites continues while potassium ions leave the cells. Even from the data represented in Fig. 2, it is impossible to derive a time independent concentration-efficiency curve. To obtain such a curve, it would have been necessary to wait until the reaction between membrane receptors and DNFB has proceeded to completion at all DNFB concentrations in the medium. Unfortunately, prolonged exposure to DNFB leads to hemolysis. Therefore, it was impossible to meet this requirement. Nevertheless, our data suggest that a permeability change can only be brought about if a certain threshold concentration is exceeded and also, that at high DNFB concentrations saturation may be achieved.

In an isotonic sodium chloride medium, potassium leaves the DNFB-poisoned cells somewhat faster than sodium enters them. Thus, the osmotic content of the cells at first decreases. When the potassium distribution between cells and medium approaches its equilibrium, Na influx still continues and the osmotic content of the cells increases. Eventually, hemolysis occurs. The addition of sucrose retards the onset of hemolysis without interfering with the cation movements (Fig. 3a). The effectiveness in

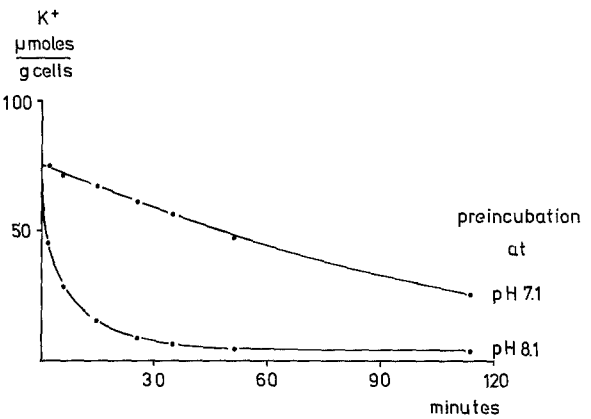

Fig. 4. Effect of dinitrophenylation at two pH values on the subsequent K^+ efflux as measured at pH 7.4. The cells were preincubated in 100 mmoles/liter KCl, 44 mmoles/liter Na_2HPO_4, pH 7.0 or 8.5. The DNFB concentration was 3.0 mmoles/liter. Cell density: 10 vol%. Determination of the pH values in the final suspensions yielded pH 7.1 and pH 8.1, respectively. After 60 min of incubation at 37 °C, the cells were centrifuged down and resuspended in 100 mmoles/liter NaCl, 44 mmoles/liter Na_2HPO_4, pH 7.4. Cell density: 10 vol%. 37 °C. *Ordinate:* Potassium content in μmoles/g cells. *Abscissa:* time in minutes

delaying hemolysis increases with increasing sucrose concentration. At a concentration of 20 mmoles/liter, sucrose retards the onset of hemolysis for about two hr (Fig. 3b). These data suggest that hemolysis is of the colloid osmotic type. However, unpublished experiments with molecules larger than sucrose (dextrane, polyvinylpyrrolidon) did not support this suggestion. Thus, it is not yet clear whether hemolysis is only a consequence of colloid osmotic swelling beyond a critical hemolytical volume or also the result of a progressive increase of the DNFB-induced membrane defect.

DNFB reacts with the uncharged form of the amino groups. If amino groups were involved in the control of ion permeability, one would expect that exposing the cells to the agent at high pH should produce a greater permeability change than at low pH. This was observed to be the case (Fig. 4): One batch of cells was dinitrophenylated at pH 8.1, another at pH 7.1, both in a high potassium medium at 37 °C for one hr. At the end of the dinitrophenylation period, the cells were centrifuged, and excess DNFB was removed by washing in high potassium medium. Subsequently, the cells of each of the two batches were resuspended in DNFB-free phosphate buffered saline of equal pH (7.4) and K^+ exit was followed. The rate of net K^+ loss from cells which were dinitrophenylated at pH 8.1 is several times higher than the rate of net K^+ loss from cells which were dinitrophenylated at pH 7.1. During incubation at 37 °C, part of the added DNFB

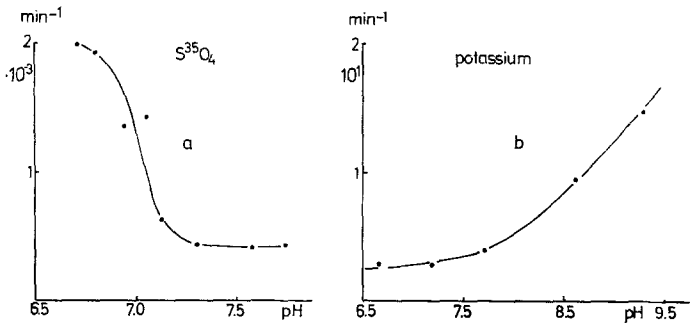

Fig. 5. (a) Effect of pH on $^{35}SO_4$ penetration in dinitrophenylated red blood cells. Red blood cells (40 vol%) were incubated at 37 °C for one hr in a solution containing 166 mmoles/liter sucrose, 68 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, and 6.0 mmoles/liter DNFB. Subsequently, the cells were washed three times in a medium which was of the described composition except that no DNFB was present. The cells were then resuspended in media containing 10 mmoles/liter Na_2SO_4, 160 mmoles/liter sucrose and sufficient NaCl, HCl, or NaOH to maintain isotonicity and to establish the desired pH values in the final cell suspensions. Hematocrit: 40%. Incubation at 37 °C was continued for one hr. Subsequently, the cell suspensions were transferred to 27 °C and kept at that temperature for another 45 min. At the end of this period, a trace of $^{35}SO_4$ was added and fluxes were measured as described by Gardos *et al.* (1969). *Ordinate:* permeability constant in min^{-1}. *Abscissa:* pH in the cell suspension. (Note: The fluxes presented in this figure are about one-tenth of those in normal red blood cells.)

(b) Effect of pH on potassium loss from dinitrophenylated red blood cells. Prior to the experiment represented in this figure, the cells were dinitrophenylated at 37 °C in a medium of the following composition: 100 mmoles/liter KCl, 66 mmoles/liter NaCl, 20 mmoles/liter sucrose, and 2 mmoles/liter DNFB. Cell density: 10 vol%. After one hr, the cells were washed once in a medium of the described composition but without DNFB and resuspended in isotonic saline containing sucrose and sufficient NaOH or HCl to give the pH values indicated on the curves. *Ordinate:* initial rate of potassium loss in percent/min. *Abscissa:* pH in the cell suspension

is spontaneously transformed into dinitrophenol. This process is favored by raising the pH. Therefore, the effective dose is smaller at pH 8.1 than at the lower pH value and hence the true effect of alkalinization should even be higher than represented in Fig. 4.

Previous investigations suggested that NH_3^+-groups play a predominant role in the maintenance of cation impermeability of the red blood cell membrane. If all of these groups were accessible to the agent, then after exhaustive dinitrophenylation of the membrane and removal of the unreacted DNFB from the medium, the rate of K^+ loss from the leaky cells should be independent of pH. For the reasons presented above it is difficult to saturate the membrane binding sites with DNFB. Nevertheless, it is remarkable that net potassium efflux from cells which were exposed to 20 mmoles/liter DNFB at pH 7.4 for one hr is quite sensitive to variations of pH (Fig. 5b).

Anion Permeability

DNFB inhibits sulfate permeability. Inhibition increases with DNFB concentration and time of exposure (Fig. 6a, b).

The action of DNFB is not restricted to sulfate ions. Table 1 shows that dinitrophenylation also reduces the penetration rates of phosphate, succinate, and lactate. The degree of inhibition is not identical for different anion species. Nevertheless, the data suggest that the modification of the membrane by DNFB does not affect a specific transport system but leads to an alteration of anion permeability in general.

The effects of DNFB on anion permeability do not represent an exact mirror image of the effects on cation permeability. Anion movements are affected at lower concentrations of DNFB than cation movements. After dinitrophenylation for 30 min at a DNFB concentration of 0.5 mmoles/liter in the medium (hematocrit 10%) sulfate flux is inhibited about 30% while

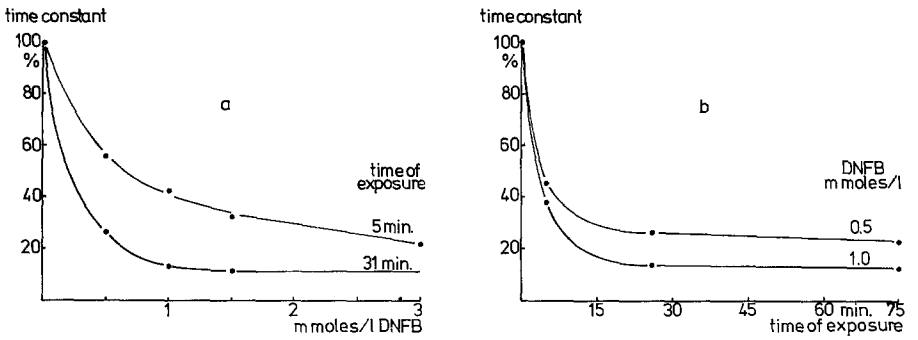

Fig. 6. Effect of DNFB on sulfate permeability. Prior to the flux measurements, the cells were loaded with $^{35}SO_4$ and then exposed to the action of DNFB.

(a) The cells were exposed to the action of the DNFB concentrations indicated on the abscissa for 5 min (upper curve) or 31 min (lower curve).

(b) The cells were exposed to the action of 0.5 (upper curve) or 1.0 (lower curve) mmoles/liter DNFB for the time periods indicated on the abscissa.

The ordinates of Fig. 6a and b represent rate constants of the $^{35}SO_4$ back exchange in percent of the uninhibited control. The absolute values of the rate constant of the controls amounted to $3.61 \cdot 10^{-2}\,min^{-1}$ (Fig. 6a) and $3.22 \cdot 10^{-2}\,min^{-1}$ (Fig. 6b). Incorporation of $^{35}SO_4$ into the cells was achieved by incubating them in 'sulfate medium' of the following composition: 151 mmoles/liter NaCl, 10 mmoles/liter Na_2SO_4, 20 mmoles/liter sucrose, and a trace of $^{35}SO_4$. Cell density: 20 vol%. After two hr at 37 °C, the suspensions were diluted 1:1 with 'sulfate media' of the composition described above (including $^{35}SO_4$). These media contained sufficient DNFB to give the final concentrations indicated on the figures. Subsequently, incubation was continued for the time periods given in the figures. Thereafter, dinitrophenylation was stopped by washing once in DNFB-free medium at 4 °C. The flux measurements were initiated by resuspending the cells in $^{35}SO_4$-free sulfate medium at 37 °C. Cell density: 2.5 vol%

Table 1. *Effect of DNFB on anion permeability*[a]

Diffusing substance	% inhibition
Sulfate	83.6
Phosphate	71.6
Succinate	62.6
Lactate	15.5

[a] The media contained 5.2 mmoles/liter of the respective anion species, 55 mmoles/liter sodium chloride, and sucrose to maintain isotonicity. pH 7.4. The cell density was 40 vol%. The time of action of DNFB (3.55 mmoles/liter) prior to the flux measurements was two hr. 37 °C. Human red blood cells.

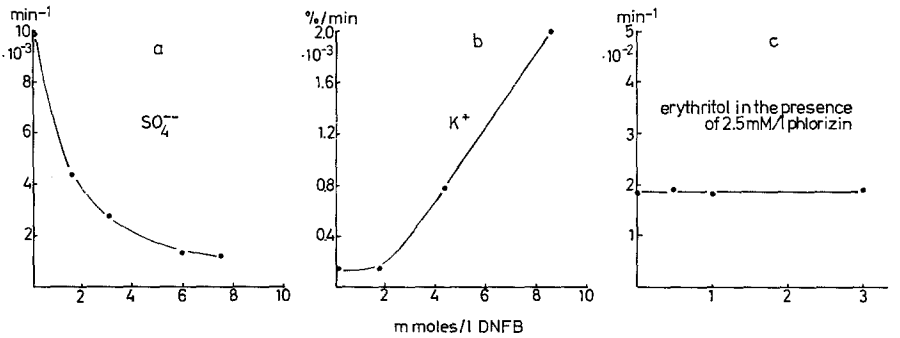

Fig. 7. Action of 1-fluoro-2,4-dinitrobenzene (DNFB) on sulfate, potassium, and erythritol permeability of human red blood cells. In all experiments, the cells were incubated in the presence of DNFB at 37 °C for two hr prior to the start of the permeability measurements.

(a) *Sulfate permeability.* The medium contained 5.2 mmoles/liter SO_4^{2-}, 85 mmoles/liter Cl^-, and sufficient sucrose to maintain isotonicity. The cell density was 40 vol%. The experiment was started by the addition of $^{35}SO_4$. *Ordinate:* permeability constant, min^{-1}. *Abscissa:* concentration of DNFB, mmoles/liter.

(b) *Potassium permeability.* The experiment was started by transferring the cells from a high KCl medium to isotonic NaCl. Final cell density: 10 vol%. *Ordinate:* rate of net K^+ loss in percent of original K^+ content per minute. *Abscissa:* concentration of DNFB, mmoles/liter.

(c) *Glucose-phlorizin insensitive fraction of erythritol permeability.* Back exchange of ^{14}C from ^{14}C-erythritol loaded cells was measured according to LaCelle and Passow (1971). The cell density was 10 vol% during the preincubation with DNFB and 2.5 vol% during ^{14}C back exchange. *Ordinate:* permeability constant, min^{-1}. *Abscissa:* concentration of DNFB, mmoles/liter

net potassium efflux is not yet significantly increased. Only if a threshold concentration of about 0.5 to 1.0 mmole/liter DNFB is exceeded does K^+ efflux assume abnormally high values. No such threshold concentration seems to exist for the inhibition of anion permeability (Figs. 2, 6 and 7).

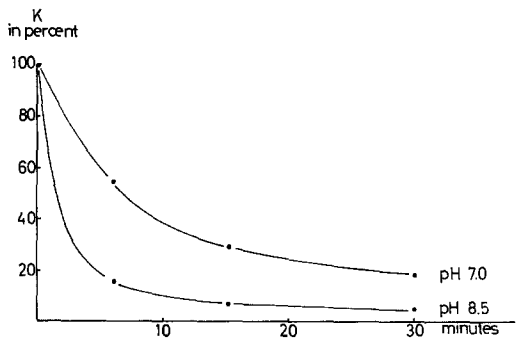

Fig. 8. Effect of pH on the interactions between DNFB and membrane sites involved in the control of sulfate permeability. $^{35}SO_4$ back exchange was measured at pH 7.0 in cells which were dinitrophenylated at pH 7.0 or 8.5 prior to the flux measurements. Red blood cells were suspended in 151 mmoles/liter NaCl, 20 mmoles/liter sucrose, 10 mmoles/liter Na_2SO_4 and a trace of $^{35}SO_4$. Cell density: 20 vol%. The pH was adjusted to 7.0 and 8.5, by the addition of HCl or NaOH, respectively. Following incubation at 37 °C for two hr, the cells were diluted 1:1 with the original 'sulfate medium' containing sufficient DNFB to give a final concentration of 1.0 mmole/liter. At the times indicated on the abscissa, the cells were centrifuged down, washed twice in unlabeled sulfate medium at 40 °C and resuspended at 37 °C in a solution containing 121 mmoles/liter NaCl, 30 mmoles/liter Tris, pH 7.0, 20 mmoles/liter sucrose, and 10 mmoles/liter Na_2SO_4 (=start of the experiment). The $^{35}SO_4$ back exchange was followed. *Ordinate:* rate constant for $^{35}SO_4$ back exchange in percent of controls. *Abscissa:* time of exposure to DNFB prior to the flux measurements

The concentrations of DNFB required to produce maximal effects are also different for anion and cation permeability. Anions respond again at lower DNFB concentration than cations. In view of the asymptotic character of the time course of development of the effects of DNFB, it is difficult to express the difference in quantitative terms. However, it is probably safe to state that anion permeability is nearly maximally inhibited at a DNFB concentration which is about one-half of that required to produce a nearly maximal facilitation of cation movements (under our experimental conditions 3.0 and 6.0 mmoles/liter DNFB, respectively).

The efficiency with which a given concentration of DNFB alters anion permeability is a function of pH. Dinitrophenylation of the cells at neutrality inhibits the ion fluxes as subsequently measured at pH 7.0 to a lesser extent than dinitrophenylation in a slightly alkaline milieu (Fig. 8). This result agrees with earlier findings of Passow (1969). However, it conflicts with a statement made by Schnell and Passow (1969) who claimed that differences of pH did not influence the DNFB effect on permeability.

A check of the original notes of the experiments of Schnell and Passow revealed that they had continued as usual to employ Tris buffer for pH

adjustment in spite of the fact that Tris reacts with DNFB. In the present experiments, pH was adjusted by the addition to the cells of Na_2HPO_4 and sufficient HCl of NaOH to establish the desired pH values. We suspect that the use of Tris buffer in our reaction mixture was responsible for our former failure to confirm one of the essential prerequisites for the stipulation of a causal relationship between DNFB action and chemical modification of amino groups. However, we could confirm another statement made by Schnell and Passow. According to the fixed charge hypothesis as formulated by Passow (1969), one would expect that a reduction of the chloride concentration in the medium by replacement of NaCl with isotonic sucrose would lead to a redistribution of anions between membrane and medium. Assuming the existence of Donnan equilibria between membrane and medium, the ratio $OH^-_{medium}:OH^-_{membrane}$ should always be equal to the ratio $Cl^-_{medium}:Cl^-_{membrane}$. For electrostatic reasons, $Cl^-_{membrane}$ should be equal to the concentration of the fixed charges and nearly independent of the chloride concentrations in the medium. Hence, a reduction of Cl^-_{medium} at constant OH^-_{medium} should increase the pH in the membrane. Consequently, one would anticipate that dinitrophenylation at equal pH but different Cl^- concentrations in the medium should influence the effects of DNFB on the permeability barrier differently. No significant effects of Cl^- concentration in the medium could be detected. However, since lowering of the ionic strength in the medium induces K^+ loss even in the absence of DNFB, we reduced Cl^-_{medium} only moderately and, perhaps insufficiently for production of a significant effect on dinitrophenylation.

In untreated red blood cells, sulfate flux decreases with increasing pH in the medium. This pH dependence is preserved in erythrocytes whose anion permeability was inhibited by dinitrophenylation at pH 7.4 with high concentrations of DNFB which produce a nearly maximal inhibition (Fig. 5a).

Erythritol Permeability

In view of the hypothesis that the effects of DNFB on ion permeability are due to dinitrophenylation of membrane bound amino groups and the ensuing change of positive membrane charge, it was of obvious interest to study the effect of dinitrophenylation of the membrane on the permeability of a nonelectrolyte. In our experiments, we used a polyalcohol, *meso*-erythritol. Part of the erythritol flux across the membrane of the human red blood cell is mediated by the glucose transfer system (Bowyer and Widdas, 1955; LaCelle and Passow, 1971). This fraction of erythritol flux can be inhibited by glucose and typical inhibitors of glucose transport, e.g. phlori-

zin. The penetration mechanism of the glucose-phlorizin insensitive fraction of erythritol flux is not yet fully understood. However, it could be shown to follow simple diffusion kinetics.

DNFB is a well known inhibitor of the glucose transport system (Bowyer and Widdas, 1956). It is also capable of inhibiting that fraction of erythritol flux which is sensitive to inhibitors of glucose transport. However, DNFB has virtually no effect on the glucose-phlorizin insensitive fraction of erythritol flux if applied at concentrations where it produces its effects on anion and cation permeability (LaCelle and Passow, 1971; Fig. 7c).

DNFB Binding to the Cell Membrane

The interpretation of the previously described experiments was based on the assumption that DNFB exerts its effects on ion permeability primarily by dinitrophenylation of amino groups in the membrane. It seemed interesting, therefore, to supplement the study of the effects of DNFB on permeability by some information on DNFB binding to the red blood cell membrane. Consequently, DNFB uptake by human red blood cells was measured under the same conditions under which the effects of DNFB on ion movements were investigated.

Preliminary experiments revealed that DNFB readily penetrates into the cells where it combines with intracellular constituents. Only a minute fraction of the total DNFB taken up by the cells is bound to the membrane (Fig. 9).

Fig. 10 shows the time course of DNFB binding to the membranes of intact cells which were exposed to DNFB in isotonic saline buffered with sodium phosphate to pH 7.4. DNFB uptake levels out after about 90 min but still continues at a reduced rate during the whole time course of the experiment (150 min).

The time course of binding is different from the time course of the development of DNFB effects on ion permeability (*c.f.* Figs. 2, 6 and 10). Alterations of anion and cation permeability level out faster than DNFB binding. The absence of a simple relationship between binding and effect on ion permeability suggests that the bulk of the DNFB binding sites in the membrane is not involved in the control of ion permeability.

The curves relating the effect of DNFB on ion fluxes to DNFB concentration in the medium were fairly reproducible. Yet, binding curves such as those depicted in Fig. 10 followed a somewhat variable course. The latter finding may not be surprising. During the incubation period, part of the DNFB is converted to 2,4-dinitrophenol. This process is enhanced by

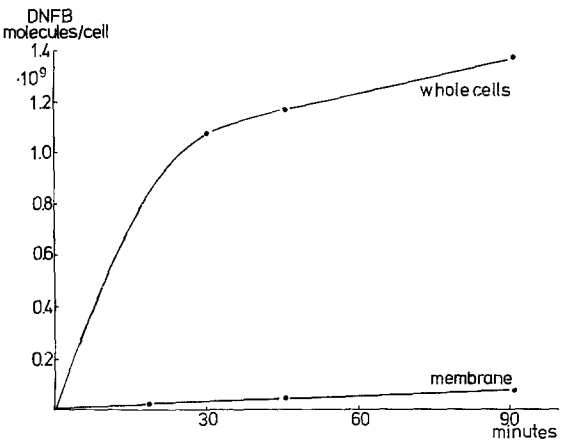

Fig. 9. DNFB-binding to intact erythrocytes and erythrocyte membranes. At the start of the experiment, the cells were suspended in isotonic saline containing 20 mmoles/liter sucrose and 2.0 mmoles/liter ^{14}C-DNFB. ^{14}C-binding to the cell membranes was determined as described under Materials and Methods. ^{14}C-uptake by the cells was estimated by following the disappearance of the radioactivity from the supernatant. 37 °C. Cell density: 5 vol%. *Ordinate:* number of DNFB molecules bound per single cell. *Abscissa:* time in minutes

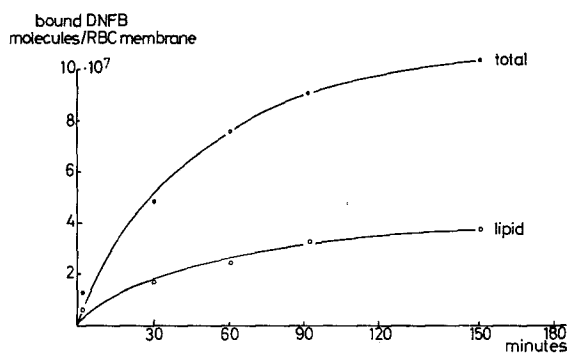

Fig. 10. Time course of DNFB binding to the red cell membrane and membrane lipids. At the start of the experiment, red blood cells were suspended in media containing 100 mmoles/liter KCl, 36 mmoles/liter NaCl, 20 mmoles/liter Na_2HPO_4, pH 7.5 and 3.0 mmoles/liter ^{14}C-DNFB. Cell density: 10 vol%. 37 °C. The binding of ^{14}C to the cell membrane was followed as described under Materials and Methods. *Ordinate:* number of DNFB molecules bound per single cell membrane. *Abscissa:* time in minutes

the intracellular carbonicanhydrase (Henkart, Guidotti & Edsall, 1968). Moreover, a large excess of intracellular hemoglobin competes with the membrane binding sites for DNFB. Hemolysis cannot always be prevented. Nevertheless, the general shape of the DNFB binding curves consistently showed the pattern represented in Fig. 10. The order of magnitude of the

number of dinitrophenyl residues incorporated into the membrane as represented in Fig. 10 is typical for a number of similar experiments.

About 30% of the total DNFB which is fixed to the membrane reacted with the membrane lipids (Fig. 10). A more detailed study of the products of the reaction between membrane lipids and DNFB revealed the dinitrophenylation of phosphatidylethanolamine (*see* Appendix). In three experiments, at 6.0 mmoles/liter DNFB in the medium, and at a pH of 7.0 (hematocrit 40%) 38, 10, and 47% of the phosphatidylethanolamine present in the membrane reacted with the agent. Under the same conditions, no binding of DNFB to phosphatidylserine could be detected.

If lipid extracts from red blood cells prepared by the method of Reed *et al.* (1960) were exposed to DNFB under conditions similar to those employed for the dinitrophenylation of intact cells, phosphatidylethanolamine as well as phosphatidylserine were dinitrophenylated. The same applies to erythrocyte ghosts prepared by the method of Dodge *et al.* (1962). Thus, in principle at least, under our experimental conditions (saline, buffered with 0.02 moles/liter phosphate to pH 7.2, two hr at 37 °C, final pH 6.9) phosphatidylethanolamine as well as phosphatidylserine are capable of reacting with DNFB.

Discussion

DNFB was found to increase cation permeability, to decrease anion permeability, and to leave unaltered the diffusional component of the nonelectrolyte, erythritol. These findings support the hypothesis that DNFB reduces the concentration of fixed ions which control passive movements of diffusible ions in the red cell membrane.

In terms of the fixed charge hypothesis, the reduction of the fixed charge density by DNFB is accompanied by a parallel decrease in the concentrations of diffusible anions within the membrane. The anion flux decreases gradually as the concentration of the fixed ions is diminished. The fact that a threshold concentration of DNFB must be exceeded in order to increase cation permeability suggests that the fixed charge density of the membrane has to be reduced below a certain critical value before the permeability changes become measurable. At high DNFB concentrations, the charge density of the membrane may have become so low that any further decrease produces little further change of anion flux. Yet, a few remaining positive fixed charges in narrow channels may still be efficient in blocking cation movements. This may explain why much higher concentrations of DNFB are required

to produce a maximal effect on cation permeability than on anion permeability.

Although DNFB is mainly an amino reactive agent, its reactivity is not confined to amino groups. As has already been stated in the Introduction, it is a fairly powerful SH reagent and can also react with imidazole and phenolic OH groups (Hirs, 1967).

Modification of SH groups in the red cell membrane has little if any effect on anion permeability (*unpublished results*). Moreover, some more sophisticated experiments performed by Knauf and Rothstein (1968) further support the contention that modification of SH groups is not the predominant feature underlying the action of DNFB. Phenolic OH groups would confer to the membrane some negative charge. A reduction of their number should, therefore, enhance anion movements and slow down cation movements, and thus produce the opposite to the observed effects. Hence, if phenolic OH groups should constitute the site of action of DNFB, then it would seem impossible to explain the ensuing effects on anion and cation permeability by means of the fixed charge hypothesis.

Experiments with 5-methoxy-2-nitrotropone (MNT), an amino reactive agent which is supposed to be much more specific than DNFB, yielded results which were similar to those described above for DNFB (Passow and Schnell, 1969). In addition, trinitrobenzene sulfonic acid (TNBS), another agent with a high specificity for amino groups, also inhibits anion permeability and enhances cation movements (*unpublished results*). In view of the similarity of the effects of MNT, TNBS, and DNFB on the permeability to anions and cations, respectively, it seems plausible to assume that DNFB causes its effect on ion permeability by reacting with amino groups. This is also suggested by the fact that alkalinization facilitates the development of membrane modification by DNFB. Furthermore, previous determinations of the pK value of those dissociable groups in the membrane which control anion movements yielded a pK of 9. This points to the presence of amino groups rather than imidazole groups in the ion permeable channels (Lepke and Passow, 1971).

DNFB is bound to membrane lipids as well as membrane proteins. Under the conditions under which DNFB exerts its effects on the permeability of intact erythrocytes, no binding of DNFB to phosphatidylserine could be observed. Hence, it is unlikely that the bulk of the amino groups of this phosphatide serves as positive fixed charge in the control of ion permeability. Interestingly enough, if erythrocyte ghosts prepared by the method of Dodge *et al.* (1962) instead of intact cells are exposed to DNFB, then phosphatidylserine easily reacts with the amino reactive agent. Ap-

parently, in the intact cells, phosphatidylserine is not accessible to DNFB or embedded in a surrounding where dinitrophenylation cannot take place. This finding is remarkable since DNFB is an uncharged molecule which is soluble in water and lipid. Without being impeded by charged groups in the membrane, DNFB can be expected to have access to hydrophilic as well as hydrophobic regions in the membrane. In the present context, it is interesting to remember that a relatively large fraction of the membrane bound phosphatidylserine belongs to the so called 'strongly bound' lipids and is more difficult to extract from red cell membranes than phosphatidylethanolamine (van Deenen, 1970; Parpart & Ballentine, 1952).

In contrast to phosphatidylserine, phosphatidylethanolamine readily reacts with DNFB in the membrane of intact erythrocytes. Experiments with pronase, a mixture of proteolytic enzymes, suggest that this cephalin can also be ruled out as site of action (Passow, 1971). Since all of the carbohydrate amino groups in the red cell membrane seem to be acylated, it is reasonable to suspect that dinitrophenylation of protein amino groups leads to the observed permeability change.

In this context, it is important to remember that even after exhaustive dinitrophenylation, when the permeability changes approach their maximum, cation as well as anion permeability are still sensitive to variations of pH. Perhaps, not all of the amino groups involved in the control of ion permeability are accessible or reactive. In addition, it is conceivable that groups other than amino groups may be responsible for the pH dependence of the residual flux across the modified cell membrane. It would even be possible that the pH dependence of the residual flux is due to the predominance of negative fixed charges in the dinitrophenylated cells where the number of positive charges is greatly reduced. Increasing the pH could increase the negative charge of the membrane and hence accelerate cation flux and diminish anion flux.

Appendix

The Chemical Nature of the Lipid Soluble Product of the Reaction between DNFB and the Erythrocyte Membrane

Since there are only two compounds containing amino groups among the lipids, i.e., phosphatidylethanolamine and phosphatidylserine[2], only two products of the reaction between DNFB and the lipids of the erythrocyte membrane could be expected. Actually, only one of them was formed, as the following study shows.

2 Our methods did not distinguish between the cephalin form and the plasmalogen form of the ethanolamine phospholipid or the serine phospholipid. Therefore, both forms are called phosphatidylethanolamine and phosphatidylserine, respectively.

The lipid extract of human red blood cells treated with DNFB was prepared as described under Materials and Methods. Its UV-spectrum showed an absorption peak at 350 nm, thus indicating the formation of a DNP–NH-compound. Separation of the lipids by thin layer chromatography in chloroform/methanol/water showed two spots having the yellow color typical for DNP-compounds. The main spot was just in front of the cephalins; the other, which was almost invisible, moved near the solvent front. In order to estimate the relative quantities of the yellow substances, we separated the lipids of red blood cells pretreated with ^{14}C-DNFB by thin layer chromatography and found that more than 90% of the total radioactivity was located in the substance in front of the cephalins. We therefore undertook to isolate and identify this substance only.

To isolate the unknown DNP-compound, preparative thin layer chromatography was used. The zone containing the yellow substance was scraped off from the chromatogram and eluted with chloroform/methanol. The purity of the eluted substance was checked by thin layer chromatography in three different solvent mixtures (*n*-propanol/25% NH_3, chloroform/ethanol, and ethylacetate/chloroform/methanol). None of these solvent systems led to further separation. The UV-spectrum of the isolated compound showed the same absorption maximum at 350 nm as the total lipid extract.

IR-spectrometry of the isolated compound revealed, in addition to the DNP-residue, the presence of an amino group and an ester bond[3]. There was no indication of a free carboxyl group. The isolated substance therefore did not contain serine and consequently, was not DNP-phosphatidylserine. But it did contain phosphate.

These findings suggested that the isolated compound was identical with DNP-phosphatidylethanolamine. This hypothesis was proven as follows:

Phosphatidylethanolamine was isolated from untreated human red blood cells and then dinitrophenylated. The authentic DNP-phosphatidylethanolamine was purified by thin layer chromatography and compared to the unknown DNP-compound. According to the following criteria, both substances are identical: (1) Thin layer chromatography in two different solvent systems (chloroform/methanol/acetone and *n*-propanol/25% NH_3) showed both substances to have the same R_f values (Fig. 11a, b); (2) After hydrolysis in 6 N HCl, the unknown compound as well as authentic DNP-phosphatidylethanolamine yielded DNP-ethanolamine. The appearance of DNP-ethanolamine was demonstrated by comparison with synthetic DNP-ethanolamine on thin layer plates (Fig. 12a, b); (3) The IR-spectra of the unknown compound and of DNP-phosphatidylethanolamine are virtually identical (Fig. 13); and (4) In DNP-phosphatidylethanolamine the ratio DNP-ethanolamine/phosphate is 1.0. Phosphorus determinations and determinations of DNP-ethanolamine in DNP-phosphatidyl-ethanolamine and in the unknown substance gave a ratio of 0.9 for DNP-phosphatidyl-ethanolamine and a ratio of 0.81 for the unknown compound.

No attempt was made to identify the other yellow compound observed in lipid extracts from dinitrophenylated red blood cells. Thin layer chromatograms showed it to be different from DNP-ethanolamine, DNP-serine, and DNP-phosphatidylserine. Since it was found in very small quantities only, it certainly did not influence the amount of fixed charges in the erythrocyte membrane. We suspect it to be a contamination by unreacted DNFB.

Thus, in the membrane of human red blood cells treated with 0.006 moles/liter DNFB under neutral conditions, no DNP-phosphatidylserine is formed, whereas DNP-phosphatidylethanolamine is readily dinitrophenylated.

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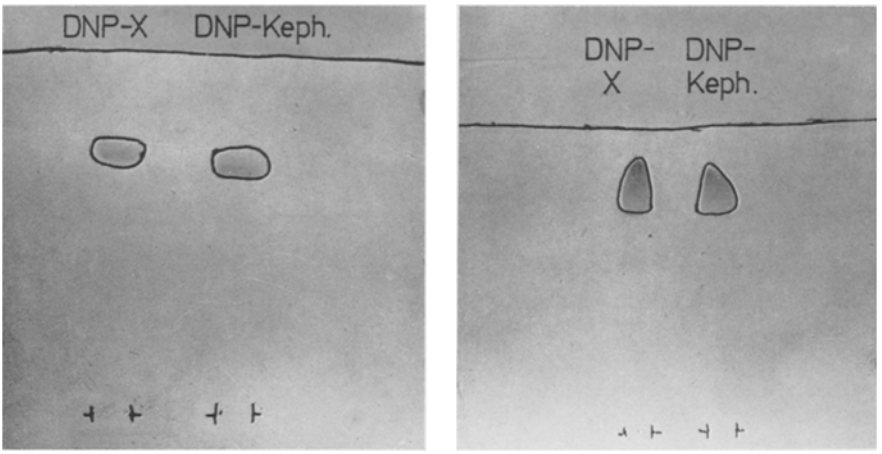
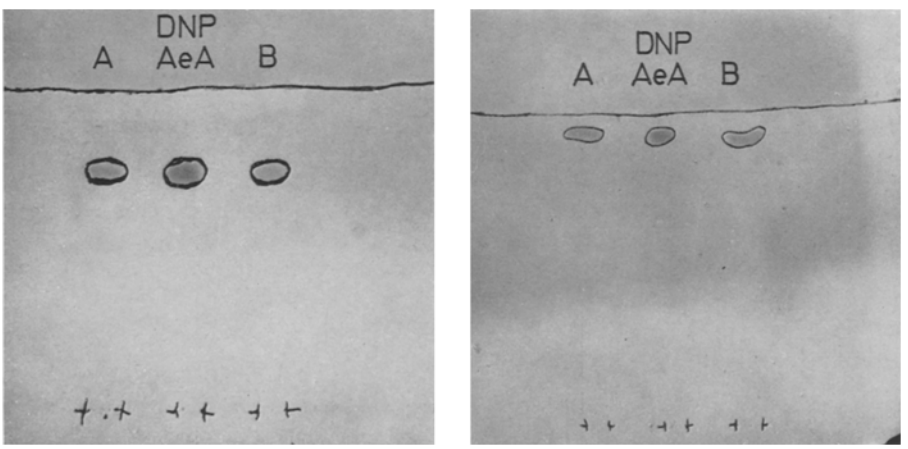
3 The IR-spectrum does not distinguish between the plasmalogen and the cephalin form of ethanolamine phospholipid, since the absorption band due to the ether bond in the plasmalogen is hidden by the absorption caused by the ester bond.



a b

Fig. 11. (a) Thin layer chromatogram of the unknown compound (DNP–X) and of DNP-phosphatidylethanolamine (DNP-keph.). Solvent mixture: chloroform/methanol/25% NH_3 = 12:4:1 (v/v/v). R_f = 0.7.

(b) Thin layer chromatogram of the unknown compound (DNP–X) and of DNP-phosphatidylethanolamine (DNP-keph.). Solvent mixture: chloroform/methanol/water = 13:6:1 (v/v/v). R_f = 0.8



a b

Fig. 12. (a) Thin layer chromatogram comparing the yellow product of acid hydrolysis of the unknown compound (A) and of DNP-phosphatidylethanolamine (B) to authentic DNP-ethanolamine (DNP–AeA). Solvent mixture: *n*-propanol/25% NH_3 = 7:3 (v/v). R_f = 0.75.

(b) Thin layer chromatogram comparing the yellow product of acid hydrolysis of the unknown compound (A) and of DNP-phosphatidylethanolamine to authentic DNP-ethanolamine (DNP–AeA). Solvent mixture: chloroform/methanol/acetone = 4:2:1 (v/v/v; +2.5% water). R_f = 0.92

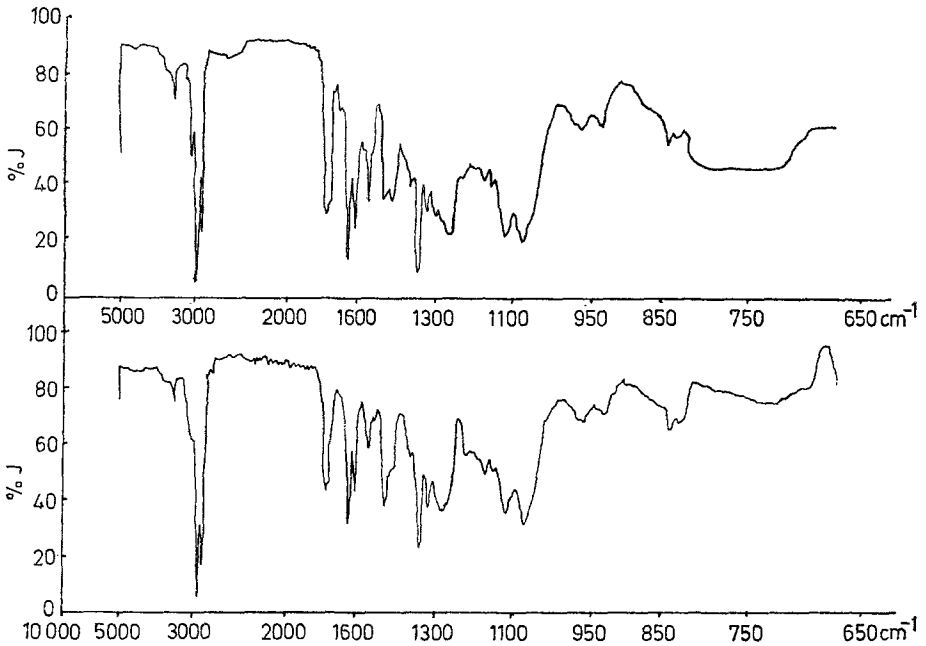


Fig. 13. IR spectra of the unknown compound (upper spectrum) and of authentic NDP-phosphatidylethanolamine (lower spectrum). (Solvent: chloroform)

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References

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466.

Berg, H. C., Diamond, J. M., Marfey, P. S. 1965. Erythrocyte membrane: chemical modification. *Science* **150**:64.

Bowyer, F., Widdas, W. F. 1955. Erythrocyte permeability to erythritol. *J. Physiol.* **129**:7P.

— — 1956. The facilitated transfer of glucose and related compounds across the erythrocyte membrane. *Disc. Faraday Soc.* **21**:251.

Dodge, J. T., Mitchell, C., Hanahan, D. 1962. The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119.

Gardos, G., Hoffman, J. F., Passow, H. 1969. Flux measurements in erythrocytes. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. p. 9. Springer-Verlag, Berlin-Heidelberg-New York.

Henkart, P., Guidotti, G., Edsall, J. T. 1968. Catalysis of the hydrolysis of 1-fluoro-2,4-dinitrobenzene by carbonic anhydrase. *J. Biol. Chem.* **243**:2447.

Hirs, C. H. W. 1967. Reactions with reactive aryl halides. *In:* Methods in Enzymology, vol. XI, p. 548. C. H. W. Hirs, editor. Academic Press, New York.

Knauf, P., Rothstein, A. 1968. *Quoted in*: A. Rothstein. Membrane permeability of erythrocytes. *In:* Metabolism and Membrane Permeability of Erythrocytes. E. Deutsch, E. Gerlach, and K. Moser, editors. p. 407. Thieme Verlag, Stuttgart.

LaCelle, P., Passow, H. 1971. Permeability of the human red blood cell to *meso*-erythritol. *J. Membrane Biol.* **4**:270.

Lepke, S., Passow, H. 1971. The permeability of the human red blood cell to sulfate ions. *J. Membrane Biol.* **6**:158.

Parpart, A. K., Ballantine, R. 1952. Molecular anatomy of the red cell plasma membrane. *In:* Modern Trends in Physiology and Biochemistry. E. S. G. Barran, editor. p. 135. Academic Press, New York.

Passow, H. 1969*a*. Passive permeability of the erythrocyte membrane. – An assessment of scope and limitations of the fixed charge hypothesis. *Progr. Biophys. Mol. Biol.* **19**:425.

– 1969*b*. Ion permeability of erythrocyte ghosts. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. p. 21. Springer-Verlag, Berlin-Heidelberg-New York.

– 1971. Effects of pronase on the passive ion permeability of the human red blood cell. *J. Membrane Biol.* **6**:233.

– Schnell, K. F. 1969. Chemical modifiers of passive ion permeability of the erythrocyte membrane. *Experientia* **25**:460.

Reed, S. F., Scott, N., Swisher, S. N., Marinetti, K. V., Eden, E. K. 1960. Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells. *J. Lab. Clin. Med.* **56**:281.

Van Deenen, L. L. M. 1969. Membrane lipids and lipophilic proteins. *In:* The Molecular Basis of Membrane Function. D. C. Tosteson, editor. p. 47. Prentice-Hall, Inc., Englewood Cliffs, N.T.