Stickland, L. H. (1934) *Biochem. J. 28*, 1746.
Stickland, L. H. (1935) *Biochem. J. 29*, 288.
Tanaka, H., & Stadtman, T. C. (1979) *J. Biol. Chem. 254*, 447.
Turner, D. C., & Stadtman, T. C. (1973) *Arch. Biochem. Biophys. 154*, 366.
Zindel, U., Freudenberg, W., Reith, M., Andreesen, J. R., Schnell, J., & Widdel, F. (1988) *Arch. Microbiol. 150*, 254.

Evidence for the Development of an Intermonomeric Asymmetry in the Covalent Binding of 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate to Human Erythrocyte Band 3†

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ABSTRACT: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) studies have identified two oligomeric forms of band 3 whose proportions on gel profiles were modulated by the particular ligand occupying the intramonomeric stilbenedisulfonate site during *intermonomeric* cross-linking by BS^3 [bis(sulfosuccinimidyl) suberate] [Salhany et al. (1990) *J. Biol. Chem. 265*, 17688–17693]. When DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) was irreversibly attached to all monomers, BS^3 covalent dimers predominated, while with DNDS (4,4′-dinitrostilbene-2,2′-disulfonate) present to protect the intramonomeric stilbenedisulfonate site from attack by BS^3, a partially cross-linked band 3 tetramer was observed. In the present study, we investigate the structure of the protected stilbenedisulfonate site within the tetrameric complex by measuring the ability of patent monomers to react irreversibly with DIDS. Our results show two main populations of band 3 monomers present after reaction with DNDS/BS^3: (a) *inactive* monomers resulting from the displacement of reversibly bound DNDS molecules and subsequent irreversible attachment of BS^3 to the intramonomeric stilbenedisulfonate site and (b) residual, *active* monomers. All of the residual activity was fully inhibitable by DIDS under conditions of reversible binding, confirming expectations that all of the monomers responsible for the residual activity have patent stilbenedisulfonate sites. However, within this active population, two subpopulations could be identified: (1) monomers which were irreversibly *reactive* toward DIDS and (2) monomers which were *refractory* toward irreversible binding of DIDS at pH 6.9, despite being capable of binding DIDS reversibly. Increasing the pH to 9.5 during treatment of DNDS/BS^3-modified cells with 300 μM DIDS did not cause increased irreversible transport inhibition relative to that seen for cells treated at pH 6.9. This result suggests that both well-defined DIDS-reactive lysine residues of the stilbenedisulfonate site have become unreactive toward DIDS within the refractory subpopulation of monomers. We suggest that the tetrameric structure generated by DNDS/BS^3 treatment is a unique quaternary state of band 3 characterized by a conformational asymmetry between active monomers at the stilbenedisulfonate binding site. In contrast, an alternate quaternary state is formed when all monomers are irreversibly bound by DIDS. This latter state yields covalent dimers on SDS–PAGE after intermonomeric cross-linking by BS^3. The significance of these two quaternary states to either the anion-exchange or the ankyrin binding functions of the porter is presently unknown.

Band 3 is the anion-exchange protein of the human erythrocyte membrane; it also binds cytosolic and cytoskeletal proteins (Passow, 1986; Salhany, 1990). We have recently identified two oligomeric forms of this porter based on in situ cross-linking with bis(sulfosuccinimidyl) suberate (BS^3)[1] (Salhany & Sloan, 1988, 1989; Salhany et al., 1990). One form was readily denatured in SDS under reducing conditions at room temperature to yield covalent[2] dimers. The other form was SDS-resistant up to 60 °C, yielding a noncovalent tetrameric complex composed of two covalent dimers. The population of the two forms was dependent on which ligand was bound to the intramonomeric stilbenedisulfonate site of band 3 during BS^3 intermonomeric cross-linking. Such structurally similar ligands as DIDS and DNDS yielded different cross-linked forms. We suggested that the two oligomeric forms might reflect different conformational states: one state (dimers) being stabilized consequent to the intro-

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[1] Abbreviations: DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; BS^3, bis(sulfosuccinimidyl) suberate; PLP, pyridoxal 5′-phosphate; Bistris, *N*,*N*-[bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FTI, fractional transport inhibition.

[2] In this paper, the term covalent means both covalent and irreversible when applied to the reactions of DIDS and BS^3 with band 3. The term reversible means the ionic binding of DIDS or BS^3 prior to the irreversible covalent reaction with band 3 lysines at the binding site. The term "DIDS-refractory" means unable to bind DIDS irreversibly but able to bind DIDS reversibly.

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duction of bulky covalent ligands such as BS^3 or DIDS at the stilbenedisulfonate site; the other state (tetramers) resulting when the monomeric stilbene site is protected from attack by BS^3 during intermonomeric cross-linking (Salhany et al., 1990). Jennings et al. (1985) have already shown that DNDS binding "functionally protects" band 3 from changes in transport activity caused by BS^3. Thus, with DNDS bound to each monomer, the slower intermonomeric cross-link (Jennings & Nicknish, 1985) "locks-in" a protected tetrameric structure.

In this study, we ask whether the structure of the stilbenedisulfonate site within the functionally protected tetrameric complex is the same as the control. In order to answer this question experimentally, we measure covalent binding of DIDS to control and to DNDS/BS^3-pretreated cells. The assumption is that DNDS will temporally protect the DIDS-reactive lysines [one of which is Passow's "lys A" (Passow, 1986); presumably lysine-539 (Bartel et al., 1989)] of the intramonomeric stilbenedisulfonate site from attack by BS^3 during the pretreatment step (Jennings et al., 1985). Support for this assumption is strong and persuasive. First, it is established that DIDS, DNDS, and BS^3 (in particular, analogues of the latter compound, when they are added at micromolar concentrations) all exclusively bind to the intramonomeric stilbene site with a 1:1 monomeric stoichiometry (Jennings & Passow, 1979; Frohlich, 1982; Beth et al., 1986; Anjaneyulu et al., 1988, 1989; Jennings & Nicknish, 1985; Jennings et al., 1985). Second, DNDS has been shown to protect "lys A" from chemical reaction with the much smaller, uncharged, and permeable reagent 1-fluoro-2,4-dinitrobenzene (Rudloff et al., 1983), and it generally protects the stilbenedisulfonate site from attack by BS^3 (Jennings & Nicknish, 1985; Jennings et al., 1985). Furthermore, the DIDS-reactive lysines are deeply buried within the membrane (Passow, 1986). For example, the unreacted isothiocyanato group of the monovalently bound H_2DIDS molecule was shown not to be reactive toward small, exogenously added agents (Jennings & Passow, 1979). The buried nature of a bound stilbenedisulfonate molecule was strongly confirmed in fluorescence energy-transfer measurements (Rao et al., 1979). This widely accepted physical picture of a stilbenedisulfonate bound to its intramonomeric site makes it extremely unlikely that the DIDS-reactive lysines could be reactive toward a large, negatively charged ligand like BS^3 when DNDS is physically bound to the stilbenedisulfonate site (Passow, 1986). Finally, with DNDS bound to the stilbene site, it has been shown that BS^3 preferentially forms *intermonomeric* cross-links involving lysines which are not a part of the intramonomeric stilbene site (Jennings & Nicknish, 1985). Indeed, one of the intermonomeric lysines can be assigned as lysine-551 of the human sequence (Tanner et al., 1988), since mouse band 3 lacks this lysine (Kopito & Lodish, 1985) and cannot be cross-linked intermonomerically by BS^3 (Jennings, 1989). This result also suggests that there are no more than two intermonomeric cross-links between band 3 monomers, each involving lysine-551 on one monomer and a lysine on the 35-kDa chymotryptic subdomain of the other monomer (Jennings & Nicknish, 1985; Staros & Kakkad, 1983).

On the basis of these facts, we expect to see two kinds of band 3 monomers after DNDS/BS^3 modification and removal of DNDS. The first population will consist of band 3 monomers which have BS^3 at the stilbene site consequent to displacement of DNDS. These monomers will be inactive in transport [under appropriate conditions (see Results and Jennings et al., 1985)], and they will be incapable of physically binding DIDS (Jennings et al., 1985). The second population will consist of active monomers which do not have BS^3 at the stilbene site. These monomers are all expected to bind DIDS reversibly. However, if there are significant differences in structure between monomers at the two DIDS-reactive lysines, then the ability of the reversibly bound DIDS molecule to actually make a covalent bond with one or both of the DIDS-reactive residues (Passow, 1986) could be compromised in some copies of band 3. This can be verified by measuring the ability to inhibit completely the residual transport activity of DNDS/BS^3-modified cells under conditions of covalent binding of DIDS.

An abstract communicating the results of this paper will appear in the *Biophysical Journal* for the society meeting to be held in San Francisco in February of 1991.

Experimental Procedures

Materials and methods were as described (Salhany et al., 1990) with pertinent specific details given in the figure and table legends of this paper.

SDS–PAGE and peak integration of gel profiles were performed as described previously (Salhany et al., 1987a).

Anion transport kinetic measurements were performed with resealed ghosts derived from variously labeled intact red cells using a dithionite transport assay (Salhany & Swanson, 1978; Salhany et al., 1980; Salhany & Gaines, 1981; Salhany, 1990) which can be continuously monitored spectrophotometrically. The specific transport conditions are given in the legends to the figures.

Total DIDS binding was measured by the centrifugation method of Frohlich (1982) using resealed ghosts derived from control and the variously modified intact red cells. Covalent binding of DIDS to band 3 was monitored by using DIDS fluorescence (Eisinger et al., 1982). Proteins were determined by using the BCA method (Pierce Chemical Co., Rockford, IL). Data fitting was performed on an IBM PC according to Leatherbarrow (1987).

Results

Transport Inhibition Studies

Reaction of BS^3 with Band 3: Preliminary Considerations. BS^3 covalently modifies band 3 by reacting with distinct lysines to form both intramonomeric and intermonomeric cross-links (Staros, 1982; Staros & Kakkad, 1983; Staros & Anjaneyulu, 1989; Anjaneyulu et al., 1988, 1989; Beth et al., 1986; Jennings & Nicknish, 1985; Jennings et al., 1985). The intramonomeric reaction occurs very rapidly at 37 °C and can proceed to completion when only micromolar concentrations of BS^3 are present (Anjaneyulu et al., 1989). One mole of BS^3 reacts per mole of band 3 under these conditions (Anjaneyulu et al., 1988, 1989), and the site of reaction physically overlaps the stilbenedisulfonate site (Jennings & Nicknish, 1985; Jennings et al., 1985; Anjaneyulu et al., 1988, 1989). Although such cross-linking of the stilbene site inhibits transport, the observed degree of inhibition depends on the pH of the transport assay buffer (Jennings et al., 1985). For the band 3 system, apparent transport p*K*s are also known to depend on the specific anion used in the assay (monovalent versus divalent) (Salhany, 1990).

For our present purpose, it was necessary to select conditions where BS^3 coverage of the stilbene site on each monomer causes near-stoichiometric inhibition of transport using our particular divalent anion transport assay system (Salhany & Swanson, 1978). To establish this correlation experimentally, we labeled band 3 with micromolar levels of BS^3 to yield

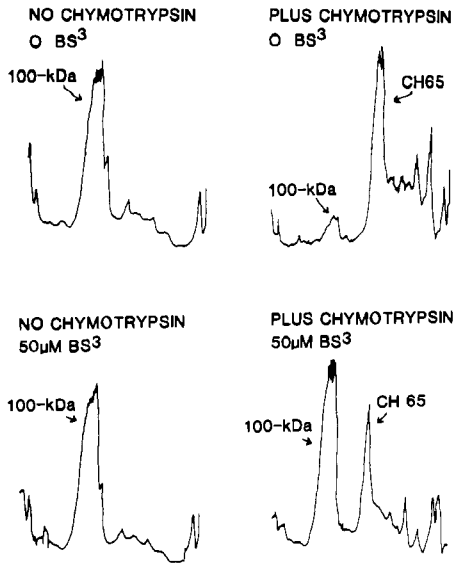

FIGURE 1: Effect of exclusive intramonomeric BS^3 cross-linking on chymotrypsin digestion of band 3. Intact red cells were washed in 106 mM sodium phosphate, pH 7.4 (buffer A), and incubated at 50% hematocrit without and with 50 μM BS^3 for 1 h at 37 °C. The reaction was quenched for both experimental and control cells as described (Salhany et al., 1990), and cells were extensively washed in buffer A. Control and BS^3-reacted cells were each split in two, and half was reacted with 200 μg/mL chymotrypsin overnight at room temperature (Steck et al., 1976). The other half of each sample was incubated under the same conditions but without chymotrypsin. The enzyme was inhibited with PMSF, and membranes were isolated, NaOH-stripped, and electrophoresed on 7.5% acrylamide gels all as described previously (Salhany et al., 1987a). One hundred micrograms of membrane protein was added per lane. The gel was stained with Coomassie Blue. The profiles in this figure show optical density scans of the gels plotted against migration distance. The predominant band seen in the absence of treatment is band 3 at approximately 100 kDa (Steck, 1974); 50 μM BS^3 did not cause intermonomeric cross-linking, since there was no evidence for a change in peak mobility. Chymotrypsin cleaved >95% of the band 3 population in control to yield the 65-kDa transmembrane (CH 65) and the 35-kDa integral (not labeled) subdomains. In contrast, 70% of the band 3 monomers were resistant to chymotrypsin after pretreatment with 50 μM BS^3, based on calculations from peak integration measurements (Salhany et al., 1987a).

intramonomeric cross-linking as the exclusive product (Figure 1). The actual fraction of intramonomeric BS^3 cross-linking was quantitated by integration of the peak areas (Salhany et al., 1987a), in Figure 1, to determine the fraction of chymotrypsin-digestible band 3 monomers as a measure of the fraction of BS^3 intramonomeric cross-linking (Staros & Kakkad, 1983). This type of experiment was performed at selected micromolar levels of BS^3, and transport was assayed as described in the legend to Figure 2. Fractional transport inhibition was then plotted against fractional intramonomeric BS^3 cross-linking to yield the correlation plot shown in Figure 2B. The points lie near the 1:1 theoretical line to within 10%. Furthermore, it should be noted that the residual transport activity seen after treatment with BS^3 can be completely (>98%) inhibited by DIDS when it is present during the transport assay (see below).

Effects of DNDS/BS^3 Pretreatment on DIDS Inhibition of Anion Transport Activity. Intermonomeric cross-linking of band 3 is observed when millimolar concentrations of BS^3 are added and cells are incubated for 1 h at 37 °C. Yet, when DNDS is present at concentrations greater than its binding constant [K_D = 2 μM at physiologic salt (Barzilay & Cabantchik, 1979)], it protects the intramonomeric stilbene site from attack by BS^3 (Jennings & Nicknish, 1985; Jennings et al., 1985). This protection allows intermonomeric cross-linking

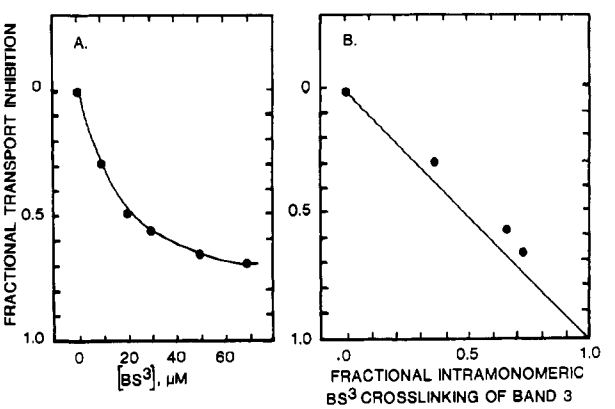

FIGURE 2: Inhibition of anion exchange by exclusive intramonomeric BS^3 cross-linking of band 3. Cells were reacted with various micromolar levels of BS^3 as indicated in Figure 1. Part of selected BS^3 samples was treated as in Figure 1, to quantitate the fraction of intramonomeric cross-linking. All samples were used to make resealed ghosts in 50 mM Bistris and 90 mM sulfate, pH 6.9 (buffer B), as described (Salhany et al., 1987b). The transport assay was performed as described (Salhany et al., 1987b) in buffer B at 10 mM sodium dithionite saturated with N_2 using ghosts at 3% hematocrit (T = 34 °C). Panel A shows a plot of fractional transport inhibition versus the concentration of BS^3 present at the time of cellular cross-linking. Fractional transport inhibition (FTI) is defined as $1 - (V/V_0)$, where V is the experimental velocity and V_0 is the velocity of control. The measured FTI values were then plotted versus the measured fractional intramonomeric cross-linking by BS^3 (Figure 2B). The data points fall within 10% of the expected 1:1 correlation line under our specific assay conditions.

to reach completion *before* the stilbene sites have been substantially modified (Jennings & Nicknish, 1985; Jennings et al., 1985). However, it is reasonable to expect BS^3 to bind covalently to the stilbene site with increasing time in DNDS/BS^3 due to the displacement of reversibly bound DNDS molecules. This slower displacement reaction will result in a reduction in the transport activity under our specific assay conditions (Figure 2), and it will also cause a reduction in DIDS covalent binding capacity as demonstrated by the radioactive labeling studies in the paper by Jennings et al. (1985).

We have studied the ability of DIDS to covalently inhibit anion exchange in resealed ghosts derived from intact red cells incubated for 1 h at 37 °C with 5 mM BS^3 plus 500 μM DNDS (Figure 3). DNDS was always added prior to addition of BS^3. Such covalent binding of DIDS to control resealed ghosts inhibited >98% of anion exchange (Figure 3A), in excellent agreement with our previous results (Salhany et al., 1987b). The DNDS/BS^3-pretreated cells showed about 20% inhibition of the original control transport activity at zero DIDS and about 60% additional inhibition due to covalent binding of DIDS (Figure 3B). However, about 20% of the original transport activity was not inhibitable by covalent binding of DIDS (Figure 3B).

We next investigated the time courses for the development of the two primary components identified in Figure 3B: (1) the component involving inhibition due to time spent in DNDS/BS^3 at 37 °C (Figure 4A) and (2) the residual component seen after pretreatment with DNDS/BS^3 which was refractory toward covalent binding of DIDS (Figure 4B). First, there was an exponential decay in the transport activity with time in DNDS/BS^3 ($t_{1/2}$ = 5 h) which was not seen in control cells without reagents (Figure 4A). Second, the component refractory toward covalent binding of DIDS (Figure 4B) developed at a rate fully 40-fold faster ($t_{1/2}$ = 8 min) than the exponential loss in activity due to DNDS/BS^3 alone (Figure 4A). The fraction of this "DIDS-refractory"

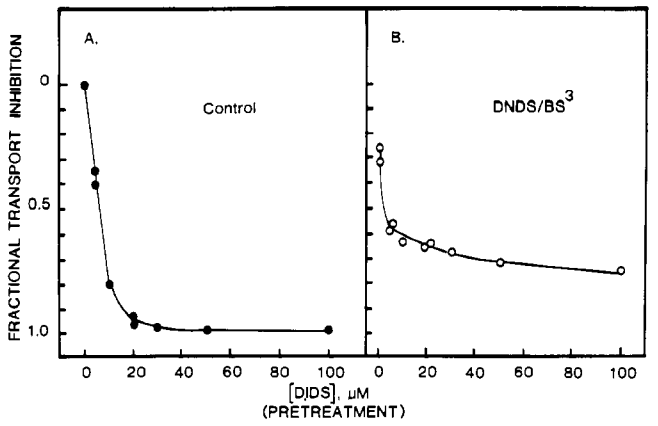

FIGURE 3: Inhibition of anion exchange by the covalent binding of DIDS to control (A) and to DNDS/BS^3-modified cells (B). Intact red cells (50% hematocrit) were reacted with 500 μM DNDS (added first) plus 5 mM BS^3 for 1 h at 37 °C, in buffer A (see Figure 1). The reaction was quenched (Salhany et al., 1990), and resealed ghosts were prepared and washed in buffer B (see Figure 2). Control ghosts were prepared and quenched similarly but without DNDS/BS^3 pretreatment. Each set of resealed ghosts was then reacted with DIDS at 50% hematocrit and at the indicated concentrations for 1 h at 37 °C in buffer B. The ghosts were washed in buffer B (including one wash in 0.5% BSA) and prepared for the transport assay. Transport was assayed as described in Figure 2. FTI was calculated as in Figure 2, using the velocity at zero DIDS for control cells to allow direct comparison.

component remained constant despite 4 h of exposure to DNDS/BS^3 through recycling of reacted cells (see legend to Figure 4 for details).

Figure 5 presents the results of a test performed to see if all of the active monomers have patent stilbenedisulfonate sites. We show the effect of reversible DIDS binding on the residual transport activity of DNDS/BS^3-modified cells. Actual time courses for the transport process are shown for control cells and for cells reacted with DNDS/BS^3 for 1 h at 37 °C. In this particular experiment, incubation with DNDS/BS^3 resulted in an initial 18% inhibition of anion exchange, while addition of DIDS to the sample during the transport assay raised the inhibitory level to ~95% of the original control activity (Figure 5). The results of Figure 5 suggest that the component which is refractory toward covalent binding of DIDS is inhibited by DIDS noncovalently. To be sure that this is the case, we prepared resealed ghosts from 1-h DNDS/BS^3 red cells and treated them with DIDS under conditions which allowed covalent binding. We then measured the time course of transport in the absence and presence of DIDS (Figure 6). The starting DNDS/BS^3/DIDS-pretreated ghosts were 73% inhibited. Addition of DIDS to these cells raised the inhibitory level to 96%, comparable to the inhibitory level reached after covalently labeling control cells with DIDS (98%) (Figure 6).

It is very well established that a bound DIDS molecule can react intramonomerically either in a monovalent or in a divalent fashion depending on the medium pH. At neutral pH, DIDS reacts monovalently with "lys A" on the 17-kDa subdomain of the monomer while at pH 9.5, the second isothiocyano group reacts with "lys B" on the 35-kDa subdomain of the same monomer, resulting in the formation of an intramonomeric cross-link (Jennings & Passow, 1979; Passow, 1986). To see if "lys B" is reactive on the monomers which are refractory toward covalent binding of DIDS at pH 6.9 (Figures 3B and 4B), we reacted resealed ghosts derived from DNDS/BS^3-pretreated red cells with 300 μM DIDS at pH 9.5 in bicarbonate buffer at 50% hematocrit (Table I). After

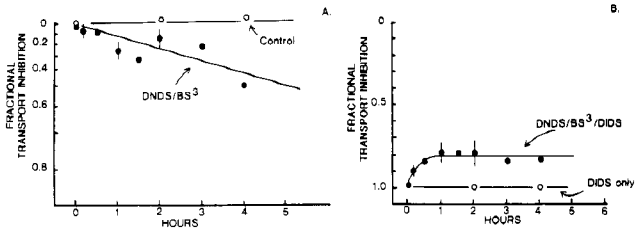

FIGURE 4: Identification of rate laws for the various processes observed in Figure 3B. Intact red cells were reacted with DNDS/BS^3 in buffer A as described in Figure 2, but for various lengths of time. The 0–2-h cells were treated as follows. At the the times indicated, portions of both experimental and control samples were removed and diluted 1:8 in ice-cold quench buffer (30 mM Tris and 150 NaCl, pH 7.4). Cells were kept cold, centrifuged, and washed in the quench buffer plus 0.5% BSA and then 2 more times in the quench buffer followed by three washes in buffer A. The 3-h point in this figure comes from cells reacted for 2 h in DNDS/BS^3, quenched as described, washed 3 times in buffer A to remove the quench buffer, resuspended in a fresh solution of DNDS/BS^3 at the same concentrations, and allowed to incubate for an additional hour at 37 °C. The reaction was quenched and resealed ghosts were prepared as described above. The 4-h point comes from 2-h cells whose reaction was stopped by placing on ice and washing 4 times in buffer A (no quench buffer). These cells were then resuspended in fresh DNDS/BS^3 at the same concentrations and incubated for an additional 2 h at 37 °C. Control cells were treated similarly but without DNDS/BS^3. A portion of the resealed ghosts derived from the above intact red cells was subsequently reacted with 50 μM DIDS for 1 h at 37 °C as described in Figure 3. The transport activity was measured under the same conditions and on the same days as the resealed ghosts which were not exposed to DIDS. FTI values in panels A and B were calculated as in Figure 3, using the same V_0 value at time = 0, from the control ghosts in (A). Panel A shows a semilog plot of the time course of transport inactivation for control cells and for cells incubated in DNDS/BS^3, including the 3- and 4-h recycled points. The DNDS/BS^3 data gave a good fit to a single-exponential function: FTI = $1 - e^{-kt}$ where $k = 0.13 \pm 0.03$ h^{-1}($t_{1/2} = 5.2$ h). Panel B shows the measured FTI for resealed ghosts derived from control cells and from cells modified for various times in DNDS/BS^3, and then reacted as resealed ghosts with DIDS under conditions leading to covalent binding as described above. These data were fit to the following equation with the resulting constants: FTI = $Ae^{-kt} + B$, where $A = 0.17 \pm 0.03$, $k = 5.1 \pm 3.1$ h^{-1}($t_{1/2} = 0.14$ h), and $B = 0.81 \pm 0.01$. The line and curve drawn through the filled circles data are based on the fits to the above equations. The vertical bars are standard deviations of means ($n = 3$).

Table I: Inhibition of Anion Exchange in Control and DNDS/BS^3-Pretreated Cells after Covalent Binding of DIDS[a]

	$1 - (V/V_0)$
control cells reacted with 50 μM DIDS at pH 6.9	0.97
DNDS/BS^3 cells reacted with 50 μM DIDS at pH 6.9	0.72
DNDS/BS^3 cells reacted with 300 μM DIDS at pH 9.5	0.71

[a] Intact red cells were treated in the absence and presence of DNDS/BS^3 exactly as described in the legend to Figure 3. Resealed ghosts were prepared, and cells were reacted with the concentrations of DIDS indicated either at pH 6.9 in buffer B or at pH 9.5 in 150 mM sodium bicarbonate for 1 h at 37 °C. The reaction was quenched, cells were washed with buffer B, and transport was assayed in buffer B (see the legends to Figures 2 and 3). The fraction of monomers inhibitable by covalent binding of DIDS was calculated by using the measured V_0 values from control cells to allow comparison with Figures 3B and 4B.

the covalent reaction was complete, the cells were washed in buffer B (Figure 2), and transport was assayed in that buffer for comparison; 50 μM DIDS was sufficient to inhibit 97% of the transport activity in control cells reacted at pH 6.9 (Table I). For DNDS/BS^3-pretreated cells, using the more extreme reaction conditions at pH 9.5 did not cause such complete inhibition of transport, but instead gave the same inhibition as that seen for similarly pretreated cells reacted with DIDS at pH 6.9 (Table I and Figure 3B). Thus, about 25% of the original transport activity remained totally re-

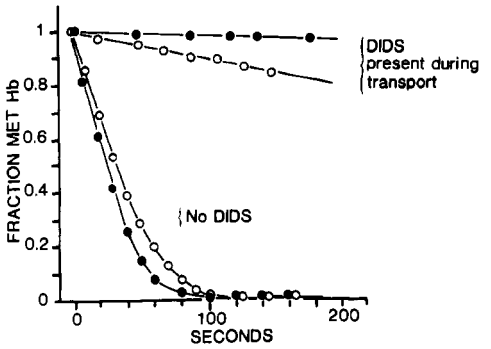

FIGURE 5: Inhibition of anion exchange by reversible DIDS binding to resealed ghosts derived from control and DNDS/BS^3-pretreated cells. (Open circles) Cells were reacted with 5 mM BS^3 in the presence of 500 μM DNDS in buffer A for 1 h at 37 °C. (Closed circles) Control cells were treated with the same procedures but without DNDS/BS^3. Resealed ghosts were prepared from DNDS/BS^3-pretreated and control cells as described in Figure 2 and were washed in buffer B. The resealed ghosts (6% hematocrit) were mixed 50:50 in the stopped flow with deoxygenated (N_2) buffer B plus 56 μM DIDS and 20 mM dithionite prepared immediately before use. The temperature of the reaction was 34 °C. The plot shows the time course for the reduction of intracellular metHb by dithionite (Salhany & Swanson, 1978; Salhany & Gaines, 1981). The y axis is expressed as the fraction of metHb based on the total absorbance change for each sample which contained matched numbers of cells with the same intracellular metHb concentration (4 mM, heme). The initial transport velocities were as follows: no DIDS present, 20 μM/s for control, 16 μM/s for DNDS/BS^3; DIDS present during transport, 0.3 μM/s for control, 1 μM/s for DNDS/BS^3. The percent inhibition of the original control velocity by reversible DIDS binding was 98% for control and 95% for DNDS/BS^3-pretreated cells.

fractory toward covalent binding of DIDS after DNDS/BS^3 pretreatment, despite the extreme reaction conditions used.

Direct DIDS Binding Studies

Covalent Binding of DIDS. We next measured the covalent binding of DIDS to control and to 1-h DNDS/BS^3-pretreated cells by mixing their respective resealed ghosts with saturating concentrations of DIDS at 37 °C and then sampling at various times by quenching the reaction on ice with albumin (Janas et al., 1989). Band 3 was isolated in pure form (Salhany et al., 1987a) and DIDS fluorescence measured on matching protein samples (Figure 7). Compared to control, 1-h DNDS/BS^3-pretreated band 3 showed a 42% reduction in covalent binding of DIDS (i.e., 58% covalently bound), in excellent agreement with the transport kinetic studies which showed about 60% DIDS-inhibitable component after 1-h incubation in DNDS/BS^3 (Figure 3B).

Total Binding of DIDS. In order to confirm the direct covalent binding studies in Figure 7, we performed total DIDS binding studies using the centrifugation technique of Frohlich (1982). With this method, resealed ghosts were simply mixed with various amounts of DIDS under stoichiometric binding conditions and then centrifuged. The amount of DIDS remaining in the supernate was then measured based on the absorbance at 340 nm (Eisinger et al., 1982), after correcting for any small amount of hemoglobin absorbance present. Here, we simply describe these confirmatory results. Pretreatment with covalent DIDS, or with covalent BS^3 alone, completely eliminates total DIDS binding, in agreement with expectations from published covalent binding studies (Jennings et al., 1985). Covalent labeling of the 1-h DNDS/BS^3-pretreated cells with excess DIDS maximally titrated only 58% of the total number of sites, in excellent agreement with the direct covalent binding measurements shown in Figure 7. This finding also agrees with the observed amount of transport inhibition seen consequent to covalent binding of DIDS to 1-h

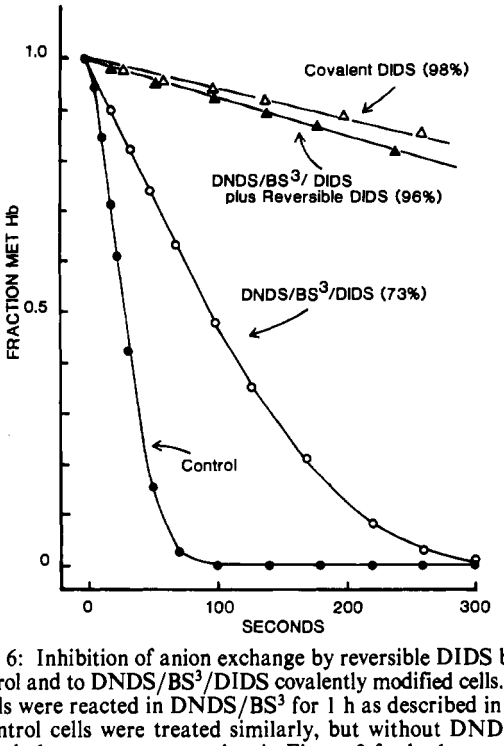

FIGURE 6: Inhibition of anion exchange by reversible DIDS binding to control and to DNDS/BS^3/DIDS covalently modified cells. Intact red cells were reacted in DNDS/BS^3 for 1 h as described in Figure 3. Control cells were treated similarly, but without DNDS/BS^3. Resealed ghosts were prepared as in Figure 2 for both sets of cells, which were then reacted with 50 μM DIDS under conditions which allow covalent binding and washed as described in Figure 3. Transport was assayed as described in Figure 5, in the absence and presence of 100 μM DIDS: (open circles) DNDS/BS^3/DIDS-prelabeled cells without DIDS in the transport assay; (closed triangles) DNDS/BS^3/DIDS-prelabeled cells with DIDS in the transport assay; (closed circles) data for control cells without DIDS in the transport assay; (open triangles) control cells which were reacted with DIDS under conditions leading to covalent binding and assayed in the absence of DIDS in the transport assay. The transport velocities (and respective FTI values) were as follows: control cells, 21 μM/s (0); DNDS/BS^3/DIDS cells, 5.7 μM/s (0.73); DNDS/BS^3/DIDS plus reversible DIDS, 0.76 μM/s (0.96); covalent DIDS, 0.32 μM/s (0.98).

cells (Figure 3B). Thus, three independent methods have shown that about 60% of the sites are covalently reactive toward DIDS after 1-h incubation in DNDS/BS^3. Finally, we could also confirm that incubation in DNDS/BS^3 caused a reduction in total DIDS binding capacity with increasing time. This loss in total binding capacity followed a similar time course to that seen in Figure 4A, confirming expectations that transport inhibition caused by DNDS/BS^3 (Figure 4A) is due to covalent binding of BS^3 to the intramonomeric stilbenedisulfonate site. Incubation of resealed ghosts without reagents for 4 h at 37 °C had no effect on total DIDS binding capacity.

Discussion

Treatment of cells with DNDS/BS^3 for 1 h at 37 °C resolved the band 3 transporter into three distinct classes of monomers (Figure 3B). Approximately 20% of the monomers lost transport activity due to incubation in DNDS/BS^3 alone. The remaining 80% of the monomers retained transport activity, but only a fraction of the original activity (~60%) could be inhibited by covalent reaction with DIDS. The remaining ~20% of the original activity was refractory toward covalent binding of DIDS at near-neutral pH (Figure 3B) where "lys A" of the stilbene site is known to be reactive in control. Those monomers were also refractory toward covalent binding of DIDS at pH 9.5 (Table I), where "lys B" is known to react with a bound DIDS molecule such that an intramonomeric DIDS cross-link forms in control band 3 (Jennings & Passow,

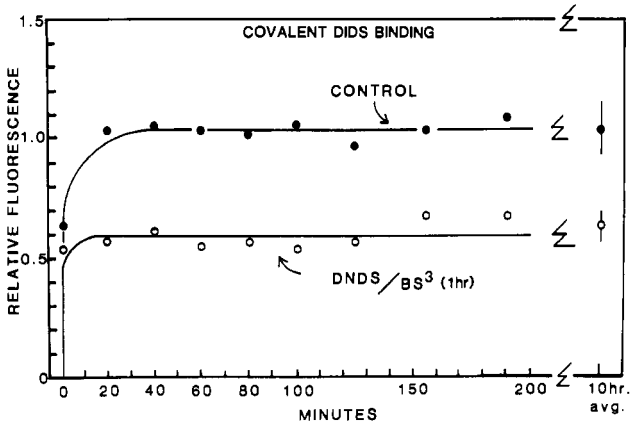

FIGURE 7: Direct measurement of the covalent binding of DIDS to band 3 protein. Resealed ghosts from control cells and cells pretreated for 1 h in DNDS/BS^3 (prepared as described in Figure 3) were preincubated for 15 min at 37 °C in buffer B and then mixed 50:50 with DIDS preincubated at the same temperature also in buffer B, to yield a final hematocrit of 50% and 100 μM DIDS. Aliquots were removed at various times up to 25 h after mixing. For each time point, the reaction was quenched by diluting an aliquot in ice-cold buffer containing 0.5% BSA. The ghosts were then extensively washed and unsealed membranes prepared in 5 mM sodium phosphate, pH 8. The band 3 was extracted in 0.5% Triton X-100 in 36 mM sodium phosphate, pH 7.5, and then isolated in pure form exactly as described previously (Yu & Steck, 1975; Salhany et al., 1987a). DIDS fluorescence emission was measured by using a Perkin-Elmer Model 650-40 fluorometer at an emission wavelength of 430 nm and an excitation wavelength 350 nm (Eisinger et al., 1982). Protein samples were adjusted to 0.042 mg/mL for each time point. The sample buffer was 82 mM sodium phosphate, pH 8.0, and 0.05% Triton X-100. Fluorescence spectra were collected on the same day using the same machine settings for these matched samples to assure valid comparison. The last points for each curve are the mean values and associated standard deviations for the last 10 h of time points which did not vary significantly. There is 42 ± 5% less DIDS fluorescence associated with the DNDS/BS^3-pretreated band 3 (open circles) as compared with control (closed circles).

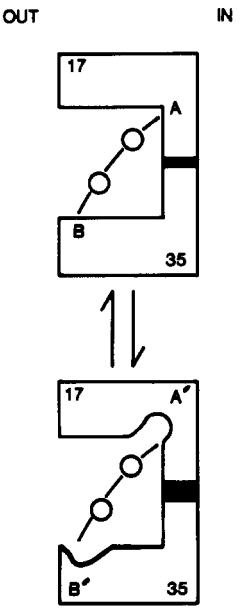

FIGURE 8: Schematic model illustrating the two conformational states of the stilbenedisulfonate site within a band 3 monomer. The double-circle structure represents a DIDS molecule. The numbers 17 and 35 refer to the 17- and 35-kDa chymotryptic subdomains of band 3. The letters A and B refer to the two DIDS-reactive lysines on band 3 (Passow, 1986), with A′ and B′ used to designate altered conformations of the two lysine residues. See the text for discussion.

1979). The level of this "DIDS-refractory" population reached a maximum during the first hour following addition of BS^3 to the DNDS-protected cells (Figure 4B), indicating that this kinetic process has come to completion within that time period.

Of the three populations of band 3 monomers identified in our experiments, the first [consisting of *inactive* band 3 monomers after DNDS/BS^3 treatment (Figure 4A)] is the simplest to understand. The appearance of this fraction results from the expected competitive displacement of reversibly bound DNDS by covalently bound BS^3 at the intramonomeric stilbene binding site on band 3. This is the only reaction, under our specific transport assay conditions, which would be expected to inactivate transport [Figure 2 and Jennings et al. (1985)]. The observed reaction proceeded in a first-order fashion throughout the 4 h over which it was monitored. Measurement of direct total binding as a function of the time cells spent in DNDS/BS^3 (see Total Binding of DIDS under Results) confirmed that there is a diminution in DIDS binding capacity, as inferred from the data in Figure 4A. Despite the high intrinsic rate of BS^3 reactivity at the stilbene site in the absence of competitors (Staros & Anjaneyulu, 1989), a slow observed rate for this reaction is predicted because of the high concentration and the preaddition of competing DNDS molecules. Thus, we ascribe the inactive population of transporters seen after DNDS/BS^3 pretreatment to what amounts to a background reaction, which occurs when protection of the stilbenedisulfonate site by DNDS is overcome.

If we identify the inactive transporters in Figures 3B and 4A with the reaction of BS^3 at the stilbene site when it is left unoccupied by DNDS, then the remaining two populations of *active* monomers must reflect reaction of BS^3 with the transporter in complex with DNDS. The two populations of active monomers are distinguishable on the basis of their ability to react covalently with DIDS. One class was DIDS-reactive, while the other was "DIDS-refractory", despite being capable of binding DIDS reversibly. Since there is only one genetic isoform of band 3 in human erythrocytes (Tanner et al., 1988), saturating concentrations of DNDS should cover all monomers equivalently. Indeed, virtually all monomers are covalently reactive toward DIDS in control human red cells (Figure 3A). If a given band 3 monomer is viewed as being both structurally identical and functionally independent with respect to its neighbor in a homopolymer, and if each monomer contains one stilbene site capable of binding one and only one bulky ligand at a time (DNDS, DIDS, or BS^3), then the simplest expectation in our experiments would have been reaction symmetry. That is, identical monomers should either have the stilbene site occupied by a single covalently bound BS^3 molecule, be inactive in transport and unreactive toward DIDS, or be empty, active in transport, and fully reactive toward covalent binding of DIDS. This simple model is not supported by the observation of two types of active monomers both of which are capable of binding DIDS reversibly but only one of which can bind DIDS covalently.

Why does the simple model fail? We suggest that this failure is the consequence of the existence of two monomeric conformational states of the stilbenedisulfonate site as illustrated in Figure 8. In one state, lysines A and B are reactive toward covalent binding of DIDS, while in the alternate state (A′ and B′) neither lysine residue is reactive. The model is supported by our finding that DIDS can bind reversibly to all active monomers of DNDS/BS^3-pretreated cells (Figure 5 and see Total Binding of DIDS under Results) but can only bind covalently to a portion of these monomers at either neutral (Figure 3B) or alkaline (Table I) pH. This behavior contrasts with control band 3, where at neutral pH "lys A" is reactive with DIDS, while at alkaline pH both "lys A" and "lys B" are

reactive toward covalent binding of DIDS [Figure 3A and Jennings and Passow (1979) and Passow (1986)]. The fact that the "DIDS-refractory" monomers remain refractory under the most reactive conditions employed (300 μM DIDS at pH 9.5) strongly suggests that a conformationally based interpretation of our data is valid. Steric hindrance arguments seem highly unlikely in light of these pH-dependent studies.

The model in Figure 8 also finds support from other studies in the literature which do not involve BS^3 cross-linking, but which do probe the reactivity of the two lysine residues in question. We refer to experiments on the kinetics of intramonomeric cross-linking of the subdomains of band 3 monomers by H_2DIDS (Kampmann et al., 1982). It was shown that the kinetics of cross-linking of lysines A and B (Figure 8) could not be fit by a simple "A–B" model [see Passow (1986) for a detailed discussion]. Instead, it was proposed that either H_2DIDS must react simultaneously with both lysines [a statistically unlikely event (Passow, 1986)] or "lys A" (or perhaps the entire stilbene site) must exist in alternate conformational states which are either reactive or unreactive toward covalent binding of H_2DIDS (Passow, 1986). Our results are consistent with the latter two-state monomeric mechanism.

If we accept a two-state monomeric mechanism to explain heterogeneity in covalent binding of DIDS to DNDS/BS^3-treated band 3, we can then consider what model would predict the observed proportions of the two monomeric states (60:20 ratio of DIDS-reactive/DIDS-refractory monomers within the 80% active population). These considerations must reconcile the functional results of the present paper with our previous BS^3 cross-linking results which showed that DIDS and DNDS yield different proportions of two BS^3 cross-linked oligomeric forms on SDS–PAGE (Salhany et al., 1990). With DIDS (or BS^3) at the stilbene site, covalent dimers were observed after BS^3 intermonomeric cross-linking. With DNDS (or PLP) bound, a tetrameric complex was observed (see the introduction). Formation of the tetrameric complex by DNDS/BS^3 occurred under the same conditions used here to produce the appearance of the "DIDS-refractory" population of monomers. Under these conditions, about 50% of the monomers exist in the tetrameric form [see Figure 4 of Salhany et al. (1990)]. In order to correlate 25% "DIDS-refractory" monomers with formation of 50% of monomers in tetramers, it is necessary to propose that the tetramer has "half-of-the-sites" intermonomeric reactivity toward covalent binding of DIDS. Thus, if 50% of band 3 exists in a quaternary state containing conformationally symmetric monomers (each fully reactive toward covalent binding of DIDS), and 50% exists in another quaternary state containing conformationally asymmetric monomers (half of which are DIDS-reactive and half of which are refractory toward covalent binding of DIDS), then 75% of the monomers in the population would be expected to be either DIDS-reactive or BS^3-reactive (intramonomerically), while 25% would be "DIDS-refractory". This two-state quaternary model is consistent with the observations of the present paper and with our previous cross-linking results (Salhany et al., 1990).

In agreement with this model, other data suggest that the tetrameric complex has "half-of-the-sites" intermonomeric reactivity. For example, partial covalent labeling of band 3 with PLP generated (or preserved) the tetrameric complex on SDS–PAGE after reaction with BS^3 (Salhany & Sloan, 1988), while ligand distribution studies showed an asymmetric distribution of PLP ligands between band 3 monomers. At half-saturation, *both* chymotryptic subdomains of band 3 were equivalently labeled by PLP (Salhany et al., 1987a) on half of the monomers, while the other half of the monomers remained virtually unlabeled by the ligand at its two classes of binding sites [see Salhany (1990) for details]. In addition, Weith et al. (1982) showed that modification of band 3 with phenylglyoxal at a single arginine on the 35-kDa subdomain caused subsequent covalent binding of DIDS to show "half-of-the-sites" reactivity. It will be interesting to see if treatment with phenylglyoxal also generates the band 3 tetrameric complex on SDS–PAGE after BS^3 intermonomeric cross-linking.

At present, the significance of the two quaternary states of band 3 to the transport mechanism is unclear. It is interesting to note that the "turnover number" for band 3 monomer anion exchange in reconstituted vesicles is 5–10 times *larger* than for band 3 within intact red cells (Ducis et al., 1988; Scheuring et al., 1988). Athough speculative, it would not be difficult to assign such drastic changes in monomeric transport activity to a change in porter quaternary state. To clarify the role of these quaternary states in transport, it will be necessary, ultimately, to develop in situ conformational "markers" to identify the two states and establish structure–function correlations. Knowledge of the three-dimensional structure of the porter oligomer would also be helpful so that the conformational environment of the stilbenedisulfonate site can be studied to see how in one state it can be reactive toward DIDS while in the other state it is unreactive. Such future advances may indicate which quaternary state is the "native" state (or whether both states exist in situ) and what role (if any) these quaternary states may play in the anion exchange or ankyrin binding functions of the porter.

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References

Anjaneyulu, P. S. R., Beth, A. H., Sweetman, B. J., Faulkner, L. A., & Staros, J. V. (1988) *Biochemistry 27*, 6844–6851.

Anjaneyulu, P. S. R., Beth, A. H., Cobb, C. E., Juliao, S. F., Sweetman, B. J., & Staros, J. V. (1989) *Biochemistry 28*, 6583–6590.

Bartel, D., Hans, H., & Passow, H. (1989) *Biochim. Biophys. Acta 985*, 355–358.

Barzilay, M., & Cabantchik, Z. I. (1979) *Membr. Biochem. 2*, 255–281.

Beth, A. H., Conturo, T. E., Venkataramu, S. D., & Staros, J. V. (1986) *Biochemistry 25*, 3824–3832.

Ducis, J., Kandrach, A., & Racker, E. (1988) *J. Biol. Chem. 263*, 8544–8550.

Eisinger, J., Flores, J., & Salhany, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 408–412.

Frohlich, O. (1982) *J. Membr. Biol. 65*, 111–123.

Janas, T., Bjerrum, P. J., Brahm, J., & Weith, J. O. (1989) *Am. J. Physiol. 257*, C601–606.

Jennings, M. L. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 397–430.

Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498–519.

Jennings, M. L., & Nicknish, J. S. (1985) *J. Biol. Chem. 260*, 5472–5479.

Jennings, M. L., Monaghan, R., Douglas, S. M., & Nicknish, J. S. (1985) *J. Gen. Physiol. 86*, 653–669.

Kampmann, L., Lepke, S., Fasold, H., Fritzsh, G., & Passow, H. (1982) *J. Membr. Biol. 70*, 199–216.

Kopito, R. R., & Lodish, H. F. (1985) *Nature 316*, 234–238.
Leatherbarrow, R. J. (1987) *Enzfitter: A nonlinear regression data analysis program for the IBM PC*, Elsevier Scientific Publishing Co., Amsterdam.
Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol. 103*, 61–203.
Rao, A., Martin, P., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505–4516.
Rudloff, V., Lepke, S., & Passow, H. (1983) *FEBS Lett. 163*, 14–21.
Salhany, J. M. (1990) *Erythrocyte Band 3 Protein*, CRC Press, Boca Raton, FL.
Salhany, J. M., & Swanson, J. C. (1978) *Biochemistry 17*, 3354–3362.
Salhany, J. M., & Gaines, E. D. (1981) *J. Biol. Chem. 256*, 11080–11085.
Salhany, J. M., & Sloan, R. L. (1988) *Biochem. Biophys. Res. Commun. 156*, 1215–1222.
Salhany, J. M., & Sloan, R. L. (1989) *Biochem. Biophys. Res. Commun. 159*, 1337–1344.
Salhany, J. M., Cordes, K. A., & Gaines, E. D. (1980) *Biochemistry 19*, 1447–1454.
Salhany, J. M., Rauenbuehler, P. B., & Sloan, R. L. (1987a) *J. Biol. Chem. 262*, 15965–15973.
Salhany, J. M., Rauenbuehler, P. B., & Sloan, R. L. (1987b) *J. Biol. Chem. 262*, 15974–15978.
Salhany, J. M., Sloan, R. L., & Cordes, K. A. (1990) *J. Biol. Chem. 265*, 17688–17693.
Scheuring, U., Lindenthal, S., Grieshaber, G., Haase, W., & Schubert, D. (1988) *FEBS Lett. 227*, 32–34.
Staros, J. V. (1982) *Biochemistry 21*, 3950–3955.
Staros, J. V., & Kakkad, B. P. (1983) *J. Membr. Biol. 74*, 247–254.
Staros, J. V., & Anjaneyulu, P. S. R. (1989) *Methods Enzymol. 172*, 609–628.
Steck, T. L. (1974) *J. Cell. Biol. 66*, 295–305.
Steck, T. L., Ramos, B., & Strapazone, E. (1976) *Biochemistry 15*, 1154–1161.
Tanner, M. J. A., Martin, P. G., & High, S. (1988) *Biochem. J. 256*, 703–712.
Weith, J. O., Andersen, O. S., Brahm, J., Bjerrum, P. J., & Borders, C. L., Jr. (1982) *Philos. Trans. R. Soc. London, B 299*, 383–399.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9170–9175.