1971

The Effects of Acute Ammonia Toxicity on Certain Homogram Parameters in Sheep

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THE EFFECTS OF ACUTE AMMONIA TOXICITY ON CERTAIN HEMOGRAM PARAMETERS IN SHEEP

BY

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

1971

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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, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser Date

Head, Entomology-Zoology Department Date

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to this country and its society for the opportunities it presents for education. 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 several other persons who made valuable contributions to this study:

To the South Dakota Animal Disease Research and Diagnostic Laboratory, for the use of their laboratory facilities.

To Dr. Gary A. Thibodeau, Assistant Professor in Entomology-Zoology, for his expert counsel, advice, and encouragement.

To Dr. W. Lee Tucker, Station Statistician, for technical assistance and advice with statistical analysis of data.

Most of all I want to thank my wife, Pat, for her confidence, patience, and encouragement throughout the preparation of this thesis, and to my daughter, Jennifer, who made it worthwhile.

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INTRODUCTION

Due to the rapidly expanding human population, one of the greatest problems facing agricultural scientists is the development of adequate food resources to meet the growing demand for quality protein. The development of more economical and higher quality feed for ruminant animals is essential since they are one of man's primary sources of animal protein.

Microorganisms in the ruminant stomach convert relatively cheap non-protein nitrogen compounds into bacterial proteins, which are digested, absorbed, and utilized by the host animal. Urea, a common non-protein nitrogen compound, is hydrolyzed into ammonia and carbon dioxide by bacterial urease. When urea is hydrolyzed faster than it is utilized by bacteria, quantities of ammonia are absorbed through the rumen wall into the bloodstream. This normally creates no problem, since the ammonia is converted into urea by the liver, however, toxicity may occur if the blood ammonium-nitrogen (BAN) level surpasses the animal's detoxification capacity.

High levels of BAN are not only toxic, but result in inefficient utilization of nitrogen for protein synthesis. There have been extensive investigations involving the physiological, nutritional, and metabolic effects of an elevated BAN level. However, inadequate information is available concerning body fluids, differential tissue ammonia content, and blood hemogram quantities resulting from toxic BAN levels.

The purpose of this investigation was to detect changes in the following hemogram parameters resulting from acute ammonia intoxication: packed cell volume (PCV), hemoglobin concentration (Hb), total red blood cell count (RBC), and total white blood cell count (WBC).

LITERATURE REVIEW

Ammonia Toxicity in the Ruminant

Becker (1924) performed one of the earliest experiments involving ammonia toxicity. Urea was administered to rabbits at 1-2 gm/kg. body weight; severe toxic symptoms and death resulted. Mortality was attributed to a primary specific toxicity.

Toxic symptoms in cattle were reported by Davis and Roberts (1959). Symptoms appeared twenty to thirty minutes after administering 14 gm. urea solution by drench/100 lbs. body weight. Signs of intoxication in sequence were as follows: restless activity, hyperventilation, muscle tremors followed by ataxia, labored breathing, vomiting, bloat, tetany, and death two hours post-dosage. Whitehair et al. (1955) noticed that bloating was a consistent symptom of ammonia intoxication in cattle and post mortem examination revealed a strong ammonia odor in the rumen.

Whitehair et al. (1955) summarized urea feeding practices that increased susceptibility to intoxication. Starved or fasted cattle consumed larger amounts of urea at a faster rate. Animals that had never been on urea diets had a lower tolerance. Improper feed mixing and addition of larger than recommended amounts of urea led to intoxication. Davis and Roberts (1959) concluded that if urea was withheld from the feed for two or more days, the animals were more susceptible

to intoxication when reestablishing a high urea diet.

Pearson and Smith (1943) discovered that urea was rapidly hydrolyzed in the rumen by bacterial urease into ammonia and carbon dioxide. Rose and Dekker (1956) concluded that the ammonia not utilized in bacterial protein synthesis was absorbed into the portal blood.

Lewis et al. (1957) demonstrated a close correlation between the concentration of ruminal ammonia and portal blood ammonia, indicating a rapid transfer across the rumen epithelium. Since no regulatory mechanism was found, they concluded that ammonia diffused through the rumen epithelium due to concentration gradients existing between the rumen and portal blood.

Chalupa (1968) stated that ammonia transfer across the rumen wall depended on both concentration gradients and pH. The pKa for ammonia at 37° C was calculated to be 9.02, according to Jacquez et al. (1959a), therefore, at a normal rumen pH below 7.0, ionized ammonia was present at a higher concentration than unionized ammonia.

Jacobs (1940) stated that the uncharged ammonia molecule could penetrate the lipid layers of the rumen more readily than the ammonium ion. A low pH opposed ammonia diffusion out of the rumen into the blood, an area of higher pH. This concept coincided with the report of Clark et al. (1951) who observed that administration of alkali decreased urea tolerance in sheep.

Hogan (1961) observed that volatile fatty acids enhanced ammonia absorption at pH 6.5. Upon decreasing pH to 4.5, ammonia absorption was absent. Ammonia transport was not affected by sodium, potassium, chloride, or water. He concluded that more ammonia appeared in the portal vein when the ruminal pH was elevated.

McDonald (1948) measured significant amounts of ammonia in the veins draining the rumen, whereas the concentration of the arterial blood serving this organ was negligible. This observation agreed with that of Folin and Denis (1912), who stated that the concentration of ammonia in portal blood was several times higher than in the peripheral arterial or venous blood. They concluded that one passage of blood through the normal liver removes virtually all of the ammonia delivered from the intestines.

When blood ammonium-nitrogen (BAN) in portal blood exceeds the liver's capacity for detoxification, ammonia appears in peripheral blood. Davis and Roberts (1959) reported that a single feeding or drench of 14-20 gm. urea/100 lbs. live weight would produce severe toxicity in cattle. None of the treated animals survived when BAN levels exceeded 4 mg.%. This value confirmed the work by Dinning et al. (1948), who administered an urea drench to steers in amounts exceeding 100 gm./animal.

Repp et al. (1955) stated that toxicity was associated with a critical peripheral BAN value of 1 mg.%. This was

confirmed by Lewis et al. (1957) who also stated that the peripheral BAN concentration did not start to rise until the rumen ammonia concentration exceeded 40-60 m-moles/liter. Later work by Lewis (1960) indicated that BAN values started to increase when ruminal ammonia concentration reached 30 m-moles/liter.

The lethal values previously described apply only to animals which had not become adapted to urea feeding. Davis and Roberts (1959) and Lewis (1960) reported that animals could safely consume higher than lethal amounts of urea upon becoming gradually adapted to increasing amounts. Lewis further stated that adaptation was likely due to changes in rumen microbial activity.

Visek (1968) stated that urea was less toxic with starch in the diet. The assumption was that starch provided available energy which facilitated rapid microbial protein synthesis. Starch diets also lowered urease activity in the rumen. Furthermore, high protein diets caused an increase in urea cycle enzyme activity in the liver.

Various attempts have been made to decrease or relieve ammonia toxicity in the ruminant. Repp et al. (1955) and Davis and Roberts (1959) reported that acetic acid, given in adequate amounts to neutralize the urea dosage, was a good therapeutic agent if given before the animals showed symptoms of tetany.

Body Distribution of Ammonia

Stabenau et al. (1959) stated that the production rate and pH gradients are the chief factors in appearance of ammonia outside the cell. Robin et al. (1960) and Visek (1968) state that ammonia exerts a partial pressure (pNH_3) and diffuses from areas of greater to lower pNH_3. Differences in pH across cell membrances influence this diffusion. With a calculated intracellular pH of 7.0 for skeletal muscle, a pressure gradient would exist favoring entry of ammonia into muscle cells bathed by extracellular fluid with a pH of 7.4. The concentration of ammonia at equilibrium in the intracellular fluid would be 2.5 times that of the extracellular fluid. This was described as diffusion trapping, since the diffusible free ammonia entered the more acidic intracellular fluid and was trapped as the nondiffusible ammonium ion.

Robin et al. (1960) proposed that procedures which change the hydrogen ion concentration of both compartments equally will produce no net shift of total ammonia from one compartment to the other. If an extracellular acidosis resulted, there would be a net shift of total ammonia from the intracellular to the extracellular fluid compartment. This hypothesis was verified by Warren and Schenker (1962). They compared fixed acid infusion and carbon dioxide inhalation for protective effects against ammonia toxicity. The fixed acid did not diffuse through the cell membrane whereas the carbon dioxide diffused readily. Acid infusion produced

a marked decrease in passage of the ammonia test dose into the brain, thus decreasing toxicity, whereas the carbon dioxide had little effect on intoxication.

The blood-brain barrier, according to Warren and Nathan (1958), was similar to the cell membrane in that it was more permeable to non-ionic ammonia than to the ammonium ion. The diffusion rate of ammonium salts was related to their different effects on blood pH. Ammonium salts that increased blood pH crossed the barrier easily, resulting in higher ammonia concentration in the cerebrospinal fluid (CSF) and greater toxicity. This was confirmed by Stabenau et al. (1959) by fixed base infusion that increased CSF, brain, and muscle ammonia concentration two to three times. They concluded that there was a direct and predictable correlation between blood pH adjustment and tissue ammonia concentration.

Caesar (1962) noted that in severe hepatic disease in man, there was a dearrangement of ammonia metabolism similar to ammonia intoxication. Control subjects had CSF ammonia concentrations less than one-fifth of arterial plasma, whereas subjects with severe hepatic disease had elevated CSF ammonia levels which occasionally exceeded plasma concentrations.

Keynes (1963) reported that less than 5% of the total body ammonia was present in the blood during liver failure, which supported earlier work by Rosado et al. (1962). Two minutes after injecting an ammonia load into the portal vein,

only 10-15% of the injected amount remained in the extracellular space. This residual amount decreased to normal in 5-20 minutes, depending on the administered dose. Most (75%) of the ammonia was removed by muscle, while liver and brain removed 2.5% and 0.5%, respectively. As BAN decreased, tissue concentration did not increase as expected, instead, liver and brain ammonia levels returned to normal within 10 minutes, whereas muscle retained 61% followed by 38% at 20 minutes. Tissue ammonia concentrations remained higher than blood ammonia concentrations.

Investigations by Keynes (1963) showed that normally ammonia concentration in canine muscle was several times higher than in blood or thoracic-duct lymph. On a tissue weight basis, normal brain, pancreas, and liver ammonia concentrations were higher than that in muscle. Investigations by Roller et al. (1969) disclosed a greater uptake of ammonium ions by uterus-and-embryo than by muscle in urea-treated rabbits in early stages of pregnancy.

Metabolism of Ammonia

Several tissues of the body were noted for their varied ammonia concentrations. Urea hydrolysis in the rumen, as described by Pearson and Smith (1943), and in the gastrointestinal tract of monogastric animals, according to Leifer et al. (1948) was a major source of ammonia. Ammonia was produced by working skeletal muscle, (Schwartz et al. 1957), and by isolated working rabbit heart, as reported by Feinberg

and Alma (1961). In addition, significant amounts of ammonia were formed by the kidney (Nash and Benedict, 1921).

Von Schroeder (1882) associated ammonia metabolism with liver function. Artz et al. (1958) demonstrated this fact by drawing blood samples from portal and hepatic veins. The hepatic vein had 81% less ammonia than the portal vein.

The following sequence of events occurred following an injection of an ammonia load, according to Rosado et al. (1962): initially, ammonia was rapidly removed from the circulation by muscle uptake; secondly, glutamine synthesis took place mainly in the liver and brain; finally, urea synthesis utilized the ammonia gradually released by muscle and by enzymatic hydrolysis of glutamine.

Flores et al. (1962) stated that the liver contained three main systems for ammonia removal: synthesis of glutamine, reductive reamination of alpha-ketoglutarate, and synthesis of N-carbamyl phosphate, a precursor of urea and pyrimidines.

The importance of glutamine synthesis in removing ammonia has been established by several authors. Handford (1961) administered urease intravenously to dogs to cause intoxication. Plasma glutamine increased from 8.4 mg.% to 76.3 mg.% in 60 minutes.

Duda and Handler (1958) administered N^{15} labeled ammonia to rats. Labeled nitrogen was incorporated into the amide position of glutamine faster than any other nitrogenous liver

compound. At low BAN levels, glutamine synthesis was more efficient than urea synthesis for ammonia detoxification. Furthermore, glutamine turnover sufficed to provide the nitrogen necessary for normal urea synthesis by the Krebs-Henseleit cycle.

Glutamine synthesis took place in brain and the rumen. Takagaki et al. (1961) infused N^{15} labeled ammonium acetate into the carotid artery of cats which caused a 50% increase in brain glutamine concentration. Hoshino et al. (1966) administered a sublethal urea dose to sheep; rumen glutamine concentration increased, indicating that glutamic acid, synthesized from alpha-ketoglutarate, was converted to glutamine.

Krebs and Henseleit (1932) demonstrated that only liver tissue had the ability to synthesize urea from ammonium salts. This established that urea synthesis was important in ammonia detoxification.

Greenstein et al. (1955) ascertained that prior injection of L-arginine resulted in complete protection from an $LD_{99.9}$ dose of ammonium acetate and accelerated urea formation in rats. Salvatore et al. (1964) noted that when L-argine, L-aspartate, and L-ornithine were injected into rats, complete protection from ammonium acetate infusion resulted. However, when the compounds were injected with alpha-methylaspartate, a strong specific inhibitor of argininosuccinate synthetase, toxicity resulted. This demonstrated that the arginine,

aspartate, ornithine mixture did indeed enhance the urea cycle and protect against ammonia intoxication.

Excretion of Ammonia

The production of ammonia for the purpose of neutralizing metabolic acid was first clearly ascribed to the kidney by Nash and Benedict (1921), who also found a higher concentration of ammonia in the renal vein than the renal artery. Robinson (1954) revealed that kidney slices from a number of mammalian species produced ammonia when allowed to respire in a suitable media.

Van Slyke et al. (1943) reported that the deamination of plasma glutamine into glutamic acid and ammonium ion accounted for as much as 60% of the urinary ammonia in the acidotic dog. Later investigation by Pilkington et al. (1964) indicated 30-40% of urinary ammonia originated from the amide nitrogen of plasma glutamine. If the concentration of plasma glutamine doubled, it accounted for 50% of the urinary nitrogen.

Another major source of urinary ammonia was from deamination of the amide nitrogen of glutamine within the renal tubular cell. Rector et al. (1955) discovered that renal glutamine was converted to glutamic acid and nonionic ammonia by glutaminase. Under normal conditions, the ammonia diffused both directions; into the peritubular capillaries and into the tubular fluid in exchange for sodium. The ammonia was trapped as the nondiffusive ammonium ion in the acidic tubular fluid.

It then combined with an anion and was excreted in the urine.

Denis et al. (1964) stated that if the renal arterial pNH_3 was the same as the renal venous pNH_3, then the pNH_3 of the tubular cell was also the same, resulting in no net flux of ammonia into the peritubular fluid from the tubular cells. An increase in the renal arterial pNH_3 would increase the rate of excretion of ammonia into the urine. This was accomplished by Pilkington et al. (1964), by infusion of N^{15} labeled glutamine, and by Balagura and Pitts (1962), by injection of ammonium chloride and ammonium acetate into the renal artery. Both investigations demonstrated an increase in urinary ammonia content.

Ammonia was the basis of a renal mechanism for secretion of excess hydrogen ions (Guyton, 1966). Hydrogen ions were produced by carbonic acid dissociating into bicarbonate ion and hydrogen ions. Ammonia was produced in the renal tubular epithelial cells and diffused into the tubular fluid to combine with hydrogen ions. The secreted ammonia was exchanged for sodium which was retained in combination with the bicarbonate ion in the extracellular fluid. This important mechanism allowed the excretion of a salt, ammonium chloride, instead of hydrochloric acid.

In vitro studies by Holmes and Patez (1930) demonstrated an increased renal ammonia production when substrate pH was lowered from 7.4 to 5.2. The effect of altered blood pH on ammonia excretion was observed by Sullivan and McVaugh (1963)

in which acid or base was infused into the circulatory system while serial urine collections were obtained. Blood pH changed before the filtered portion of the infusion appeared in the urine. Infused base raised urine ammonia concentration when urine pH remained constant. Later, as base appeared in the urine, pH increased and ammonia concentration declined. Acid infusion had the opposite effect.

There was one other significant route for excretion of blood ammonia. Robin et al. (1959) and Jacquez et al. (1959b) detected measurable amounts of ammonia in expired air from dogs infused with ammonium acetate or having portocaval shunts. Since ammonia was 1500 times more diffusible than carbon dioxide, the pNH_3 of the pulmonary capillaries and the alveoli must equilibrate rapidly. No measureable amount of ammonia was found in expired air during control periods.

Mechanism of Ammonia Toxicity

Controversy existed concerning the active agent in urea toxicity. Clark et al. (1951) failed to produce toxic symptoms by intravenous injection of urea or ammonia solution. This led some investigators to believe that the carbamate ion was the toxic agent, rather than ammonia. Gorin (1959) proved that carbamate ion was produced both in vivo and in vitro by ruminal microorganisms.

Hale and King (1955) proposed that the toxic action of urea was not due to the absorption of ammonia, but due to the

absorption of ammonium carbamate formed by combination of ammonia and carbon dioxide in the rumen.

Wilson et al. (1968) determined LD_{50} and LD_{99} values for ammonium carbamate, ammonium carbonate, and ammonium bicarbonate in young mice. One mole of ammonium carbonate and one mole of ammonium carbamate both yielded two moles of ammonia at pH 7.0. One mole of ammonium bicarbonate yielded one mole of ammonia. If the ammonia was the toxic agent, the toxicity of ammonium carbonate and ammonium carbamate would be equal and twice as toxic as ammonium bicarbonate. They confirmed these predictions.

Changes in acid-base balance have been observed by a number of investigators. Roberts et al. (1956) infused ammonium acetate into dogs and measured blood pH and carbon dioxide concentration. Respiratory alkalosis resulted due to a decreased concentration of carbon dioxide and an increased blood pH. A tolerance to the ammonia was noted in animals receiving 10% carbon dioxide. This suggested that respiratory alkalosis could enhance toxicity.

Toxicity caused by ammonium chloride, ammonium acetate, and urea followed different metabolic pathways, according to Lewis (1960). In the case of ammonium chloride, there was a distinct metabolic acidosis; a low blood pH preceded respiratory hyperventilation and a consequent low blood bicarbonate level. The administration of ammonium acetate was different. Although there was a decline in blood bicarbonate level,

there was relatively little change in blood pH. The change in blood pH following administration of urea was not as pronounced as that of ammonium chloride. There seemed to be an initial metabolic alkalosis, followed by an apparent recovery to normal pH level, at the time when the most obvious signs of toxicity were observed.

Similar observations were noted by Roller (1966) upon administration of urea to cattle. He observed an initial metabolic alkalosis, followed by a distinct metabolic acidosis, and an increase in packed cell volume at the time of death. It was concluded that death resulted from metabolic acidosis due to high levels of ammonium ions interferring with metabolic reactions.

Schwartz et al. (1953) studied patients in various stages of hepatic coma. There was no abnormality in blood pH, plasma sodium, chloride, and potassium concentration. An elevated BAN level was the only parameter that could be correlated with the degree of coma. In a few patients however, an elevated plasma glutamine concentration was noted.

Roller (1966) measured changes in certain plasma and red blood cell electrolytes after urea administration to cattle. Plasma sodium, calcium, and magnesium levels were unchanged while plasma potassium values were increased. Red blood cell sodium level was lowered and red cell potassium values were increased.

Ammonia toxicity caused various alterations in metabolism.

A hypothesis for the chemical pathology associated with hepatic coma was presented by Bessman and Bessman (1955). Ammonia intoxication involved depletion of alpha-ketoglutarate from the tricarboxylic acid cycle; but they were unable to confirm this.

Evidence for the above hypothesis was presented by Clark and Eiseman (1958) who studied biochemical changes in brain tissue of dogs during ammonia-induced coma. There was a significant decrease in brain alpha-ketoglutarate and an increase in pyruvate and glutamine. Blockage of the tricarboxylic acid cycle due to depletion of alpha-ketoglutarate could decrease energy derived from the respiratory chain in the brain.

Schenker and Mendelson (1964) failed to demonstrate a decline in cerebral cortex adenosine triphosphate (ATP) concentration in rats with ammonia-induced coma. They proposed however, that there may be a decrease in certain localized areas of the brain or central nervous system. There should be a decrease in ATP synthesis, due to the inhibitory action of ammonia on cerebral oxidative mechanisms, and an increase in ATP utilization in the formation of glutamine from glutamic acid. Schenker et al. (1967) later proved this to be correct by determining cortex and basilar ATP concentrations in ammonia toxic rats. Even though the ammonia uptake was the same for the two areas, basilar ATP concentration decreased, whereas cortical ATP remained unchanged. They also associated

the neurological symptoms, associated with hepatic encephalopathy, with malfunctions of structures in the base of the brain and their cortical connections.

Experimentation by Clifford et al. (1969b) indicated that ammonia intoxication in rats caused a depletion of reduced pyridine nucleotides. There was a 16% increase in liver nicotinamide adenine dinucleotide (NAD), and a 23% decrease in reduced nicotinamide adenine dinucleotide (NADH). Only 60% of the decrease in NADH could be accounted for by the increase in NAD. They ascribed the remaining 40% to an increase in oxidative cleavage of NADH to nicotinamide.

Investigators have associated additional observations with ammonia toxicity. Koenig and Koenig (1949) infused toxic doses of ammonium salts into rats, guinea pigs, and cats and produced acute pulmonary edema, congestion, and hemorrhage. Acidosis was ruled out as a primary factor because pulmonary edema was not produced when acidosis was induced by other means.

Warren and Schenker (1960) noted that hypoxia potentiated the toxic effect of an ammonium chloride infusion in albino mice. When exposed to 7% oxygen, the intensity of the toxicity was greater than when exposed to 21% oxygen.

Schenker and Warren (1962) showed that hyperthermia augmented ammonia toxicity and hypothermia decreased toxicity in mice. They ascribed differences in ammonia toxicity with changes in body temperature to be a result of changes in the rate of ammonia uptake by the brain, a variation in the rate

of cerebral ammonia detoxification, and a direct effect on the metabolic functions of the cerebral parenchyma.

MATERIALS AND METHODS

Experimental Animals

The present study was undertaken to gain basic information concerning certain hemogram parameters during ammonia toxicity. The experimental animals were seven young Southdown wether sheep, weighing between 21.4 and 39.1 kilograms. Examination of each animal demonstrated that they were alert, active, and apparently in good health. The sheep were sheared and housed indoors. A mixture of alfalfa and grass hay was provided *ad libitum* with water. Feed and water were withheld 24 hours prior to experimentation.

Experimental Procedures

The sheep were randomly selected for experimentation and weighed. An urea solution was prepared for each experimental animal by dissolving 2 gm. urea/kg. body weight in 300 ml. of tap water. This concentration was determined, during previous experimentation, to be sufficient to cause acute ammonia toxicity. A total of 300 ml. was selected for convenience in dosing and complete dissolution of the urea.

Blood for analysis was drawn via jugular venipuncture. Following a pre-treatment blood withdrawal, the urea solution was administered with a dose syringe, and the dosage time recorded as zero experimental time. Subsequently, blood was drawn at ten-minute intervals for three aliquots, then at 30-minute intervals for the next three aliquots. Blood was also

drawn immediately before death.

The blood was collected in test tubes previously prepared with 10% sodium EDTA (di-sodium salt of diethylene diamine tetraacetic acid) to prevent coagulation after gentle, but thorough mixing. An adequate amount of blood was drawn to allow duplicate analysis for BAN, PCV, Hb, RBC count, and WBC count.

Analytical Procedures

Packed Cell Volume and Blood Ammonia

The microhematocrit method was used to determine packed cell volume. The capillary tubes were centrifuged for five minutes in an Adams Autocrit Centrifuge.[1]

"Blood ammonia" represents total ammonia (NH_3 + NH_4^+) present in whole blood as blood ammonium-nitrogen (BAN).

The cation-exchange method of Hutchinson and Labby (1962) was modified by reducing blood volume to 0.5 ml. for each determination. Consequently, volumes of resin suspension, Nessler's reagent, ammonium working standard, and water were reduced by one-fourth.

Ammonium ions were exchanged for sodium and potassium ions on cation exchange resin, Dowex 50W-X8, 50-100 mesh, hydrogen ion form.[2] Resin was rendered ammonia free and converted to

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[1] Clay Adams, Inc., New York, New York 10010.

[2] J. T. Baker Chemical Company, Phillipsburg, New Jersey 08865.

the sodium-potassium form before use. All glassware used in the procedure was also rendered ammonia free before use by washing with 0.1N NaOH.

Standard ammonium sulfate stock solution (1 mg. NH_4^+-N/ml.) was made by weighing 4.7166 gm. of ammonium sulfate analytic reagent and adding it to a liter volumetric flask containing approximately 500 ml. of double distilled deionized water; 5.6 ml. of concentrated sulfuric acid was introduced and the flask filled to the liter mark with double distilled deionized water. The stock solution was diluted 0.5 to 100 and 1 to 100 daily to yield working standards of five and 10 µg. NH_4^+-N/ml. One-half milliliter of working standard was added to each of two 5-ml. glass stoppered graduated conical centrifuge tubes, previously prepared with 1 ml. of resin suspension. Standards were subjected to the same procedure as the whole blood aliquots. These concentrations were chosen to represent the expected median of the BAN values expected in the intoxicated animals.

Blood for BAN determination was drawn from the collection tube into a sterile 1-ml. tuberculin syringe. The syringe was filled to the 1 ml. mark and 0.5 ml. expressed into each of two resin tubes. These tubes were shaken for three minutes to ensure complete ammonium adsorption on the resin and lysis of the blood cells. The laked blood was then decanted and the resin washed four times with double distilled deionized water.

Elution of the ammonia from the resin and color development was accomplished in one step with Nessler's reagent, formula of Bock and Benedict, as described by Hawk et al. (1954). One and one-half milliliters of a filtered 1:5 dilution of stock Nessler's reagent were added to each resin tube. The ammonia-Nessler's complex was transferred to a 5 mm. cuvette, and optical density (OD) readings taken against a water blank at 450 millimicrons in a Beckman Model DB-G Grating Spectrophotometer.[3]

Contamination errors were essentially eliminated by subtracting OD of a method blank from OD of blood and standards before calculating BAN concentrations. Duplicate method blanks were prepared by adding 0.5 ml. of double distilled deionized water to each of two resin tubes in place of standard or blood. Subsequent handling of the method blanks was identical to that of the blood and standard.

BAN values in μg. NH_4^+-N/ml. were calculated according to the following simplified formula where 0.5 ml. and 1.0 ml. of working standard equals 5 and 10 μg. NH_4^+-N, respectively.

$$\mu g.\ NH_4^+\text{-N/ml. of blood} = \frac{(5 \text{ or } 10) \times \text{OD unknown}}{\text{OD of standard}}$$

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[3]Beckman Instruments, Inc., Fullerton, California 92634

Blood Cell Count and Hemoglobin Concentration

The total red blood cell counts were made by utilizing a Coulter Counter Model F[4] which was standardized for sheep red blood cells with the aperture set at eight, attenuation at one, and threshold at six. The 1 to 50,000 dilution was accomplished by a two-step method. In the first step, 40 lambda of blood was added to 20 ml. of Isoton[5] by means of a Coulter Counter Dual-Diluter.[6] In the second step, 200 lambda of the above solution was added to 20 ml. of Isoton by using a 200 lambda Oxford Sampler.[7] The red blood cell counts were repeated. If an aliquot had variable values, a new solution was made. The background count of the Isoton was determined and subtracted from the total Coulter Counter count prior to final enumeration of the red blood cells. To correct counting errors caused by more than one cell passing through the aperture at one time, a Coulter Counter Coincidence Correction Chart[8] was employed to give the final red blood cell count.

The total white blood cell counts were made by utilizing the Coulter Counter standardized for sheep white blood cells

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[4]Coulter Electronics, Inc., Hialeah, Florida 33010.

[5]Coulter Diagnostic, Inc., Miami Springs, Florida (0.9% NaCl plus 0.1% Sodium Azide).

[6]Coulter Electronics, Inc., Hialeah, Florida 33010.

[7]Oxford Laboratories, San Mateo, California 94401.

[8]Coulter Electronics, Inc., Hialeah, Florida 33010.

with the aperture set at 16, attenuation at one, and threshold at eight. The 1 to 500 dilution was accomplished by adding 40 lambda of blood, to 20 ml. of Isoton by means of a Coulter Counter Dual-Diluter. Five drops of Zap-Isoton,[9] a lysing agent, were added to the solution to hemolyze the red blood cells. The white blood cell counts were determined in duplicate by the same procedure used for red blood cell counts.

Hemoglobin concentration was obtained by utilizing the cyanmethemoglobin (Hycel) technique. Appropriate proportions of blood and Hycel[10] were pipetted using a calibrated Hycel Cuvette Chemistry System.[10] Photometric examination was made with a Coleman Junior II Spectrophotometer Model 6L20[11] at 540 millimicrons.

The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from the following formulas:

$$\text{MCV } (c\mu) = \frac{\text{packed cell volume x 10}}{\text{erythrocytes, million/cmm.}}$$

$$\text{MCH } (\mu\mu g.) = \frac{\text{hemoglobin concentration, Gm./100 ml. blood x 10}}{\text{erthrocytes, million/cmm.}}$$

$$\text{MCHC } (\%) = \frac{\text{hemoglobin concentration, Gm./100 ml. blood x 100}}{\text{packed cell volume}}$$

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[9] Coulter Diagnostics, Inc., Miami Springs, Florida.

[10] Hycel Inc., Houston, Texas 77036.

[11] Coleman Instruments, Maywood, Illinois 60153.

RESULTS

The results of this study indicate that many physiological changes in certain hemogram values may be induced by ammonia intoxication in sheep. Lines of regression with time revealed significant changes in BAN, red blood cell count (RBC), white blood cell count (WBC), packed cell volume (PCV), and hemoglobin (Hb). Lines of regression with time could not be plotted for mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) due to excessive random variability.

An electronic computer was employed in statistical analysis using raw data listed in the appendix, table 4 through 11. Cards were punched for: animal number, time from urea dosage, time from death, WBC count, Hb, PCV, RBC count, BAN, MCV, MCH, and MCHC.

Linear, quadratic, cubic, and polynomial equations for regression lines were computed for each parameter to obtain change in relation to time. The equations were tested to determine whether a significant difference existed between pre-treatment values and death (see appendix). Since time of death varied among the experimental animals, regression lines were determined for time intervals prior to death, instead of time intervals following treatment.

Parameters having significant lines of regression were plotted in figures 1 through 5. Since lines of regression

revealed no significant changes with time for MCV, MCH, and MCHC, they were not plotted.

Lines of regression demonstrated a steady and highly significant rise in BAN values as time progressed. Lines of regression demonstrate a steady increase in RBC count and Hb. Initially, PCV increased at a steady rate, followed by a slower rate of increase as time progressed. Lines of regression demonstrate a steady decline in WBC count as intoxication progressed.

Computed significant percent changes at death from tables 1 and 2 gave the following results: BAN, RBC count, PCV, and Hb increased 1504.0%, 7.9%, 11.2%, and 7.9% respectively. White blood cell count decreased 27.5%.

Table 1: Overall mean values for blood ammonium-nitrogen (BAN) * and white blood cell (WBC) count.**

	BAN ($\mu g.\ NH_4^+$-N/ml.)	White Blood Cell Count (mm^3)
Pre-treatment	2.10	7,406
Post-treatment		
10 minutes	2.47	7,335
20 minutes	3.18	7,285
30 minutes	4.65	6,796
60 minutes	8.92	6,605
90 minutes	21.78	6,048
Death	33.68	5,367

*$p < .01$
**$p < .05$

Table 2: Overall mean values for red blood cell count (RBC),* packed cell volume (PCV),* and hemoglobin concentration (Hb).*

	RBC Count (cmm.)	PCV (%)	Hb (Gm./100ml.)
Pre-treatment	10,843,600	38.61	13.87
Post-treatment			
10 minutes	10,873,300	39.74	13.98
20 minutes	10,250,700	38.12	12.89
30 minutes	10,140,700	38.68	13.32
60 minutes	11,032,900	41.89	14.11
90 minutes	11,673,100	43.24	14.46
Death	11,703,600	42.95	14.96

*$p < .05$

Table 3: Overall mean values for mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

	MCV (cu.μ.)	MCH (μ.μ.gm.)	MCHC (%)
Pre-treatment	36.58	12.86	35.11
Post-treatment			
10 minutes	36.98	12.87	34.92
20 minutes	37.56	12.67	33.80
30 minutes	38.25	13.18	34.62
60 minutes	37.90	12.80	33.83
90 minutes	37.32	12.42	33.37
Death	36.77	12.81	34.88

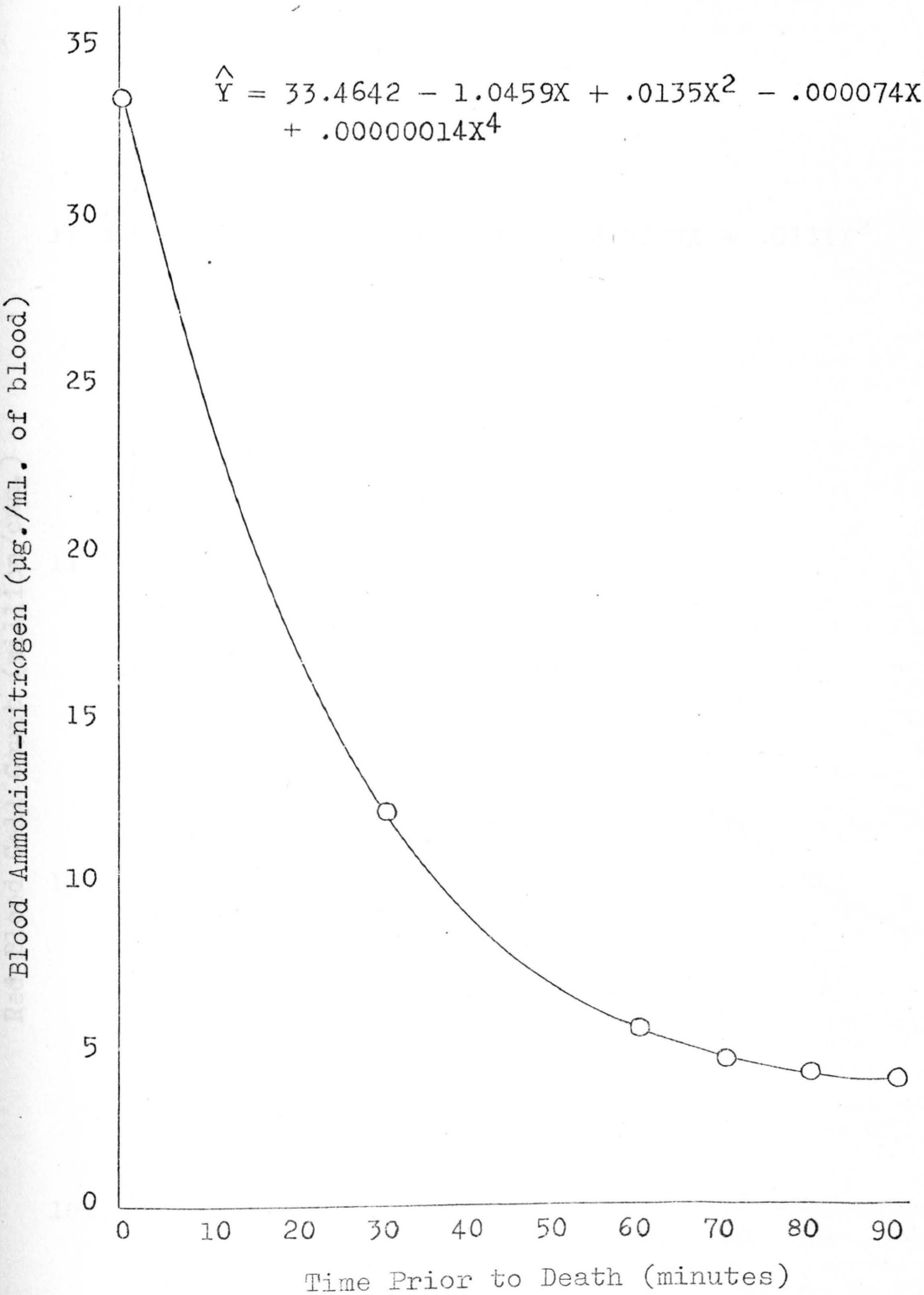

Figure 1: Regression of blood ammonium-nitrogen levels with time prior to death for urea-intoxicated animals.

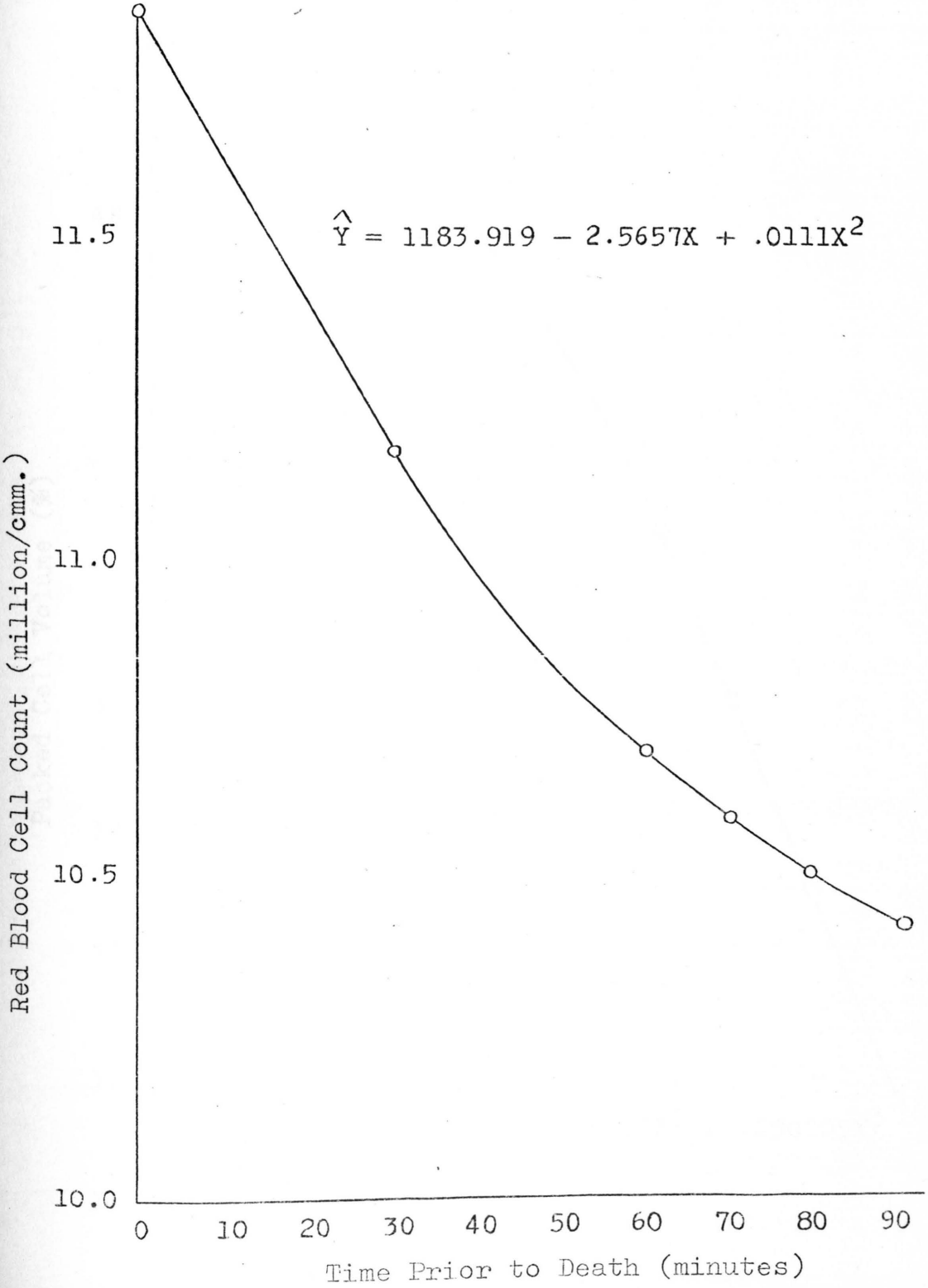

Figure 2: Regression of red blood cell counts with time prior to death for urea-intoxicated animals.

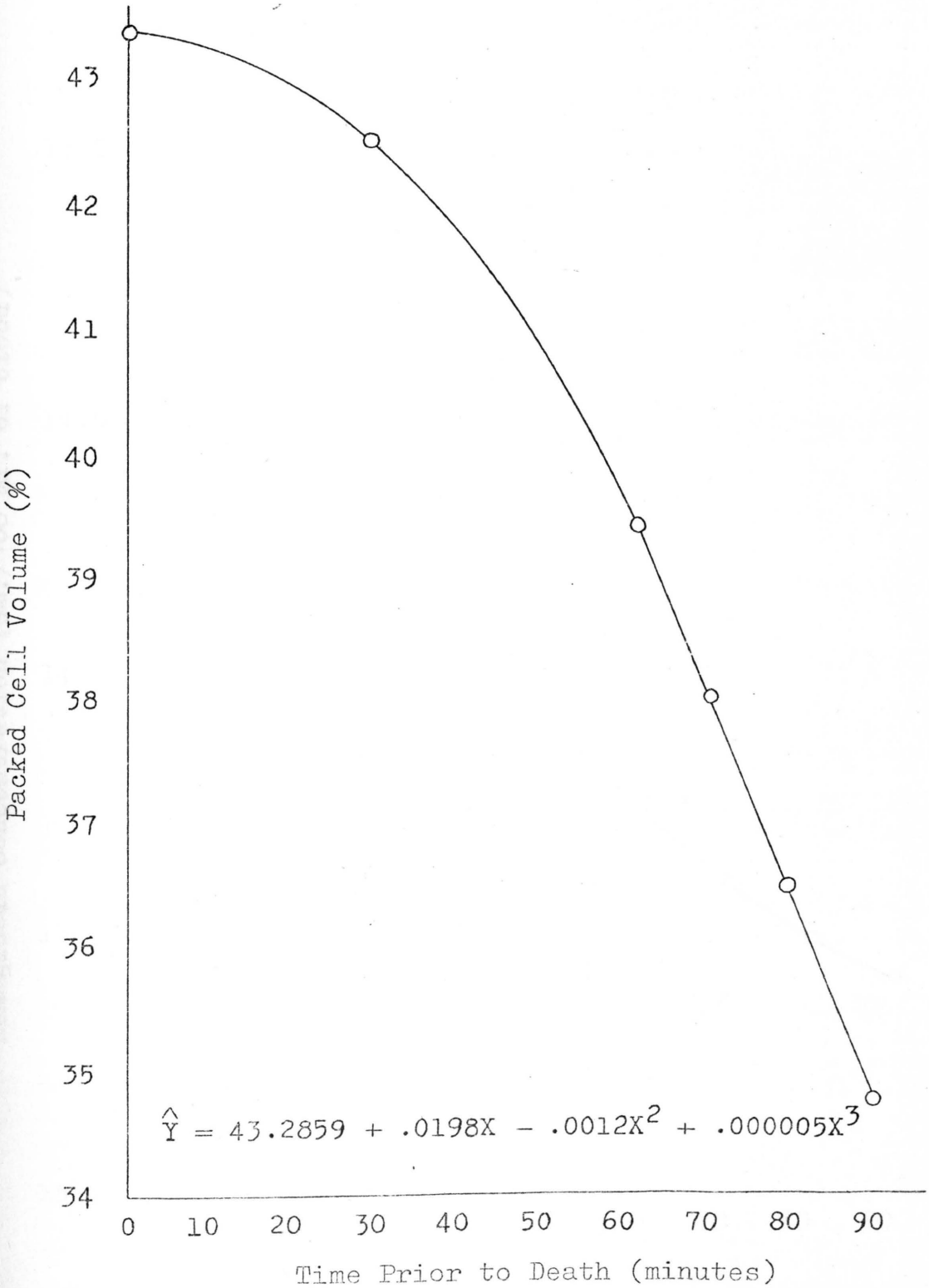

Figure 3: Regression of packed cell volumes with time prior to death for urea-intoxicated animals.

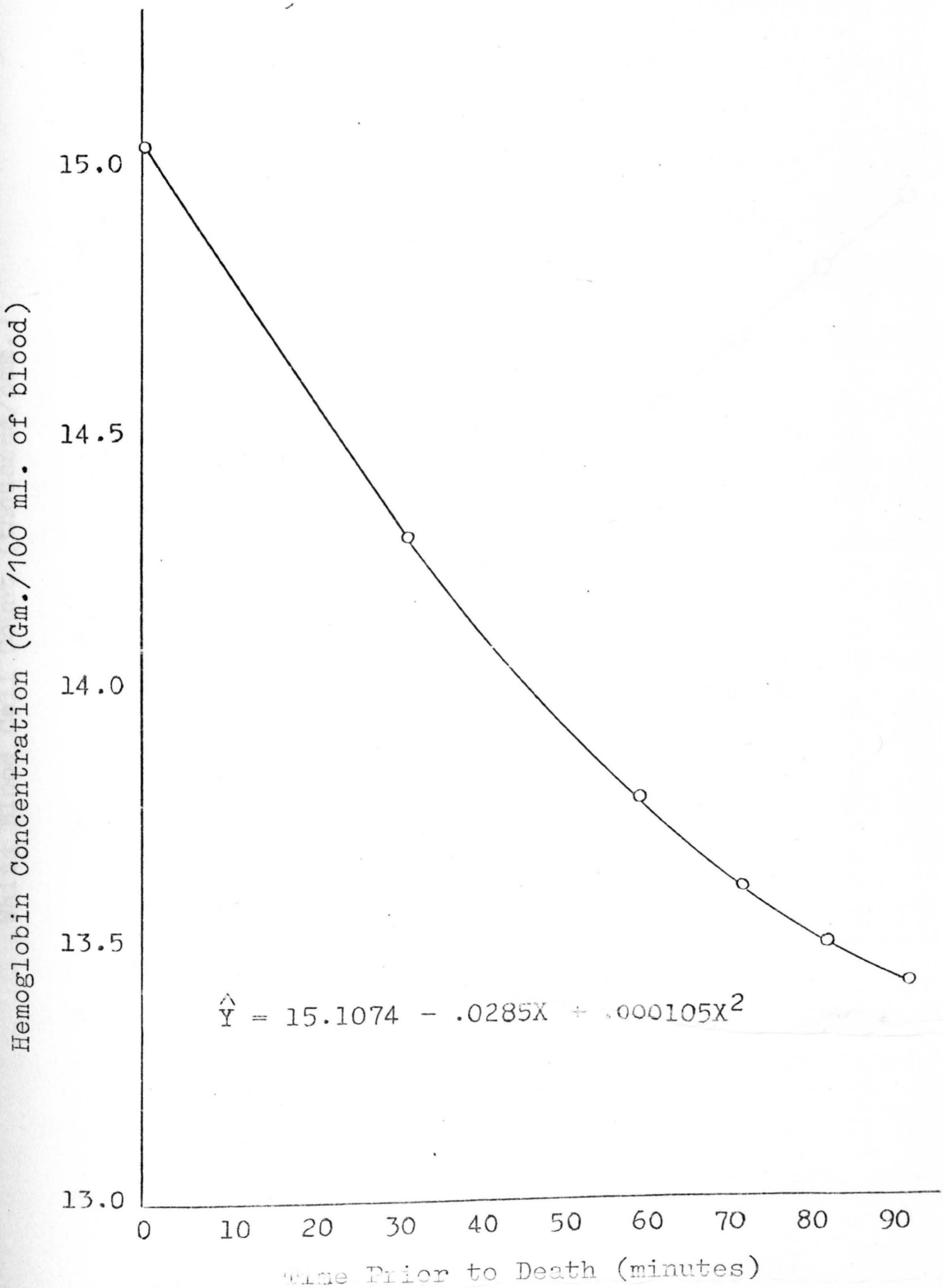

Figure 4: Regression of hemoglobin concentrations with time prior to death for urea-intoxicated animals.

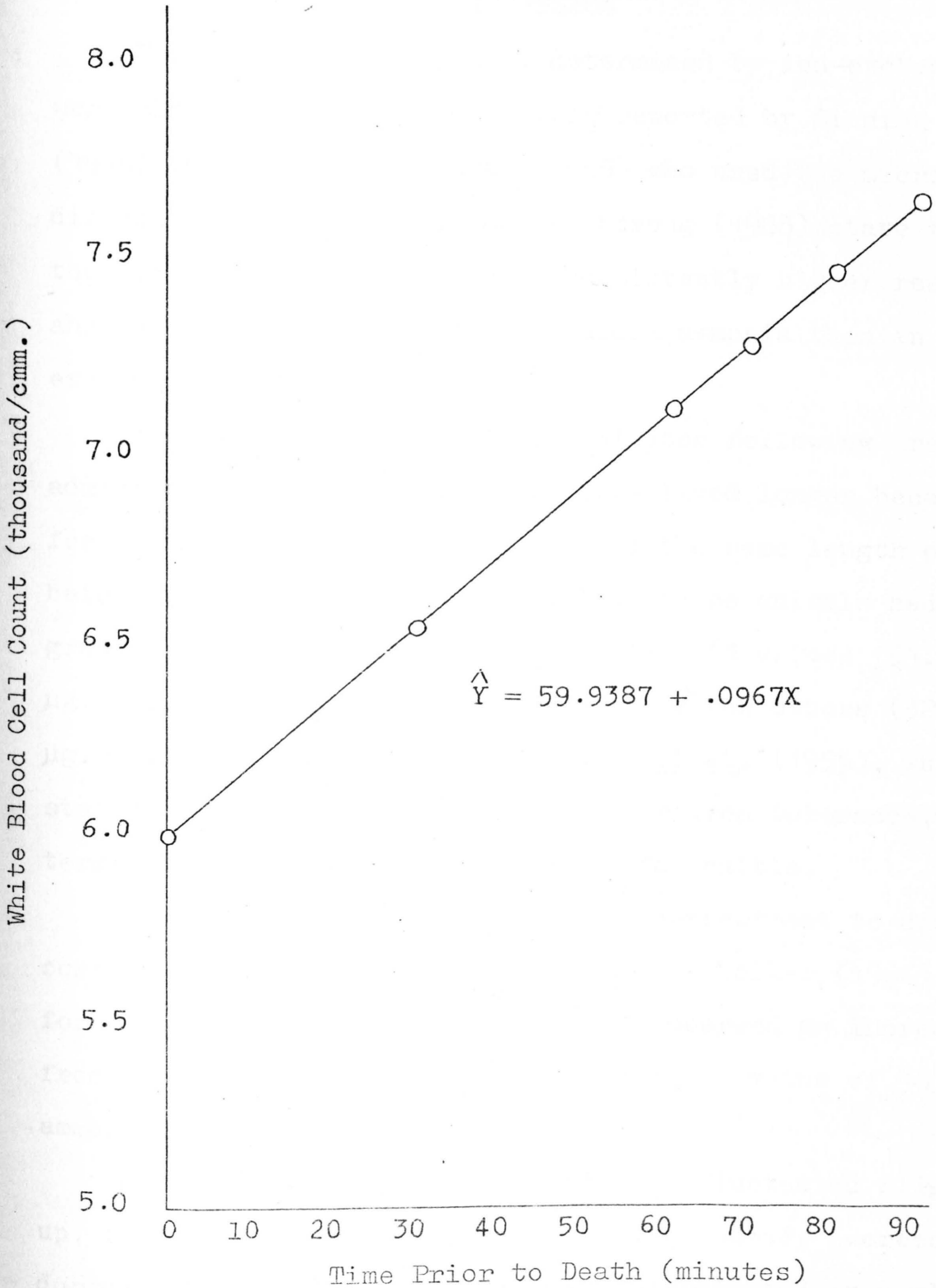

Figure 5: Regression of white blood cell counts with time prior to death for urea-intoxicated animals.

DISCUSSION

Terminal ammonia values, determined by ion-exchange, were similar to those from cattle reported by Dinning et al. (1948) and Davis and Roberts (1959) who used the micro-diffusion technique. Acland and Strong (1968) state that the microdiffusion method gave consistently higher results and less consistent recovery of added ammonia than an ion-exchange method.

Four animals died within 90 minutes following urea administration. The remaining three lived longer because feed and water had not been withheld the same length of time before experimentation. Even though these animals had a greater urea tolerance, their terminal BAN values (34.90 µg./ml.) were approximately the same as the others (32.77 µg./ml.). This agrees with Whitehair et al. (1955), who stated that fasted cattle have a lower urea tolerance, but terminal BAN values are the same as fed cattle.

An 11.2% increase in PCV from pre-treatment to death corresponds with an increase reported by Roller (1966) for cattle. Clifford et al. (1969a) observed an increase from an initial value of 47% to a terminal value of 48% in ammonia intoxicated rats.

Mean values (Table 2) indicate PCV fluctuated slightly up, then down 10 minutes post-dosage. A steady increase during the balance of the experiment terminated in a slight

drop at death. Roller (1966) noticed similar fluctuations with time as BAN values increased. He observed a slight decrease five minutes after urea administration followed by a steady increase with time.

Roller (1966) suggested that dilution was an initial response of the blood vascular system to elevated BAN levels. Ammonia is known to be irritating and dilution is a physiological means of reducing the intensity of the irritant.

Labby and Hoagland (1947) and Bateman et al. (1949) reported an expanded plasma volume and thiocyanate space in patients with severe hepatic failure. The BAN values from those patients approximated those reported in this study 20 minutes post-treatment, when a decreased PCV was evident.

The subsequent increase in PCV may be the result of redistribution of body fluid, splenic contracture, or both. Post-mortem examination of ammonia intoxicated dogs (Handford, 1961) revealed excessive serous fluid in body cavities. The liver, kidneys, and lungs were congested and the spleen contracted. Excessive mucous and saliva were present in the trachea and bronchi.

Koenig and Koenig (1949) reported massive pulmonary congestion following ammonia intoxication in rats, guinea pigs, and cats. Assuming the ammonia distribution in sheep to be similar to that of the rat, as reported by Rosado et al. (1962) and Bessman and Bradley (1955), the redistribution of blood

and tissue fluids would be predominantly into the skeletal muscle with minor increases in the liver and brain.

Significant lines of regression reveal a steady rise in RBC count and Hb concentration that correlates with time as BAN values increase in ammonia intoxicated sheep. Careful literature search failed to provide any published research endeavors describing changes in RBC count and Hb concentration during acute ammonia toxicity. Data from tables 1 through 3 show that fluctuations in RBC count and Hb values followed changes in PCV. This probably resulted from redistribution of body fluids as described above.

Hunter (1968) reported that human RBC swelled when suspended in 1% sodium chloride with successive additions of 0.555M ammonium chloride. The amount of swelling was proportional to the amount of ammonium chloride in the suspension. An increase in MCV from 95 to 120 cubic microns was demonstrated by Labby and Hoagland (1947) in patients with acute liver failure. The size of the RBC returned to normal as the patient's health improved. This phenomenon was not demonstrated in this study since changes in MCV varied randomly.

Careful literature search failed to provide any published research endeavors describing changes in WBC count when BAN values were elevated. This study demonstrated a significant decrease in WBC with time, as BAN values increased. These experiments did not show where the cells went. Possibly,

as ammonia was distributed to other tissues, as described by Rosado *et al.* (1962) and Bessman and Bradley (1955), ammonia stimulated margination of WBC and increased capillary permeability resulting in a chemotaxic response. This in turn, might enhance WBC migration from the cardio-vascular system into the tissues.

SUMMARY

The effects of acute ammonia toxicity on certain hemogram parameters in sheep were investigated. Seven Southdown wether sheep were maintained on mixed alfalfa and grass hay, and water ad libitum prior to experimentation. Feed and water were withheld 24 hours and an aqueous urea solution was administered with a dose syringe at a rate of 2 Gm./kg. of body weight.

Blood for analysis was drawn by jugular venipuncture at regular time intervals throughout the experiment. Analyses were made for blood ammonium-nitrogen (BAN), packed cell volume (PCV), red and white blood cell count (RBC and WBC), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Statistically significant lines of regression with time demonstrated a steady rise in BAN, PCV, Hb, and RBC count following urea dosage. Lines of regression with time showed a significant decline in WBC count as BAN values increased. No significant lines of regression could be plotted for MCV, MCH, and MCHC.

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APPENDIX

Determination of statistically valid equations for lines of regression.

The sum of squares of the cubic equation was subtracted from the sum of squares of the polynomial equation. The difference was subjected to an "F"-test by dividing it by the error mean squares to see if the difference was statistically significant. If not, no validity would be lost by using the cubic, rather than the polynomial equation. The difference between sums of squares of quadratic and cubic equations was treated in the same manner to determine whether validity was lost by using the quadratic equation in place of the cubic. If not, the difference between sums of squares of linear and quadratic equations was treated in the same manner to determine if validity was lost by using the linear equation in place of the quadratic. If not, the sum of squares of the linear equation was divided by the appropriate mean squares to determine whether the linear equation was valid. If not, it was not used and no regression line could be plotted.

Table 4: Pre- and post-treatment BAN* values from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	0.73	0.27	3.73	2.41	1.32	3.62	2.63
Post-treatment							
10 minutes	2.14	1.05	3.45	2.09	1.59	3.17	3.79
20 minutes	2.55	2.09	4.09	2.82	1.86	4.38	4.51
30 minutes	3.77	5.14	4.09	3.36	4.82	5.80	5.58
60 minutes	14.88	10.48	4.82	4.77	7.90	10.40	9.20
90 minutes	33.29**	33.17**	8.68	4.59	8.12	27.52**	37.08**
120 minutes	-----	-----	11.56	7.86	35.91**	-----	-----
180 minutes	-----	-----	32.54**	15.91	-----	-----	-----
200 minutes	-----	-----	-----	36.27**	-----	-----	-----

*blood ammonium-nitrogen in µg./ml.
**death occurred

Table 5: Pre- and post-treatment RBC counts* from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	9.285	11.350	11.090	11.650	10.200	10.980	11.350
Post-treatment							
10 minutes	10.890	------	10.450	11.900	10.575	10.675	10.750
20 minutes	10.480	------	10.050	10.630	9.195	10.900	12.200
30 minutes	10.980	9.565	10.860	10.225	9.290	10.725	9.340
60 minutes	12.050	10.960	10.175	9.455	10.790	12.300	11.500
90 minutes	12.650**	12.650**	10.460	11.425	10.875	11.500**	11.900**
120 minutes	------	------	10.980	10.495	10.840**	------	------
180 minutes	------	------	12.050**	11.650	------	------	------
200 minutes	------	------	------	10.335**	------	------	------

*red blood cell count (million/cmm.)
**death occurred

Table 6: Pre- and post-treatment PCV* values from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	37.34	33.67	38.75	41.75	38.50	39.75	40.50
Post-treatment							
10 minutes	43.00	37.67	39.50	40.25	34.25	39.50	44.00
20 minutes	37.67	37.17	37.25	38.00	35.50	38.50	42.75
30 minutes	44.67	36.34	35.50	36.50	35.75	39.50	42.50
60 minutes	50.00	44.50	34.50	35.75	42.00	43.50	43.00
90 minutes	49.00**	42.67**	39.00	37.00	45.00	45.00**	45.00**
120 minutes	-----	-----	43.00	41.00	39.00**	-----	-----
180 minutes	-----	-----	41.50**	42.25	-----	-----	-----
200 minutes	-----	-----	-----	38.50**	-----	-----	-----

*packed cell volume (%)
**death occurred

Table 7: Pre- and post-treatment hemoglobin (Hb)* values from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	14.10	14.30	13.25	14.35	13.40	14.15	13.55
Post-treatment							
10 minutes	14.40	-----	14.20	14.00	12.80	13.90	14.55
20 minutes	14.15	-----	12.95	12.80	11.70	14.00	12.95
30 minutes	14.55	13.10	12.85	13.80	11.80	12.95	14.20
60 minutes	15.50	15.25	12.35	12.35	13.70	14.80	14.80
90 minutes	15.75**	15.50**	12.50	12.35	14.00	16.00**	15.10**
120 minutes	-----	-----	14.20	14.10	14.15**	-----	-----
180 minutes	-----	-----	14.50**	13.85	-----	-----	-----
200 minutes	-----	-----	-----	13.70**	-----	-----	-----

*Gm./100 ml. of blood
**death occurred

Table 8: Pre- and post-treatment WBC counts* from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	6,971	6,687	10,076	7,620	5,797	6,613	8,075
Post-treatment							
10 minutes	7,541	-----	8,746	7,826	5,949	6,004	7,941
20 minutes	7,385	-----	9,296	7,632	5,126	6,192	8,081
30 minutes	6,392	6,749	8,788	7,145	4,930	6,092	7,479
60 minutes	7,333	6,562	8,380	6,479	4,994	5,258	7,231
90 minutes	6,780**	3,483**	9,065	8,073	5,028	3,335**	6,571**
120 minutes	-----	-----	8,234	7,371	5,388**	-----	-----
180 minutes	-----	-----	6,853**	7,419	-----	-----	-----
200 minutes	-----	-----	-----	5,160**	-----	-----	-----

*white blood cell counts (cmm.)
**death occurred

Table 9: Pre- and post-treatment MCV values* from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	40.39	35.24	34.95	35.84	37.75	36.20	35.68
Post-treatment							
10 minutes	39.95	-----	37.80	33.82	32.39	37.01	40.93
20 minutes	36.26	-----	37.06	35.75	38.61	35.32	35.06
30 minutes	40.53	37.99	32.69	35.70	38.48	36.83	45.50
60 minutes	41.08	40.83	33.91	37.81	38.93	35.37	37.39
90 minutes	38.74**	33.60**	37.29	32.39	41.84	39.13**	38.24**
120 minutes	-----	-----	39.16	39.07	35.98**	-----	-----
180 minutes	-----	-----	34.44**	36.27	-----	-----	-----
200 minutes	-----	-----	-----	37.25**	-----	-----	-----

*mean corpuscular volume (cμ)
**death occurred

Table 10: Pre- and post-treatment MCH values* from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	15.19	12.60	11.95	12.32	13.14	12.88	11.94
Post-treatment							
10 minutes	13.22	-----	13.59	11.76	12.10	13.02	13.54
20 minutes	13.50	-----	12.89	12.04	12.72	12.84	10.61
30 minutes	13.23	13.70	11.83	13.50	12.70	12.08	15.20
60 minutes	12.86	13.91	12.14	13.06	12.70	12.03	12.87
90 minutes	12.45**	12.25**	11.95	10.81	12.87	13.91**	12.69**
120 minutes	-----	-----	12.93	13.43	13.05**	-----	-----
180 minutes	-----	-----	12.03**	11.89	-----	-----	-----
200 minutes	-----	-----	-----	13.26**	-----	-----	-----

*mean corpuscular hemoglobin (µµg.)
**death occurred

Table 11: Pre- and post-treatment MCHC* values from urea-intoxicated sheep.

	Sheep number						
	1	2	3	4	5	6	7
Pre-treatment	37.61	35.75	34.19	34.37	34.81	35.60	33.47
Post-treatment							
10 minutes	33.11	-----	35.96	34.78	37.38	35.20	33.07
20 minutes	37.24	-----	34.77	33.68	32.97	36.37	30.30
30 minutes	32.70	36.42	36.19	37.82	33.01	32.80	33.42
60 minutes	31.32	34.08	35.80	34.55	32.62	34.03	34.42
90 minutes	32.14**	36.48**	32.05	33.38	30.77	35.56**	33.19**
120 minutes	-----	-----	33.02	34.39	36.28**	-----	-----
180 minutes	-----	-----	34.94**	32.78	-----	-----	-----
200 minutes	-----	-----	-----	35.59**	-----	-----	-----

*mean corpuscular hemoglobin concentration (%)
**death occurred