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Characterization of BADS-Binding Proteins in Epithelial Plasma Membranes

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Summary. When a fluorescent stilbene was added to epithelial plasma membrane suspension the emission spectrum showed a broad peak containing overlapping emissions resulting from different adducts. By focusing on a specific emission wavelength a common site having a dissociation constant of $\approx 5\mu$M was calculated in the rat kidney, small intestine, pancreatic islets and shark rectal gland. This binding could be displaced by loop diuretics, (e.g., furosemide with an IC_{50} of 40 μM), DIDS (k_i 1 μM) and thiocyanate. These results pose certain questions such as: (i) whether the evidence for multiple peaks are due to specific interactions representing multiple binding affinities and (ii) whether the binding of stilbene and the observed displacement can be identified on a specific protein. Separating the proteins present in the purified basolateral and brush-border membranes by SDS-PAGE, transfer of these proteins onto nitrocellulose paper and labeling of the nitrocellulose strips by radioactive BADS (4-benzamido-4'aminostilbene-2-2'disulphonic acid) and bumetanide could identify labeled proteins. These experiments showed that whereas some proteins bound either BADS or bumetanide, one protein with a molecular weight of ≈ 100 or 130,000 D appeared to bind both. This protein was found on the basolateral membrane in the rat kidney cortex and medulla and the shark rectal gland and in the basolateral and brush-border membranes of the small intestine. Displacement of the protein-bound stilbene by loop diuretics could not be quantitated on the nitrocellulose transfer strips for this protein. Antibodies raised against the cytoplasmic fragment of band 3 reacted with the stilbene-labeled 100–130,000 D proteins indicating sufficient immuno-cross-reactivity between the separate species. These experiments involving binding of BADS and bumetanide and cross-reactivity with the human band 3 antibody suggest that these kilodalton proteins could structurally resemble human band 3.

Key Words band 3 · stilbene · bumetanide · rat kidney · small intestine · shark rectal gland

Introduction

Binding interactions of stilbenes with plasma membranes were examined previously using fluorescence measurements with an extrinsic probe BADS[1] (Pearce & Zadunaisky, 1990). Addition of BADS to plasma membranes from the rat kidney cortex, kidney medulla, small intestine and pancreatic islets, shark rectal gland vesicles and frog corneal epithelium showed a broad emission spectrum representing multiple peaks of bound BADS. Although a saturable binding to BADS with a dissociation constant of $\approx 5\mu$M was obtained by narrowing the measurements to one discrete wavelength, there were other fluorescence emission peaks that could represent stilbene-sensitive sites of interest.

The stilbene binding was displaced by submillimolar to micromolar concentrations of loop diuretics (furosemide, bumetanide and piretanide), DIDS and thiocyanate in all of the above tissues except the frog corneal epithelium (displacement was not documented in the pancreatic islets), and the affinity for BADS binding was reduced in the presence of excess chloride except in the case of the frog cornea where it remained unchanged. This displacement of BADS by loop diuretics could have been observed as a result of electrostatic interactions and has not been reported before. Apart from the questions raised above there are additional points that need to be answered such as (i) whether BADS and the loop diuretics bound to the same protein or to two separate proteins acting synergistically to show the displacement, (ii) if the molecular weights of these proteins were similar to band 3 or band 3 analogs reported in the literature, (ii) whether they were located on BLM or BBM, and (iv) if there were additional proteins labeled by either BADS or

[1] *Abbreviations*: BADS: (4-benzamido-4'aminostilbene-2-2'disulphonic acid); SITS: (4-amino-4'isothiocyanostilbene-2-2'-disulphonic acid); DIDS: (4-4'diisothiocyanostilbene-2-2'disulphonic acid); BBM: brush-border membranes; BLM: basolateral membranes; RBC: red blood cell ghosts or human erythrocyte ghosts; RHS: right-hand side; LHS: left-hand side.

bumetanide. To answer these questions the following approaches were applied.

Membrane proteins were separated by gel electrophoresis, transferred onto nitrocellulose and incubated with radioactive BADS and/or bumetanide to identify binding of these substances to one or more proteins. Thus, the proteins that bind either or both radiolabeled BADS and bumetanide could be identified. The molecular weights of the labeled proteins could be calculated by comparing with known standards. Displacement of radiolabeled BADS by bumetanide could also be carried out on nitrocellulose sheets with loop diuretics and DIDS. In addition to the binding studies discussed previously, if similarities were found between these labeled proteins to band 3 of the red cell one could expect some sequence homology between species. So the separated proteins were tested on a Western blot for binding to the antibody raised against the cytoplasmic fragment of band 3. Previously, antibody labeling studies have been carried out with kidney plasma membranes (Drenckhahn & Schluter, 1985) and in the dark epithelial cells of turtle bladder (Drenckhahn et al., 1987) where the relative molecular weights were 130,000 and 110,000 Da, respectively. These authors have stated that these proteins are anion exchangers like band 3 of the red cell. Our studies showed one predominant protein, in all the epithelia studied except in the frog cornea, which bound both BADS and bumetanide and had a molecular weight in the kilodalton range. This protein showed varied cross-reactivity against the antibody from the cytoplasmic fragment from human band 3. These data suggests that the protein identified could be structurally similar to band 3, but does not necessarily conclude that its function resembles band 3.

Materials and Methods

All buffer preparations were carried out with salts obtained from Sigma Chemical. Radiolabeled BADS was prepared as described previously (Pearce & Zadunaisky, 1990), and its activity was 13.5 μCi/μM. Radiolabeled bumetanide was a gift from Dr. Kinne (Max Planck Institute, Dortmund), and its activity was 15 Ci/mM. Cultured SV-40 (HIT) cells was a gift from Drs. Steve Richardson and Norman Altschuler (New York University, School of Medicine, New York).

Membrane Preparation

Human erythrocyte ghosts were prepared from freshly outdated blood as previously described (Steck & Kant, 1974) by hypotonic lysis.

The kidney and intestinal membranes were separated into BLM and BBM vesicles. The rationale behind attempts to isolate the luminal and contraluminal areas of the cell membrane of epithelial cells was based on the different composition of the membranes. The methods used to separate BBM utilize Mg or Ca divalent cations to crosslink with BBM and to change the density of the membranes. The BLMs which resemble plasma membranes from less polarized cells were isolated by differential centrifugation followed by a density-gradient centrifugation. Sprague-Dawley rats (NYU Berg facility) 180–200 g body wt were used for obtaining kidney and small intestinal membranes and pancreatic islets. Intestinal mucosa was gathered and suspended in 50 ml of 250 mM sucrose, 10 mM triethanolamine hydrochloride at pH 7.6 and homogenized. BBMs were isolated by calcium precipitation followed by purification using density-gradient centrifugation (Schmitz et al., 1975). The BLMs were also isolated using density-gradient centrifugation as previously described (Scalera et al., 1980).

The excised kidneys were separated into renal cortex and medulla; the tissues were homogenized independently in 250 mM sucrose, 10 mM HEPES/Tris, pH. 7.6. The BBMs were prepared by calcium precipitation, and the BLM of the kidney cortex and medulla were isolated as described by Kinsella et al. (1979). The membranes were purified by sucrose-gradient centrifugation. Gradient fractions collected from the intestinal and kidney preparations were assayed for alkaline phosphatase (E.C.3.1.3.1, BBM marker) and Na-K ATPase (E.C.3.6.1.3, BLM marker) using the methods described by Berner and Kinne (1976). Those fractions containing the purified BBM with the highest alkaline phosphatase and the lowest Na-K ATPase activities were pooled. similarly, those fractions containing the purified BLM with the highest Na-K ATPase and the lowest alkaline phosphatase activities were pooled. The membranes were also assayed for 5′ nucleotidase (E.C.3.1.3.2, plasma membrane marker) and succinate dehydrogenase (E.C.1.3.99.1, mitochondrial membrane marker) (Evers et al., 1978).

Marker enzyme activities measured were similar to the ones reported in the literature (Evers et al., 1978; Chen & Verkman, 1987). The BBM were enriched in alkaline phosphatase activity 18-fold over the crude homogenate in the kidney and in the intestine, whereas the Na-K ATPase activity was reduced to 0.3-fold. Ouabain-inhibitable Na-K ATPase activity in the BLM was enriched 15-fold in the kidney and 12-fold in the intestine, and the alkaline phosphatase activity was reduced to 0.25-fold over the homogenate. The activity of 5′ nucleotidase was present in all the membranes, and succinate dehydrogenase activity was reduced to 5% in all the membranes.

The rectal gland from dogfish *Squalus acanthias* was removed and placed in shark Ringer's for vesicle preparation. The membranes were isolated as previously described by Hannafin et al. (1983). Analysis of Na-K ATPase activity showed a four fold increase of enzyme activity of membrane pellet over homogenate. The membranes were frozen at −70°C until used.

Pancreatic islets were prepared by the technique of tissue dissociation with collagenase (Neilsen & Lernmark, 1983) and subsequent incubation in culture medium. The islets were homogenized in buffered 250 mM sucrose, and the plasma membranes were isolated by an initial low-speed spin (750 × g) to remove intracellular material, followed by a high-speed spin at 45,000 × g.

The culture medium was poured off the SV-40 (HIT) cells, and they were washed with 5 ml of 10X PBS–1 mM EDTA. The cells were dislocated from the culture flask by shaking in PBS-EDTA. The cells were collected by centrifugation at 2,500 × g. They were then ruptured by sonication in buffered sucrose, a low-speed spin (750 × g) removed intracellular material, and the membranes were isolated at 45,000 × g.

The frog corneal cells were obtained by a method of Gipson and Grill (1982) where, twelve frogs, *Rana catesbiana* were sacrificed by decapitation and pithing. The cells, dislodged from the stroma by the action of Dispase, were washed repeatedly with PBS to remove Dispase and were subsequently ruptured by sonication, and the membranes were isolated using the procedure for the HIT cells. The membranes of the pancreatic islets, the frog cornea and the HIT cells were not separated into BBM and BLM as the quantities of membranes obtained were very small.

Polyacrylamide Gel Electrophoresis in SDS

Electrophoresis was performed using gels containing 7.5 or 10% acrylamide including bis, 0.2% SDS following the procedure of Laemmli (1970) which was further adapted by Fairbanks, Steck and Wallach (1971). Samples were prepared for electrophoresis by adding the following to the stated final concentrations: 1% SDS, 50% glycerol, 10 mM Tris-HCl at pH 8, 1 mM EDTA at pH 8, 40 mM β-mercaptoethanol and 10 mM bromophenol blue, a tracking dye. The solutions were incubated at 37°C for 15–20 min to promote reduction of disulphide bonds. Molecular weight markers were treated in the same manner. Electrophoresis was carried out using clear sample solutions in a slab gel apparatus with 1 liter of noncirculating buffer. Gels were stained using Coomassie brillant blue. The proteins were observable in sufficient density so as to avoid silver staining. Apparent molecular weights were obtained by comparing the separated bands with standards by plotting graphically the molecular weights against the R_f's. Calculated values were taken to be useful estimates rather than true molecular weights owing to the limitation of SDS-PAGE (Steck, Ramos & Strapazon, 1976).

Electrophoretic Transfer and Autoradiography of SDS Gels

Detection and analysis of electrophoretically resolved proteins were performed by transferring these molecules onto nitrocellulose paper and further labeling these molecules with ^{14}[C] BADS and ^{3}[H] bumetanide, or alternatively, by reacting them with rabbit polyclonal antibodies raised against the cytoplasmic fragment of human band 3. The transfer and labeling of bands from acrylamide gels (Western blotting) has been extensively described (Erickson, Minier & Lasher, 1982; Gershoni & Palade, 1982). Samples were run in triplicate. The strips were washed in PBS to remove blotting buffer. One set of strips was stained where the staining established the efficiency of transfer and the position of the bands. The remaining strips were not blocked with BSA before incubation with radiolabeled compounds as both BADS and bumetanide showed binding to BSA. These strips were incubated in PBS containing 100,000 cpm of ^{14}[C] BADS or ^{3}[H] bumetanide for 30 min. The strips were removed and washed once with PBS containing 5% BSA and twice with PBS or three times with PBS alone.

One set of strips was stained with fast green. Another set of strips was air dried, sprayed with *Enhance*® (National Diagnostics) and placed against an X-ray sensitive film at −70°C to obtain autoradiographs (Laskey & Mills, 1975). The third set of strips was sliced into 1-mm pieces and counted in scintillation vials containing scintillation fluid 10 ml of (*Liquiscint*®, National Diagnostics). The bands in all three samples were compared to determine those that were labeled.

Rabbit polyclonal antibodies were raised against the chymotryptic fragment from the cytoplasmic region of the protein band 3 from the human erythrocyte membrane (Fukuda et al., 1978). They were purified (Fukuda et al., 1978; Pasternack et al., 1985) and incubated with the transfer strips obtained from the SDS-PAGE. The antibodies, bound to the blocked nitrocellulose transfer strips, were further cross-reacted with goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate and stained using the standard HRP color development procedure (Towbin, Staehlin & Gordon, 1979; Karcher et al., 1981).

Displacement of Membrane-Bound BADS

Displacement of BADS by bumetanide or DIDS was examined on the nitrocellulose transfer strips containing the proteins. The displacements were measured by incubating the nitrocellulose strips with varying concentrations of either bumetanide or DIDS (10^{-6}–10^{-3} M) for 30 min, washing either once with PBS containing 5% BSA and twice with PBS or three times with PBS and subsequently adding 5 μM BADS. The strips were incubated in BADS for 30 min and then washed twice with PBS containing 5% BSA and three times with PBS alone. The strips were cut into 1-mm pieces and then counted using both ratio ^{3}[H]:^{14}[C] and single-channel modes.

Results

SDS-Polyacrylamide Gel Electrophoresis, Transfer, Labeling and Autoradiography

Electrophoresis of membrane proteins in 10 and 7.5% SDS gels showed multiple bands in all of the tissues examined. These bands were reproducible if the conditions of the electrophoresis were strictly adhered to. The intensity and the sharpness of some of the minor bands were affected by changes in pH and buffer composition. The gel profile indicated that, when compared to the red cell, the composition of proteins in the plasma membranes was much more complicated, and the ratio of BADS-sensitive protein to other membrane proteins was reduced. Molecular weight estimates for components labeled were obtained by rigorous calibration of gels with molecular weight markers. The SDS-PAGE gels were transferred onto nitrocellulose paper, and the paper was treated with labeled ^{14}[C] BADS or ^{3}[H] bumetanide in PBS. The densitometer readings of the autoradiographs from the nitrocellulose strips were automatically corrected for the opacity of the background. Background counts (60–200 cpm) were obtained, either from nitrocellulose strips that did not contain protein or contained a minor trace of protein.

If the strips obtained after transfer were not washed with 5% BSA there were many more proteins labeled by both BADS and bumetanide (Fig. 1). There are a significant number of sites labeled that may or may not have functional significance. For example, in Fig. 1 both BADS and bumetanide

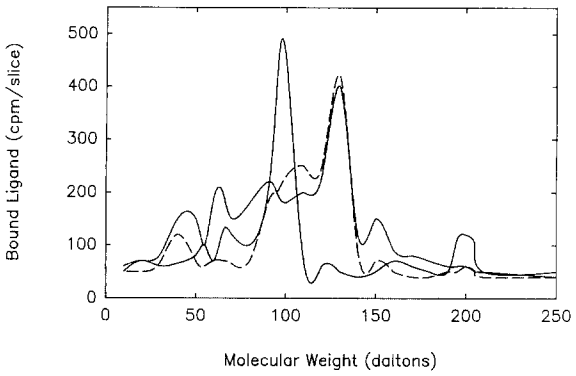

Fig. 1. Labeling of gels with ^{14}[C] BADS or ^{3}[H] bumetanide. The gels are incubated with either radiolabeled BADS or bumetanide, and in this case, they are not washed with 5% BSA. The red cell labeled with BADS (—) and the kidney cortex BLM labeled with BADS (- - -) and with bumetanide (– · –) are shown.

appear to label a number of proteins in the rat kidney cortex BLM. The red cell also shows a larger background for BADS-bound strips. If the strips were washed with 5% BSA those proteins with a $K_d > K_d$ of BSA will be unlabeled. Thus, some of the low affinity (nonspecific) labeling can be removed by washing with 5% BSA, and the remaining bands are shown in Fig. 2.

In the red cell (Fig. 2*A*) the major protein labeled by BADS and bumetanide is the broad diffuse band at 95,000 ± 6,000 Da. The diagram to the left shows densitometer readings from the ^{14}[C] autoradiograph and the counts obtained from labeled bumetanide and BADS strips. The ^{14}[C] autoradiograph (Fig. 2*A*;*b*) shows that band 3 is labeled. These autoradiographs are not distinct, as ^{14}[C], a β emitter, appears to produce weak bands. The ^{3}[H] bumetanide autoradiographs were not clear, in spite of the high activity of bumetanide; therefore, only the data from the counted strips are shown. The results are similar to those reported in the literature (Drickamer, 1980). In the rat kidney BLM (Fig. 2*B*) BADS and bumetanide both bind to a large mol. wt. protein at 120,000 Da. Bumetanide also shows low-level binding to proteins at 90,000, 75,000 and 50,000 Da; BADS to a protein at 45,000 Da. The proteins at 90,000 and 75,000 Da can loose their radiolabel by washing with 5% BSA; (Fig. 2*B*) the remaining radiolabel on these proteins is very low. Similar information is obtained for the small intestinal BLM (Fig. 2*C*) and the shark rectal gland (Fig. 2*D*). In the case of the shark the major protein labeled lies at ≈100,000 Da. There are other proteins that react with bumetanide in the rectal gland; they lie at 180,000, 90,000 and 48,000 Da (Fig. 2*D*). The autoradiographs of the gels shown in Fig. 2 have all been washed with 5% BSA, and hence, only show the relative high affinity radiolabels. The rat intesti-

Fig. 2. Densitometer readings from ^{14}[C] autoradiographs and counts from ^{14}[C]- and ^{3}[H]-labeled nitrocellulose transfer strips and 10% gels from SDS-PAGE. The graphs and gels shown are taken from one set of experiments. The numbers used are the average from all the sets of experiments. These gels and nitrocellulose strips shown below have been washed with 5% BSA; therefore, the additional bands indicated in the text, which can be washed away by BSA, are not visible. This weak binding has been illustrated for two tissues in Fig. 1. The graphs are plotted with molecular weights on the *x*-axis *vs.* radioactivity or absorbance on the *y*-axis. The counts for the binding of ^{3}[H] bumetanide (-●-●-), ^{14}[C] BADS (-▲-), and densitometer readings from the autoradiographs (- - -) are shown. (*A*) The red cell ghosts show bindings of both BADS and bumetanide to band 3 at 92,000 Da. The 10% SDS gel (*a*), its ^{14}[C] autoradiograph which shows labeling predominantly of the 95,000 ± 6,000 Da protein and (*c*) molecular weight standards are shown. (*B*) The kidney plasma membranes (*a*), the purified BLM (*b*); the ^{14}[C] autoradiograph and a molecular weight (*d*) standards appear to the right of the line drawings of the labeled 120,000 ± 6,000 Da protein on the kidney BLM. (*C*) The line drawings of the small intestinal BLM show binding of BADS and bumetanide to a protein at 120,000 ± 6,000 Da on the LHS. The gels of the small intestinal plasma membranes (*a*), small intestinal purified BLM (*b*), the autoradiograph of bound BADS (*c*) and the molecular weight standards (*d*) are on the RHS. (*D*) The shark rectal gland shows binding of both BADS and bumetanide to a protein which appears at 100,000 ± 5,000 Da, whereas bumetanide also labels protein at 200,000, 90,000 and 48,000 Da. The SDS gels of the basolateral membrane of the rectal gland (*a*); the autoradiograph from bound BADS (*b*) and the molecular weight standards (*c*) are shown. There are other smaller molecular weight proteins labeled by both BADS and bumetanide in all of these other tissues, and they appear in the range of 50,000–45,000 Da.

nal BBM (Fig. 3*a*), the stilbene-labeled protein, appears to have a slightly slower mobility than that observed for the kidney and small intestinal BLM and gives a mol. wt. of 125,000 Da. The rat kidney medulla BLM (Fig. 3*d*) shows labeling of a 120,000 and a 45,000 Da protein, respectively. The frog cornea (Fig. 3*c*) and the kidney cortex BBM (Fig. 3*b*) do not show labeling of a kDa protein but of a low mol. wt. protein of > 50,000 Da. Bumetanide appears to bind to a minor protein of 48,000 dalton mol. wt. in the rat kidney cortex BBM and in the frog cornea. There is also a band in the rat intestinal BBM with weak affinity to bumetanide and appears at 45,000 Da. The pancreatic islets (Fig. 3*e*) and the β cell are both labeled albeit weakly at 120,000 Da. The overall binding of BADS and bumetanide to rat pancreatic islets (Fig. 3*e*) is very weak and is dependent on the amount of protein in the membrane which appears to be correspondingly small. The estimated molecular weights of all the proteins labeled are listed in the Table.

The *y*-axis in Figs. 2 and 3 cannot be compared quantitatively since these figures are obtained from different gels and there are variations in the quantities of protein loaded on each gel. It is deceptive

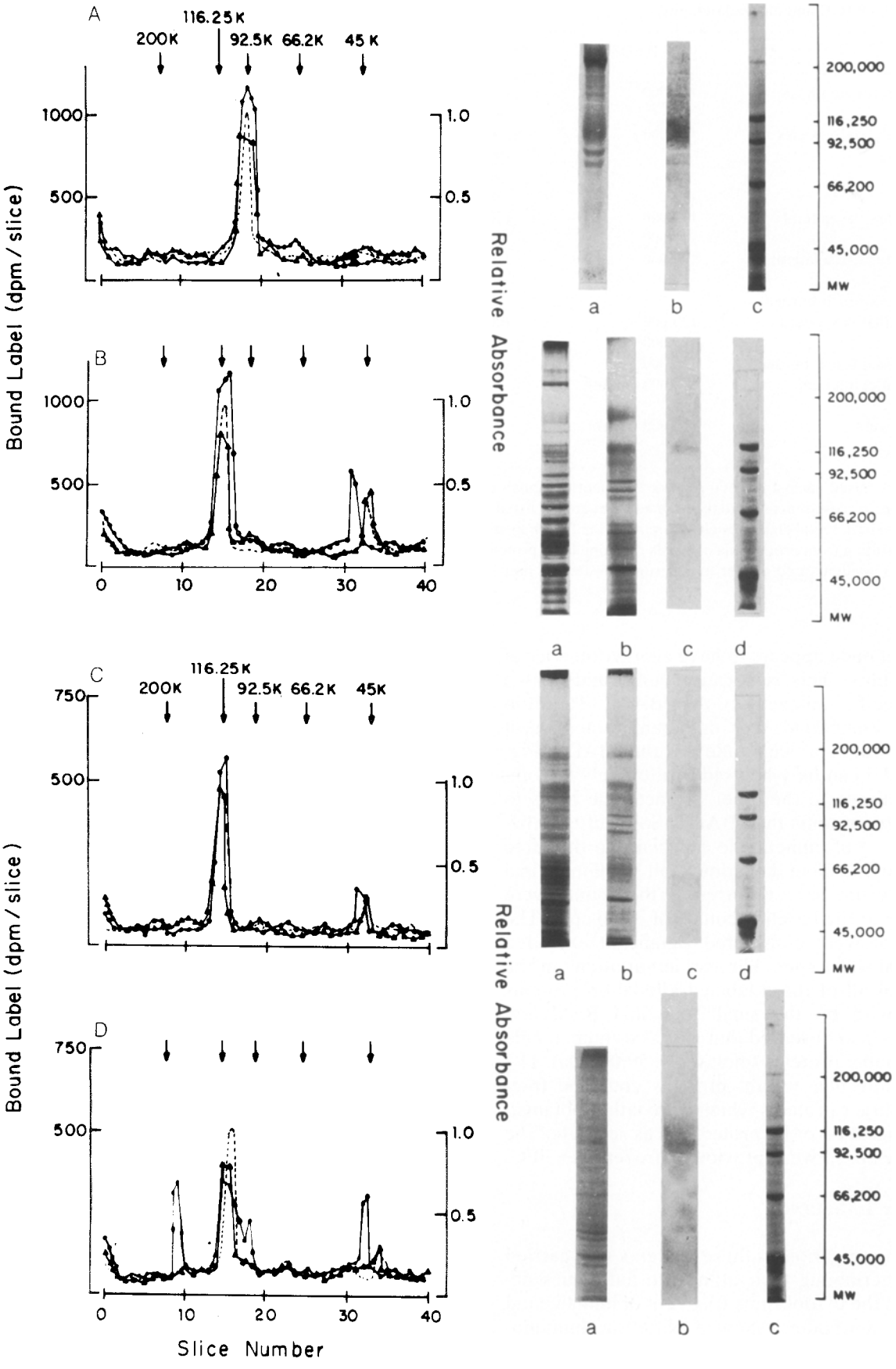
A
116.25K
200K
92.5K
66.2K
45K
1000
500
1.0
0.5
B
C
750
D
Bound Label (dpm / slice)
0
10
20
30
40
Slice Number
Relative Absorbance
a
b
c
d
200,000
116,250
92,500
66,200
45,000
MW

Table 1. SDS-PAGE and autoradiography

Membranes	[^{14}C] BADS	[^{3}H] Bumetanide	Antibody[a]
Human erythrocyte ghosts	100,000	100,000	100,000
		48,000	
Shark rectal gland vesicles[b]	100,000	>200,000 (weak)	100,000
		100,000	
		90,000 (weak)	
		48,000	
Kidney cortex basolateral	120,000	120,000	120,000
	45,000	50,000	
Kidney medulla basolateral	120,000	120,000	120,000
		45,000	
Kidney cortex brush border		48,000 (weak)	
Small intestinal basolateral	120,000	120,000	120,000 (weak)
	42,000	45,000	
Small intestinal brush border	125,000	125,000	125,000 (weak)
Frog corneal epithelium	40,000 (weak)	90,000 (weak)	
		48,000	
Pancreatic islets	120,000 (weak)		120,000 (weak)
β cell			120,000 (weak)

[a] Antibody is raised against the cytoplasmic fragment of human erythrocyte band 3. There are other bands that show cross-reactivity to the antibody that are not listed in the table but can be seen in Fig. 4.
[b] Gels of the shark rectal gland vesicles were run from frozen membranes. It is possible that there may be bands resulting from proteolysis or bands resulting from protein labile in SDS. These gels were not compared to nondenatured gels or to gels run from freshly prepared membranes.

that bumetanide appears to have a larger number of bound counts. This is because bumetanide has a higher specific radioactivity than BADS. The ratio of bound bumetanide per milligram protein is at least 2–3 orders of magnitude less than BADS. Figures 2 and 3 can only be used qualitatively to compare one tissue to the other. Bumetanide binds to many more proteins than BADS. Some of the low-level binding of bumetanide especially to the three bands near 75,000 in the kidney and small intestinal BLM have not been reported as the counts were very close to the baseline counts of ≈200 cpm. The counts that were considered significant extended from ≈400–1200 cpm. The subtle variations in the mobility of all of these labeled kilodalton proteins are exhibited by the small intestinal BLM and BBM. It is also observed that BADS or bumetanide bind to smaller proteins (mol. wt. > 50,000 Da). The smaller molecular weight proteins could be fragments of larger proteins which were either obtained by SDS digestion or by proteolysis as several of the membranes used were previously frozen at −70°C.

Antibody Labeling

Further investigation of these proteins was carried out by determining their affinity to antibodies derived from the cytoplasmic fragment of human band 3. These antibodies showed differing immuno-cross-reactivity to the previously BADS labeled proteins in the separated gel. In Fig. 4 all of the membranes except the red cell ghosts were incubated for 45 min in the HRP color developing solution. The red cell ghost membranes were incubated for 15 min and showed immediate development of color. Among nonerythroid membranes the shark rectal gland vesicles show the strongest cross-reactivity to the antibody. The kidney cortex and medulla BLM show much less cross-reactivity than band 3. The pancreatic islets and the intestinal BLM and BBM were incubated for 45 min and showed very weak cross-reactivity. The frog cornea and the kidney BBM did not react at all. Binding affinity for the antibody for the human erythrocyte ghosts ⩾ the shark rectal gland > rat kidney cortex > kidney medulla BLM > the rat intestinal BBM > the rat intestinal BLM > the pancreatic islets (Fig. 4). This is based on comparing the intensities of staining the protein with either Coomassie blue or fast green with the intensities of the staining obtained from the antibody reactivity. These results show that the intestinal membranes and the pancreatic islets show negligible reactivity to the band 3 antibody, indicating that there is probably species or sequence differences between them.

Displacement of Membrane-Bound BADS

Displacements of bound BADS was examined with radiolabeled bumetanide, excess cold bumetanide

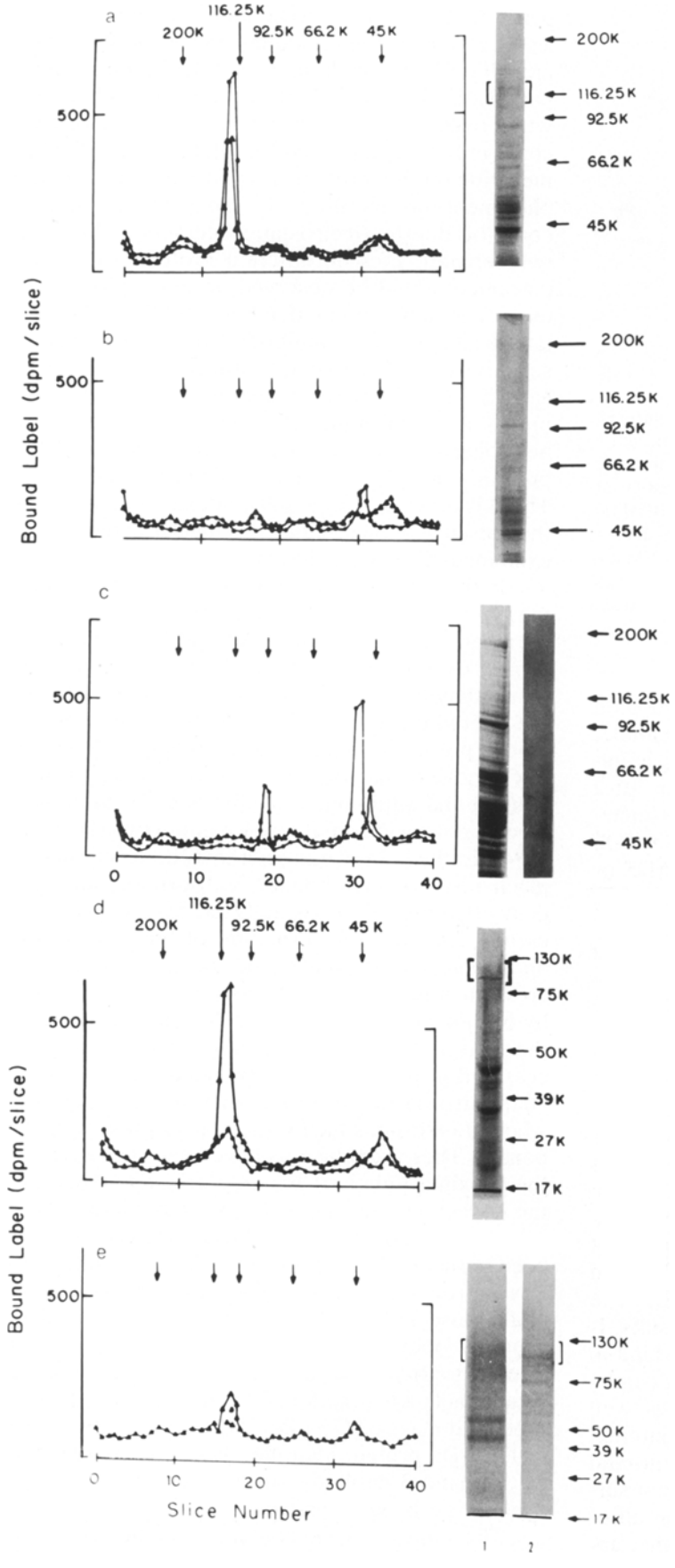

Fig. 3. SDS-PAGE of BBMs and counts from nitrocellulose strips are plotted. Counts from the binding of ^{3}[H] bumetanide (-●-●-) and ^{14}[C] BADS (-▲-) to nitrocellulose strips are plotted with molecular weight on the *x*-axis and counts on the *y*-axis. Also represented on the side of the graphs are the gels from the remaining membranes. Intestinal brush border (*a*) shows labeling of a large protein at 125,000 ± 5000 Da by both BADS and bumetanide. The kidney medulla BLM (*d*) also shows labeling of a large protein at 120,000 Da. The kidney cortex BBM (*b*) and the frog cornea (*c*) show an absence of binding to a large molecular weight protein by BADS; however, many smaller proteins are labeled by both BADS and bumetanide. The pancreatic islets and the cultured β cell (*e*) show a weak and diffuse band at 120,000 Da which is labeled by BADS. *C* shows a lane on the extreme RHS which is a ^{14}C BADS autoradiograph. There is no labeling at the high end. The film is dark due to prolonged exposure and only a weak band is observed near 45,000 Da. The extreme RHS lane in *e* shows a gel of cultured HIT cells. There is a broad, diffuse and weak band similar to that observed from islet cells at 120,000 ± 5000 Da.

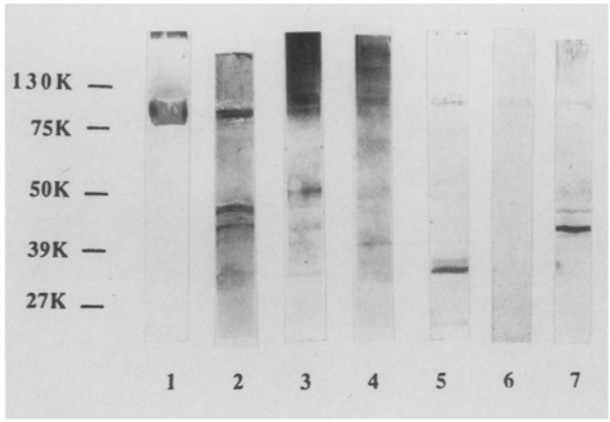

Fig. 4. Antibody labeling of epithelial membranes. The nitrocellulose strips incubated with the antibody obtained from the cytoplasmic fragment of band 3 are shown. Human erythrocyte ghosts (*1*), shark rectal gland vesicles (*2*), kidney cortex BLM (*3*), kidney medulla BLM (*4*), small intestinal BBM (*5*), BLM (*6*) and cultured β cell (*7*) are shown. Those membranes that did not show any reactivity such as the frog cornea and kidney BBM are not included in this figure. It can be seen that the small intestinal BBM, BLM and the pancreatic islets show very low level reactivity and could be interpreted as unreactive.

and DIDS. The nitrocellulose strips when incubated with varying concentrations of ^{3}[H] bumetanide, and subsequently, with BADS did not yield a credible displacement curve. It appeared that after BADS bound to the immobilized protein the bumetanide label could be more easily washed away. On the addition of excess cold DIDS neither BADS or bumetanide could bind to the nitrocellulose strips. Adding increasing concentration of DIDS to membranes in 5 μM BADS a displacement curve with an IC_{50} of 10 μM was obtained showing a lower affinity than that observed in the filter binding assay (Pearce & Zadunaisky, 1990).

Discussion

Previous studies showed that the fluorescent stilbene BADS binds to the epithelial membranes showing saturable kinetics. This binding was abolished in the presence of excess DIDS. In all epithelia studied except the frog cornea, it was found that BADS could be displaced by loop diuretics and thiocyanate (Pearce & Zadunaisky, 1990). The present studies expand on these previous results. It was found that both BADS and bumetanide bind to several proteins, where some of the binding can be eliminated by washing with 5% BSA. It is well known that BADS and bumetanide both interact with hydrophobic sites; therefore, they could bind to several nonspecific sites. Therefore, it is not surprising that several proteins were labeled in all of the epithelia studied. There is one protein that appears to bind both BADS and bumetanide, to a lesser extent, in the rat kidney cortex BLM, kidney medulla BLM, small intestinal BLM and BBM, pancreatic islets and shark rectal gland with a mol. wt. between 130,000 and 100,000 Da. Displacement could only be quantitated for DIDS and not for bumetanide on this protein. It is possible that the displacement observed in the fluorescence studies could be due to electrostatic interactions between two separate sites on different proteins as no displacement could be observed on immobilized proteins. The same protein that binds BADS and bumetanide also binds the antibody from human band 3; sensitivity to this antibody appears to vary with each tissue. The tissue preparations contain membranes from different cell types and are therefore heterogeneous. Conclusions on the location of the protein can only be made in similar broad terms. There is other experimental evidence that indicates the presence of bicarbonate exchanger in the tissues examined. These are discussed below.

In the *kidney* cortex, the proximal tubule under normal circumstances resorbs about 50 to 66% of the filtered fluid by both active and passive mechanisms. Complete inhibition of renal enzyme carbonic anhydrase results in excretion of only 30% of the filtered bicarbonate load. A study based on the kinetic properties of carbonic anhydrase of dog kidneys showed that the enzyme was active in both luminal and antiluminal membranes and there was no differences in activity between the two (Dubose, 1984). In the kidney it is seen that there is a second mechanism of bicarbonate reabsorption independent of catalyzed or uncatalyzed hydroxylation of carbon dioxide. The formation of bicarbonate ion on the blood side would indicate, in a logical progression, the presence of band 3 analogue on the blood side of the proximal tubule where it assists in the transport of bicarbonate in the reabsorption process and in pH regulation. By similar argument, it could also be found in the cortical collecting duct where it is responsible for the reabsorption of bicarbonate. These conclusions have been suggested for the proximal tubule (Chen & Verkman, 1987) and the collecting duct (Drenckhahn & Schluter, 1985) in previous experiments. These conclusions have been borne out by experiments where multiple transcripts from band 3 gene, which are expressed in rat and mouse kidney, have now been expressed in *Xenopus* oocytes and are able to function in anion transport (Brosius et al., 1989). This band 3-related protein is lacking the first 79 amino acids and has a predicted mass of 137 kDa (Alper et al., 1988). The 120,000-Da protein identified here and its reaction to the band 3 antibody strongly suggests that this protein is a band 3 analogue.

The *small intestine* contains complicated trans-

port pathways where absorption and secretion of amino acids and sugars are the major transport activities, but along with them there are ion regulation requirements. Transfer of the proteins separated by SDS-PAGE showed a protein that was labeled by both BADS and bumetanide on the basolateral fraction with a mol. wt. of 120,000 Da. The apical membranes contained a protein of lesser concentration which was labeled by both BADS and bumetanide whose mol. wt. was 125,000 Da. The small intestine was not separated into the ileum and jejunum; and at this point, the distribution of this protein cannot be pinpointed to exist on the apical side in the jejunum and on the basolateral side in the ileum or vice versa as there may be cross-contamination. However, the protein on the apical surface showed a greater affinity for the antibody from human band 3 in comparison to the BLM protein, but both these proteins showed very little cross-reactivity overall when compared to the kidney cortex BLM. Perhaps this may be due to either insignificant sequence homology between these intestinal proteins and band 3 or a lack of interspecies cross-reactivity. Although the apical membrane in the small intestine is shown to have a bicarbonate exchange mechanism (Liedtke & Hopfer, 1982), these proteins will have to be studied functionally to associate them conclusively with this mechanism.

The serum sodium of the shark is higher than that of man, approximately 260–290 mEq/liter (Epstein, Silva & Stoff, 1981). The osmotic concentration of mineral salts and urea balances the osmotic concentration of seawater, preventing dehydration. A stilbene, 4 amino-4′-isothiocyanostilbene-2-2′ disulphonate (SITS), which blocks anion exchange mechanisms in other systems at concentrations of 1 mM reduced the potential difference by 36% (Greger & Schlatter, 1984). Cotransport of sodium and chloride is the favored mechanism responsible for the balance of chloride concentration in the rectal gland (Eveloff et al., 1978). The results obtained on the binding studies of BADS were only seen with open vesicles (vesicles lysed with hyptonic solution and sonication) which indicated that the BADS site was on the inside of the vesicle, or that this protein has a reverse orientation for the BADS binding site when compared to other epithelia. There is also a great deal of cross-reactivity between the antibody produced from band 3 and the shark rectal gland vesicles. This finding is curious; the only explanation for this difference could be a retention of the characteristics of band 3 through evolution from the shark to human. There is a large concentration of carbonic anhydrase in the rectal gland. This protein is responsible for the concentrations of bicarbonate within the cell; however, carbonic anhydrase inhibitors do not seem to affect chloride secretion (Swenson & Maren, 1984). The presence of the BADS-sensitive protein does not contradict the data already accrued on chloride secretion. Radiolabeled bumetanide binds other membrane proteins on the gel. These could be the Na/K 2Cl cotransporter or Na/HCO_3 transporter or the chloride channel. However, for efficient binding of bumetanide to the cotransporter there should be Na, K and Cl ions in the reaction buffer.

The *cornea* does not show protein binding to BADS in the 100,000 Da range. The protein affected by BADS appears near 40,000 Da. Bumetanide on the other hand, shows significant binding to a protein in the 45,000 Da range. There also seems to be a lightly labeled band at 90,000, and future studies may show the significance of these bands. These lightly labeled bands were also seen in the kidney apical membrane vesicles. We can conclude that the frog cornea does not contain the same protein identified in the other epithelia. Previous studies have indicated the absence of bicarbonate transport in the frog cornea (Zadunaisky, 1966). The lack of abundant tissue is hampering biochemical studies; however, the use of cultured cells will assist in future studies.

The studies on the *pancreatic islets* appear to yield the least conclusive data. The difficulty exists in the low concentration of the protein in the islet preparation. The cultured cells show negligible cross-reactivity with the antibody obtained from human band 3, but this is not particularly conclusive evidence that this protein is present in the β-cell.

Lack of reactivity to human band 3 need not necessarily indicate that band 3 is absent. Monospecific polyclonal antibodies raised against total human band 3 which recognize determinants within a M_r 20,000 fragment of the cytoplasmic domain does not show cross-reactivity to murine band 3. There is only 57% homology between human and mouse in residues 125–138, whereas residues flanking either side is said to be 90–95% homologous. This observation is consistent with the lack of cross-reactivity of anti-human band 3 antisera with mouse protein and vice versa (Kopito & Lodish, 1985). An immunological study using anti-human band 3 to label erythrocyte band 3 from dog, monkey, goat, ferret, sheep, rat, mouse, duck and rabbit showed an immunoprecipitate only with monkey erythrocyte band 3 (England, Gunn & Steck, 1980). Our results of cross-reactivity between rat kidney and shark rectal gland and antibody from human band 3 is anomalous. We do not have any definitive explanation for these results. These anomalies indicate the complexity of the system under investigation.

There are many band 3-like proteins in cells other than the systems studied here, which have been obtained from a family of related genes. The

functions of these proteins have not been clearly established in any other tissue except the human red cell (Passow et al., 1989). Moreover, Passow et al. (1989) has indicated that in a cell line from human leukemic cells, a nonerythroid band 3 having high sequence homology to erythroid band 3 shows many functional variations from the red cell band 3. One of the major differences is its inability to bind covalently to ^{2}[H] DIDS near its site of noncovalent binding, unlike its erythroid counterpart. To determine the functional role of these BADS- or bumetanide-binding proteins, these proteins should be isolated and incorporated into a system such as *Xenopus* oocytes so that transport can be measured. Inhibition of Cl/HCO_3 exchange or sulphate uptake, or binding to the protein by BADS and/or bumetanide can be further documented. Other proteins of mol. wt. 200,000–150,000, 90,000 and 45,000 which bind bumetanide need to be investigated. It has been shown recently that chloride channels from kidney and trachea have similar molecular weights to those identified by bumetanide binding (Landry et al., 1989). Further studies will reveal whether there are greater functional differences or similarities between erythroid and nonerythroid band 3 in spite of probable sequence homology.

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