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N-Hydroxysulfosuccinimido Active Esters and the L-(+)-Lactate Transport Protein in Rabbit Erythrocytes†

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ABSTRACT: Esters of *N*-hydroxysulfosuccinimide strongly inhibit L-(+)-lactate transport in rabbit erythrocytes, probably by acylating amino groups on the transport protein. Lactate transport studies using bis(sulfosuccinimido) suberate (BS^3), bis(sulfosuccinimido) adipate (BS^2A), bis(sulfosuccinimido) dithiobis(propionate), and a variety of monocarboxylate esters suggest that an exofacial amino group of the lactate transport protein is essential for lactate transport. Also, reductive methylation studies show that even when positive charge is preserved in modified amino groups, the transport is strongly inhibited. At pH <6, band 3 mediated inorganic anion transport is enhanced in BS^3-treated cells, while at pH >6, it is inhibited. BS^3-induced inhibition of L-(+)-lactate transport does not have this pH dependence. BS^3 reduces the labeling of a 40–50-kDa membrane polypeptide (band R) by tritiated 4,4′-diisothiocyanato-2,2-dihydrostilbenedisulfonate ($[^3H]H_2DIDS$) and by tritiated bis(sulfosuccinimido) adipate ($[^3H]BS^2A$). Tritiated sulfosuccinimido acetate ($S^2[^3H]$acetate) also labels band R, over a range of concentrations where lactate transport is inhibited in a dose-dependent manner by S^2acetate. BS^3 is a known impermeant protein cross-linker. S^2acetate permeates rabbit red cell membranes by an H_2DIDS-inhibitable mechanism. BS^3 cross-links the proteolytic fragments of rabbit band 3 produced by extracellular chymotrypsin. These labeling experiments support an association between band R and specific monocarboxylate transport.

Evidence of protein-mediated monocarboxylate transport exists for a wide variety of biological systems. Specific monocarboxylate transporters are present in bacteria (Harold & Levin, 1974), mitochondria (Thomas & Halestrap, 1981) and the plasma membranes from a spectrum of mammalian tissues, including erythrocytes (Halestrap, 1976; Dubinsky & Racker, 1978; Deuticke et al., 1978; Deuticke, 1982), Ehrlich ascites tumor cells (Spencer & Lehninger, 1976; Johnson et al., 1980), thymocytes (Anderson et al., 1978; Regen & Tarpley, 1978), renal brush border (Barac-Nieto et al., 1980), small intestine (Lamers & Hülsmann, 1975), liver (Schwab et al., 1979; Fafournoux et al., 1985), brain capillary epithelium (Oldendorf, 1973), and smooth (Kutchai et al., 1978), skeletal

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(Jorfeldt et al., 1976), and cardiac muscle (Watts & Randle, 1967). The kinetics and inhibitor sensitivity of the lactate transporter from red blood cells are fairly well characterized (Deuticke, 1982). Erythrocytes from humans, rats, and rabbits all have a specific lactate transport system that is strongly dependent on extracellular pH and is sensitive to inhibition by sulfhydryl reagents (Deuticke et al., 1978). At least 95% of the total lactate transport across the rabbit red cell membrane is mediated by the *p*-(chloromercuri)benzenesulfonic acid (pCMBS) sensitive specific monocarboxylate carrier (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985); the remainder of the flux is carried by band 3 and via simple passive diffusion across the bilayer. In rabbit red cells, the specific transport is inhibited by 4,4′-diisothiocyanato-2,2-dihydrostilbenedisulfonate (H_2DIDS) (Deuticke et al., 1982; Jennings & Adams-Lackey, 1982) and isobutylcarbonyl lactyl anhydride (iBCLA) (Johnson et al., 1980; Donovan & Jennings, 1985). These agents probably act by covalently modifying amino groups on the polypeptide chain. In estimations from kinetics (Deuticke et al., 1978), rabbit erythrocytes appear to have an abundance of the lactate transport protein, perhaps 10^5 copies per cell. Thus, rabbit red cell membranes are a likely model for the identification and isolation of this transport protein. In our laboratory, chemical labeling with [^{3}H]H_2DIDS at concentrations that inhibit specific lactate exchange suggests that a 40–50-kDa integral membrane polypeptide (band R) is associated with lactate transport in rabbit erythrocytes (Jennings & Adams-Lackey, 1982) and in spectrin-free vesicles budded from rabbit erythrocytes (Donovan, 1985). We have also shown that the site of [^{3}H]H_2DIDS labeling on band R is protected by the potent lactate transport inhibitor iBCLA (Donovan & Jennings, 1985). This further supports an association between band R and lactate transport.

Staros (1982a,b) has synthesized active esters of *N*-hydroxysulfosuccinimide. Bis(sulfosuccinimido)esters of dicarboxylic acids are membrane-impermeant protein cross-linkers (Staros et al., 1980; Staros, 1982a,b) and very likely act by acylating amino groups (Staros, 1982b). Staros (1982b) has shown that bis(sulfosuccinimido) suberate (BS^3) and bis(sulfosuccinimido) dithiobis (propionate) (DTSSP) cross-link human erythrocyte band 3 into covalent dimers on gel electrophoresis. Staros and Kakkad (1983) showed that treating human red cells with BS^3 or DTSSP results in both intermolecular and intramolecular cross-links in band 3. Further, the intramolecular linkage modifies the function of band 3 as an inorganic anion transporter (Jennings et al., 1985).

The lactate transport system in rabbit red cells is susceptible to inhibition by modification with amino-reactive agents (Deuticke et al., 1982; Halestrap, 1976; Deuticke, 1982). We took advantage of the amino group specific active esters of *N*-hydroxysulfosuccinimide (Staros, 1982a,b) in our investigation of structural and functional properties of this transport protein. In this paper, we describe the effects of BS^3 on the specific lactate transporter and the inorganic anion transporter in rabbit erythrocytes. We find that lactate transport is inhibited irreversibly by BS^3 in a concentration-dependent manner; however, unlike inorganic anion transport, this inhibition is not sensitive to the pH of the extracellular flux medium. From the original protocol of Staros (1982b), we prepared the bis(sulfosuccinimido) ester of [^{3}H]adipic acid, [^{3}H]BS^2A, as well as the monocarboxylate active esters of acetic acid (sulfosuccinimido acetate or S^2acetate), butanoic acid (S^2butanoate), hexanoic acid (S^2hexanoate), and octanoic acid (S^2octanoate). From an adaptation of this protocol, we synthesized the tritiated ester, S^2[^{3}H]acetate. We then used BS^3, H_2DIDS, [^{3}H]H_2DIDS, [^{3}H]BS^2A, and S^2[^{3}H]acetate in a series of direct and indirect labeling experiments that provide further evidence that band R is associated with lactate transport. BS^3, DTSSP, and the unlabeled monocarboxylate active esters, all good inhibitors of lactate exchange, were used in a variety of functional experiments that investigate the nature of the active ester induced modification of the lactate transport protein.

Experimental Procedures

Materials. Red blood cells (ethylenediaminetetraacetic acid anticoagulant) were collected by ear vein phlebotomy from New Zealand white rabbits. Cells were used after a maximum of 2 days storage at 4 °C. L-(+)-[^{14}C]lactic acid (90 mCi/mmol) was purchased from ICN Radiochemicals. H^{36}Cl was purchased from ICN and neutralized with NaOH before use. H_2DIDS and [^{3}H]H_2DIDS were made in our laboratory (Jennings et al., 1984) according to methods adapted from Lepke et al. (1976) and Levinson et al. (1979). BS^3 was made according to the method of Staros (1982b) or was purchased from Pierce. DTSSP was from Pierce. BS^2A was made according to the original protocol of Staros (1982b), except that adipic acid was substituted for suberic acid. [^{3}H]BS^2A was also prepared by this procedure; tritiated adipic acid was made by reducing hydromuconic acid with tritium gas generated from Na[^{3}H]BH_4 over charcoal/Pd catalyst. S^2acetate, S^2butanoate, S^2hexanoate, and S^2octanoate were made according to the procedure of Staros (1982b). S^2[^{3}H]acetate (50 mCi/mmol) was made from [^{3}H]acetic anhydride (100 mCi/mmol) in an adaptation of the original procedure. All other chemicals were of at least reagent grade.

L-[^{14}*C*]*Lactate Efflux* (*Jennings & Adams-Lackey, 1982*; *Donovan & Jennings, 1985*). Cells were washed 3 times in 145 mM KCl and 20 mM sodium phosphate, plus 5 mM sodium L-lactate (medium A), pH 7.4, with 10-min incubations at room temperature before each centrifugation. Cells were loaded with L-[^{14}C]lactate by suspending them at room temperature for 5 min in an equal volume of the same buffer plus 5 μCi of L-[^{14}C]lactate/mL. The cells were pelleted by 2-min centrifugation at 7000*g*, the supernatant was removed, and the ^{14}C-loaded cells were chilled on ice. L-[^{14}C]Lactate efflux was initiated (time zero) by resuspending 100 μL of packed cells in 10 mL of ice-cold medium A at a variety of pH values: 5.4, 6.4, 7.4, and 8.2. At appropriate time points (30, 60, 90, and 120 s), 0.5-mL aliquots of the gently mixed suspension were placed into 0.5 mL of ice-cold 200 μM phloretin in phosphate-buffered saline, pH 6.0. When the four paired time points were collected, the samples were centrifuged in an Eppendorf microcentrifuge, and the radioactivity in 0.7 mL of the supernatant was determined by liquid scintillation counting. Extracellular radioactivity at infinite time was estimated by lysing a 0.5-mL aliquot of the flux suspension in 0.5 mL of trichloroacetic acid (9%). Again, the samples were centrifuged and 0.7-mL aliquots of the supernatants counted.

^{36}*Cl Efflux*. Band 3 mediated inorganic anion transport was measured as the exchange of intracellular ^{36}Cl for extracellular Br. Cells washed in 145 mM KCl and 10 mM sodium phosphate, pH 7.4 (PBK 7.4), were loaded with ^{36}Cl by incubating them for several minutes in PBK 7.4 plus ^{36}Cl (2 μCi/mL) at room temperature. The loaded cells were then centrifuged, the supernatant was removed, and the pellet was chilled on ice. Efflux was initiated (time zero) by resuspending 100 μL of the packed cells into 8 mL of ice-cold 150 mM KBr,

10 mM potassium gluconate, and 10 mM potassium phosphate. Extracellular flux pH values were 4.4, 5.4, 6.4, 7.4, and 8.2. At appropriate time points (30, 60, 90 and 120 s), 1-mL aliquots of the gently mixed suspension were centifuged immediately, and 0.5 mL of the supernatant was counted for ^{36}Cl by liquid scintillation counting. Infinity time points were measured by using 9% trichloroacetic acid, just as for L-[^{14}C]lactate efflux.

H_2DIDS and [^{3}H]H_2DIDS Treatment (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985). Cells washed in PBK 7.4 were incubated at 10% hematocrit in PBK 7.4 plus 4 μM H_2DIDS for 80 min at 37 °C. Alternatively, cells were treated with 25 μM H_2DIDS or 25 μM [^{3}H]H_2DIDS (specific activity, 54 mCi/mmol) at 10% hematocrit in PBK 7.4 at 37 °C for 60 min. The treated cells were then centrifuged, and the cells were resuspended in PBK 7.4 plus 50 mM glycine and incubated at room temperature for 5 min. The cells were then washed in PBK 7.4 plus 0.2% bovine serum albumin (BSA), followed by three washes in PBK 7.4.

Sulfosuccinimido Ester Treatment. Cells washed in PBK 7.4 were treated with BS^3, DTSSP, or the monocarboxylate esters at 5% hematocrit in PBK 7.4 for 30 min at room temperature (Staros, 1982b). Cells treated with DTSSP were subsequently treated with 100 mM dithiothreitol (DTT) for 1 h at 37 °C to reduce the disulfide bond in the cross-link. We found that DTT under these conditions is sufficient to reduce most of the DTSSP-induced disulfide bonds linking chymotryptic fragments of rabbit band 3 (not shown). Alternatively, PBK-washed cells were treated with 500 μM BS^{2}A or 500 μM [^{3}H]BS^{2}A (18 mCi/mmol) at 15% hematocrit in PBK 7.4 for 30 min at room temperature. Following treatment with the esters, the cells were incubated in PBK 7.4 plus 50 mM glycine for 5 min and were then washed in PBK 7.4 plus BSA, followed by three washes in PBK 7.4.

Phenylglyoxal Modification (Takahashi, 1968; Bjerrum et al., 1983). PBK 7.0 washed rabbit red cells were treated as a 20% suspension in 100 mM sodium phosphate, pH 10, with 10 mM phenylglyoxal for 90 s at room temperature. The reaction was quenched in 10 volumes of PBK 6.0, and the cells were washed twice in PBK 7.0. This cycle was repeated 5 times, with cells reserved after each treatment for lactate exchange flux studies.

Reductive Methylation (Means & Feeney, 1968; Jones & Vidaver, 1981; Jennings, 1982). Rabbit red cells (5% hematocrit in PBK 7.0) were subjected to three cycles of reductive methylation. At time zero, 100 mM sodium borate was added to bring the suspension to 2.5 mM borate (final pH, 8.5). At 1 min, 2 M formaldehyde was added to bring the final concentration to 16 mM formaldehyde. At 3, 4, and 5 min, equal portions of 100 mM sodium borohydride were added to bring the final concentration to 5 mM borohydride. After 6 min, the cells were pelleted by centrifugation and then were washed twice in PBK 7.0/0.5% BSA and then 4 times in PBK 7.0 before the next cycle or before being used in lactate flux studies.

Membrane Isolation and Polyacrylamide Gel Electrophoresis. Cells treated with H_2DIDS, [^{3}H]H_2DIDS, BS^3, and/or [^{3}H]BS^{2}A, in the concentrations and combinations shown in Figures 2–4, were washed in ice-cold 150 mM $NaHCO_3$ and were then lysed osmotically in ice-cold 5 mM $NaHCO_3$. Membranes (ghosts) were prepared for electrophoresis on 6–18% polyacrylamide gels (Laemmli, 1970), exactly as described by Jennings and Adams-Lackey (1982). The protein present in membrane aliquots was determined according to the method of Lowry et al. (1951) as modified by Peterson (1977). The gel lanes on which isotope-labeled membranes were electrophoresed were, in turn, excised, cut into 2.5-mm segments, digested, and counted by liquid scintillation counting as previously described (Donovan & Jennings, 1985).

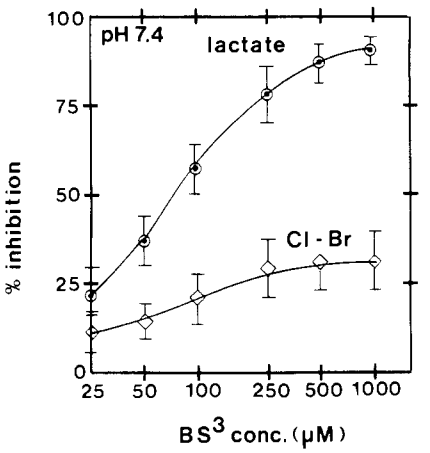

FIGURE 1: Inhibition by BS^3 of lactate–lactate (⊙) and chloride–bromide (◇) exchange in rabbit erythrocytes. Rabbit red cells were treated with BS^3 and subjected to the flux assays described under Experimental Procedures. Percent inhibition was calculated by the method described by Jennings and Adams-Lackey (1982). Error bars show the standard deviation for three experiments (n = 3).

Chymotrypsin Treatment. Cells were treated with chymotrypsin either prior to or following treatment with 1000 μM BS^3. PBK-washed cells were exposed at 10% hematocrit to 1 mg/mL chymotrypsin in PBK 7.4 for 60 min at 37 °C. Cells were then washed thoroughly in PBK 7.4. Isolated membranes from treated cells were electrophoresed as described above. These slab gels were then transferred to nitrocellulose membranes according to the method of Towbin et al. (1979) or stained with Coomassie Blue G. The nitrocellulose transfers were treated with the IV-F-12 anti-human band 3 monoclonal antibody (Jennings et al., 1986), followed by treatment with a peroxidase-linked goat anti-mouse IgG secondary antibody, according to the method of Hawkes et al. (1982). The blots were developed in 4-chloro-1-naphthol/H_2O_2.

Influx of S^2[^{3}H]acetate into Intact Rabbit Red Cells. One millilter of washed rabbit red cells was suspended in PBK 7.4 in a 20-mL total volume at 25 °C. At time zero, solid S^2-[^{3}H]acetate was added with stirring to bring the final concentration to 500 μM. At 5, 10, 20, 30, 60, and 90 min, 3-mL aliquots of the suspension were drawn and pelleted by brief centrifugation at 5000g. Pellets were immediately resuspended in PBK 7.4 plus 50 mM glycine and incubated for 5 min. The pellets were then washed in PBK 7.4 plus 0.5% bovine serum albumin, followed by four washes in 150 mM $NaHCO_3$. The washed red cell pellets were then lysed in 3 mL of ice-cold 5 mM $NaHCO_3$. aliquots of 50 μL of the lysed cell suspension were set aside for liquid scintillation counting. The lysed cells were pelleted by centrifugation for 15 min at 50000g. Fifty-microliter aliquots of the lysate supernatants were set aside for counting. The remaining supernatants were aspirated, taking care not to disturb the pink ghost pellets. The ghost pellet was then brought to volume by adding 3 mL of 5 mM $NaHCO_3$, and 50-μL aliquots were reserved for counting. The 50-μL aliquots from the total, supernatant, and pellet suspension were bleached in 0.5 mL of 30% H_2O_2 before being counted for tritium.

RESULTS

[^{14}C]Lactate–Lactate Exchange and ^{36}Cl–Br Exchange. BS^3 treatment results in a concentration-dependent inhibition

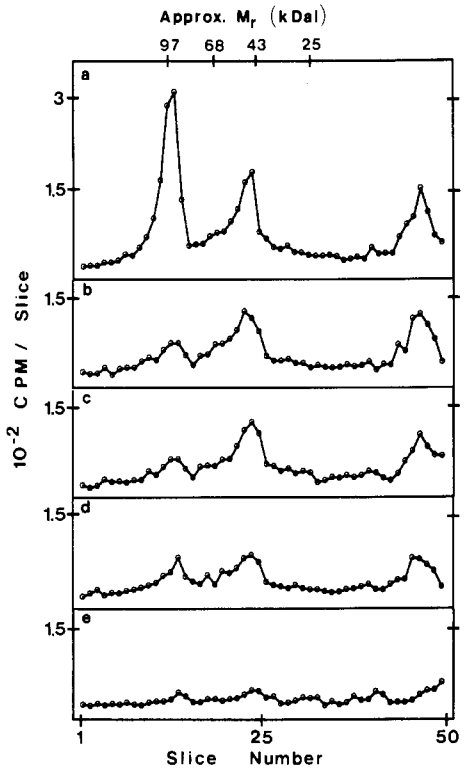

FIGURE 2: Labeling of rabbit erythrocyte membranes by [^{3}H]BS^{2}A. Cells were first treated with 0 (a) or 4 μM (b–e) H_2DIDS for 80 min at 37 °C, washed, then treated with 0 (a,b), 50 (c), 100 (d), or 500 μM (e) BS^3 for 30 min at room temperature, washed, then labeled with 500 μM [^{3}H]BS^{2}A for 30 min at room temperature, and washed. Aliquots of known protein concentration (0.17 mg of total protein per lane) were electrophoresed, and the gel lanes were sliced, digested, and counted for ^{3}H as described under Experimental Procedures. Comparisons to molecular weight standards (not shown) demonstrate that [^{3}H]BS^{2}A labels band 3 and band R (a). A 4 μM concentration of H_2DIDS eliminates most of the labeling of band 3, resulting in a preferential labeling of band R, which is somewhat attenuated (b). A 50 μM concentration of BS^3 (c) does not further diminish the labeling of band R, while 100 (d) and 500 μM (e) BS^3 reduce the labeling of this band.

of lactate–lactate exchange in rabbit red cells (Figure 1). The inhibition of lactate transport by 100 μM BS^3 is not dependent on extracellular flux pH for the pH values investigated (Figure 2). Also, lactate self-exchange is more strongly inhibited than chloride–bromide exchange over the entire range of BS^3 concentrations at extracellular flux pH 7.4 (Figure 1). Half-maximal inhibition for both the lactate exchange and the inorganic anion exchange occurs following treatment with about 75 μM BS^3. A 1000 μM concentration of BS^3 inhibits lactate self-exchange about 90% but chloride–bromide exchange only about 30% at pH 7.4. The pH dependence of ^{36}Cl–Br exchange observed in BS^3-treated human red cells (Jennings et al., 1985) is also found in rabbit red cells (not shown). At extracellular pH 4.4 and 5.4, ^{36}Cl–Br exchange is enhanced, while at pH 6.4, 7.4, and 8.2, this exchange is inhibited when compared to fluxes in untreated cells. Jennings et al. (1985) have shown that at least some of the BS^3-induced enhancement of the inorganic anion transport is the result of the blockage of a self-inhibitory anion binding site.

DTSSP, Monocarboxylate Active Esters, and [^{14}C]Lactate–Lactate Exchange. The importance of intramolecular cross-linking for the inhibition of lactate transport by the active esters was studied by using the cleavable cross-linker DTSSP and a variety of monofunctional esters. At 1 mM concentration, the treatment of rabbit red cells with DTSSP, S^2-acetate, S^{2}butanoate, S^{2}hexanoate, or S^{2}octanoate results in more than 90% inhibition of lactate transport (not shown). When cells so treated with DTSSP are subsequently exposed to 100 mM DTT for 1 h at 37 °C (conditions sufficient to reduce the disulfide linkage), the transport remains more than 90% inhibited. Cells treated with a range of concentrations of S^{2}acetate have a concentration-dependent inhibition of lactate–lactate exchange (not shown) similar to that of BS^3-treated cells (Figure 1).

Phenylglyoxal Treatment, Reductive Methylation, and [^{14}C]Lactate–Lactate Exchange. The question as to whether the modification of arginyl residues of the transporter can strongly inhibit lactate exchange was studied by using phenylglyoxal (Takahashi, 1968; Bjerrum et al., 1983). The values for the inhibition of lactate exchange at pH 7.4 for cells reserved after each of five consecutive cycles of 10 mM phenylglyoxal treatment are as follows: 28%, 41%, 66%, 68%, and 67% (expressed as a percentage of the slope of the log approach to equilibrium for untreated cells; two experiments). Reductive methylation was used to study the effects on lactate exchange of this highly specific amino-reactive modification (Means & Feeney, 1968; Jones & Vidaver, 1981). The percentages of inhibition of lactate exchange in cells reserved after each of three consecutive cycles of reductive methylation with 16 mM formaldehyde/5 mM borohydride are as follows: 66%, 82%, and 87%.

Labeling of Rabbit Erythrocyte Membranes with [^{3}H]BS^{2}A. Cells were treated successively with 4μM H_2DIDS, then with 0, 50, 100, or 500 μM BS^3, and then with 500 μM [^{3}H]BS^{2}A. The membranes were isolated, solubilized, and electrophoresed as described under Experimental Procedures. Solubilized membranes from cells treated only with [^{3}H]BS^{2}A were also electrophoresed on the same gel (Figure 2). A 4 μM concentration of H_2DIDS strongly diminishes the labeling of band 3, leaving band R preferentially labeled. The increasing concentrations of BS^3, which correspond to increasing inhibition of lactate–lactate exchange (Figure 1), also diminish the labeling of band R in a concentration-dependent manner (Figure 2).

Labeling of Rabbit Erythrocyte Membranes with [^{3}H]-H_2DIDS. Cells were treated successively with 4 μM H_2DIDS, then with 0, 50, 100, and 500 μM BS^3, and then with 25 μM [^{3}H]H_2DIDS. Ghosts were isolated, solubilized, and electrophoresed as under Experimental Procedures. The preferential labeling of band R by 25 μM [^{3}H]H_2DIDS following treatment with 4 μM H_2DIDS is just as reported for rabbit red cells (Jennings & Adams-Lackey, 1982) (Figure 3). In the present investigation, BS^3 reduces the labeling of band R by [^{3}H]H_2DIDS in a concentration-dependent manner. However, some labeling by [^{3}H]H_2DIDS of lower molecular weight bands is seen following treatment with the higher BS^3 concentrations. Counts in the lysate supernatants from the preparation of these isolated labeled ghosts are trichloroacetic acid (TCA) precipitable. The counts in these supernatants are in a ratio of about 3:4 to the counts in the same volume of the suspended washed ghosts.

In the above labeling experiments, there is no evidence of intermolecular cross-linking of band R or band 3 by the bis-(sulfosuccinimido) esters on Coomassie Blue G stained electrophoresis gels. In experiments where rabbit red cells were exposed to 5 mM BS^3 for 30 min at room temperature, no intermolecular cross-linking of band 3 into dimer is seen on Coomassie Blue G stained electrophoresis gels (not shown). However, evidence for intermolecular cross-linking of rabbit band 3 by these concentrations of BS^3 is seen using the anti-human band 3 monoclonal antibody IV-F-12 on Western

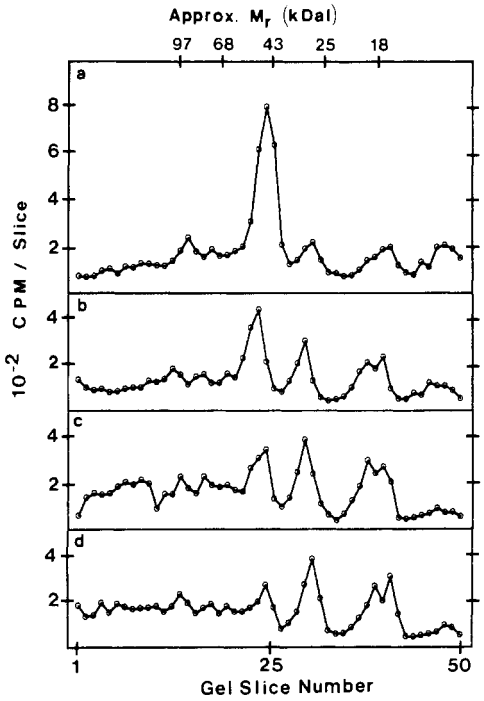

FIGURE 3: Labeling of rabbit erythrocyte membranes by [^{3}H]H_2DIDS following pretreatment with H_2DIDS and BS^3. Cells were first treated with 4 μM H_2DIDS for 80 min at 37 °C, washed, then treated with 0 (a), 50 (b), 100 (c), and 500 μM (d) BS^3 for 30 min at room temperature, washed, and then labeled with 25 μM [^{3}H]H_2DIDS for 60 min at 37 °C. Aliquots of isolated membranes were electrophoresed (0.17 mg of total protein per lane), and the gel lanes were excised, sliced, digested, and counted by liquid scintillation counting, as described under Experimental Procedures. Comparisons to molecular weight standards run on the same gel (not shown) show that the preferential labeling of band R is diminished by BS^3. Also seen is labeling of lower molecular weight bands by [^{3}H]H_2DIDS following treatment with BS^3.

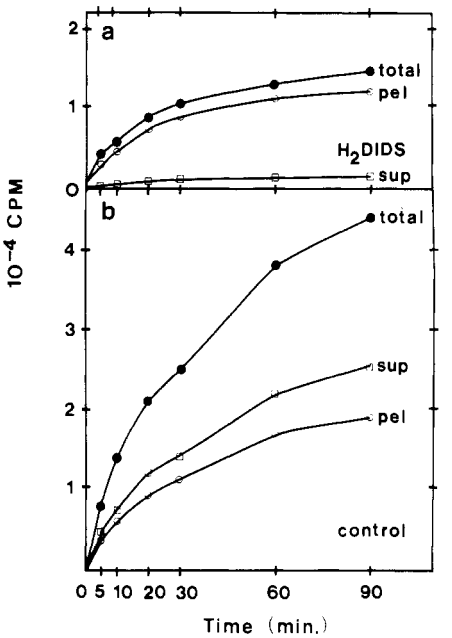

FIGURE 4: Influx of S^2[^{3}H]acetate into rabbit erythrocytes. Five micromolar H_2DIDS treated cells (a) and untreated cells (b) were suspended at 25 °C in PBK 7.4 plus 500 μM S^2[^{3}H]acetate at time zero. At the time points indicated, 3-mL aliquots of the suspension were stopped in PBK 7.4 plus 50 mM glycine and washed by repeated centrifugation. Washed cells (250-μL packed volume) were lysed in 3 mL of ice-cold 5 mM $NaHCO_3$, and a 50-μL aliquot of the total suspension (●) was counted for tritium as described under Experimental Procedures. The lysed cell suspension was centrifuged to separate supernatant from the pellet. A 50-μL aliquot of the lysate supernatant was counted for each time point (□). The counts in 50 μL of *supernatant, representing the intracellular contents*, were 95% precipitable in 0.5 mL of 21% trichloroacetic acid (aqueous) for both control and H_2DIDS-treated cells (not shown). The supernatant was aspirated, the pellet was resuspended in 3 mL of 5 mM $NaHCO_3$, and a 50-μL aliquot was again counted for each time point (○). The fraction of total counts in the supernatant was reduced by more than 80% in the H_2DIDS-treated cells. To convert counts per minute (cpm) to moles of S^2[^{3}H]acetate per milliliter of cells, multiply by 1.08 × 10^{-11} mol/(cpm·mL).

blots (Towbin et al., 1979) of electrophoresed rabbit ghosts (not shown). In human red cells, most copies of band 3 are cross-linked to dimer under these conditions (Staros, 1982b). Rabbit band 3 chymotrypsin proteolysis fragments are cross-linked in situ by BS^3 and DTSSP (not shown), just as is reported for human band 3 (Staros & Kakkad, 1983).

Influx of S^2[^{3}H]acetate into Intact Rabbit Red Cells (Figure 4). Before we proceeded with the direct labeling of red cells with S^2[^{3}H]acetate, the influx of this compound into intact rabbit red cells was studied in order to determine whether, and under what conditions, this label is membrane-impermeant. In the flux experiment described under Experimental Procedures, control cells have more than half of the total counts from S^2[^{3}H]acetate in the lysate supernatant (representing the intracellular contents) over the course of incubation. These counts are more than 95% precipitable in TCA (21%). Pretreatment with 4 μM H_2DIDS reduces the fraction of total counts in the lysate supernatant by more than 80%.

Labeling of Rabbit Erythrocyte Membranes with S^2[^{3}H]-acetate. Cells were treated with 4 μM H_2DIDS, washed, and then treated with 50, 100, 250, and 500 μM S^2[^{3}H]acetate. The cells were then incubated in PBK/glycine and washed in PBK/BSA as described. Ghosts were isolated, solubilized, and electrophoresed as described under Experimental Procedures. There is a concentration-dependent labeling of Band R by S^2[^{3}H]acetate over a range of concentrations where lactate transport is inhibited in a dose-dependent fashion by S^{2}acetate (Figure 5). The H_2DIDS pretreatment markedly reduces the labeling of band 3 by the tritiated active ester. Some labeling of ~25- and ~18-kDa polypeptides is also seen. There is no detectable labeling of spectrin even in the cells not pretreated with H_2DIDS.

Labeling of Rabbit Red Cells by Reductive Methylation Using Tritiated Borohydride. The characteristic tritium-labeling pattern of band 3 and band R, as seen in membranes from cells treated with 25 μM [^{3}H]H_2DIDS (Jennings & Adams-Lackey, 1982), 500 μM [^{3}H]BS^{2}A (Figure 2), or 500 μM S^2[^{3}H]acetate (Figure 5), is also seen for cells treated with a single cycle of reductive methylation using 16 mM formaldehyde and 5 mM sodium [^{3}H]borohydride (not shown).

Discussion

Lactate exchange and band 3 mediated inorganic anion exchange are both sensitive to modification with BS^3. The responses of the two systems are qualitatively quite different. Lactate transport is inhibited in a concentration-dependent manner, and this inhibition does not appear to be dependent on the pH of the extracellular flux medium. In contrast, the inorganic anion transport is less strongly inhibited over the same range of concentrations at pH 7.4, and the extracellular flux pH profoundly affects the anion flux, so that at lower pH (<6) the flux is enhanced and at higher pH (>6) the flux is inhibited. The effects of BS^3 on chloride–bromide exchange in human red cells have been studied in detail by Jennings et al. (1985). Functionally, these effects on rabbit red cell in-

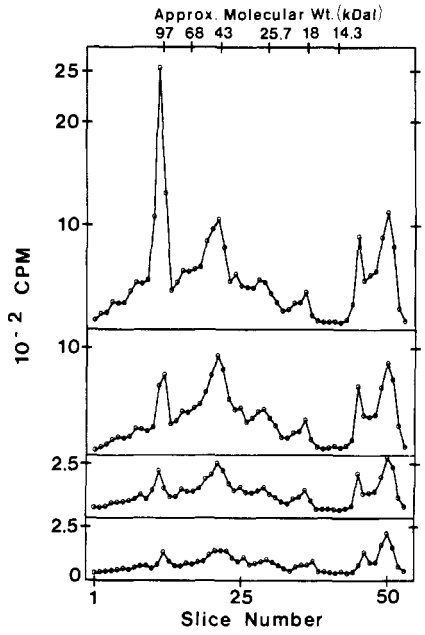

FIGURE 5: Labeling of rabbit erythrocyte membranes by $S^2[^3H]$-acetate. Cells were treated with 0 (top) or 4 μM H_2DIDS (bottom three plots) prior to treatment with 500, 500, 100, and 50 μM S^2-[^{3}H]acetate (top to bottom) at 25 °C for 30 min. Samples of known protein concentration were electrophoresed (0.17 mg of total protein per lane), and the gel lanes were sliced and counted as described under Experimental Procedures. $S^2[^3H]$acetate labels band R and band 3; the band 3 labeling is strongly diminished by the H_2DIDS pretreatment. The concentration-dependent increase in the labeling of band R occurs over a range where S^2acetate increasingly inhibits lactate–lactate exchange (see Results).

organic anion transport are similar (see Results).

The *N*-hydroxysulfosuccinimido esters presumably act by acylating amino groups (Staros, 1982b). The sensitivity of the monocarboxylate transporter to inhibition by amino group modification is well-known (Deuticke, 1982; Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985). Deuticke (1982) has proposed that an amino group at the active site provides the positive charge that allows initial binding of the anionic substrate to the transport protein. Acylation of amino groups by the sulfosuccinimido esters would eliminate this positive charge on the protein. Since a question may exist as to whether the positive charge carrying guanadino group is also functionally involved in lactate transport, the importance of arginyl residues in the transport protein was investigated by phenylglyoxal modification (Takahashi, 1968; Bjerrum et al., 1983). Repeated treatments of intact red cells with this agent brought about a maximal inhibition of the transport of about 67% (see Results), suggesting that arginyl residues may be related, but not essential, to the function of the transporter. Finally, reductive methylation minimally modifies amino groups by replacing hydrogens with methyl groups; it preserves the positive charge carried by these amino groups. We find that this method also brings about a strong inhibition of lactate transport (see Results). This indicates that even the elimination of the charge carried by the amino group is not strictly a necessary condition for inhibition and suggests that subtle alterations in the structure and conformation of this localized region of the active site of the transporter have far-reaching functional consequences.

Staros (1982b) has shown that the bis active esters BS^3 and DTSSP are membrane-impermeable. We investigated the permeability of our new labeled $S^2[^3H]$acetate (Figure 4). Over a course of incubation, a significant number of the counts appear to partition into the intracellular space, on the basis of counts in the supernatant of the lysed cells. These supernatant counts precipitate in 21% trichloroacetic acid, suggesting that they are associated with protein (probably hemoglobin, by its molar excess). If the cells are first treated with a low concentration of H_2DIDS, the fraction of total counts appearing in the lysate supernatant is reduced by 80%. This suggests that the anionic S^2acetate is a substrate for band 3. It is also noteworthy that when $S^2[^3H]$acetate is used to label intact rabbit red cells, no appreciable labeling of spectrin is seen (Figure 5), even in the cells not pretreated with H_2DIDS. Since a significant amount of the labeled S^2acetate appears to enter the cell by an H_2DIDS-sensitive mechanism (Figure 4), the absence of labeling of this abundant intracellular cytoskeletal protein is not of itself evidence that the label is membrane-impermeant. The low (undetectable) level of spectrin labeling is probably a consequence of the vast molar excess of hemoglobin in the intracellular compartment.

H_2DIDS is a known inhibitor of inorganic anion (Shami et al., 1978) and lactate transport (Deuticke et al., 1982; Jennings & Adams-Lackey, 1982) in rabbit erythrocytes. [^{3}H]H_2DIDS has been used to label band R preferentially in a concentration-dependent manner over a range of concentrations where H_2DIDS increasingly inhibits lactate transport (Jennings & Adams-Lackey, 1982). This suggests that band R is involved in lactate transport. Also, iBCLA, a lactate analogue and a potent inhibitor of lactate exchange (Johnson et al., 1980), reduces the labeling of band R by [^{3}H]H_2DIDS, which provides further evidence that band R is involved in lactate transport (Donovan & Jennings, 1985). In the present work, an analogous experiment using BS^3 instead of iBCLA gives a completely consistent result (Figure 3). That is, BS^3 reduces the labeling of band R by [^{3}H]H_2DIDS in a concentration-dependent manner over a range of BS^3 concentrations that increasingly inhibit lactate exchange. Also evident in Figure 3 is the labeling of lower molecular weight bands by [^{3}H]-H_2DIDS following BS^3 treatment. It is clear from Figure 3 that the BS^3-dependent appearance of labeling in these other bands does not have the BS^3 concentration dependence seen in the labeling of band R. One possibility is that BS^3 may alter higher order protein structure (Staros, 1982b), which might unmask potential [^{3}H]H_2DIDS binding sites on other smaller or less abundant membrane proteins. However, the positions of these bands also correspond to the expected positions for hemoglobin and hemoglobin dimer, which raises a possibility that BS^3 may potentiate the cross-linking by tritiated H_2DIDS of hemoglobin to amino-containing lipid in the membrane; BS^3 may also influence covalent and noncovalent associations of labeled hemoglobin to other components of the membrane. It is known for human red cells that some H_2DIDS binds to the cells and is not removed by albumin washes but comes off when the cells are hypotonically lysed (Passow et al., 1982). The significant number of TCA-precipitable counts that are associated with the supernatants following lysis of the labeled cells (see Results) is consistent with the labeling of cytoplasmic protein by [^{3}H]H_2DIDS.

BS^2A behaves much like BS^3 in red cells. It is a good inhibitor of lactate transport in rabbit, and it cross-links human band 3 into dimer (not shown). [^{3}H]BS^2A at 500 μM concentration labels band 3 and band R in control rabbit red cells; it labels band R preferentially in cells pretreated with 4 μM H_2DIDS. BS^3, in concentrations that increasingly inhibit lactate transport, also diminishes this labeling of band R in a dose-dependent manner. This provides additional evidence that band R is associated with specific lactate transport. The monocarboxylate label $S^2[^3H]$acetate gives consistent results:

The labeling of band 3 is diminished by a low concentration of H_2DIDS, and the dose-dependent labeling of band R occurs over a range of S^2acetate concentrations where the lactate transport is increasingly inhibited. The concentration-dependent labeling of ~25- and ~18-kDa polypeptides by S^2-[^{3}H]acetate technically associates these bands with the concurrent inhibition of lactate transport. However, these bands are not labeled by either [^{3}H]H_2DIDS (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985) or [^{3}H]BS^2A (Figure 2) under conditions where lactate transport is inhibited. The active esters and H_2DIDS are not affinity labels but simply covalent chemical modifiers that preferentially react with amino groups. Thus, it is possible that a relatively small molecule, such as S^2acetate, might have access to amino groups on polypeptides that are not accessible to the larger bis esters.

Rabbit band 3 chymotrypsin proteolysis fragments are cross-linked in situ by BS^3 and DTSSP (not shown), just as is reported for human band 3 (Staros & Kakkad, 1983). Thus, at least one intramolecular BS^3 cross-link is present in rabbit band 3. An intramolecular cross-link is related to the functional alteration of anion exchange in human red cells (Jennings et al., 1985).

The [^{3}H]H_2DIDS, [^{3}H]BS^2A, and S^2[^{3}H]acetate labeling experiments provide additional evidence that band R is associated with lactate transport in rabbit erythrocytes. The sensitivity of the lactate transport system to agents known to modify amino groups on other polypeptides [e.g., pyridoxal phosphate (Deuticke et al., 1978); fluorodinitrobenzene (Halestrap, 1976), and H_2DIDS (Jennings & Adams-Lackey, 1982)] suggests that amino groups on the lactate transport protein are critical in facilitating the transport (Deuticke, 1982). While it is evident from this and many other studies that specific monocarboxylate transport and inorganic anion transport are distinct processes (Dubinsky & Racker, 1978; Anderson et al., 1978; Deuticke et al., 1982), they bear a similarity in that both systems mediate the transfer of negatively charged entities across the bilayer. The importance of lysine residues to this process in human band 3 is well-known (Cabantchik et al., 1975; Passow et al., 1980; Jennings, 1982; Rudloff et al., 1983; Jennings et al., 1985). It is reasonable to expect that amino side chains also have a functional role in the transport of monocarboxylates by the specific carrier. The present investigation suggests that the details of this role in the two transport systems are not the same; the pH dependence and extent of inhibition of Cl–Br exchange and lactate–lactate exchange in BS^3-treated cells (Figure 1) are quite different. A definitive demonstration of the contribution of this functional group in the particular protein responsible for monocarboxylate exchange will depend on the positive identification of this carrier protein in the membrane. Perhaps the best criterion for this identification is the isolation and reconstitution into a lipid bilayer of a protein that performs this function. This has yet to be accomplished. These chemical labeling experiments give direction to future work in this area.

Registry No. BS^2A, 100367-07-5; BS^3, 82436-77-9; DTSSP, 81069-02-5; S^2[^{3}H]acetate, 100367-08-6; Cl^-, 16887-00-6; Br^-, 24959-67-9; L-(+)-lactic acid, 79-33-4; *N*-hydroxysulfosuccinimide, 82436-78-0.

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Neonatal Human Foreskin Keratinocytes Produce 1,25-Dihydroxyvitamin D_3†

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ABSTRACT: Primary cultures of neonatal human foreskin keratinocytes converted 25-hydroxyvitamin D in high yield to a metabolite with the chromatographic behavior of 1,25-dihydroxyvitamin D_3. The identity of this metabolite as 1,25-dihydroxyvitamin D_3 was confirmed both by its potency in displacing 1,25-dihydroxyvitamin D_3 in the chick cytosol receptor assay and by mass spectral analysis. These results suggest that 1,25-dihydroxyvitamin D_3 may be formed in the epidermis to regulate vitamin D production by the epidermis and to provide an alternative to 1,25-dihydroxyvitamin D_3 production by the kidneys.

Vitamin D_3 is produced in the epidermis from 7-dehydrocholesterol under the influence of ultraviolet irradiation (Holick et al., 1980). This process has been studied in vitro by using primary cultures of human foreskin keratinocytes (Nemanic et al., 1983b). Although the production of vitamin D_3 from 7-dehydrocholesterol is viewed as a sequence of photochemical and thermal reactions without hormonal regulation, some data suggest that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$],[1] the most biologically active metabolite of vitamin D_3, regulates the amount of 7-dehydrocholesterol available for conversion to vitamin D_3 (Nemanic et al., 1983a; Esvelt et al., 1980). This possibility is supported by the presence of high-affinity receptors for 1,25$(OH)_2D_3$ in the skin (Stumpf et al., 1979; Feldman et al., 1980). Furthermore, 1,25$(OH)_2D_3$ has been shown to influence differentiation of the keratinocytes (Hosomi et al., 1983).

1,25$(OH)_2D_3$ is produced primarily if not exclusively in the kidney under normal physiologic circumstances (Fraser et al., 1970; Reeve et al., 1983; Shultz et al., 1983; Brumbaugh et al., 1974). However, some studies suggest that 1,25$(OH)_2D_3$ may also be produced by human bone cells in culture (Howard et al., 1981), in melanomas (Frankel et al., 1983), in sarcoid tissue (Barbour et al., 1981; Mason et al., 1984), and in placenta (Gray et al., 1979; Whitsett et al., 1981; Weisman et al. 1979). Furthermore, anephric humans (Lambert et al., 1982) and anephric pigs (Littledike et al., 1982) have been noted to have circulating levels of what appears to be 1,25$(OH)_2D_3$ which are most readily detected after vitamin D_3 or 25-hydroxyvitamin D_3 (25OHD_3) administration, although other recent studies (Manolagas et al., 1983; Reinhardt et al., 1984) have not detected 1,25$(OH)_2D_3$ in anephric humans. Therefore, the possibility that other tissues produce 1,25$(OH)_2D_3$ when production by the kidney is reduced needs further consideration. We tested the possibility that cells from the epidermis produce 1,25$(OH)_2D_3$—a mechanism that could provide a means to regulate vitamin D_3 production in the epidermis as well as an alternative source of 1,25$(OH)_2D_3$ production in patients with renal failure.

Experimental Procedures

Fresh human neonatal foreskin was obtained and keratinocytes (readily distinguished by morphologic criteria) were prepared by the method of Rheinwald and Green (1975) using a mitomycin-treated 3T3 cell feeder layer and Dulbecco's modified Eagle's medium (DME-H21) containing 20% fetal calf serum. The cells were studied after one or two passages. Just prior to use, contaminating cells were removed (>95%) with 0.1% ethylenediaminetetraacetate.

To assay for 25OHD_3 metabolism, the cells were grown to confluence in plates 3 cm in diameter. The growth medium was then replaced with 1 mL of serum-free medium for 36–40 h before [^{3}H]25OHD_3 was added to a final concentration of 7×10^{-10} M (0.1 μCi per well; specific activity, 148 Ci/mmol). After a defined period of incubation at 37 °C under 1 atm of 5% CO_2–air, the cells and medium were extracted by the

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[1] Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25OHD_3, 25-hydroxyvitamin D_3; DME-H21, Dulbecco's modified Eagle's medium.

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