Ammonia Levels in Maternal and Fetal Tissues and Blood of Urea-Treated Sheep

Charles C. Yelverton

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AMMONIA LEVELS IN MATERNAL AND FETAL TISSUES
AND BLOOD OF UREA-TREATED SHEEP

BY

CHARLES C. YELVERTON

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Zoology, South Dakota
State University

1974
AMMONIA LEVELS IN MATERNAL AND FETAL TISSUES
AND BLOOD OF UREA-TREATED SHEEP

This thesis is approved as a creditable and independent
investigation by a candidate for the degree, Master of Science,
and is acceptable as meeting the thesis requirements for this
degree. Acceptance of this thesis does not imply that the
conclusions reached by the candidate are necessarily the
conclusions of the major department.

Thesis Adviser              Date

Head, Entomology-Zoology    Date
Department
ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to the State of South Dakota and South Dakota State University for making this educational experience possible. I would like to give thanks to Drs. Michael H. Roller and Robert N. Swanson for their expert guidance, assistance, and encouragement throughout the research for, and preparation of, this thesis. In addition to the staff of the Entomology-Zoology Department, I would also like to express my appreciation to those persons who made valuable contributions to this study: Mr. Rex Riis, Graduate Research Assistant in Zoology, for his assistance in the collection of data; Dr. W. Lee Tucker, Station Statistician, for technical assistance and advice with statistical analysis of data.

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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>2</td>
</tr>
<tr>
<td>Ammonia Toxicity</td>
<td>2</td>
</tr>
<tr>
<td>Ammonia Production</td>
<td>3</td>
</tr>
<tr>
<td>Tissue Ammonia</td>
<td>5</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>8</td>
</tr>
<tr>
<td>Experimental Animals</td>
<td>8</td>
</tr>
<tr>
<td>Preparation</td>
<td>8</td>
</tr>
<tr>
<td>Experimental Procedure</td>
<td>10</td>
</tr>
<tr>
<td>Analytical Procedure</td>
<td>11</td>
</tr>
<tr>
<td>RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>18</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>20</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>21</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>24</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table | Page
---|---
1. Mean ammonium nitrogen concentrations in whole blood of ewes and fetuses | 15
2. Mean tissue ammonium nitrogen concentrations in ewes and fetuses | 16

Appendix

3. Whole blood ammonium nitrogen | 25
4. Tissue ammonium nitrogen | 26
INTRODUCTION

Ammonia toxicosis is known to be a problem of nonprotein nitrogen or urea feeding in ruminant animals. Urea is hydrolyzed in the gastrointestinal tract in the presence of bacterial urease to ammonia and carbon dioxide. Diffusion of ammonia into the circulatory system normally causes no problems since the ammonia is converted into urea by the liver. The harmful effects arise when ammonia concentrations are excessive, normal detoxication processes become impaired, or ammonia is introduced too rapidly.

Investigators have concerned themselves with metabolic alterations and cellular activity resulting from ammonia intoxication. The economic advantages of feeding urea to ruminants and the toxic effects due to hydrolytic release of ammonia provide compelling reasons for obtaining accurate knowledge of varied ammonia concentrations in fetal and maternal tissues. Information concerning tissue ammonia concentration is meager, especially concerning fetal tissues.

The purpose of this study was to determine the ammonium nitrogen concentration of fetal tissues and blood following ammonia intoxication of the dam.
LITERATURE REVIEW

Ammonia Toxicity

Lawrence et al. (1957) observed that ammonia toxicity was correlated with intracellular ammonia concentrations and was determined by total ammonia concentration and the pressure gradient across the cell membrane, which in turn was determined by differences in pH. Nonionic ammonia readily diffused through a cell membrane impermeable to ammonium ions. Bessman, Rudo, and Cowley (1960) described the relationship between pH and ammonia toxicity. With the pH lowered, urea synthesis slowed, blood ammonia increased, and tissues retained ammonia. When the pH rose, urea synthesis improved, the arterial level of ammonia decreased, and less ammonia was retained by the tissues. Tissue retention was the product of blood flow and the arterial-venous blood ammonia concentration difference. Prior and Visek (1972) described the intensity of intoxication as a function of duration of exposure times tissue ammonia concentration.

Rosado et al. (1962) gave rats a high concentration of ammonia. After ten minutes the blood ammonia concentration decreased, while liver and brain tissue ammonia concentrations returned to normal. This fact indicated glutamine synthesis, which increased in blood, liver, and brain, but not in muscle. The ammonia did not diffuse evenly into the tissues, with muscle taking up twice as much as liver and brain. Because the ammonia concentration in muscle did not decrease when the blood ammonia concentration returned to normal,
a mechanism for active cell retention of ammonium ion was suggested with the pH gradient being an important component.

Walker and Schenker (1970) postulated on mechanisms for intracerebral ammonia toxicity by suggesting that ammonia interfered with pyruvate entry into the Krebs's cycle, NADH and \( \alpha \)-ketoglutarate were depleted, and ATP consumption was increased because of increased glutamine formation. ATPase activity increased and cerebral acetylcholine decreased.

Ammonia Production

Tashiro (1922) demonstrated that ammonia was produced at myoneural junctions in both resting and stimulated nerves. Nerve stimulation resulted in a twofold ammonia increase and a simultaneous carbon dioxide increase.

Weil-Malherbe (1950) demonstrated that ammonia was produced by cells and Brown et al. (1957) described ammonia as a toxic compound which existed in tissues at low concentrations because of detoxication mechanisms performed in the liver.

Schoenheimer (1942) observed that \( ^{15} \text{N} \) in animals as ammonia or as \( \alpha \)-amino acids may be incorporated with urea and with amino acids of tissue protein. Duda and Handler (1958) used the same isotope to discover that ammonia was incorporated with the amide of glutamine more rapidly than with any other nitrogenous liver components. The major fraction of ammonia formed in kidney tissues was derived from amide nitrogen of glutamine (Pilkington, Preuss, and Pitts, 1964). Carter, Lifton, and Welch (1973) stated that production of urea in
liver and glutamine in brain could be the reason for accumulation of higher $^{13}$NH$_3$-$^{13}$NH$_4$ activity in these organs as compared with blood, skull, or muscle tissue.

Investigations by Bessman and Bradley (1955) demonstrated that muscle removed approximately 40% of the ammonia in arterial blood or twice that of brain. They suggested a lesion in the Kreb's cycle of the brain as the determining factor. Bessman and Bessman (1955) also reported a parallel between uptake of ammonia by muscle and increased arterial levels of ammonia in hepatic disease. Brain took up ammonia when blood ammonia concentrations rose above 1 μg./ml. Bessman, Rudo, and Cowley (1960) placed less emphasis on the effect of pH on ammonia transfer across cell membranes by conducting tests where movement of ammonia into tissues was apparently governed by the arterial level of ammonia.

Rosado et al. (1962) measured ammonia in liver, muscle, and brain of rats and discovered normal concentrations to be 22, 11, and 8 μg. NH$_3$-N/Gm. of the respective tissues. The normal concentration for blood was 3.5 μg. NH$_3$-N/ml. Seventy-five percent of ammonia removed from blood in intoxicated rats was accounted for by muscle uptake, 2.5% by liver, and 0.5% by brain tissue.

Ingle and Williams-Ashman (1962) reported that with 70% of the liver removed rats could tolerate concentrations of blood ammonia which were in excess of those found under conditions of normal metabolism. In an experiment with perfused liver, Linzell, Setchell, and Lindsay (1971) observed that 20 mM NH$_3$/hour/100 Gm. of liver was
the limit of the detoxicating capacity in sheep. This was equivalent to 6 to 7 mg NH$_3$/100 ml. of portal blood.

McDonald (1948) discussed the importance of ammonia production in protein degradation and the ready absorption of ammonia from the rumen of anesthetized sheep. Lewis, Hill, and Annison (1957) reported that as rumen ammonia concentration reached 60-100 mM/L, a leakage of ammonia occurred into peripheral venous blood. As the blood ammonia concentration exceeded 0.6-0.9 mM/L, toxic symptoms developed. When the portal blood ammonia concentration exceeded 0.8 mM/L, the arterial ammonia concentration increased. Chalupa (1972) stated that nonprotein nitrogen sources would be most efficient when utilized to produce an ammonia concentration in the rumen optimal for bacterial protein synthesis. When the rumen ammonia concentration was elevated above an undetermined optimal level, increased absorption of ammonia caused toxicity.

**Tissue Ammonia**

It has been observed (Handford, 1961) that ammonia intoxicated dogs had hard congested livers, contracted spleens, dark and congested kidneys, and wet lungs.

Keynes (1963) discovered that blood urea and ammonia concentrations rose during renal and hepatic failure. In dogs, normal muscle ammonia was many times higher than in blood or lymph and ammonia in brain, pancreas, and liver was higher still. Dogs with rising blood ammonia had ammonia in muscle, brain, and pancreas increased twice to three times normal. Prior, Clifford, and Visek
(1970) discovered 2.25 times as much ammonia in kidney tissues of urease injected rats as in control rats. Plasma ammonia concentrations averaged 5 to 6 times those from the control group and liver ammonia concentrations were 1.6 times those of control. Tissue ammonium nitrogen (TAN) values of uterus with embryo were greater (P < 0.01) in intoxicated rabbits than in control rabbits (Roller, Swanson and Lang, 1970). TAN values of psoas muscle were greater (P < 0.05) in urea treated pregnant rabbits than in controls.

Carter, Lifton, and Welch (1973) used \(^{13}\text{N}H_3-^{13}\text{NH}_4\) to measure tissue ammonia accumulation. Liver accumulation exceeded that in blood by a factor of 3.8 and brain accumulation exceeded that in blood by a factor of 1.6. They emphasized the sensitivity of brain ammonia accumulation to minor changes in blood pH. Liver ammonia uptake was less sensitive to blood pH.

Kirkpatrick, Roller, and Swanson (1972) reported that TAN values at death were higher for intoxicated ewe sheep than for controls. TAN values of liver, kidney, psoas muscle, and heart muscle were greater (P < 0.01) than control values. In adrenal gland, TAN values were greater in principal than control tissues at the P < 0.05 level. Jaquette (1972) compared ammonium nitrogen concentrations in tongue, liver, and kidney from control and ammonia intoxicated lambs. Tissue samples were collected at the time of death and 2, 4, 8, 16, and 24 hours afterwards. Although tissue ammonia concentrations in both groups of lambs increased during the 24-hour postmortem period, concentration differences between control lambs and principal lambs
were statistically significant at the P 0.01 level for all collection periods.

Urea was excreted by sheep fetuses at approximately 0.54 mg./minute which was equivalent to 0.25 mg./kg. of fetal body weight (Gresham et al., 1971). This amount of urea production would require 1.6 ml. of O₂ consumption per minute which represented approximately 25% of fetal O₂ uptake. Production of urea by adult sheep and man were approximately 0.14 and 0.25 mg./minute/kg. of body weight, respectively. Oja, von Bonsdorff, and Lindroos (1966) found that the ammonia concentration of immature rat brain was 0.47 μmoles/Gm. of fresh brain tissue and decreased with maturation. Colombo and Richterich (1968) discovered urea cycle enzyme activity in the human fetal liver beginning from the fiftieth day of pregnancy, sustaining the hypothesis of an in vivo urea production in the fetal organism.
MATERIALS AND METHODS

Experimental Animals

Twelve Southdown sheep, between 80 and 85 days pregnant were divided into two groups. Seven animals in the principal group were intoxicated by drenching with urea solution, and five animals in the control group were drenched with water. At least one animal from each group was used on each of five test days. All the animals were sheared, housed, and fed indoors before testing and each appeared alert and healthy on test day.

Preparation

Extraneous ammonia was removed from water and all apparatus (5- and 10-ml. centrifuge tubes, glass stoppers, plastic and glass 1-ml. tuberculin syringes, and 0.5-, 1-, 2-, 4-, and 10-ml. volumetric pipettes) by washing with detergent\(^a\) and water, rinsing thoroughly with deionized and distilled water (ammonia-free), then soaking in 0.1\( \text{N} \) sodium hydroxide solution for 1 to 24 hours. Final treatment was at least five rinses in ammonia-free water before oven drying. Storage before use was in ammonia-free containers.

A modification of the method reported by Miller and Rice (1963) was used to measure the ammonium nitrogen concentration in whole blood and tissues. This involved the use of ion-exchange resin\(^b\)

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\(^a\)Haemo-Sol, Scientific Products, Evanston, IL 60201

\(^b\)C-244, 30-30 mesh, J. T. Baker Chemical Company, Phillipsburg, NJ 08865
which was converted to a sodium and potassium ion form by a process reported by Hutchinson and Labby (1962). A standard stock solution of ammonium ions was prepared to serve as a known source of ammonia for comparison with subsequent unknown solutions. This was prepared by adding 4.7166 Gm. of dried ammonium sulfate to a one-liter mark. The result was a solution with an ammonium nitrogen concentration of 1 mg./ml. Working standards to compare with unknowns were made from the stock solution in 100-ml. portions of 20 and 40 µg./ml. ammonium nitrogen concentrations. These concentrations represented the range of those expected in test materials. One liter of phenol color reagent was prepared by adding ammonia-free water to 10 Gm. of phenol and 50 mg. of sodium nitroprusside in a volumetric flask. Alkaline hypochlorite was prepared by mixing 6 ml. of 6% sodium hypochlorite, 5 Gm. of sodium hydroxide pellets, and ammonia-free water added to the one-liter mark of a volumetric flask. Resin suspension was prepared by washing 50 Gm. of resin three times with 0.1 N sodium hydroxide, five times with ammonia-free water, three times with 0.2 N sodium-potassium phosphate buffer (pH 7.4), and finally three washes with ammonia-free water. The resin was stored in an amber reagent bottle with water.

Resin tubes were prepared by pipetting 0.35 ml. of resin suspension into each 5-ml centrifuge tube. Ammonia-free water was added until total resin suspension was 1 ml. Tubes were stoppered with glass stoppers and were ready for use. Duplicate tubes were
properly labeled for samples of blood, water blank, and working standard.

Experimental Procedure

Ewes were weighed and bled by jugular venipuncture with a 14-gauge hypodermic needle and tuberculin syringe before drenching. One-half ml. of blood was added to each of two resin tubes and mixed for one minute with a mechanical mixer\(^{c}\). Ewes in the principal group were given (drench) 12.5 ml. of 3.3 \(M\) urea solution per kilogram of body weight, and water was given to ewes in the control group at the same rate. They were bled at 30, 90, and 150 minutes after drenching and at the time of death. Principal ewes died by intoxication and control ewes died by exsanguination at a time lapse from drenching equal to death by intoxication. After death, a mid-ventral incision was made to expose visceral organs. Five-gram slices of liver, kidney, spleen, and medial muscle of the thigh were taken from ewes and fetuses. Tissues were placed in plastic bags\(^{d}\), immersed in liquid nitrogen for quick freezing, and stored at -20 C until analytical procedures were performed. Fetal blood was drawn from the umbilical vein with a 20-gauge hypodermic needle attached to a tuberculin syringe. Values from duplicate blood and tissue ammonium nitrogen analyses were averaged and used as one value.

\(^{c}\)Vortex Genie Mixer, Scientific Industries, Inc., Springfield, MAS 01103

\(^{d}\)Whirl Pak, Nasco, Fort Atkinson, WI 53538
Analytical Procedure

Whole blood ammonium nitrogen concentrations (BAN) were measured after the last samples were acquired. The water blank was prepared in duplicate by adding 0.5 ml. of ammonia-free water to resin tubes and the duplicate standard was prepared by adding 0.5 ml. of the 20 μg./ml. ammonium sulfate working standard to resin tubes. The resin-whole blood mixtures were mixed for one minute and, after allowing the resin to settle, the supernate was aspirated and discarded with a Pasteur pipette attached by flexible tubing to a Clay Adams apparatus. Three-ml. aliquots of ammonia-free water were used for washing the resin repeatedly until the supernate appeared clear and free of foam. One ml. of 0.1 N sodium hydroxide was added to each tube and mixed for one minute to elute the ammonium ions. One-half ml. of this solution was transferred via pipette to a 10-ml. centrifuge tube, and 1 ml. each of phenol color reagent and alkaline hypochlorite was added. The volume was brought to 10-ml. with ammonia-free water and mixed by inversion. All of the 10-ml. tubes, including standard and water blank duplicates, were placed in a 37 C water bath for at least 30 minutes so that a blue color complex would develop. The optical density of each solution was determined with a spectrophotometer<sup>6</sup> operated at 640 nm. wavelength. The calculation for ammonium nitrogen concentration was determined

<sup>6</sup>Beckman Model DB-G, Beckman Instruments, Inc., Fullerton, CA 92634
from the optical densities recorded and the following formula:

\[
\frac{[20 \text{ (correct O.D. of unknown)]/corrected O.D. of standard}}{\text{mg. of } \text{NH}_4\text{-N/ml.}}. \text{ The } 20 \text{ in the formula represents the ammonium nitrogen concentration of the standard. Corrected O.D.'s are determined by subtracting the O.D. of the water blank from the O.D. of the standard and of the unknowns.}
\]

The analytical procedure for ammonium nitrogen in tissues was basically the same as for whole blood. Two grams of tissue in its frozen state were placed in the homogenization unit of the variable speed apparatus used by Jaquette (1972) and ground for approximately 30 seconds. About 150 mg. of the homogenate was placed in tared 5-ml. centrifuge tubes with 1 ml. of resin suspension and reweighed. The total weight of the tissue was recorded and enough ammonia-free water added to bring the volume to 1.5 ml. Water blanks and working standards of 20 and 40 \(\mu g./ml.\) were used with unknowns as in the whole blood analysis. Blood and tissue sample analyses were identical in procedure where washing and aspiration begin. When optical densities were recorded and corrected with the water blank, the following formula was used to determine concentrations:

\[
\frac{[20 \text{ or } 40 \text{ (corrected O.D. of unknown)]}100/\text{corrected O.D. of standard/mg. of tissue analyzed}}{\text{mg. of NH}_4\text{-N/100 mg. of tissue}}. \text{ The } 20 \text{ and } 40 \text{ represent the concentrations of ammonium nitrogen standards in } \mu g./ml. \text{ The } 20 \text{ was used with control ewe and fetal tissues and principal fetal tissues; the } 40 \text{ was used to measure principal ewe tissues. The } 100 \text{ represents the basis of } 100 \text{ mg. of tissue.}
RESULTS

Two ewes in each group were not pregnant, therefore, ammonia values were reported for five principal ewes and three control ewes with their fetuses. Blood ammonium nitrogen concentrations for ewes and fetuses in principal and control groups were recorded in Table 1. There was a gradual BAN increase until death of the ewes in the principal group (Table 1-A). All principal group fetuses were alive after death of their dams and their BAN concentrations were lower than those of their dams at death (Table 1-A, 1-B). Principal group ewe blood had higher BAN concentrations than that of the control group, which changed very little during the experiment. Control group fetuses had slightly higher BAN concentrations than their dams.

Tissue ammonium nitrogen concentrations for liver, kidney, spleen, and muscle of ewes and fetuses in principal and control groups were recorded in Table 2. Ewes had higher TAN concentrations than their fetuses (Table 2-B). Liver had the highest TAN concentration of ewe tissues and muscle tissues in ewes and fetuses represented the lowest TAN concentrations of the principal groups. Control group TAN concentrations were higher in the ewes than in their fetuses (Table 2-B).

Highly significant differences (P<0.01) determined by analysis of variance occurred between BAN concentrations of principal and control groups at 150 minutes after drenching and for blood drawn from ewes and fetuses at death of the ewe (Table 1-A). The difference in BAN concentrations between principal group ewes and their fetuses was
highly significant but there was no such difference in a similar comparison of control ewes and their fetuses (Table 1-B).

Differences between TAN concentrations of principal ewes and fetuses were highly significant for liver, kidney, spleen, and muscle tissues (Table 2-B). The same comparisons in control group tissues were significant at the $P<0.05$ level for spleen, highly significant ($P<0.01$) for kidney and muscle, but no significant difference was present for liver (Table 2-B).

TAN concentrations in principal fetuses were higher ($P<0.01$) than in control fetuses except for liver tissue which was higher at the $P<0.05$ level (Table 2-A). Principal ewe TAN concentrations were higher ($P<0.01$) than in control ewes except for muscle tissue which was higher at the $P<0.05$ level.
Table 1.—Mean ammonium nitrogen concentrations in whole blood (μg. NH₄-N/ml.) of ewes and fetuses. Five principal ewes were given (drench) 12.5 ml. of 3.3 M urea solution per kilogram of body weight and three control ewes were given water at the same rate.

### A

<table>
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<th>EWES</th>
<th>Control group</th>
<th>Principal group</th>
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<td>pretreatment</td>
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<td>2.3±0.7</td>
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<td>NS</td>
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<tr>
<td>30 minutes</td>
<td>2.1±0.4</td>
<td>3.8±1.7</td>
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<td>90 minutes</td>
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<td>150 minutes</td>
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<td>at death</td>
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<td>15.4±2.8</td>
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<td>Fetuses</td>
<td>2.1±0.7</td>
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### B

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<td>15.4±2.8</td>
<td>10.0±1.8</td>
<td>5.4</td>
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Data are expressed as mean ± standard deviation.

*Analysis of variance for ammonia values.
Table 2.—Mean tissue ammonium nitrogen concentrations (μg. NH₄-N/100 mg.) in ewes and fetuses. Five principal ewes were given (drench) 12.5 ml. of 3.3 N urea solution per kilogram of body weight and three control ewes were given water at the same rate.

<table>
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<th>Principal group</th>
<th>Principal minus control</th>
<th><em>P</em></th>
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Table 2.--(Continued)

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</tr>
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<td>Muscle</td>
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<td>Principal group</td>
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<tr>
<td>Liver</td>
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<td>Kidney</td>
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<td>0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.

*Analysis of variance for ammonia values.
DISCUSSION

In this investigation BAN concentrations of principal group ewes increased with time, which supported evidence gathered by Jaquette (1972) and Kirkpatrick, Roller, and Swanson (1973). The fetuses were all alive after their dams died. This fact implied a degree of maternal protection of the fetuses against ammonia intoxication. Tissue ammonium nitrogen concentrations of the principal group fetuses were 85% higher than in fetuses of the control group, so the protection was not complete. In an identical comparison of ewe tissue, the principal group had 125% higher TAN concentrations than the control group. Ammonia toxicity produced more ammonium nitrogen uptake in the ewe tissues than in the fetal tissues. Control ewes had 78% and principal ewes 118% higher TAN concentrations than their fetuses.

Urea cycle enzymes in the sheep fetus make the production of urea a major metabolic event in the 130-146 day-old fetus (Gresham et al. 1972). Protection from ammonia intoxication may depend on both fetal urea production capabilities and ewe tissue ammonium nitrogen uptake. Fetuses in this study were not as old as those in the report by Gresham. The fetal urea production may not be as great in less mature fetuses. This may be a reason for the control fetal BAN concentration being higher than the control ewe BAN concentration at death. Urea cycle enzyme activity in fetal liver may be a factor in the nonsignificant difference between control ewe and fetal liver tissue.

Ammonium nitrogen concentrations of ewe liver and kidney and their differences between principal and control groups were similar to
results described by Jaquette (1972). A search of the literature failed to produce information relative to fetal ammonium nitrogen concentrations. Further evidence is necessary to understand the limits and degree of maternal protection against ammonia toxicity.
SUMMARY

Eight pregnant Southdown ewes were drenched with 12.5 ml. of 3.3 M urea solution per kilogram of body weight and the ammonium nitrogen concentration of the blood and tissues of the ewes and their fetuses were measured and compared with control ewes (drenched with water) and their fetuses.

Blood ammonium nitrogen (BAN) and tissue ammonium nitrogen (TAN) concentrations for liver, kidney, spleen, and muscle of ewes and fetuses were determined by an ion exchange procedure. Samples of blood were collected before treatment, 30, 90, and 150 minutes after treatment and at death of the dam.

The principal group ewes had increasing BAN concentrations with time after drenching and their fetuses had higher BAN concentrations than control group fetuses (P<0.01).

All fetuses were alive after death of their dams and had lower TAN values than their dams. The ammonia differences between ewes and fetuses were larger in the principal group than in the control group. Except for ewe muscle and fetal liver, all principal group tissues had highly significantly greater (P<0.01) TAN concentrations than control group tissues. The principal group ewe muscle and fetal liver tissues had significantly (P<0.05) greater TAN concentrations than control group tissues.
LITERATURE CITED


Table 3.--Whole blood ammonium nitrogen. Principal and control sheep were drenched with 12.5 ml. of 3.3 \text{M} \text{urea} \text{solution} \text{and} 12.5 \text{ml.} \text{of water per kilogram of body weight, respectively.}

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<thead>
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<td>6.5</td>
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<tr>
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<td>---</td>
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<td>2.8**</td>
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<td>10.4</td>
<td>7.1</td>
<td>10.7*</td>
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*average of twins.
**average of triplets.
Table 4.--Tissue ammonium nitrogen. Principal and control animals were drenched with 12.5 ml. of 3.3 M urea solution and 12.5 ml. of water per kilogram of body weight, respectively.

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