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Nicole Jorissen  
Minnesota State University, Mankato

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## **The Effect of Maternal Aldosterone Levels on the Expression of 11 $\beta$ -HSD Isoenzymes in Normal and Hypertensive Rat Placentas**

Nicole Jorissen (Chemistry and Geology)

Terry Salerno, Faculty Mentor (Chemistry and Geology)

### **ABSTRACT**

In this project, we developed a Western blotting procedure to semi-quantitate levels of 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 in whole cell extracts. Then, we applied this technique to analyze the effect of reduced maternal aldosterone levels on the expression of 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 isoenzymes in the placental tissue in both normal and hypertensive rats. These enzymes control levels of glucocorticoids which compete for aldosterone's mineralocorticoid receptor. Overstimulation of this receptor results in hypertension. If aldosterone levels decrease, levels of the enzymes controlling active glucocorticoid concentrations might change to compensate for the lowered aldosterone levels. Decreased placental 11 $\beta$ -HSD2 expression could affect hypertension in the offspring. The Western blotting procedure was optimized for the detection of the two isoenzymes in their dimeric forms. The use of multiple protein levels on the blot was useful to obtain a more reliable semi-quantitation. Because of the presence of multiple bands, it was impossible to use an internal beta actin standard to normalize the data from one blot to another. This along with the low expression levels and uneven blot backgrounds made it difficult to obtain enough data on the expression of the two isoenzymes. Very limited data do not support differences in the placental expression of the two isoenzymes when maternal aldosterone levels are lowered.

## INTRODUCTION

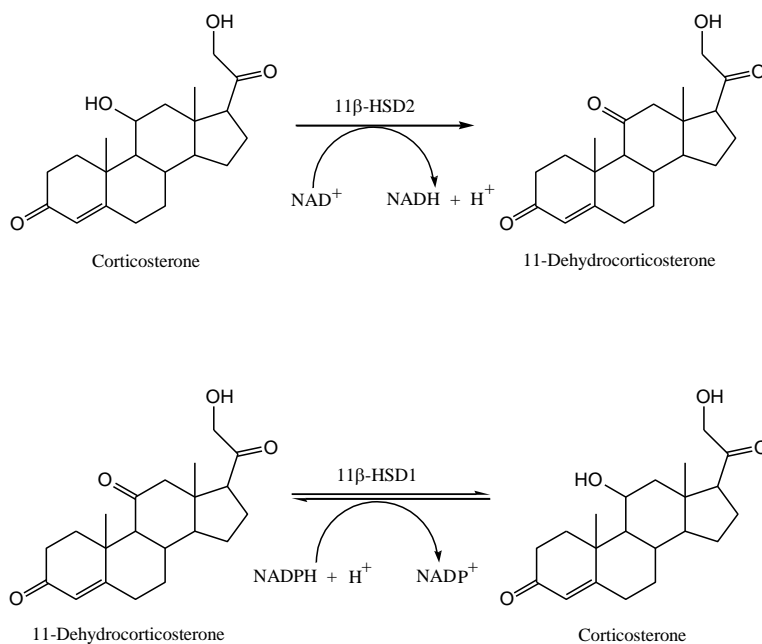
The enzymes 11- $\beta$  HSD1 and HSD2 are very important in steroid hormone metabolism. The roles of 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 are to interconvert cortisol and the inactive glucocorticoid, cortisone in humans. They also convert corticosterone and 11-dehydrocorticosterone in rodents [Gomez-Sanchez, 2003]. There are two different forms of the enzyme 11 $\beta$ -HSD. The first one, 11 $\beta$ -HSD1 acts as an oxidoreductase to form the active glucocorticoid cortisol or corticosterone in rats. The second isoform is 11 $\beta$ -HSD2 which produces inactive 11-keto metabolites. The reactions for the isoenzymes are given in Figure 1 on the next page.

Active glucocorticoids increase the synthesis of glucose and its storage polysaccharide form, glycogen. Because they can bind to the mineralocorticoid receptor, they can act like aldosterone and increase the absorption of sodium ions. Therefore, it is important to have sufficient 11- $\beta$  HSD2 activity in the placentas to protect against high levels of maternal active glucocorticoids [Staud, 2006]. If excessive amounts of maternal corticosteroids accumulate in the fetal tissues, this can lead to higher risks for hypertension and other diseases in adulthood [Struwe, 2007].

This study addresses a related problem. If maternal aldosterone levels are decreased through cryodestruction of adrenal glands, will this lower active maternal glucocorticoid levels and subsequently affect placental levels of the HSD enzymes? Which of the two HSD placental enzymes are affected and in what way? If the lowering of maternal aldosterone levels decreases hypertension in the mother, is this programmed into the offspring? What is the role of the HSD isoenzymes in these mechanisms? This study is part of larger study currently being done by Dr.

Knoblich and co-workers. They are studying the hormonal and blood pressure effects of lowering maternal aldosterone, and this study is designed to look at the enzymatic effects.

**Figure 1.** Reactions of the HSD isoenzymes



The 11β-HSD1 enzyme catalyzes the largest production of active glucocorticoids from inactive 11-keto metabolites and many of these reactions occur in the liver microsomes [Lloyd, 1999]. It generally uses NADPH to carry out the reduction reaction to produce active glucocorticoids ( Figure 1). However, it is also capable of catalyzing the reverse reaction. The molecular weight of the monomer unit is approximately 31.9 kDa. Because this enzyme is a glycosylated protein, the masses may vary [Dotsch, 2002]. Limited evidence exists for the active dimer forms which are capable of showing positive cooperativity in their kinetic properties [Maser, 2002].

The 11  $\beta$ -HSD1 enzyme is also produced in the rat kidney where there are high amounts present. It is unclear what its role is in human kidney although recent studies support a dehydrogenase function [Gong, 2008].

The second of the isoenzymes is 11 $\beta$ -HSD2. This enzyme is considered unidirectional as a dehydrogenase using the coenzyme NAD<sup>+</sup>. It inactivates the glucocorticoids through their oxidation (Figure 1). The molecular weight of the monomer unit is approximately 43.7 kDa. There are limited studies which suggest that this isoenzyme may also dimerize because of oxidation creating disulfide linkages, but unlike HSD1, it becomes inactive in this dimer form. [Gomez-Sanchez, 2001]

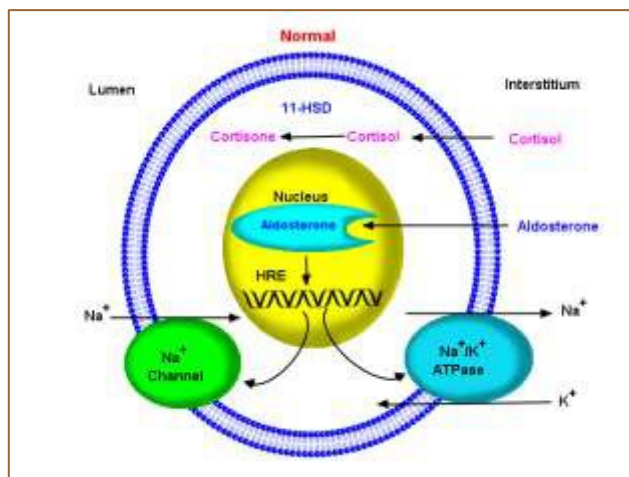
A summary comparing the two isoenzymes is given in Table 1 below:

**Table 1.** A Comparison of the 11 $\beta$ -HSD Isoenzymes

<b>11<math>\beta</math>-HSD 1</b>	<b>11<math>\beta</math>-HSD 2</b>
<b>31.9 kDa (varies with glycosylation)</b>	43.7 kDa
<b>Functions bi-directionally</b>	Functions unidirectionally
<b>Can act as a reductase with NADPH to produce active glucocorticoids</b>	Acts an oxidase with NAD <sup>+</sup> to produce inactive glucocorticoids
<b>Can act as an oxidase to produce inactive glucocorticoids</b>	Allows aldosterone access to mineralocorticoid receptor
<b>Liver and kidney, placenta, others</b>	Kidney and liver, placenta, others

These two enzymes are important in controlling active glucocorticoid levels. The active glucocorticoids can compete with the hormone aldosterone for the mineralocorticoid receptor. Either of the two hormones can bind to the mineralocorticoid receptor (MR) leading to the transcription and translation of the sodium ion channel protein and the sodium/potassium ATPase. The net effect of this hormonal cascade is to increase sodium reabsorption and to increase hypertension [Bocchi, 2003]. HSD2 is known to function in the kidney to inactivate cortisol or corticosterone (See Figure 2 below.) and prevent hypertension which could result from the glucocorticoids occupying the MR. [Lloyd, 1999].

**Figure 2.** Hormonal Mechanisms at the Mineralcorticoid Receptor



Placental 11 $\beta$ -HSD2 protects the fetus from the high circulating levels of the maternal glucocorticoids. When the cortisol crosses the placenta of the animal it becomes inactivated and doesn't hurt the growing embryo. In rodents 11 $\beta$ -HSD2 expression is very high and is related to fetal weight at midgestation, but drops dramatically toward the end of the term [Krozowski, 1999]. Chronic stress in the maternal rats does not allow for increased 11 $\beta$ -HSD2 activity although acute stress near term did increase levels of the enzyme to protect the fetus from increased levels of maternal corticosterone [Welberg, 2005].

11  $\beta$  -HSD2 also plays a role in hormonal programming. When fetal and placental 11  $\beta$  -HSD2 levels are insufficient, this can promote hypertension in adulthood [Fujisawa, 2004]. The offspring of rats treated with synthetic glucocorticoid, dexamethasone, during late pregnancy had lower birth weights than offspring of control rats and were shown to increase their expression of 11 $\beta$  -HSD1 [Wan, 2005 ]. The results of these experiments indicated that 11 $\beta$ -HSD1 plays a

critical role in the control of normal fetal growth and the subsequent development of adult disease.

In another study, diabetes was drug induced in pregnant normotensive rats and the maternal corticosterone levels were reduced [Fujisawa, 2004]. The researchers found that  $11\beta$ -HSD 2 mRNAs decreased significantly in the experimental placentas and fetal kidneys. Because diabetic mothers are known to imprint high blood pressure in their offspring, the authors suggested that this may be due in part to the lowered expression of the HSD2 enzyme.

Other studies suggest that there is a correlation between hypertension in adulthood and embryo growth restriction in the human womb. In their study, Struwe and coworkers [2007] not only found that  $11\beta$ -HSD2 gene expression was reduced in placentae of smaller than normal neonates, there was also a corresponding decrease in the expression of  $11\beta$ -HSD1 gene expression. The authors speculated that this could be a possible mechanism to counteract the loss of inactivation of the cortisol hormone because of lowered amounts of HSD2.

This work is part of a larger study by Dr. Knoblich and researchers, who are using cryodestruction of the outer layer of the maternal adrenal gland to lower aldosterone levels and measure hypertension in the offspring. Because the programming is known to occur in the fetus and because HSD placental enzymes control active glucocorticoid levels, their expressions were measured in both normotensive and hypertensive rat placentas. To accomplish this, the feasibility of using a Western blotting technique to semiquantitate HSD isoenzymes from whole cell extracts (not microsomes) was examined. Then, the methods were used to measure the expression of the HSD isoenzymes in the placentae of rats with lowered aldosterone levels.

## MATERIALS AND METHODS

### Treatment of the rats

Four to five week old rats were fasted for 16 hours before surgery. Under anesthesia, the right adrenal gland was removed in the experimental rats. The left adrenal gland was frozen using a metal spatula dipped in liquid nitrogen, with each area of the gland's surface frozen three times in succession. Control rats underwent a mock or sham surgery with the identical incisions, but no removal or freezing procedures were done on the adrenals. This part of the project was conducted by Dr. Knoblich and her students and has IRB approval.

### Isolation of placental tissue

The rats were mated after a recovery period and placentas were removed by Dr. Knoblich on the 20<sup>th</sup> day of gestation. They were washed in saline and cut in quarters followed by quick freezing in liquid nitrogen. They were stored in a -80 freezer.

### Isolation of the crude protein extracts

The rat placentas were frozen in liquid nitrogen and then ground to a powder and weighed out to give 100 mg of frozen tissue. The frozen powder was mixed with 4 volumes of extraction buffer which consisted of 25mM Tris-HCl, pH 8.0, 200mM Sucrose, 154mM KCl, and 20mM DTT. The protein was then vortexed for 3 minutes with a one minute rest in between. The mixture was then centrifuged for 15 minutes at 10,000 x g. The crude protein extract was then diluted into Laemmli sample buffer to give a final concentration of 300mM DTT and 1.3% SDS. Four different concentrations of protein in the range of approximately .5-2.5 mg/ml were prepared. The samples were heated for 6 minutes at 100 degree C and 20 ul of sample was quantitatively applied to respective wells.



### Gel Electrophoresis and Blotting

The sample mixtures were run on a 12.5% Laemmli gel (BIO-RAD Criterion), equilibrated in 1X Laemmli Buffer, at 35 milliamps for a half hour and then at 40 milliamps for two hours and twenty minutes. The gels were blotted on to polyvinyl-difluoride (PVDF) membranes. The blotting was run in 1X Towbin containing 20% methanol at 85 volts for 75 minutes. The membranes were taken out and placed in a blocking solution consisting of 4% bovine serum albumin (BSA) in a 1X Tris buffered saline, pH 8.0 (TBST) solution containing .10 % Tween 20 and then agitated for 60 minutes. The primary antibodies to HSD1 and HSD2 (Santa Cruz no. 20175 and 20176, respectively ), were put onto the gels at a dilution of 1:1000 in blocking buffer, and were agitated for 15 minutes and then put into a cold room overnight. The following morning they were agitated for one more hour at room temperature.

The gels were then washed in a 1X TBST solution 8 times for 5 minutes each. The secondary antibody (conjugated to peroxidase) was diluted into blocking buffer, at a dilution of 1:120,000. The membranes were then agitated for an additional 60 minutes. The membranes were then washed again 8 times for 5 minutes with the 1X TBST. The membranes were then soaked in 6.5- 7.0 ml of the SuperSignal West Femto Maximum Sensitivity substrate (Pierce) for 5 minutes. They were then put onto clear transparency films and sealed with all air bubbles being removed. The membranes were then developed using ECL film for 2- 5 seconds.

### Determination of protein concentrations and protein band sizes

The Kaleidoscope protein standards (BIO-RAD) were used to obtain a standard curve for molecular weights and to estimate the size of the major protein bands on the blot.

The Bradford assay (BIO-RAD) was used to determine the ug of protein in each well. An average of four samples was taken and BSA was used as the protein for the standard curve.

### Semi-quantitation of the HSD isoenzymes

The developed film was quantitated using a Hoeffler densitometer and the areas under the peaks were determined using Gaussian integration. The width of the lane was measured and used to normalize the sample areas. These areas were then plotted against the ug of protein used. Line equations were then used to determine the total area for 50ug of protein and the error associated with the number could be estimated from the line fit.

## RESULTS

### I. Optimization of the Blot

The HSD isoenzymes are not abundant proteins. Most western blots in the literature represent the analyses of microsomal extracts. Because of time and equipment restrictions, this project attempted to use whole cell extracts along with a very sensitive chemiluminescent substrate to detect these relatively rare enzymes.

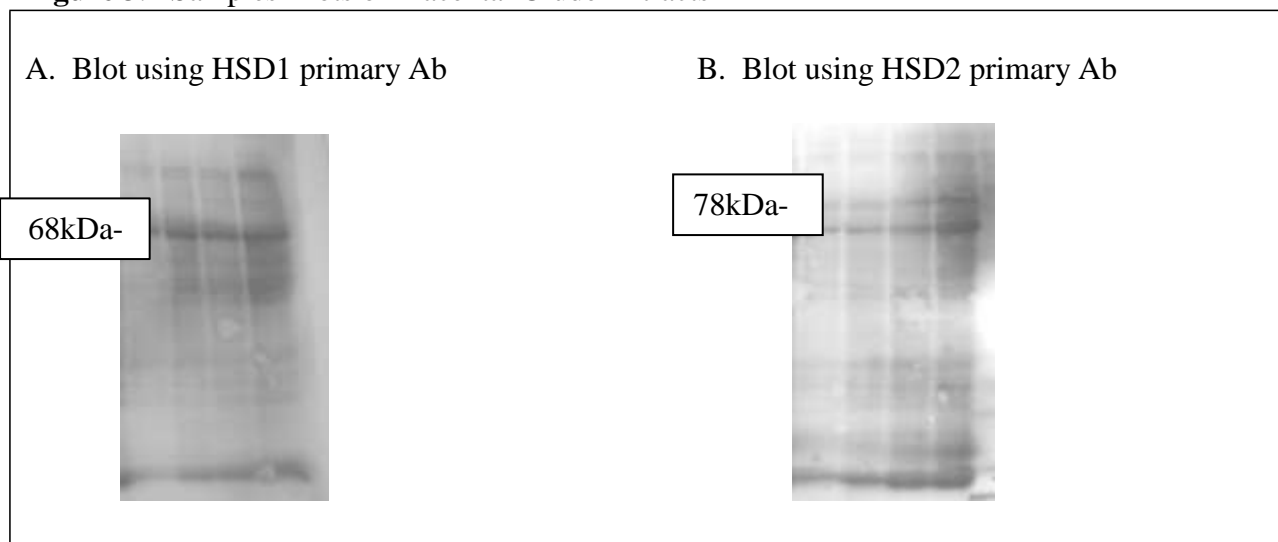
Several blots were run initially to optimize conditions. The extraction buffer and secondary antibody concentrations as well as the blocking protein were varied. Also, 10% polyacrylamide gels were compared to 12.5% gels for the best resolution of protein bands. The optimal conditions are given in the materials and methods. Beta actin was initially used for an internal standard. However, because of the multiple bands seen with the other antibodies, it was difficult to detect so its use had to be discontinued. Unfortunately, without the use of an internal standard to normalize the areas, it was impossible to compare the numbers among blots.

None of the conditions gave major bands at the desired molecular weights. Multiple bands were seen but the major bands present were a 68kD protein on the HSD1 film and a band

representing a 78kD protein on the HSD2 film (Figure 3). The HSD 2 film also showed a major 68 kD protein. This was disturbing since the sequence comparison in the region to which the antibody was directed was not highly homologous between the two isoenzymes. However, insufficient funds did not allow for the purchase of additional primary antibodies.

Analysis of adult kidney and liver samples showed multiple bands as well with different molecular weights. Some of the variability can be due to processing and glycosylation differences in different tissues. Because of the literature documentation of dimerization in older samples, it was assumed that the dimer forms at 68 kDa and 78 kDa represented HSD 1 and HSD 2 and since these could be quantitated in all blots, these were the bands which were analyzed.

**Figure 3.** Samples Blots of Placental Crude Extracts



### II.A comparison of HSD 1 and HSD 2 levels in the treated vs. control WKY samples

A limited number of WKY samples were tested for HSD1 and HSD2 protein expression (Table 2). The results of blot 1 showed no significant differences and those of blot 2 suggest a decrease in HSD 1 expression in the experimental placentae. However, one of the experimental

samples (J) does not show a significantly reduced maternal aldosterone level. Therefore, the very limited preliminary data do not support differences in the expression of the HSD1 enzyme.

**Table 2.** A Comparison of HSD 1 Expression in Control vs. Experimental WKY Placentas

	<i>Control WKY Sample</i>	<i>HSD 1 area</i>	<i>Experimental WKY Sample</i>	<i>HSD 1 area</i>
<b>Blot 1</b>	D (20.9)	19559 ± 7260	B (4.72)	23967 ± 4868
	G (10.7)	15084 ± 1365	F (39.5)	18623 ± 1130
	average	17321 ± 4312	average	21295 ± 2999
<b>Blot 2</b>	E (21.6)	15678 ± 1390	I (2.92)	6027 ± 370
	A (10.3)	11931 ± 1550	J (19.3)	8873 ± 2584
	average	13804 ± 1470	average	7450 ± 1477
<b>Blot 4</b>	DD(15.0)	6732 ± 7269	BB (1.25)	30102 ± 4540

Numbers in parentheses represent aldosterone levels in ng/dl (Knoblich, personal communication)

In blot 1, when HSD2 protein expressions were compared, there were no significant differences (Table 3). In blot 2 there appears to be a decrease in the expression of HSD2 in the experimental samples. However, sample J does not show a reduced maternal aldosterone level. Therefore, the limited preliminary data do not support differences in the expression of the HSD2 enzyme in the placentae of WKY rats with lowered aldosterone levels.

**Table 3.** A Comparison of HSD2 Expression in Control vs. Experimental WKY Placentae

	<i>Control WKY Sample</i>	<i>HSD 2 area</i>	<i>Experimental WKY Sample</i>	<i>HSD 2 area</i>
<b>Blot 1</b>	D (20.9)	12914 ± 8739	B (4.70)	5921 ± 836
	G (10.7)	2727 ± 719	F (39.5)	4066 ± 160
	average	7820 ± 4729	average	4993 ± 498
<b>Blot 2</b>	E (21.6)	5041 ± 2115	I (2.92)	1420 ± 86
	A (10.3)	3491 ± 913	J (19.3)	1791 ± 187
	average	4266 ± 1514	average	1605 ± 136
<b>Blot 4</b>	DD (15.0)	9588 ± 4428	BB (1.25)	7430 ± 1209

Numbers in parentheses represent aldosterone levels in ng/dl (Knoblich, personal communication)

**III. A comparison of HSD 1 and HSD 2 levels in the treated vs. control SHR samples**

The results for the SHR samples (Tables 4 and 5) showed no significant change in the protein expression of either isoenzyme. The error and the inadequate number of experimental samples, with reduced maternal aldosterone levels, made it difficult to develop any conclusions.

**Table 4.** A Comparison of HSD1 Expression in Control vs. Experimental SHR Placentas.

	Control SHR Sample	HSD 1 area	Experimental SHR Sample	HSD1 area
<b>Blot 3</b>	M (13.4)	24740 ± 19548	S (15.2)	9367 ± 1112
	O (19.5)	11720 ± 3615	N (3.46)	18870 ± 2231
	average	18230 ± 11581	average	14118 ± 1671
<b>Blot 4</b>	Q (14.4)	10171 ± 1445	T (3.35)	14856 ± 3450

Numbers in parentheses represent aldosterone levels in ng/dl (Knoblich, personal communication)

**Table 5.** A Comparison of HSD2 Expression in Control vs. Experimental SHR Placentas.

	Control SHR Sample	HSD 2 area	Experimental SHR Sample	HSD 2 area
<b>Blot 3</b>	M (13.4)	1596 ± 170	S (15.2)	4341 ± 924
	O (19.5)	4075 ± 2037	N (3.46)	4753 ± 1521
	average	2785 ± 1103	average	4547 ± 1222
<b>Blot 4</b>	Q (14.4)	16100 ± 1506	T (3.35)	15063 ± 2324

Numbers in parentheses represent aldosterone levels in ng/dl (Knoblich, personal communication)

**DISCUSSION**

The limitations of western blotting as a quantitative tool were evident in this project. When the background was uneven or bubbles occurred in the blot, it made quantitation difficult and the error values were high. The use of multiple concentrations helped but limited the number of samples which could be analyzed. The lack of the internal standard made it impossible to average sample numbers from one set of blots to the next. Because of variability in the rat data, this was a serious limitation in the design and needs to be corrected before further

studies are conducted. Better primary antibodies may solve this problem. The only affordable primary antibodies were polyclonal and gave multiple bands.

The presence of the dimers has been previously documented in the literature. It would be useful to verify with another source of primary antibody. It may be that cytosolic forms of the enzyme exist in precursor or dimer forms or that aging of the samples causes crosslinks that cannot be disrupted by a reducing agent.

The best approach to measure expression changes may be the measurement of messenger RNA (mRNA) levels using reverse transcription and quantitative polymerase chain reaction (RT-qPCR). This technology will soon become available at MSU with the purchase of a real time PCR instrument. However, this technique cannot measure changes in expression which occur later at the protein level.

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#### Student Biography: Nicole Jorissen

Nicole Jorissen is currently a junior at Minnesota State University, Mankato studying Biochemistry. She is involved in several clubs on campus and is the treasurer of the MSU Chem Club on campus. She also does work study with the Chemistry faculty and works in the labs most time gaining experience that will be helpful for grad school. She plans to attend Purdue or the University of Pennsylvania in the fall of 2010. She hopes to obtain her PhD. in molecular genetics with an emphasis in biochemical reactions. She is also a current McNair scholar and has presented at two national conferences. She recently presented at the NCUR conference in MD and the URC conference at MSU Mankato. She currently resides in Mankato, MN.

#### Mentor Biography: Terry Salerno

Dr. Terry Salerno received an A.B. degree in Biochemistry from Mt. Holyoke College and a Ph.D degree in Biochemistry from the University of Wisconsin, Madison. She has been a professor at Minnesota State University since the fall of 1986 where her primary course responsibilities are allied health chemistry and the biochemistry courses. In the last ten years she has mentored several undergraduate research students. She has collaborated with Dr. Rife and Dr. Knoblich on the study of HSD isoenzymes for the past couple of years. This is the first project to study expression at the protein level.