AN ABSTRACT FOR THE THESIS OF

Jennifer Ziegler for the Master of Science Degree in

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Title: The role of various ion transport mechanisms on erythrocyte osmotic resistance to lysing in the American bullfrog (*Rana catesbeiana*)

Abstract approved: David K. Saunders

Previous studies have shown that the erythrocytes of amphibians are much less likely to lyse in hyposmotic conditions as are the erythrocytes of mammals. Such resistance to lysing may be the result of volume regulating membrane transport systems in amphibian erythrocytes. To determine the role of these membrane transport systems, blood was collected from American bullfrogs (*Rana catesbeiana*) and mice (*Mus musculus*) and erythrocytes were treated with one of the following specific membrane transport inhibitors: furosemide, 4,4'-diidothiocyanatostilbene-2,2'-disulphonic acid (DIDS), and [(dihydroindenyl)oxy] alkanoic acid (DIOA). After exposure to furosemide, DIDS, DIOA, or all the inhibitors together the osmolality of the solution at which 50 percent of the erythrocytes had undergone lysis (50% lysis) was determined for both bullfrogs and mice. Furosemide, an inhibitor of the K^+/Cl^- cotransporter and the $Na^+/K^+/Cl^-$ cotransporter, and DIDS, an inhibitor of anion exchange, caused a significant increase in the lysing of mouse erythrocytes, but had no significant effect on bullfrog erythrocytes. However, exposure of erythrocytes to DIOA, a potent inhibitor of the K^+/Cl^- cotransporter, resulted in increased lysing of both mouse and bullfrog erythrocytes, with the effect being greater for mouse erythrocytes. Trials using all the

inhibitors together also resulted in increased bullfrog erythrocyte lysing. Thus, the disabling of only specific membrane transport systems in frog erythrocytes hinders the cells' ability to volume regulate. Additionally, the bullfrog erythrocytes appear to be less affected by the inhibition of membrane transport mechanisms than mouse erythrocytes. This suggests that bullfrog erythrocytes possess either additional volume regulatory membrane transport mechanisms or other means by which they tolerate hyposmotic environments.

THE ROLE OF VARIOUS MEMBRANE TRANSPORT MECHANISMS ON ERYTHROCYTE OSMOTIC RESISTANCE TO LYSING IN THE AMERICAN BULLFROG (*RANA CATESBEIANA*)

A Thesis

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by

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PREFACE

My thesis was written in the style according to the instructions for submission to the Journal of Experimental Zoology.

TABLE OF CONTENTS

LIST OF TABLES

Page

INTRODUCTION

When cells are exposed to osmotically challenging conditions, they undergo processes of volume regulation. When cells are hyposmotically challenged, or placed in a solution that has a solute concentration that is less than the solute concentration of the inside of the cell, they will take on water through osmosis, which causes swelling. This swelling triggers the cell to undergo a process termed regulatory volume decrease (RVD). During RVD, the cell activates membrane transport systems, which work by pumping osmotically active solutes, such as ions, from the cytoplasm out of the cell. Conversely, when these molecules leave the cell, water follows, thus decreasing the cell's volume. When cells are hyperosmotically challenged, or placed in a solution that has a solute concentration that is higher than the solute concentration of the inside of cell, water will leave the cell, thus causing shrinkage. As with swelling, cell shrinkage causes the activation of membrane transport systems, except these transporters mediate the gain of solutes into the cell. Water follows these solutes and cell volume increases. This process is termed regulatory volume increase (RVI) (For reviews on cell volume regulation see McManus et al., '95; Lang et al., '98; Hoffman, '00).

There are many different types of membrane transport systems that are believed to be utilized in volume regulation. These transport systems mainly differ in the specific ion or osmotically active molecule transported. In addition, transport systems utilized in volume regulation are known to vary between different cell types and between species. Most research supports the idea that the main transport mechanisms used in RVD are the K^+/Cl^- cotransporter (O'Neill, '89; Gibson et al., '93; Armsby et al., '95; Godart et al., '99), separate K^+ channels (Cannon et al., '98), anion channels that are not only

responsible for expelling Cl^-, but also other negatively charged molecules (Motais et al., '91; Nilius et al., '94; Bogdanova et al., '98), and channels that mediate the loss of organic osmolytes (Garcia-Romeu et al., '91; Culliford et al., '95; Davis-Amaral et al., '97). RVI is somewhat more complicated than RVD, given that more transport systems are used and they vary more depending on the cell type. The chief membrane transport systems used in RVI are the Na^+/H^+ exchange system (Armsby et al., '95), the $Na^+/K^+/Cl^-$ cotransporter (Garay et al., '88) and the Na^+/K^+ antiporter (Zicha and Duhm, '90).

Because of its simple structure and its lack of internal membranes, the red blood cell (RBC), or erythrocyte, has been an ideal subject for investigating volume regulation. RBC volume and shape are important determinates for blood viscosity and oxygen carrying capability (Chein et al., '71; Benga et al., '83).

Much has been learned about the mechanisms used in RVD by exploring the impact that certain membrane transport inhibitors have on volume regulation. Loop diuretics, such as furosemide and bumetanide, have been shown to be strong inhibitors of the $Na^+/K^+/Cl^-$ cotransporter (Garay et al., '88). Although the $Na^+/K^+/Cl^-$ cotransporter is not believed to be utilized in RVD, some evidence has shown that furosemide may also have an effect on the K^+/Cl^- cotransporter (Garcia-Romeu et al., '91; Gusev et al., '99). 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), which is part of the stilbene sulphonates inhibitors, is a specific inhibitor of anion exchange systems located in erythrocyte membranes (Garcia-Romeu et al., '91; Gusev et al., '99). [(dihydroindenyl)oxy] alkanoic acid (DIOA), has been found to be a potent inhibitor of the K^+/Cl^- cotransporter (Garay et al., '88; Gusev et al., '99) and has been found to have no effect on the $Na^+/K^+/Cl^-$ cotransporter in RBCs (Garay et al., '88).

Many ectotherms, such as reptiles and amphibians, overwinter or hibernate in or around water (Ultsch '89; Ultsch and Wasser, '90). This exposure leads to excess uptake of water and weight gains have been reported due to water uptake (Bentley and Yorio, '79; Ultsch, '89; Ultsch and Wasser, '90; Crocker et al., '00). This excess water in the blood causes a concentration gradient, which induces movement of water into the RBCs. Significant increases in cell water should cause the RBCs to swell and/or even rupture. If rupturing occurs in sufficient concentrations, the animal would have a decreased ability to carry oxygen to the tissues, which could result in death. However, overwintering reptiles and amphibians are able to survive in spite of the animals taking on excess water (Bentley and Yorio '79; Ultsch, '89; Ultsch and Wasser, '90; Crocker et al., '00).

Aldrich and Saunders ('01) showed that RBCs of amphibians and reptiles are much less likely to burst in high concentrations of water than RBCs of mammals and birds. This may be due to a better ability of the RBCs of amphibians and reptiles to undergo RVD. Thus, my research examined if the added osmotic strength of bullfrog erythrocytes is due to membrane transport systems by inhibiting certain transport systems to reveal if the cells have altered osmotic fragility when subjected to increased water concentrations.

MATERIALS AND METHODS

This research was conducted at Emporia State University, from May of 2001 until May of 2002, and all animals used in this research were handled according to guidelines established by the Emporia State Animal Care and Use Committee.

Blood Collection and Preparation

Animals were anesthetized with approximately 1 ml of Halothane. Blood volumes of approximately 1 ml were collected from bullfrogs (*Rana catesbeiana*) (n = 19) and mice (*Mus muscrilus*) (n = 24) via heart puncture using a heparinized syringe and placed in heparinized vacutainers.

Immediately after collection, the blood was centrifuged at 2000 *g* for approximately 1 minute. Plasma was removed and kept for osmolality determination (plasma osmolality was determined using a 5520 Wescor Vapro Vaper Pressure Osmometer). The erythrocytes were washed 3 times in an isosmotic solution that was species-specific. The bullfrog isosmotic solution contained (mmol/L): 65 NaCl; 5 KCl; 1 $MgCl^2$; 10 tris-HCl; 80 sucrose; 0.1 ouabain (Sigma lot 30K1207). The mouse isosmotic solution contained (mmol/L): 140 NaCl; 10 glucose; 5 KCl; 0.1 ouabain; 10 Na-phosphate. Two solutions were made using either monobasic or dibasic Na-phosphate and then they were mixed until the pH of the final solution was 7.6 for the bullfrog and 7.4 for the mouse. After each wash the RBC suspension was centrifuged (2000 g), and the wash supernatant was discarded. RBCs were then placed in the appropriate test solution.

Test Solutions and Incubation

Each individual animal's RBCs were tested in a total of three different incubation solutions. A control solution, which was identical to the species-specific isosmotic solutions. A test solution, which contained an inhibitor. The test solutions contained one of the following inhibitors: furosemide (Sigma lot 69H1237) (5 mmol/L for the mouse and bullfrog); DIDS (Sigma lot 31K1719) (9.5 mmol/L for the mouse and bullfrog); or

DIOA (Sigma lot 107H4730) (1.5 mmol/L for the bullfrog and varied between 0.7 mmol/L and 3 mmol/L for the mouse). The last was a solubilizer solution and it was made by adding the same concentrations of the alcohol in which the inhibitors were solubilized to the isosmotic solutions. Furosemide and DIOA were solubilized in ethanol and DIDS was solubilized in methanol. RBCs were added to the appropriate incubation solution to a final packed cell volume of between 5% and 15%. Packed cell volumes were determined by the microhematocrit method. Erythrocytes were allowed to incubate in the solutions for 30 min.

Erythrocyte Osmotic Fragility Determination

After incubation, erythrocyte osmotic fragility (EOF) was determined for each sample. Samples were vortexed to produce a well-mixed solution and immediately after vortexing, 20 µl of the sample was added to 1 ml of each of the EOF solutions. Erythrocyte osmotic fragility solutions were made by taking the species-specific isosmotic solutions and diluting them with distilled water. This created a gradient of solutions of varying osmotic strength. For each trial there were ten solutions ranging in osmotic strength of distilled water (approximately 0 mOsm) to isosmotic (approximately 200 mOsm for the bullfrog and approximately 320 mOsm for the mouse). The solutions were not evenly distributed. More solutions were clustered around previously estimated 50% hemolysis values. Three EOF trials were run on each blood sample: control, solubilizer, and inhibitor trials. The solutions were mixed well and allowed to sit for 30 min. After 30 min, each sample was mixed and then centrifuged for 5 min at 2000 *g*. The supernatant was removed and hemoglobin concentrations were determined using a Milton Roy Spectronic 301 spectophotometer to measure absorbance at 540 nm. The

following equation was used to find the percent lysis of the erythrocytes at each EOF solution:

$$\% \text{ Hemolysis} = \frac{OD(x) - OD(i)}{OD(o) - OD(i)}$$

where OD stands for the optical density or absorbance, (x) is the solution from which the percent hemolysis was obtained, (i) is the absorbance of the isotonic solution and (o) is the absorbance of the distilled water solution. Absorbance values indicate how much lysis took place in a solution by measuring how much hemoglobin is in the solution. Percent hemolysis versus osmolality of the solution was graphed using Microsoft Excel. A sample graph is shown in Figure 1. Using these graphs, 50% hemolysis values for each trial were determined. Fifty percent hemolysis values could not be obtained for the mouse DIOA trial group, due to the considerable amount of lysing that took place for each inhibitor concentration. A sample graph of this trial is shown in Figure 2. For this trial, absorbance values at the isosmotic solutions were used for statistical comparisons.

Statistical Analysis

The 50% hemolysis values for the all the trials were compared using one-way analysis of variance with repeated measures, except for the mouse DIOA group. For the mouse DIOA group, 50% hemolysis values were not obtained due to the considerable amount of lysing in the solutions, the lysis values at the isosmotic solution were compared using a one-way analysis of variance with repeated measures. Multiple comparison tests were then performed to determine which groups differed significantly. Differences were considered significant at $p \leq 0.05$.

Trial with All Inhibitors

A fourth trial was completed on the bullfrog erythrocytes using all the inhibitors combined. Trials were not run on mouse blood, because previous trials using DIOA indicated that 50% hemolysis values probably could not be obtained. Inhibitor and solubilizer concentrations used in this trial were the same as the other trials as stated previously. The procedure used for this trial was the same as the previous trials, aside from the following differences; only three bullfrogs were used, 50% hemolysis values were not obtained due to extensive hemolysis for each solution, and statistical tests were not performed on the data obtained.

Figure 1. Sample graph of percent hemolysis versus osmolality.

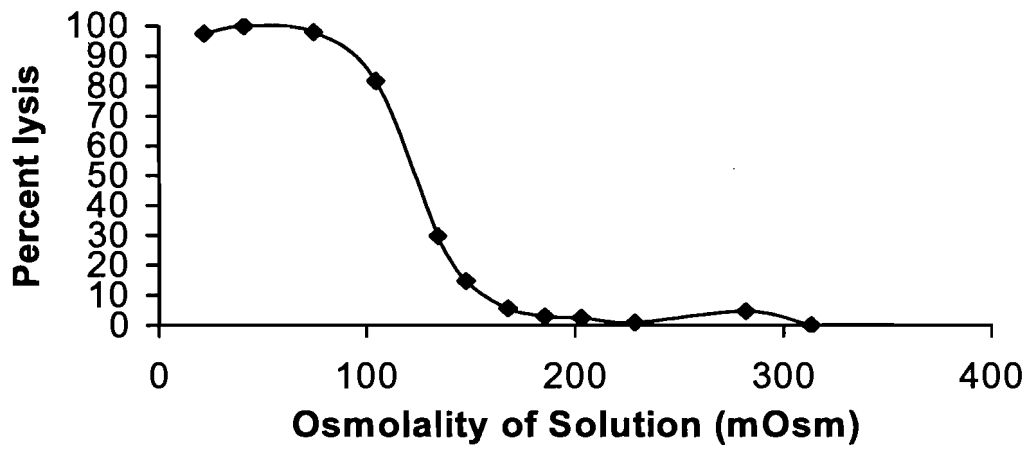
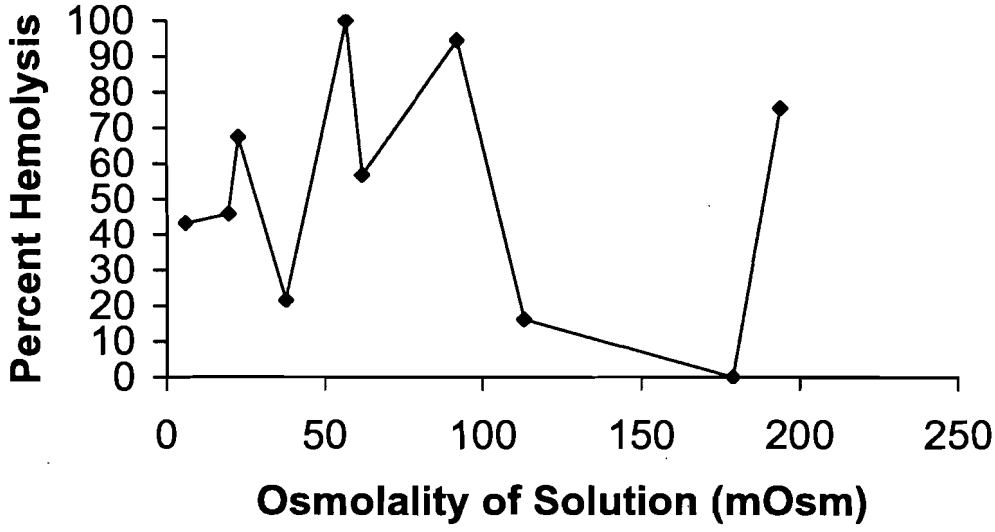

Percent lysis
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
Osmolality of Solution (mOsm)

Figure 2. Sample graph of percent hemolysis versus osmolality showing an extensive amount of lysing.


Percent Hemolysis
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
Osmolality of Solution (mOsm)

RESULTS

Furosemide

Significant differences in lysing were found in the mouse furosemide trial ($F = 13.060$; d.f. = 10; $p < 0.001$) (Table 1). Significant increases in lysing were found between the mouse furosemide treated RBCs and both the control and ethanol treated RBCs (Table 1). Significant differences in lysing were also found in the bullfrog furosemide trial ($F = 6.524$; d.f. = 6; $p = 0.012$) (Table 2). Although this cannot be attributed to furosemide, as that there was only a significant difference between the ethanol and control treated RBCs (Table 2).

DIDS

DIDS significantly increased RBC lysing in mice ($F = 22.033$; d.f. = 7; $p < 0.001$) (Table 3). In the bullfrog DIDS trial significant differences in hemolysis were found ($F = 41.832$; d.f. = 5; $p < 0.001$) (Table 4). A significant increase in hemolysis was found between the DIDS treated RBCs and the control treated RBCs, but not between the DIDS treated RBCs and the methanol treated RBCs (Table 4).

DIOA

Significant differences in lysing were found in the mouse DIOA trial ($F = 20.015$; d.f. = 6; $p < 0.001$) (Table 5). There was a significant increase in the extent of mouse RBC lysing in the DIOA group as compared to both the control and ethanol groups (Table 5). Significant differences in lysing also were found in the bullfrog DIOA trial ($F = 65.306$; d.f. = 5; $p < 0.001$) (Table 6). There were significant increases in the amount of lysing in the bullfrog DIOA treated RBCs as compared to both the control and ethanol trials (Table 6).

All Inhibitors

Statistical tests were not completed on the trial using all the inhibitors together due to the small sample size. However, the group with all the inhibitors had increased hemolysis when compared to both the control and solubilizer groups (Table 7), although it is not known if this increase is significant.

TABLE 1. Means and standard deviations (mOsm) of mouse 50% hemolysis values for control, ethanol, and furosemide treated RBCs (n = 11).

Control	136.522 (±7.567)[a]
Ethanol	135.241 (±8.272)[a]
Furosemide	144.393 (±8.113)[b]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 2. Means and standard deviations (mOsm) of bullfrog 50% hemolysis values for control, ethanol, and furosemide treated RBCs (n = 7).

Control	33.733 (±3.024)[a]
Ethanol	39.571 (±6.261)[b]
Furosemide	35.541 (±3.586)[ab]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 3. Means and standard deviations (mOsm) of mouse 50% hemolysis values for control, methanol, and DIDS treated RBCs (n = 8).

Control	132.231 (±8.844)[a]
Methanol	130.179 (±8.381)[a]
DIDS	142.630 (±5.536)[b]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 4. Means and standard deviations (mOsm) of bullfrog 50% hemolysis values for control, methanol, and DIDS treated RBCs (n = 6).

Control	24.072 (±4.052)[a]
Methanol	32.893 (±2.789)[b]
DIDS	33.583 (±1.357)[b]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 5. Means and standard deviations of mouse absorbance values at isosmotic solution for control, ethanol, and DIOA treated RBCs (n = 7).

Control	0.723 (±0.0159)[a]
Ethanol	0.741 (±0.0194)[a]
DIOA	0.891 (±0.0976)[b]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 6. Means and standard deviations (mOsm) of bullfrog 50% hemolysis values for control, ethanol, and DIOA treated RBCs (n = 6).

Control	15.098 (±2.303)[a]
Ethanol	18.043 (±3.591)[a]
DIOA	42.182 (±8.740)[b]

Those with the same letter in a column are not significantly different ($p < 0.05$).

TABLE 7. Means and standard deviation of bullfrog absorbance values at isosmotic solution for control, solubilizer, and all inhibitors treated RBCs (n = 3).

Control	0.759 (±0.193)
Solubilizer	0.777 (±0.0145)
All Inhibitors	0.922 (±0.125)

DISCUSSION

The objective of this research was to investigate if the increased resistance to lysing possessed by bullfrog RBCs relative to mouse RBCs is due to membrane transport systems rather than some property of the membrane itself. To test this I inhibited various membrane transport systems and subjected the RBCs to hyposmotic conditions to determine if they had altered osmotic fragility.

To date, little research on the effect of various membrane transport system inhibitors on RBC volume regulating abilities has been performed using bullfrogs. Considerably more research has been done on the role of these inhibitors on the volume regulating abilities of rodent RBCs. As such, I used the mouse trials to ensure the inhibitors were effective.

Furosemide is a loop diuretic that has been used for characterization of the $Na^+/K^+/Cl^-$ cotransporter in erythrocyte membranes (Garay et al., '88). Furosemide, along with other loop diuretics such as, bumetanide, has been found to block the action of the $Na^+/K^+/Cl^-$ cotransporter by competing with Cl^- for an anion receptor site on the transporter (Garay et al., '88). When mouse RBCs were placed in solutions containing furosemide, there was increased lysing when the cells were subjected to hyposmotic conditions. This indicates that furosemide causes a significant weakening of the volume regulating abilities of mouse RBCs. The $Na^+/K^+/Cl^-$ cotransporter has not been shown to be used in RVD, but rather in RVI. Various research has elucidated that in some animals, such as sheep, duck, toadfish, and humans, furosemide also blocks the K^+/Cl^- cotransporter (Lauf et al., '92). This may also explain the reduced ability of mouse RBCs to undergo RVD when exposed to furosemide.

When bullfrog RBCs were treated with furosemide they showed no significant increase in the amount of lysis, which suggests that furosemide did not interfere with the mechanisms of bullfrog RBC RVD. Gusev et al. ('97) suggested that the RBC membrane of the frog *Rana temporaria* does not contain the $Na^+/K^+/Cl^-$ cotransporter. They also found that after osmotic swelling, bumetanide, a loop diuretic like furosemide, did not affect K^+ efflux from frog RBCs (Gusev et al., '97; Gusev et al., '99). These results may indicate that loop diuretics like furosemide do not affect the K^+/Cl^- cotransporter in ranids and thus, have no effect on RVD. My results support the findings of Gusev et al. ('97 and '99) and may provide an explanation as to why furosemide interfered with mouse RVD and not with bullfrog erythrocyte RVD.

DIDS, a stilbene derivative, is a powerful inhibitor of the band-3-mediated anion exchange system in RBC membranes. Evidence supports the thought that anion channels play a role in RVD of RBCs (Motais et al., '91; Nilius et al., '94; Bogdanova et al., '98). DIDS is effective at inhibiting Cl^--dependent K^+ fluxes in RBCs (Garcia-Romeu et al., '91; Delpire and Lauf, '92). I found there was a significant increase in lysing between the control and DIDS treated RBCs, but not between the methanol and DIDS treated RBCs. This significant difference most likely can be attributed to the interference of methanol and not due to the effect of DIDS on RVD. These results are not surprising, as Gusev et al. ('99) reported in frog RBCs, DIDS caused an almost equal increase in Rb^+ influx and K^+ efflux, thus resulting in no net gain or loss of ions and would have no impact on cell volume. Rb^+ is a substitute for K^+, and the transporters will move Rb^+ as they would K^+, if K^+ is not present extracellularly.

For the mouse RBCs treated with DIDS, there were significant increases in lysing between the DIDS treated RBCs and both the control and methanol treated RBCs. These results indicate DIDS is causing some weakening in the mouse RBCs' ability to undergo RVD. It is unclear if this perturbation in RVD is attributed to the inhibition of the anion channels, the K^+/Cl^- cotransporter, or both transport systems, as the data do not provide evidence for distinguishing between transporters.

Garay et al. ('88) suggest DIOA is the most potent inhibitor of the K^+/Cl^- cotransporter in human RBCs. DIOA has no effect on the $Na^+/K^+/Cl^-$ cotransporter in RBCs and it can be used to characterize K^+/Cl^- cotransport in various cells (Garay et al, '88). DIOA is now known to inhibit the K^+/Cl^- cotransporter in many different cell types (Saitta et al., '90; Olivieri et al., '91; De Silva and Aronson, '97; Gusev et al., '99). Armsby et al. ('95) found that DIOA inhibited the K^+/Cl^- cotransporter of mouse RBCs, however this was to a lesser degree than what was previously demonstrated in human RBCs. In this study, mouse DIOA treated RBCs lysed to such a large extent I was not able to obtain 50% lysis values. These results seem to indicate DIOA severely decreases the ability of mouse RBCs to undergo RVD. Because 50% lysis values were unobtainable, statistical procedures using the lysis values of the isosmotic solutions were used instead. Significant increases in lysing were found in DIOA treated mouse RBCs relative to both the ethanol and control treated RBCs. Because DIOA has no effect on other membrane transport systems (Garay et al, '88), my results seem to indicate that inhibiting the K^+/Cl^- cotransporter in mouse RBCs markedly decreases the cells' ability to carry out RVD.

Gusev et al. ('97) discovered DIOA blocked Cl^--dependent K^+ efflux from hypotonically swollen *R. temporaria* RBCs. This result indicates that the K^+/Cl^- cotransporter is used in RVD of frog RBCs and can be inhibited by DIOA. Later Gusev et al. ('99) completed another study that supported their previous findings. In my study, there was a significant increase in the amount of lysing between DIOA, and both control and ethanol treated bullfrog RBCs. These results indicate DIOA causes a significant weakening in the ability of bullfrog RBCs to undergo RVD. These findings support previous research performed by Gusev et al. ('97 and '99), in that the K^+/Cl^- cotransporter is essential for RVD in *Rana* erythrocytes and disabling it hinders the volume regulating process that occurs when RBCs are placed in hypotonic media.

In the DIOA bullfrog trial the 50% hemolysis values of the control and ethanol groups were substantially lower than the control and ethanol groups of the furosemide and DIDS trials. For the furosemide and DIDS trials, the lowest osmolality EOF solution was 23 mOsm. For the DIOA trial the lowest osmolality EOF solution was 6 mOsm. I believe the lower value in the DIOA trial could have caused the 50% hemolysis values to be moved to lower osmolalities. I do not believe the discrepancy in the 50% hemolysis values has a significant bearing on the conclusion made from these results. Comparisons of 50% hemolysis values are of importance between inhibitor trials and their solubilizer and control trials, while comparisons between the furosemide, DIDS and DIOA trials are of little value.

If the increase in resistance to lysing shown in amphibian RBCs is due solely to the transport systems located in their plasma membranes, then after their inhibition bullfrog RBCs should lyse at osmolalities similar to that of mouse RBCs. However,

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when transport systems in bullfrog RBCs were inhibited one at a time, this did not occur. Even after inhibition, bullfrog RBCs are still much more resistant to lysing than are mouse RBCs. Two possible explanations may exist for this. One, other membrane transport systems are compensating for the inhibited systems and so RVD can still take place, though at a decreased level. Alternatively, amphibian RBC resistance to lysing is due, at least in part, to integral properties of the plasma membrane itself, meaning, bullfrog RBC membranes are stronger than mouse RBC membranes when exposed to equal hyposmotic conditions. To test this, trials were run using all the inhibitors on bullfrog RBCs. Using all the inhibitors caused such a considerable amount of lysing, 50% lysis values could not be determined. Instead, the hemolysis values at the isosmotic solutions were used (Table 7). Because of a small sample size, statistical comparisons could not be done, but the results of this study suggest there is potentially some compensation of other membrane transport systems when only one membrane transport system is inhibited.

In conclusion, this study indicates that mouse RBCs are dependent on anion exchange systems, and the K^+/Cl^- cotransporter for RVD and disabling these membrane transport systems does significantly weaken mouse RBCs' ability to volume regulate in hyposmotic conditions. Trials using the inhibitors furosemide and DIDS showed no significant weakening of bullfrog RBCs when exposed to hyposmotic conditions. Significant weakening only occurred in bullfrog RBCs when DIOA was used. This result seems to indicate, and is supported by previous research (Gusev et al., '97 and '99), that the K^+/Cl^- cotransporter is the most important membrane transport system used in RVD of bullfrog RBCs and inhibiting it causes a severe decrease in the cells' ability to volume

regulate when exposed to hyposmotic conditions. Aldrich and Saunders ('01) showed amphibian RBCs had greater osmotic strength when exposed to hyposmotic conditions than did rodent RBCs. This is not surprising since bullfrog RBCs are exposed to hyposmotic conditions by the uptake of water through their skin. This study seems to show that this increased osmotic strength is due, to some extent, to the membrane transport systems located in the bullfrog RBC membrane, because inhibiting these transport systems causes a decline in the cells' ability to undergo RVD. This study did not address the reasons why these membrane transport systems are better. It is possible that these transport systems are able to move ions more efficiently or it could be there are more transport systems in bullfrog RBC membranes than in mouse RBC membranes. This study also showed bullfrog RBCs still have a greater ability to volume regulate even when all the inhibitors were used. Perhaps, other transport systems are used in bullfrog RVD, ones that were not inhibited in this study. This result also could be due, at least in part, to some integral characteristic of the RBC membrane itself, which leads to the greater osmotic strength of bullfrog RBCs.

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