Biochimica et Biophysica Acta, 649 (1981) 557–571
Elsevier/North-Holland Biomedical Press

BBA 79469

RELATION BETWEEN RED CELL MEMBRANE ($Na^+ + K^+$)-ATPase AND BAND 3 PROTEIN

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(Received June 29th, 1981)

Key words: Band 3 protein; ($Na^+ + K^+$)-ATPase; Cation transport; Anion transport; ^{31}P-NMR; (Human erythrocyte)

This study is designed to examine the participation of the major red cell membrane protein, band 3 protein, in the chain which transmits information from the cardiac glycoside site on the external face of the cell ($Na^+ + K^+$)-ATPase to the megadalton glycolytic enzyme complex within the cell. The experiments show that the anion transport inhibitor, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, affects the resonance of 2,3-diphosphoglycerate, as does the cardiac glycoside cation transport inhibitor, ouabain. Resonance shifts induced by the cardiac glycoside alone are modulated by addition of the anion transport inhibitor which indicates that there is coupling in the red cell between the ($Na^+ + K^+$)-ATPase and band 3 protein. Band 3 protein was separated from the membrane and partially purified following the technique of Yu and Steck ((1975) J. Biol. Chem. 250, 9170–9175). When glyceraldehyde-3-phosphate dehydrogenase was added to the separated band 3 protein preparation, addition of cardiac glycosides caused shifts in the ^{31}P resonance of glyceraldehyde 3-phosphate. These experiments indicate that there is coupling between the ($Na^+ + K^+$)-ATPase and band 3 protein in the separated preparation and suggest that the anion and cation transport systems may be closely related spatially and functionally in the intact red cell.

Introduction

Evidence has previously been presented that shows a functional linkage between the ($Na^+ + K^+$)-ATPase which spans the human red cell membrane and two intracellular glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and monophosphoglycerate mutase (EC 2.7.5.3) [1,2]. We have further suggested that the three components (($Na^+ + K^+$)-ATPase/band 3 protein/glyceraldehyde-3-phosphate dehydrogenase) are linked to one another so closely that conformational information can be transferred through this complex from the outside of the cell to the inside. At the time this suggestion was made, it was pointed out that the case of a linkage between the first and third members of the association was strong, whereas the evidence that band 3 protein was an essential component in the information transfer chain was less compelling. The present experiments are designed to show that band 3 protein is the protein responsible for information transfer between a site located on the external face of the ($Na^+ + K^+$)-ATPase and the glycolytic enzymes in the cytoplasm.

Experimental methods

Nuclear Magnetic Resonance (NMR)

NMR experiments were either carried out on a JEOL FX-60 Fourier transform NMR spectrometer

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

0005-2736/81/0000–0000/$02.50 © 1981 Elsevier/North-Holland Biomedical Press

(JEOL Analytical Instruments, Inc., Cranford, NJ) operating at 24.16 MHz for ^{31}P in 10 mm (o.d.) tubes, or on a Nicolet NT-360 wide bore Fourier transform spectrometer (Nicolet Instrument Co., Mountain View, CA) operating at 145.75 MHz for ^{31}P in 20 mm (o.d.) sample tubes for experiments in Tables IV–VI. The spinner was not used in either instrument in order to avoid centrifugal separation of the suspensions. The 90° resonance frequency pulse width was 20 μs for the FX-60 and 37 μs for the NT-360. Pulse angles of 45°–60° were routinely used. Spectral widths of 3 KHz were employed with 8 K data tables for the FX-60 (8 KHz width and 16 K data tables for the NT-360). The signal to noise ratio was improved by digital filtering (1.5 Hz line broadening on the FX-60 and 3 Hz on the NT-360). Broad band noise-modulated proton decoupling was used on the FX-60 (1 KHz at 12.5 W). Broad band noise modulated CHIRP proton decoupling (4 W) was used on the NT-360. Both the JEOL FX-60 and the NT-360 spectrometers were locked on the deuterium signal of $^{2}H_2O$ so that $^{2}H_2O$ automatically acted as an internal reference standard. Both the JEOL FX-60 and the Bruker NT-360 keep the magnetic field strength constant by a 'field frequency lock'. As discussed in the succeeding paragraphs, the P_i measurements are extremely reproducible so that P_i, in turn, serves as an internal reference standard.

A few preliminary experiments were also carried out on a Varian XL-100 spectrometer operating at 40.27 MHz for ^{31}P and two of the experiments in Table IV and one in Table VII were carried out at 109.3 MHz on the Bruker HX-270 Fourier transform spectrometer which is situated in the Francis Bitter National Magnet Laboratory. All experiments were carried out at 24–25°C.

Fig. 1 shows a comparison of the spectra of control and depleted red cells at 145.75 MHz in the NT-360. The NT-360 has a computer program which determines the position of the resonances by fitting the data points to a curve. Since the resonances at 24.16 MHz were also sharp, it was possible to place 12–25 data points (usually spaced 0.25 Hz apart) over the upper half of the peak at this frequency and one data point clearly defined the maximum. The presence of paramagnetic impurities can clearly be seen by peak broadening. From time to time paramagnetic substances, often introduced from our

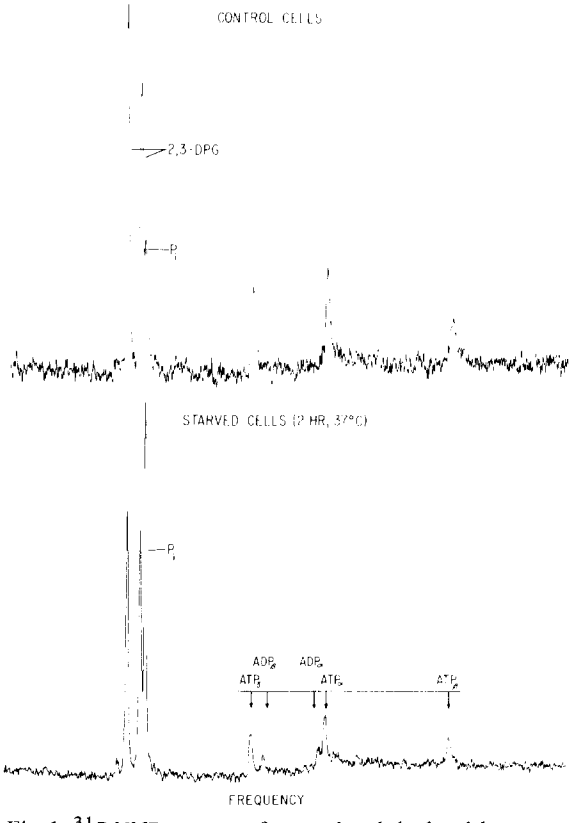

Fig. 1. ^{31}P-NMR spectra of control and depleted human red cells at 145.75 MHz.

'de-ionized' water, would appear in our system; when this happened, the experiments were discarded. In normal successful experiments the half-widths of the 2,3-diphosphoglycerate resonances were about 4 Hz at 24.16 MHz. In these experiments, 0.1 mM EDTA was occasionally added to the buffer. The absence of any subsequent change in half-width confirmed that the acceptable experimental preparations were free of paramagnetic impurities.

A number of control experiments were carried out in order to check the reproducibility of the system and to illustrate the dependence of the 2,3-diphosphoglycerate resonances on pH. The top half of Table I shows the results of one experiment, typical of three, in which the pH dependence of quintuplicate samples of NMR buffer solutions were measured. The pH dependence both of P_i and 2,3-diphosphoglycerate agree with those obtained by Moon and Richards [3] in rabbit red cells and those obtained by Burt et

TABLE I

EFFECT OF pH (a) AND OTHER AGENTS (b) ON ^{31}P RESONANCES AT 24.16 MHz

(a) Buffer contents (mM): 2,3-diphosphoglycerate, 5; Mg^{2+}, 5; P_i, 2; Hepes, 17; measurements in quintuplicate of a single solution at each pH. The data are given relative to an initial P_i of 0.0 at pH 7.20.
(b) Buffer contents (mM): 2,3-diphosphoglycerate, 5; Mg^{2+}, 5; P_i, 5; Hepes, 17; pH, 7.4. Myoglobin was 50 μM. Quintuplicate measurements of a single solution to which additions were made. The values for P_i give the midpoint and the maximum deviations, relative to an initial P_i of 0.0 at pH 7.40.

	P_i	2-P	3-P
(a) pH 7.2	−0.1 ± 0.1	−17.3 ± 0.2	−41.2 ± 0.2
7.4	−4.4 ± 0.1	−23.6 ± 0.2	−45.7 ± 0.1
7.6	−9.3 ± 0.1	−33.6 ± 0.1	−49.4 ± 0.1
ΔHz/0.01 pH	−0.23	−0.41	−0.21
(b) Control	0.0 ± 0.0	−18.3 ± 0.2	−38.7 ± 0.1
+ 5 μM ouabain	−0.1 ± 0.1	−18.5 ± 0.1	−38.8 ± 0.1
+ myoglobin	−0.2 ± 0.2	−18.6 ± 0.1	−38.5 ± 0.3
+ 42 μM DIDS	−0.1 ± 0.1	−18.6 ± 0.1	−38.0 ± 0.1
+ myoglobin	−0.2 ± 0.2	−18.6 ± 0.1	−37.6 ± 0.1

al. [4] in solution. The dependence of the 3-P resonance on pH is exactly the same as that of P_i, so that measurements of resonance shift of the 3-P relative to the P_i resonance should be independent of pH over this range, whereas those of the 2-P resonance should show some dependence on pH. These data show that the P_i values are very reproducible and can serve as an index of pH change. P_i was measured in virtually all the experiments; the data given either in the Tables, or in the legends, indicate that pH was closely controlled, as determined either by the P_i resonance or by a pH meter, or both.

In order to determine the reproducibility of our data five duplicate samples of NMR buffer (Hepes) were prepared at pH 7.4 and five measurements were made at 24.16 MHz of the P_i, 2-P and 3-P resonances in each aliquot. The standard deviations of the individual resonance shifts were ±0.2 Hz for P_i, ±0.2 Hz for the 2-P and ±0.4 Hz for the 3-P resonance in one experiment, typical of two. These figures are in reasonable agreement with the errors shown in Table I and indicate that differences of 0.5 to 1.0 Hz are significant at about the 1% level.

The bottom section of Table I shows the results of additional control experiments at 24.16 MHz indicating that the 2,3-diphosphoglycerate resonances are not affected by the addition of ouabain, the stilbene inhibitor DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid), either alone, or in combination with myoglobin. The differences between the control values in these experiments and the values at pH 7.40 in the top section of the table (after subtraction of the P_i reference resonance shifts) has been attributed to the difference in P_i in the two buffers. It should be pointed out that the resonance shifts in Table I are dependent also on the concentration of Mg and the nature of the buffer.

Preparation of red cells, band 3 protein and inside-out vesicles

Whole red cells were drawn from healthy young adults into heparin (10 USP units/ml blood) centrifuged and aspirated to remove components other than packed red cells. The cells were washed twice and then suspended, at about a 50% hematocrit, in an isosmolal buffered saline solution of the following concentration (mM): NaCl, 142; KCl, 4.4; $CaCl_2$, 1.2; $MgCl_2$, 0.5; Na_2HPO_4 plus NaH_2PO_4, 5 (to make the final pH 7.4); total osmolality, 290 mosM. 15 mM glucose was added to one aliquot (the normal control) and no substrate was added to the other. Both the normal control and the experimental aliquots were then divided into two parts each and usually incubated for 90 min in a water bath at 37°C. Ouabain, usually at a concentration of 10^{-6} M, was then added to one aliquot in each class and the 37°C incubation was continued for an additional 30 min. Depleted cells were characterized by the presence of a P_i resonance whose peak height was 1- to 1.8-times that of the 2,3-diphosphoglycerate resonances. In acceptable preparations, the P_i content was large enough to produce this characteristic resonance peak ratio and such cells displayed characteristic 'depleted red cell' behavior. Since the glucose content in normal humans is very variable, blood from some donors did not exhibit the higher P_i resonance, and the experiment was terminated, or occasionally, the blood was incubated for a longer time. For the experiments at 145.75 MHz in Table II, the P_i/2,3-diphosphoglycerate resonance ratio was assayed (at 24.16 MHz) and the incubation continued until ratios of 1.5 to 1.6 were obtained. Following incubation, the cells

TABLE II

SYNERGISTIC EFFECT OF OUABAIN AND STILBENE INHIBITORS ON THE 2,3-DIPHOSPHOGLYCERATE RESONANCE SHIFT AT 145.75 MHz

Average of two experiments, each in duplicate. The cells were starved at 37°C until the resonance peak height ratio of P_i/2,3-diphosphoglycerate was 1.5 in one experiment and 1.6 in the other. P_i was measured in all experiments and the extreme variation was from +0.6 to -0.5 Hz; pH was measured in all experiments and the total excursion was from pH 7.34 to 7.45 including all experiments and all conditions.

	3-P resonance shift (Hz)	
	DNDS	DIDS
Depleted red cells		
+ ouabain	−10.9 ± 0.3	−10.9 ± 0.3
+ stilbene inhibitor	+13.3 ± 0.6	+ 4.2 ± 0.6
if additive	+ 2.4 ± 0.1	− 6.7 ± 1
+ ouabain and stilbene inhibitors (observed)	+18.1 ± 1.2	+14.1 ± 0.6

were then washed twice in NMR buffer of the following composition (mM): NaCl, 150; KCl, 5; Tris, 17; 20% ^2H_2O, pH 7.40; NMR spectra were obtained on cells suspended at 50% hematocrit. In the experiments reported in Tables I, IV, V and VI, the Tris was replaced by 17 mM Hepes.

Band 3 protein was obtained from red cell ghosts prepared from bank blood, out-dated by no more than three days. The ghosts were depleted of band 6 and then treated with 0.5% Triton X-100, according to the procedure of Yu and Steck [5], followed by overnight incubation at 4°C with SM-2 Bio-Beads to remove the detergent. Our partial purification stopped before the treatment with *p*-chloromercuribenzoate and ion exchange chromatography. Consequently this partially purified preparation contains bands PAS 1-3 and up to 50% of the phospholipids as well as some band 4.2. SDS polyacrylamide gel electrophoresis carried out by standard methods [6] showed band 3 protein and a small amount of band 4.2. This band 3 protein preparation was then dialyzed (in tubing that had been exhaustively washed) against three changes of NMR buffer and was then observed in the same buffer at a concentration of 0.2 to 0.4 mg protein/ml.

Inside-out vesicles were produced from dated bank blood by the method of Steck and Kant [7]. The sidedness of the inside-out vesicles was routinely measured, as described by these authors, and the vesicles were found to be 80–90% inaccessible to acetylcholinesterase. Vesicles were observed within 1–2 days after preparation at a concentration of 0.3 units acetylcholinesterase activity/ml (approx. 0.1 mg protein/ml) in the NMR buffer whose composition has already been given. Their pH was checked and adjusted, if necessary, to 7.40 just before they were added to the enzyme solutions.

Concentrated glyceraldehyde-3-phosphate dehydrogenase (or other enzyme) solutions were dialyzed (in exhaustively washed dialysis tubing) three times against NMR buffer (Tris, to which 5 mM Mg had been added unless otherwise specified) prior to use. The enzymes were used within 1 day after dialysis. Solutions of glyceraldehyde-3-phosphate were prepared in this buffer and pH was re-adjusted to 7.40.

4,4′-Diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) was either supplied as a gift from Dr. Aser Rothstein or Dr. Philip Knauf or purchased from Pierce Chemical Co., Rockford, IL. To prepare DIDS-treated red cells, fresh human blood was first washed three times in phosphate-buffered saline buffer ($(NaH_2PO_4 + Na_2HPO_4)$, 5 mM; NaCl, 150 mM, pH 8.0). Washed cells at 25% hematocrit were then incubated with the compound at a concentration of 10 μM for 30 min in the dark with phosphate-buffered saline buffer at 37°C, as described by Grinstein et al. [8]. Control and DIDS-treated cells were then washed three times in 1% bovine serum albumin in phosphate-buffered saline buffer, pH 8, to remove unreacted inhibitor. In order to make sure that the anion transport system was completely inhibited, red cell permeability to sulfate was measured photometrically at 30°C after three washes with isosmolar NaCl and titration to pH 6.3 with 1 M HCl as described by Jennings (private communication). This procedure depends on the volume shift induced by sulfate-chloride exchange at pH 6.3. Resonance shift studies were only carried out on cells in which the sulfate influx had fallen to a rate too small to measure.

Concanavalin A was obtained from the Miles Laboratory, Elkhart, IN and succinylated by the method of Gunther et al. [9]. *Maclura aurantiaca* lectin was prepared from *Maclura pomifera* seeds obtained from

F.W. Schumacher Co., Sandwich, MA by the method of Tanner and Anstee [10]. *allo*-Emicymarin was the gift of Professor T. Reichstein. All phosphorus containing molecules were purchased from the Sigma Chemical Co., St. Louis, MO.

Results and Discussion

Evidence that band 3 protein transmits conformational changes across the membrane

The purpose of the present study is to establish the role of band 3 protein in the association ((Na^+ + K^+)-ATPase/band 3 protein/glycolytic enzyme complex). The experiments described in this section of the paper were carried out to demonstrate one link in this association, that between band 3 protein and the cellular glycolytic enzyme complex. Such a linkage is to be expected because band 3 protein has been shown by Strapazon and Steck [11] and McDaniel et al. [12] to have binding sites for each of the two glycolytic enzymes, aldolase (EC 4.1.2.13) and glyceraldehyde-3-phosphate dehydrogenase; Kliman and Steck [13] have measured the glyceraldehyde-3-phosphate dehydrogenase binding constant. We have previously shown [14] that the cellular glycolytic enzymes are linked together in a megadalton complex and are bound tightly enough so that conformational changes can be passed along from one member of the complex to another. We used the ^{31}P resonances of 2,3-diphosphoglycerate as a nuclear magnetic resonance probe for the conformation of monophosphoglycerate mutase, which uses 2,3-diphosphoglycerate as an obligatory intermediate in the mutase reaction, and found that extracellular ouabain modulates these conformational changes.

Our initial experiments [1] which demonstrated the ouabain effect made use of whole red cells. Addition of 10^{-6} M ouabain to the outside of red cells, previously depleted of substrate for 2 h, produced a ^{31}P resonance shift in intracellular 2,3-diphosphoglycerate. Recently, Momsen et al. [15] have reported their inability to reproduce our result in their spectrometer at a resonance frequency of 40.16 MHz. Since our published observations [1] are the basis of many of the experiments that will be presented below, it is important to demonstrate that the resonance shifts we previously reported are real and may be reproduced. For this purpose, we have used the Nicolet NT-360 spectrometer which has recently become available to us. The higher field strength of this spectrometer was expected to increase the resonance shifts by a factor of about six. The results that have been obtained (Table VI, appendix) show the expected increase and generally confirm our published observations. A full discussion of these and other related experiments is reserved for the appendix, which also contains a discussion of other differences between the results of Momsen et al. [15] and our own.

The participation of band 3 protein in the 2,3-diphosphoglycerate resonance shift has been supported by the observation that reduced nicotinamide adenosine dinucleotide and adenosine triphosphate reduce the resonance shifts [2] observed in inside-out vesicles. This observation agrees with the finding of Kant and Steck [16] that these agents markedly reduced the binding of glyceraldehyde-3-phosphate dehydrogenase to band 3 protein. We have examined the role of band 3 protein further by taking advantage of its role in anion transport (see Cabantchik et al. [17]). The disulfonic stilbene reagent DIDS is an impermeable amino reagent which has been shown by Cabantchik and Rothstein [18] to be a specific inhibitor of band 3 protein anion transport. This inhibitor binds covalently to band 3 protein on the outside of the red cell membrane with a very high specificity. When DIDS is bound covalently to the red cell surface, more than 90% of the inhibitor is bound to band 3 protein and anion transport is 100% inhibited when the number of stilbene inhibitor molecules bound to the cell equals the number of copies of band 3 protein [19].

DIDS can also inhibit anion transport non-covalently as can another closely related anion transport inhibitor DNDS [18,19]. We have observed that non-covalent interactions with both of these inhibitors cause saturable chemical shifts in the ^{31}P resonances of 2,3-diphosphoglycerate at 145.75 MHz in 2 h substrate depleted red cells as shown in Fig. 2. Similar results were obtained in three experiments at 24.16 MHz. The half-maximum chemical shift produced by the diisothiocyano inhibitor shown in Fig. 2 is observed at a total inhibitor concentration of 0.5 μM and the half-maximum shift for the dinitro compound is observed at 7 μM. These are not true K_i values since no correction has been made for the differ-

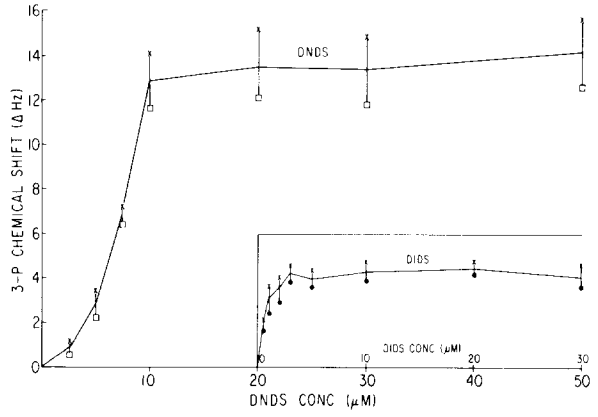

Fig. 2. Resonance shifts produced by anion transport inhibitors in the 3-phosphate resonance of 2,3-diphosphoglycerate at 145.75 MHz in depleted red cells. The points marked × are the results from an experiment in which the ratio of the resonance of P_i to 2,3-diphosphoglycerate was 1.8; for those marked • and □, the ratio was 1.2. The figure gives results of two experiments with the inhibitor DNDS and the insert shows results with the inhibitor DIDS. Similar results were observed for the 2-P resonance for both inhibitors, and also in three experiments at 24.16 MHz. pH was measured before and after the experiments and found to be 7.38, with a maximum excursion of 7.36 to 7.39. The P_i resonance was set at 0 and the maximum excursions were +0.4 and −0.3 Hz. In both cases saturation was observed at high inhibitor concentrations. Since the hematocrit in these experiments is about 50%, most of the inhibitor is bound and these data can not be used to provide values for the inhibitor concentration which produces the half-maximum resonance shift.

ence between free and total inhibitor concentration. Cabantchik and Rothstein [19] observed 50% inhibition of anion transport in the presence of 0.1 μM DIDS bound covalently and we have observed 50% inhibition (Dix et al., private communication) in the presence of 0.5 μM inhibitor, without covalent binding. In the case of DNDS, which does not react covalently, Rao et al. [20] observed 50% inhibition at 14 μM whereas Barzilay et al. [21] report 50% inhibition at 2 μM and Dix et al. (private communication) found a half-value of 8 ± 2 μM. The qualitative agreement between these values and the data in Fig. 2 is illusory since the band 3 protein concentration in our experiments was much greater than the stilbene concentration, so that the free stilbene concentration was much smaller than the total concentration given on the abscissa.

As Fig. 2 shows, the half-maximum resonance shift is very much larger with the inhibitor DNDS, which indicates that each inhibitor produces a different effect at the 2,3-diphosphoglycerate site within the cell and suggests that the binding site for the inhibitor at the outside of the cell adopts different conformations to fit the different inhibitors. This observation also suggests that the resonance shift is to be attributed to specific interactions of the stilbene inhibitor with its receptor and not to nonspecific binding.

The concentrations of inhibitor and band 3 protein are very different under the conditions of the NMR experiment. The total amount of DIDS present in the system is about 0.3 nmol/(ml of suspending medium) for the half-maximum resonance shift. For each ml of suspending medium there are 0.5 ml of packed red blood cells, which contain 8 nmoles of band 3 protein monomer. Thus the half-maximum resonance shift is produced when no more than about 4% of the band 3 protein molecules have interacted with inhibitor. This indicates either, that only a small fraction of the band 3 protein molecules are involved in the resonance shift, or that all of the molecules are involved and that there is a mechanism for transmitting conformational information from one band 3 protein molecule to another as has been previously suggested [22].

The stilbene inhibitors are specific probes of a functional site on band 3 protein. There is another site on band 3 protein which can be specifically recognized by lectin binding, and we have studied this interaction in a further investigation of the specificity of the 2,3-diphosphoglycerate resonance shift. Concanavalin A has been shown by Tanner and Anstee [10] to bind to band 3 protein but not to other components on the human red cell membrane, specifically not to glycophorin (bands PAS 1–3) and the region of bands 4.2 to 5. The observation of Findlay [23] that concanavalin A binds to only about 15% of the band 3 protein on the red cell surface is probably to be attributed to heterogeneity of the sugars in band 3 protein. Treatment of depleted red cells with succinylated concanavalin A causes a shift of the resonances of the 2-phosphate of 2,3-diphosphoglycerate by an average of −1.3 Hz (−1.2 Hz and −1.4 Hz; P_i constant to −0.1 Hz). This shift is specific to binding of concanavalin A to the red cell surface since repeated washing of the red cells with 0.2 M α-methyl-D-mannoside reduced the resonance shift

progressively until, after the 4th wash, the shift had fallen to zero (+0.2 Hz). Concanavalin A is known to bind to mannose and Tanner and Anstee have shown that α-methyl-D-mannoside inhibits the binding of concanavalin A to band 3 protein. These lectin experiments suggest that the 2,3-diphosphoglycerate resonance shift is linked to a mannose receptor that binds specifically to band 3 protein.

It would, however, be possible that some generalized binding of lectins to the cell surface could be responsible for the resonance shifts. Accordingly we carried out experiments with the lectin from *M. aurantiaca* which Tanner and Anstee [10] had shown to bind to the glycosylated transmembrane proteins, bands PAS 1–3, but not to band 3 protein. *M. aurantiaca* lectin is without effect on the resonances of the phosphates of 2,3-diphosphoglycerate (two experiments, Δ Hz = 0.1 and 0.2). One explanation would be that the *M. aurantiaca* lectin did not bind to the cell under our conditions. Consequently we measured the agglutination titre of three lectins. With the *ricinus communis* lectin (2.2 mg/ml) we observed an agglutination titre of 1 : 1024 which agrees with the value given by Sharon and Liss [24]. The agglutination titre for concanavalin A (3 mg/ml) was 1 : 64 and that for the *M. aurantiaca* lectin (0.6 mg/ml) was 1 : 16. In the lectin resonance shift experiments, we used 30 μg/ml of each lectin which compares with the agglutination titre of 46 μg/ml of concanavalin A and 38 μg/ml of the *M. aurantiaca* lectin. Hence, these control experiments indicate that both concanavalin A and the *M. aurantiaca* lectin interact with the red cell membrane at the concentrations used to obtain the resonance shifts given above.

Though these lectin binding experiments strengthen the argument that it is band 3 protein which transmits the signal across the membrane, they still fall short of demonstrating conclusively that band 3 protein must be responsible for the information transfer, because they do not rule out the possibility that the concanavalin A effect is caused by reaction with a transmembrane protein, such as the $(Na^+ + K^+)$-ATPase. Though $(Na^+ + K^+)$-ATPase is present in amounts too small to be visible on a gel, it is known to interact with concanavalin A [25,26]. Consideration of the magnitude of the observed ^{31}P resonance shifts, however, shows that the concanavalin A effect is not the result of direct action on the $(Na^+ + K^+)$-ATPase. The typical size of the resonance shifts is given by the data from the two experiments with inside-out vesicles in Table VI. The average inside-out vesicle concentration in these experiments is 1 unit of acetylcholinesterase activity/ml of suspension. Cohen and Solomon [27] report that there are 10 units of acetylcholinesterase activity/ml of packed cells. Assuming that there is no loss of acetylcholinesterase when the inside-out vesicles are prepared, as Cohen and Solomon do, and that there are 200 copies of $(Na^+ + K^+)$-ATPase/cell (see Hoffman [28] and Drickamer [29]) this concentration of inside-out vesicles is equivalent to $3.3 \cdot 10^{-13}$ moles of $(Na^+ + K^+)$-ATPase/ml of suspension. The average observed resonance shift is about 25 Hz at 145.75 MHz, or 0.17 ppm; the 2,3-diphosphoglycerate concentration is 1 mM. Assume that each copy of the $(Na^+ + K^+)$-ATPase binds one glyceraldehyde-3-phosphate dehydrogenase to which one copy of monophosphoglycerate mutase and one molecule of 2,3-diphosphoglycerate are bound. The fraction of 2,3-diphosphoglycerate molecules bound would then be $3.2 \cdot 10^{-7}$. Assuming fast exchange, the resonance shift would be an average of the shifts of bound and unbound 2,3-diphosphoglycerate, so that the resonance shift of each bound ^{31}P would have to be $5 \cdot 10^5$ ppm. This number is orders of magnitude greater than the ^{31}P resonance shifts observed by Gorenstein [30] for model compounds even after making allowance for possible additional shifts due to changes of polarity (Lerner and Kearns [31]). It is also four orders of magnitude greater than the value of 54 ppm observed when 2,3-diphosphoglycerate is bound to the monophosphoglycerate mutase/phosphoglycerate kinase complex [22]. Hence it does not seem possible for the binding of an enzyme to the $(Na^+ + K^+)$-ATPase on a 1 : 1 basis to account for the 2,3-diphosphoglycerate resonance shift that we have observed. These calculations, coupled with the observation of the resonance shifts induced by the specific stilbene inhibitors and concanavalin A, provide very strong support for the conclusion that band 3 protein is responsible for the 2,3-diphosphoglycerate resonance shift.

Synergistic effect of anion and cation transport inhibitors on 2,3-diphosphoglycerate resonance shifts

The data in Fig. 2 show that anion transport inhibitors produce upfield (positive) displacements of the

2,3-diphosphoglycerate resonances. We have previously shown that the cation transport inhibitor, ouabain, produces a downfield (negative) displacement of the 2,3-diphosphoglycerate resonances in depleted red cells [1]. If these two different inhibitors, the first specific for band 3 protein and the second specific to the $(Na^+ + K^+)$-ATPase, act independently, the two displacements should be additive. The results of two experiments at 145.75 MHz shown in Table II indicate that the effects are not additive. When stilbene is added to ouabain-treated cells, the resonance, far from being shifted downfield, is shifted even further upfield than when the stilbene is added by itself. Similar results have been obtained with the 2-P resonance and in two other experiments of a similar kind at 24.16 MHz. These experiments indicate that there must be a common element in the pathway leading from the disparate sites of action of these two different classes of inhibitors to the observed shift of 2,3-diphosphoglycerate resonances. We have interpreted this common element as strong evidence that there is a link between the $(Na^+ + K^+)$-ATPase and band 3 protein.

Specificity of cardiac glycoside reaction with $(Na^+ + K^+)$-ATPase

It is a characteristic of cardiac glycoside inhibition of red cell cation transport that the extent of ion transport inhibition depends upon the exact configuration of the cardiac glycoside, as was shown by Solomon et al. [32] and by Glynn [33]. Ouabain was used in a preliminary NMR experiment designed to relate the dose response curve of cardiac glycoside inhibition of red cell cation transport to the 2,3-diphosphoglycerate resonance shift. At 40.27 MHz, we observed a resonance shift of –1.9 Hz at 10^{-7} M ouabain, –2.8 Hz at 10^{-6} M and –3.0 Hz at 10^{-5} M for the 3-phosphate of 2,3-diphosphoglycerate. These figures are consistent with the finding of Solomon et al. [32] that the half value for ouabain inhibition of red cell K transport was $5.6 \cdot 10^{-8}$ M and that the inhibition was about 90% complete at 10^{-6} M and completely saturated in the range of 10^{-5} to 10^{-4} M.

An important feature of cardiac glycoside inhibition is the molecular specificity of the effect; because of the pharmacological implications, the specificity was studied in great detail by Chen [34] in a classical series of studies on cat heart muscle. Chen showed that the cardiac glycoside effect depended both on the position of the hydrogen bonding groups and on the aspect of the lactone ring which is an essential feature for such cardiac action. Solomon et al. [32] showed that the mean lethal dose for the cat was linearly related to the dissociation constant for cardiac glycoside binding to the human red cell, thus extending by implication the results for molecular specificity on the cat heart to the human red cell. Glynn [33] also studied the effect of molecular configuration on red cell cation transport inhibition. In particular, Glynn showed that the position of the lactone ring was crucial by comparing the effect of *allo*-emicymarin, in which the lactone ring is found in the β position on carbon 17 with that of digoxin in which the lactone ring is in the normal α position on carbon 17. Glynn found that *allo*-emicymarin produced 30% inhibition at a concentration of 10^{-5} M, a concentration at which ouabain inhibits almost all of the cardiac glycoside inhibitable flux. We have chosen to use *allo*-emicymarin to explore the dependence of the ^{31}P resonances in 2,3-diphosphoglycerate on the molecular configuration of the cardiac glycosides.

The top line of Table III shows that ouabain causes a downfield displacement of the 2,3-diphosphoglycerate resonances in depleted red cells, whereas *allo*-emicymarin causes an upfield displacement. The bottom section of Table III shows that neither glycoside produces a significant resonance shift in control, non-starved cells in agreement with our expectations and previous findings [1]. The cardiac glycoside resonance shifts in depleted cells depend upon the exact molecular configuration of the cardiac glycoside similar to the molecular specificity exhibited by the action of the stilbene inhibitors on band 3 protein. The environment of the 2,3-diphosphoglycerate within the cell is sensitively controlled by the way the cardiac glycoside fits into its receptor on the other side of the membrane, providing further evidence of the tight coupling which links the cardiac glycoside receptor on the outside of the membrane to the glycolytic enzyme complex on the inside.

An additional purpose of the experiments in Table III is to look for a common element in the pathways of cardiac glycoside and lectin action. Comparison of the results expected if the effects were additive (line 3), and those actually observed (line 4) indicates that such a common element exists in the actions of

TABLE III

EFFECTS OF CONCANAVALIN A AND CARDIAC GLYCOSIDES ON 2,3-DIPHOSPHOGLYCERATE RESONANCES AT 24.16 MHz

Average of duplicate experiments. The concentrations of ouabain and *allo*-emicymarin were 10^{-6} M and the glycoside was added 30 min before the end of the incubation of the cells at 37°C. In the lectin experiments 30 μg/ml of concanavalin A was added 20 min before the end of the incubation. P_i resonances were measured for most of the experiments with the depleted red cells and varied between +0.2 Hz and −0.2 Hz.

	Resonance shift (Hz)			
	Ouabain		*allo*-Emicymarin	
	2-P	3-P	2-P	3-P
Depleted red cells				
+ cardiac glycoside	−1.2	−1.3	1.1	2.4
+ concanavalin A	−1.2	−1.3	−1.2	−1.3
if additive	−2.4	−2.6	−0.1	1.1
+ cardiac glycoside and concanavalin A (observed)	−2.1	−1.6	1.3	2.6
Control red cells				
+ concanavalin A	0.0	0.0	0.0	0.0
+ cardiac glycoside	0.2	0.2	0.0	0.0

the cardiac glycoside and the lectin, but the evidence is less compelling than in the stilbene experiments at 145.75 MHz. Nonetheless, these observations add further support to the conclusion that band 3 protein and the $(Na^+ + K^+)$-ATPase are physically coupled in the red cell membrane.

Interaction of separated band 3 protein and $(Na^+ + K^+)$-ATPase

The experiments reported in the previous section demonstrate a linkage between band 3 protein and the $(Na^+ + K^+)$-ATPase, but do not indicate whether other membrane components, for instance spectrin, are also involved. Hence we have carried out further experiments on a separated band 3 protein preparation, which has been partially purified as described in Methods and contains glycophorin, a small amount of band 4.2 protein and about half of the phospholipids. This list of components does not exclude the $(Na^+ + K^+)$-ATPase which Drickamer [29] has shown to be present in so small an amount that it would not produce a visible band in the SDS gel, and whose phosphorylated intermediate co-migrates with band 3 protein.

The experiments in Table IV used glyceraldehyde 3-phosphate to probe the conformation of glyceraldehyde-3-phosphate dehydrogenase which was added, with Mg, to the partially purified band 3 protein. They were carried out in buffer containing 155 mM choline instead of the 155 mM $(Na^+ + K^+)$ in the normal NMR buffer because we have found empirically that choline increases the resonance shifts. Recently, Kliman and Steck [13] have determined the dissociation constant for the binding of glyceraldehyde-3-phosphate dehydrogenase both to red cell ghosts and to inside out vesicles, as a function of ionic strength. At our ionic strength, which is 0.16, we have computed that $K_D = 8.5 \cdot 10^{-6}$ M, and have assumed that this value may be applied to the separated band 3 protein preparation. This assumption seems reasonable since Yu and Steck [5] have previously shown that glyceraldehyde-3-phosphate dehydrogenase binding to band 3 protein depends on ionic strength. Under our experimental conditions there is normally a large molar excess of band 3 protein; using a typical band 3 protein concentration of 0.2 mg/ml (2.1 μM) our calculations indicate that 19% of the glyceraldehyde-3-phosphate dehydrogenase should be bound to band 3 protein. Table IV shows that the addition of band 3 protein to a solution containing glyceraldehyde-3-phosphate dehydrogenase, glyceraldehyde 3-phosphate and Mg produces a resonance shift of 12 to 14 Hz at 145.75 MHz. When ouabain is added there is an additional resonance shift of 4–6 Hz which varies from preparation to preparation. This may be due to differences in band 3 protein aggregation, as discussed below. The specificity of the effect to the molecular composition of the cardiac glycoside is shown by the experiments with *allo*-emicymarin which causes a smaller resonance shift * than

* In Expt. 1 in Table IV, the difference between the resonance shifts induced by *allo*-emicymarin and ouabain is smaller than in Expts. 3 and 4. In Expt. 1, the inhibitor concentrations were 10^{-5} M rather than 10^{-6} M as in the other experiments and the difference may possibly be ascribed to differences in the dose response curve between the two cardiac glycosides.

TABLE IV

INTERACTION OF OUABAIN WITH BAND 3 PROTEIN

The experiments were carried out with NMR buffer (Hepes), modified by replacing the Na and K with 155 mM choline chloride. Ouabain and *allo*-emicymarin were 10^{-6} M except in experiments 1 and 2 where their concentrations were 10^{-5} M. Band 3 protein concentration was: Expt. 1, 0.18 mg/ml; Expt. 2, 0.30 mg/ml; Expt. 4, 0.22 mg/ml. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was added at 0.1 mg/ml with 2 mM (Expts. 1 and 2) or 5 mM (Expts. 3 and 4) glyceraldehye 3-phosphate (G3P) and 5 mM Mg. Myoglobin concentration was 2.6 μM. Reduced nicotinamide adenine dinucleotide (NADH) concentration was 1 mM. 2 mM P_i was added to the buffer in Expt. 1 and 2; the limits for the P_i resonance shift were +0.4 Hz and −0.4 Hz.

	G3P resonance shift (Hz)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
^{31}P resonance frequency (Mhz)	145.75	145.75	109.3	109.3
Control experiments				
G3P (control	0.0	0.0		
+ G3PDH	0.8	1.0		
+ G3PDH + band 3 protein	12.4	13.8		
+ G3PDH + band 3 protein + NADH	4.2	5.9		
+ G3PDH + myoglobin	–	0.8		
+ G3PDH + myoglobin + NADH	–	1.2		
Ouabain experiments				
G3P + G3PDH + band 3 protein	0.0	0.0	0.0	0.0
+ ouabain	4.4	3.7	6.3	5.3
+ *allo*-emicymarin	2.9	–	2.8	1.6

ouabain, particularly in Expts. 3 and 4.

A number of control experiments were carried out to confirm the assignment of the resonance shift in Table IV to interactions of glyceraldehyde-3-phosphate dehydrogenase, when it is bound to band 3 protein. The specificity of this association to band 3 protein was tested by substituting an equimolar concentration of myoglobin for band 3 protein. This protein was chosen because it is not known to bind ions; no resonance shift was observed, as Table IV shows (Expt. 2). Furthermore, addition of 1 mM reduced nicotinamide adenine dinucleotide, which Yu and Steck [5] have shown to dissociate glyceraldehyde-3-phosphate dehydrogenase from band 3 protein in the separated preparation, reduces the band 3 protein resonance shift by about 70% (Expts. 1 and 2) similar to its effect on inside-out vesicles previously discussed. There may be a small reduced nicotinamide adenine dinucleotide effect on myoglobin (Expt. 2); if so, it is in the opposite direction. We conclude that the experiments in Table IV indicate that binding of a cardiac glycoside to a specific receptor in the partially purified band 3 protein preparation induces a conformational change which is reflected in the environment to which glyceraldehyde 3-phosphate is bound. Since there is no spectrin in this preparation, spectrin is not required to transmit this conformational change.

A separate set of experiments was carried out to confirm that the anion transport system was involved in the ^{31}P resonance shifts of separated band 3 protein. Two preparations of band 3 protein were made from the same sample of blood, one after the anion transport inhibitor DIDS had been covalently bound to the anion transport inhibition site on band 3 protein to which it is known to bind specifically (see Knauf [35]). The experiments in Table V show that the covalent binding of the stilbene inhibitor to band 3 protein produces resonance shifts in glyceraldehyde 3-phosphate. This observation is in agreement with the findings of Salhany et al. [36] who used a light-scattering method to detect similar effects of stilbene inhibitors on hemoglobin binding to band 3 protein.

The evidence that the link between the $(Na^+ + K^+)$-ATPase and glyceraldehyde-3-phosphate dehydrogenase is band 3 protein may be summarized as follows:

TABLE V

RESONANCE SHIFTS AT 145.75 MHz DUE TO INTERACTION OF DIDS WITH BAND 3 PROTEIN

Cells treated with DIDS as described in Methods were used to prepare DIDS-treated band 3 protein, as also described in the same section. The composition of the NMR buffer (Hepes) is given in the methods sections; choline chloride at 155 mM replaced the NaCl and KCl in the choline buffers. Band 3 protein concentration was 0.32 mg/ml in Expt. 1, and 0.24 mg/ml (for controls in choline buffer) and 0.30 mg/ml (for the DIDS-treated cells in choline buffer) in Expt. 2. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was added at 0.1 mg/ml with 5 mM glyceraldehyde 3-phosphate (G3P), 5 mM Mg and 2 mM P_i. Values are resonance shifts, relative to P_i, expressed in Hz. The maximal limits of the P_i resonance shifts were +0.8 to −0.8 Hz, which corresponds to a maximum pH shift of 0.015 units between the extremes.

	Resonance shift (Hz)			
	NMR buffer		Choline buffer	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
G3P + G3PDH + band 3 protein	0.0	0.0	23.4	18.0
+ band 3 protein (DIDS-treated cells)	5.2	6.1	26.5	21.4
Difference due to DIDS treatment	5.2	6.1	3.1	3.4

(1) reduced nicotinamide adenine dinucleotide, which is known to dissociate glyceraldehyde-3-phosphate dehydrogenase from band 3 protein, decreases the observed resonance shift by 70%; (2) covalent binding of DIDS to the red cells, from which the band 3 protein preparation was obtained, changes the glyceraldehyde 3-phosphate resonance shift. Consequently, these experiments with the partially purified band 3 protein preparation support the ($(Na^+ + K^+)$-ATPase/band 3 protein/glyceraldehyde-3-phosphate dehydrogenase) linkage and virtually exclude the possible contribution of the other components present in the preparation.

It is desirable to confirm the association between the $(Na^+ + K^+)$-ATPase and band 3 protein by other means and to measure the amount of enzyme bound. The 200 copies of the $(Na^+ + K^+)$-ATPase present in the native red cell [28,29] are a small fraction of the $5 \cdot 10^5$ copies of the band 3 protein dimer. In an initial attempt to measure the ratio of $(Na^+ + K^+)$-ATPase to band 3 protein we used [^{3}H]ouabain and applied the forced dialysis technique of Cantley and Hammes [37] to measure the binding directly. In four experiments, we were unable to measure any [^{3}H]ouabain binding in the band 3 protein preparation. It is difficult to determine the lower limit of detectability, since there is no measure of the association constant of ouabain to separated red cell $(Na^+ + K^+)$-ATPase. If we accept the association constant of $1.69 \cdot 10^{-8}$ mol/l blood given by Solomon et al. [32], the detection limit of the method would be about 1000 copies of $(Na^+ + K^+)$-ATPase for the $5 \cdot 10^5$ dimers of band 3 protein. This concentration ratio would have given us a detectable increase in radioactivity of 14%, whereas the 200 copies expected would not have been detectable by this method. Nonetheless, these experiments do show that the fraction of $(Na^+ + K^+)$-ATPase molecules is significantly less than 10^{-3}/band 3 protein molecule provided the association constant for the intact red cell is applicable.

We next measured the ouabain inhibitable dephosphorylation of adenosine triphosphate in our preparation. Dr. Lewis Cantley was kind enough to make these measurements for us in a solution of the following composition (mM): NaCl, 100; KCl, 20; adenosine triphosphate, 2.5; Mg, 5; EGTA, 1; Hepes, 10; pH 7.4. The ouabain inhibitable enzyme activity is taken as the difference with and without 10^{-4} M ouabain. These are the optimal conditions for measuring the activity of dog kidney $(Na^+ + K^+)$-ATPase which, if pure, would have an activity of 1.2 mmol/mg per h. In three duplicate experiments, our band 3 protein preparation had an activity of $(2.0 \pm 0.5) \cdot 10^{-5}$ mmol/mg per h. In this preparation only 10–20% of the total ATPase is ouabain inhibitable, as compared with almost 100% for the pure dog kidney enzyme. On the basis of one ouabain site for a 270 kdalton

oligomer of $(Na^+ + K^+)$-ATPase given by Jørgensen [38] and a molecular weight of 95 000 for each band 3 protein monomer, the phosphorylase activity would correspond to about 6 copies of $(Na^+ + K^+)$-ATPase per $5 \cdot 10^5$ band 3 protein dimers. This is a small fraction of the expected 200 copies, possibly due to substantial loss of ATPase activity consequent upon purification, as observed by Powell and Cantley [39]. Furthermore, the computation is based on dog kidney $(Na^+ + K^+)$-ATPase enzyme specific activity rather than red cell. Nonetheless these experiments confirm that $(Na^+ + K^+)$-ATPase is present in our partially purified band 3 protein preparation, in accordance with the NMR data presented in Table V.

Our previous order-of-magnitude calculations [22] have indicated that some 10^3 to 10^5 copies of the enzymes, glyceraldehyde-3-phosphate dehydrogenase and monophosphoglycerate mutase, are taking part in the resonance shift, consistent with the participation of most, if not all, of the band 3 protein dimers. Thus there appears to be an amplification of the effect of a few molecules of $(Na^+ + K^+)$-ATPase by some orders of magnitude. The mechanism by which this amplification can take place in the isolated preparation of band 3 probably involves solution aggregation. Band 3 protein is known to aggregate in solution, but it is difficult to measure the aggregation in the samples used in the NMR measurements, since they have to be concentrated to permit molecular weight determination by Sepharose filtration. Concentration, by itself, induces association so that the results of our Sepharose filtration experiment which showed the band 3 protein preparation to be in the void volume, equivalent to a molecular weight greater than $4 \cdot 10^6$, are consistent with, but do not prove, that there was solution aggregation in the NMR samples.

The existence of enzyme complexes linking ion transport to the metabolic enzymes that form ATP is not limited to red cells and may be of general occurrence in nature. In cardiac muscle, Grosse et al. [40] have found an interaction between the $(Na^+ + K^+)$-ATPase and creatinephosphokinase that exhibits many of the properties that characterize the enzyme complex in the human red cell. As these authors point out, there are significant advantages in efficiency from the apposition of the energy production and transport steps in cardiac muscle which, of course, apply equally to the red cell. In the case of the red cell, Parker and Hoffman [41] and Mercer and Dunham [42] have shown that the pump ATP is in a pool segregated from the rest of the ATP in the cytoplasm. The juxtaposition of the glycolytic enzyme complex and the $(Na^+ + K^+)$-ATPase could place the phosphoglycerate kinase which produces the ATP close to the phosphorylating site on the ATPase. The anion transport properties of band 3 protein could deliver the P_i to the site where it is needed for the phosphorylation reaction.

The experiments reported in Table IV are concerned with the cation transport properties of the $(Na^+ + K^+)$-ATPase in the separated band 3 protein preparation whereas those in Table V are concerned with the anion transport aspects of band 3 protein. Taken together, these results indicate that specific inhibitors for the two processes exercise an effect on the partially purified band 3 protein. Since Hoffman et al. [43] have shown that the Na^+/K^+ transport mechanism in the red cell is electrogenic, there appears to be no requirement that each net cation that crosses the membrane be accompanied by a net anion. Nonetheless, our results indicate that there is a close spatial and conformational relationship between the proteins most closely associated with cation and anion transport.

Appendix

As pointed out in the Results and Discussion section, Momsen et al. [15] gave been unable to repeat some of our published observations [1] which had been made at a ^{31}P resonance frequency of 24.16 MHz (JEOL FX-60). We have therefore repeated several of the experiments using the Nicolet NT-360 spectrometer whose higher resonance frequency (145.75 MHz for ^{31}P) should cause the resonance shifts to increase by a factor of about six. We first repeated our experiments showing that ouabain causes a shift in the resonances of 2,3-diphosphoglycerate in 2 h depleted human red cells and obtained the results in Table VI. The resonance shifts are given relative to P_i in order to compensate for any pH shifts or other changes in the intracellular environment as discussed in connection with Table I. Following the suggestion of Momsen et al. [15] we have also replaced Tris in our buffer with Hepes, whose pK_a of 7.55 is much closer to our normal experimental pH of

TABLE VI

EFFECT OF OUABAIN ON 2,3-DIPHOSPHOGLYCERATE RESONANCES AT 145.75 MHz

	Inside-out vesicle concn. [a] (units ACE), units/ml	Resonance shifts (Δ Hz)		
		Relative to P_i		P_i
		2-P	3-P	
Control red cells [b]		0.0	0.0	0.0
+ 10^{-6} M ouabain, Expt. 1		+0.6	−1.0	−0.3
Expt. 2		−0.4	0.6	–
Depleted red cells [b]		0.0	0.0	0.0
+ 10^{-6} M ouabain, Expt. 1		−9.3	−3.7	−0.1
Expt. 2		−9.9	−8.5	0.3
Inside-out vesicles				
Expt. 1 [c]	1.1	−29.2 ± 3	−26.5 ± 2.2	−1.1 ± 1
+ 10^{-6} M ouabain	1.7	−0.2 ± 1	−0.6 ± 0.6	−0.4 ± 0.4
Expt. 2 [c]	1.0	−25.3 ± 0.9	−22.0 ± 0.7	−1.2 ± 1
+ 10^{-6} M ouabain	0.9	−0.2 ± 0.5	−1.2 ± 2	−0.2 ± 1

[a] The acetylcholinesterase (ACE) assay is expressed in units of acetylcholinesterase activity per min.

[b] The reported resonance shifts are relative to control cell resonances (row 1) and depleted cell resonances (row 4) which have been set at 0.

[c] The reported resonance shifts are relative to the solution to which the inside-out vesicles were added. It was composed of NMR buffer (Hepes) to which was added: 2,3-diphosphoglycerate, 1 mM; Mg, 5 mM; monophosphoglycerate mutase, 0.25 mg/ml; phosphoglycerate kinase, 0.6 mg/ml. The resonance shifts were measured five times in each experiment and the standard deviations were computed from these quintuplicate sets of data.

7.4. The resonance for the 2-phosphate in whole red cells shifted by −9.3 Hz and −9.9 Hz, just about 6-times greater than the value of −1.6 Hz previously found (see Table VII), whereas the shift in the 3-phosphate resonance (−1.7 Hz at 24.16 MHz, see Table VII) was 5-times greater in Expt. 2, though only about twice as great in Expt. 1 (for reasons that are not clear). The data in column 5 (top section) show that the P_i resonance did not shift more than ±0.3 Hz. At 145.75 MHz, this means that the cellular pH remained essentially constant throughout these experiments.

The wide bore of the magnet of the NT-360 makes it possible to accomodate 5 ml of solution rather than the 2 ml which is used in the JEOL FX-60 spectrometer. This increase in volume has enabled us to decrease the 2,3-diphosphoglycerate concentration to 1 mM and still obtain sufficient signal intensity. Depending upon the dissociation constant for 2,3-diphosphoglycerate, this reduction can produce up to a 5-fold increase in the resonance shift, over and above the increase due to the increased magnetic field strength. Hence we have chosen to confirm the ouabain effect with inside-out vesicles in which we can set the external (cellular face) 2,3-diphosphoglycerate concentration at 1 mM rather than being constrained to work with the 4.3 mM concentration characteristic of whole cells. The two experiments with ouabain produced resonance shifts of −22 to −29 Hz as shown in the bottom section of Table VI. In order to facilitate comparisons with the data previously obtained, we have gathered the relevant published data in Table VII (Refs. 1 and 11). These data show that the resonance shifts in inside-out vesicles increased from −1 to −2 Hz at 24.16 MHz to −4 to −6 Hz at 109.3 MHz so that the present results confirm the published trend.

The results of these experiments, both in red cells and inside-out vesicles, generally confirm our published data and show that our reported resonance shifts take place when the internal red cell pH is held rigorously constant. We have learned that the exact

TABLE VII

RECAPITULATION OF PREVIOUSLY PUBLISHED OBSERVATIONS

	^{31}P resonance frequence (MHz)	Inside-out vesicle concn. (units ACE/ml)	No of expts.	Resonance shift (Hz)		Ref.
				2-P	3-P	
Depleted red cells	24.16	–	4	0.0	0.0	1
+ 10^{-6} M ouabain		–	4	−1.6 ± 0.4	−1.7 ± 0.4	
Inside-out vesicles [a]	24.16	–	2	−1.8	−1.1	1
+ 10^{-6} M ouabain			5	0.0	0.0	
Inside-out vesicles [b]	109.3	0.66 ± 0.14	4	−5.6 ± 0.3	−3.8 ± 0.3	11
+ 10^{-6} M ouabain			4	−0.3 ± 0.1	−0.3 ± 0.1	

[a] The acetylcholinesterase (ACE) assay in these experiments was not recorded; all the inside-out vesicles prepared from 700 μl of washed cells were used. In the experiments at 109.3 MHz in the next two rows the starting material was 800 μl of washed cells, so the vesicle concentration should have been comparable.

[b] The resonance shifts reported here are the averaged of data from two duplicate experiments, one pair given in row 1 of Table III (of ref. 11), and the second pair given in row 5. The first pair were done on one inside-out vesicle preparation and the second pair on another preparation.

conditions under which the red cells are depleted of substrate are very important. They were not described in detail in our previously published papers and we are of the opinion that the difference between the results of Momsen et al. [15] and ourselves may lie in the exact metabolic condition of the red cell when the NMR experiment was carried out.

We also reported previously on the interaction of the glycolytic enzymes, phosphoglycerate kinase and monophosphoglycerate mutase, in solution [1]. Resonance shifts were observed when phosphoglycerate kinase was added to monophosphoglycerate mutase in the presence of 2,3-diphosphoglycerate and Mg (2.3 Hz for the 2-P and 2.0 Hz for the 3-P; average of 3 experiments at 24.16 MHz) and we interpreted these shifts as evidence of a (phosphoglycerate kinase/monophosphoglycerate mutase) complex. At that time we confirmed the existence of the complex by eluting the enzyme mixture on a Sepharose 6B column and finding a peak corresponding to a 1 : 1 complex of the two enzymes. Momsen et al. [15] were unable to reproduce either the NMR or Sepharose filtration results using higher concentrations of enzymes obtained from a different supplier. They ascribed our NMR results to an imputed pH shift, even though our experiments were made in buffered solutions whose pH was controlled.

We repeated the experiment in the NT-360 spectrometer, reducing the 2,3-diphosphoglycerate concentration to 2 mM in place of the 5 mM originally used, and we also reduced the enzyme concentration by one-third. The resonance shifts were expected to increase by a factor of $10(5/2 \times 2/3 \times 360/60)$ and were found to do so, 21.7 Hz for the 2-phosphate and 20.4 Hz for the 3-phosphate as compared with values of 2.3 and 2.0 Hz at 24.16 MHz.

We have also repeated the Sepharose filtration experiment and have confirmed the existence of the third peak which is ascribed to the complex. Momsen et al. [15] used a Sephadex G-150 superfine column instead of the Sepharose 6B column we used. Our filtration was complete in less than 30 min, whereas the column of Momsen et al., ran for about 45 h, long enough to allow the complex to dissociate as it moved down the column.

Acknowledgements

This research has been supported in part by Grants 5 R01 GM 15692 and HL 26215 from the National Institutes of Health, in part by a grant-in-aid from the American Heart Association, 76-900, and in part by Contract C-670 from the National Science Foundation. This work was done during the tenure of one of us (E.T.F.) of an Established Investigatorship of the

American Heart Association. We would like to express our deep appreciation to Mr. Michael Toon and Ms. Lucille Pothier for their technical assistance which has been essential to the completion of the research. We would also like to thank Dr. Lewis Cantley for the ATPase assays and to acknowledge Dr. Michael Lukacovic's contribution to our understanding of band 3 protein separation. We are also grateful for the use of the Bruker HX-270 Fourier transform magnetic resonance spectrometer at the Francis Bitter National Magnet Laboratory.

References

1 Fossel, E.T. and Solomon, A.K. (1977) Biochim. Biophys. Acta 464, 82–92
2 Fossel, E.T. and Solomon, A.K. (1979) Biochim. Biophys. Acta 553, 142–153
3 Moon, R.B. and Richards, J.H. (1973) J. Biol. Chem. 248, 7276–7278
4 Burt, C.T., Glonek, T. and Barany, M. (1976) J. Biol. Chem. 251, 2584–2591
5 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170–9175
6 Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 26, pp. 3–27, Academic Press, New York
7 Steck, T.L. and Kant, J.A. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. 31, pp. 172–180, Academic Press, New York
8 Grinstein, S., Ship, S. and Rothstein, A. (1978) Biochim. Biophys. Acta 507, 294–304
9 Gunther, G.R., Wang, J.L., Yahara, I., Cunningham, B.A. and Edelman, G.M. (1973) Proc. Natl. Acad. Sci. USA 70, 1012–1016
10 Tanner, M.J.A. and Anstee, D.J. (1976) Biochem. J. 153, 265–270
11 Strapazon, E. and Steck, T.L. (1976) Biochemistry 15, 1421–1424
12 McDaniel, C.F., Kirtley, M.E. and Tanner, M.J.A. (1974) J. Biol. Chem. 249, 6478–6485
13 Kliman, H.J. and Steck, T.L. (1980) J. Biol. Chem. 255, 6314–6321
14 Fossel, E.T. and Solomon, A.K. (1978) Biochim. Biophys. Acta 510, 99–111
15 Momsen, G., Rose, Z.B. and Gupta, R.K. (1979) Biochem. Biophys. Res. Commun. 91, 651–657
16 Kant, J.A. and Steck, T.L. (1973) J. Biol. Chem. 248, 8457–8464
17 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
18 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
19 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330
20 Rao, A., Martin, P., Reithmeier, R.A.F. and Cantley, L.C. (1979) Biochemistry 18, 4505–4516
21 Barzilay, M., Ship, S. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 227–254
22 Solomon, A.K. (1978) Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 31–59, Raven Press, New York
23 Findlay, J.B.C. (1974) J. Biol. Chem. 249, 4398–4403
24 Sharon, N. and Lis, H. (1972) Science 177, 949–959
25 Marshall, M.O. (1976) Biochim. Biophys. Acta 455, 837–848
26 Esmann, M. (1980) Anal. Biochem. 108, 83–85
27 Cohen, C.M. and Solomon, A.K. (1976) J. Membrane Biol. 29, 345–372
28 Hoffman, J.F. (1969) J. Gen. Physiol. 54, 343s–350s
29 Drickamer, L.K. (1975) J. Biol. Chem. 250, 1952–1954
30 Gorenstein, D.G. (1975) J. Am. Chem. Soc. 97, 898–900
31 Lerner, D.B. and Kearns, D.R. (1980) J. Am. Chem. Soc. 102, 7611–7612
32 Solomon, A.K., Gill, T.J. and Gold. G.L. (1956) J. Gen. Physiol. 40, 327–350
33 Glynn, I.M. (1957) J. Physiol. (Lond) 136, 148–173
34 Chen, K.K. (1945) Annu. Rev. Physiol. 7, 677–706
35 Knauf, P.A. (1979) Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 12, pp. 249–363, Academic Press, New York
36 Salhany, J.M., Cordes, K.A. and Gaines, E.D. (1980) Biochemistry 19, 1447–1454
37 Cantley, L.C. and Hammes, G.G. (1973) Biochemistry 12, 4900–4904
38 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97–108
39 Powell, L.D. and Cantley, L.C. (1980) Biochim. Biophys. Acta 599, 436–447
40 Grosse, R., Spitzer, E., Kupriyanov, V.V., Saks, V.A. and Repke, K.R.H. (1980) Biochim. Biophys. Acta 603, 142–156
41 Parker, J.C. and Hoffman, J.F. (1967) J. Gen. Physiol. 50, 893–916
42 Mercer, R.W. and Dunham, P.B. (1981) Fed. Proc. 40, 613
43 Hoffman, J.F. Kaplan, J.H. and Callahan, T.J. (1979) Fed. Proc. 38, 2440–2441