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Tissue Ammonium Nitrogen in Sheep Tongue, Liver and Kidney Following Death by Ammonia Intoxication

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TISSUE AMMONIUM NITROGEN IN SHEEP TONGUE, LIVER, AND KIDNEY FOLLOWING DEATH BY AMMONIA INTOXICATION

BY

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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. 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 Department Date

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INTRODUCTION

Urea was introduced as an economical nonprotein nitrogen source for ruminant animals. In the gastrointestinal tract, urea is hydrolyzed in the presence of bacterial urease to ammonia and carbon dioxide. The liberated ammonia is utilized by rumen microorganisms for amino acid synthesis. Ammonia not used by microorganisms diffuses through the gastrointestinal lining and is transported to the liver by blood, where enzymes synthesize urea by combining two moles of ammonia and one mole of carbon dioxide. The nontoxic urea is excreted by the kidney or secreted with saliva and other digestive fluids for recycling.

Ammonia intoxication occurs when more ammonia is produced and absorbed than can be detoxified by the liver. Ammonia, predominately in the form of ammonium ion, produces toxicity symptoms which include hyperpnea, ataxia, muscle tremors, convulsions, tetany, and death.

Numerous investigations have been conducted concerning the metabolic alterations and clinical signs in acute ammonia intoxication, however there is inadequate information concerning stability of ammonia in postmortem tissues.

The purpose of this study was to investigate the postmortem stability of ammonia in sheep tongue, liver, and kidney tissues following acute ammonia intoxication. Liver and kidney tissues were collected because of their metabolic significance. Tongue tissues were collected because of easy access, which would provide a simple method of obtaining diagnostic tissues without opening the carcass.

LITERATURE REVIEW

Ammonia in Biological Fluids

Existence of ammonia in vivo

Early investigators, Conway and Cook (1939) and White, et al. (1955) reported that no free ammonia existed in blood in vivo. They stated that ammonia being measured was artifactual and was formed after the sample was collected. Calkins (1956) presented data that disagreed with the earlier reports. Conducting blood ammonia tests at 10-second to 20-minute intervals after withdrawal, he found that no significant change in blood ammonia occurred. Calkins concluded that a significant amount of ammonia was present in the blood of normal persons in vivo. Ninety three percent of normal subjects had blood ammonia values from 0.5 to 1.1 μg./ml. Seligson and Hirahara (1957) confirmed the presence of ammonia in circulating blood and reported a mean plasma ammonia value of 0.78 μg./ml. for human patients free of liver disease. Kirkpatrick (1971) reported a mean plasma ammonia value of 2.1 μg./ml. in sheep.

Form of ammonia in vivo

Jacobs and Parpart (1938) demonstrated that erythrocytes were permeable to nonionized ammonia and relatively impermeable to the ammonium ion. Warren (1962) stated that ammonia existed in two forms in biological fluids, the relative proportions of which were determined primarily by the pH of the solution. The above two reports were used to account for the fact that ammonia at low pH was toxic only if present

in large quantities, whereas at high pH, much smaller amounts were lethal. The pKa of ammonia in blood plasma has been reported to be 9.02 with a range of 9.1 at 32 C. to 8.91 at 41 C. (Warren, 1962). Using a pKa value of 9.0, the percentages of nonionized ammonia at pH 6, 7, 8 and 9 were approximately 0.1, 1, 10, and 50, respectively.

Formation of Artifactual Ammonia

After it had been established that ammonia was present in circulating blood of normal individuals, a large variation in the reported values became evident. Irregardless of which method was used for ammonia detection, the formation of artifactual ammonia led to erroneous results.

Storage of Samples

It was known from the results of a number of investigations, that if allowed to stand at room temperature, ammonia concentration in blood increased within 15 to 60 minutes after collection.

Effects of temperature variation on ammonia concentration in stored blood were reported by Conn and Kuljian (1964). Refrigeration of blood at 4 C. resulted in a tremendous increase in ammonia concentration after two to four days. There was no significant increase in ammonia during this time period when blood was frozen at -15 C. It was noted that ammonia concentration in frozen blood at -15 C. increased significantly after four days of storage. No significant difference was found between the rate of ammonia formation in blood from normal patients and blood with an elevated ammonia concentration. An

ethanol-dry ice mixture inhibited ammonia formation two days, and the ammonia concentration in blood stored in liquid nitrogen remained constant for three to four days. Freezing and thawing had no significant effect on the ammonia concentration if the samples were analyzed immediately after thawing. This supported the observations of Merchant (1960), who had previously reported the value of alcohol-dry ice in the preservation of blood for ammonia analysis.

Jacquez, et al. (1959) reported that the ammonia concentration in blood stored in an ice bath was stable for two hours after collection. A significant rise in ammonia occurred after this period. Merchant (1960) concurred with Jacquez, but Ternberg and Hershey (1960) noted a significant increase in ammonia as early as one hour after blood collection.

Nathan and Rodkey (1957) added whole blood to an ice cold solution of trichloroacetic acid and noted that the ammonia concentration was stable for up to 80 minutes if kept frozen. Wilder (1962), using the technique of Nathan and Rodkey, reported a significant increase in ammonia within 24 hours after collection, and concluded that freezing was an unsatisfactory method of preservation.

Miller and Rice (1963) used an ion exchange resin to inhibit formation of artifactual ammonia. After the resin was washed free of plasma and allowed to stand at room temperature for as long as three hours, there was no significant change in ammonia concentration.

Anticoagulants

Using commercial heparin containing phenol and purified heparin, Dimond (1955) demonstrated that the enzyme adenylic deaminase was inhibited. Jacquez, et al. (1959) recognized this property of heparin as beneficial to ammonia analysis and recommended its use.

Conn (1962), using the Seligson-Hirahara method, found no significant difference in blood ammonia concentration when heparin or ethylenediaminetetraacetic acid (EDTA) was used. There were higher ammonia values when sodium citrate, potassium oxalate, and sodium fluoride were used as anticoagulants. It was also demonstrated that the ammonia concentration in noncoagulated blood was slightly higher than in heparinized blood. Dienst (1961) reported that some heparin preparations contained significant amounts of ammonia.

Analytical Methods

Microdiffusion

Microdiffusion involves alkalinization of whole blood or plasma to convert ammonium ion, predominating at physiological pH, to diffusable ammonia gas which is liberated from solution. Ammonia moves by gaseous diffusion to an acid medium where it is reconverted to the ionized form. The ammonia concentration is then determined colorimetrically or by titration.

Preston (1969) compared the microdiffusion methods of Seligson and Hirahara (1957), Nathan and Rodkey (1957), and Reinhold and Chung (1961) while determining ammonia nitrogen in sheep blood. Values obtained were comparable and all of the procedures appeared to be valid.

Microdiffusion has been criticized by many workers in the field. Jacquez, et al. (1959) reported the formation of ammonia from protein decomposition during alkalinization of blood. A later investigation by Reinhold and Chung (1961) supported this conclusion and emphasized that neither a short diffusion period nor extrapolation were acceptable methods to compensate for ammonia formation during alkalinization.

Acland and Strong (1968) compared the microdiffusion method of Seligson and Hirahara (1957) with an ion exchange method reported by Fenton and Williams (1968) for blood ammonia tests on man. The ion exchange method was demonstrated to be superior because of more consistent recovery of added ammonia.

Ion Exchange

The ion exchange principle consists of adsorption of ammonium ions, which predominate at physiological pH, on a cation exchange resin. After elution from the resin, the concentration of ammonium nitrogen is determined colorimetrically.

The capacity of clays for ammonia adsorption was reported by Thompson (1850). The first application of this adsorptive capacity to analytical chemistry was reported by Folin and Bell (1917). In this procedure, urinary ammonia was adsorbed by zeolite, liberated with sodium hydroxide, and subjected to Nesslerization.

Current ion exchange methods utilize a strongly acidic cation exchange resin. Conversion of resin to sodium-potassium form was recommended by Hutchinson and Labby (1962) because of a threefold increase

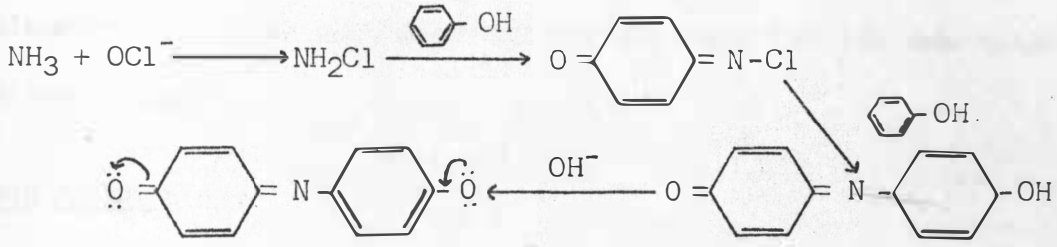
in mean venous blood ammonia when the hydrogen ion form was used. By the process of elimination, they identified glutamine as the sole contributor of the excess ammonia. The method of Hutchinson and Labby was adapted by Kirkpatrick (1971) to be effective at high levels of blood ammonia, as would be expected in acute ammonia intoxication in ruminant animals.

Colorimetry and Titration

The final step of microdiffusion and ion exchange methods involves titration or a colorimetric procedure. Titration has been criticized by Miller and Rice (1963) because estimation of the end point was difficult when titrating against strong acid. Conway (1963) summarized operator error and variable glass errors involved with titration.

Colorimetric procedures involving Nessler's reagent and the indophenol reaction have become widely used. Nesslerization has been discounted by many researchers. Miller and Rice (1963) reported that Nessler's reagent was easily influenced by pH changes and time allowed for color development. Dienst (1961) stated that most color formation with Nessler's took place within three to four minutes, after which the color density increased, presumably due to alkaline hydrolysis of protein remaining in the sample. Kirkpatrick, et al. (1972) reported an excessive variation in adrenal gland tissue ammonium nitrogen values because, upon Nesslerization, the color mixture became cloudy. He noted that the intensity of the cloudy material appeared to vary with the amount of ammonia present.

A proposed mechanism for the reaction of ammonia, hypochlorite, phenol, and sodium hydrozide to form the blue indophenol was reported by Bolleter, et al. (1961).



They stated that hypochlorite must be added before phenol to get color development. Noble (1955), concluded that phenol must be added before sodium hypochlorite. Wearne (1963) reported that if hypochlorite is added first, the reaction may take place with amino acids remaining in the sample at low pH.

Recently, investigators have demonstrated the value of adding sodium nitroprusside as a catalyst to hasten the formation of indophenol. Chaney and Marbach (1962) confirmed that sodium nitroprusside was effective as a catalyst and reported that maximum blue color was obtained at room temperature in approximately 30 minutes. He stated that the absorbance remained unchanged for at least 24 hours. Miller and Rice (1963) and Dienst and Morris (1964) recommended heating the final solution for 15 to 30 minutes in a water bath at 37 C.

Leffler (1967) demonstrated that a final pH of 11.7 produced maximal density and that too little sodium hypochlorite would not allow maximal density to occur. Substitution of Clorox[1] for sodium hypochlorite resulted in equally good color density.

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[1]Clorox Company, Oakland, California, 94623.

Chaykin (1969) reported that during ammonia detection in the presence of nicotinamide, the concentration of sodium nitroprusside was crucial. Too much or not enough nitroprusside blocked color development, and each nicotinamide concentration had its own nitroprusside optimum.

Tissue Ammonia

Tashiro (1922) demonstrated the production of ammonia at the myoneural junction in both resting and stimulated nerves. There was a twofold increase in ammonia production with nerve stimulation and a simultaneous increase in carbon dioxide production.

Feinberg and Alma (1961) reported the first simultaneous observation of ammonia production and oxygen consumption. Alterations in pH did not affect the ratio of ammonia production/oxygen consumption.

Studying blood ammonia in human subjects with liver disease, Bessman and Bessman (1955) demonstrated that ammonia was removed by the brain when concentrations exceeded 1 μg./ml. It was also noted that ammonia removal by muscle coincided with reduced arterial ammonia. Schwartz, et al. (1958) reported an increase in peripheral blood ammonia following muscular activity such as voluntary exercise or artificially induced convulsions. Bessman and Bradley (1955) stated that muscle removed approximately 40 percent of the ammonia from arterial blood, which was twice the amount removed by the brain. After administering ammonium chloride to rats, Rosado, et al. (1962) observed that most of the ammonia was removed by muscle. Liver and brain tissue ammonia was much lower than in muscle tissue.

Keynes (1963) stated that muscle ammonia in normal dogs was many times greater than ammonia in blood or lymph, and the amounts in normal brain, pancreas, and liver were greater still. In animals with rising blood ammonia from acute liver failure, the ammonia in muscle, brain, and pancreas reached two to three times the amount measured in normal dogs. On the basis of this data, Keynes calculated that less than 5 percent of total body ammonia was present in blood during liver failure.

Roller, et al. (1970) noted a significant ammonia increase in rabbit uterus, embryo, and skeletal muscle tissues following the administration of 5 Gm. urea/kg. body weight.

Kirkpatrick, et al. (1972) reported a significant ammonia increase in sheep liver, kidney, psoas muscle, and cardiac muscle tissues following administration of 2 Gm. urea/kg. body weight. The greatest increase was noted in liver and kidney tissues, which increased 88.8 percent and 88.4 percent, respectively.

Stability of Ammonia Postmortem

A search of the literature failed to reveal a significant amount of information concerning postmortem ammonia concentrations. Brown, et al. (1957) reported a rapid increase in postmortem liver ammonia, but the collection terminated 90 seconds after death. Faulkner (1958) reported that postmortem blood ammonia was extremely elevated.

Appearance of Postmortem Tissues

Hart, et al. (1939) observed that feeding a high level urea ration resulted in liver necrosis and hyalinized kidneys. Handford (1961)

described dog livers as hard and congested and kidneys as dark and congested after the intravenous administration of 15 mg. urease/kg. body weight.

MATERIALS AND METHODS

Experimental Animals

Ten Southdown wether lambs, between 186 and 305 days of age and weighing 23.6 to 45 kg., were used in this study. The lambs were sheared and housed indoors where mixed alfalfa and brome grass hay and water were provided ad libitum until one hour prior to the experiment. All experimental animals appeared to be alert, active, and in good health.

The animals were randomly divided into two groups. Group I consisted of three control animals and group II consisted of seven principal animals.

Preparation

Ammonia-free Water

Ammonia-free water was prepared by passing distilled water through a Barnstead[1] mixed bed hose-type cartridge and redistilling with a Loughborough[2] all-glass water still. After distillation, the ammonia-free water was stored in covered plastic containers.

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[1]Barnstead Still and Sterilizer Company, Boston, Massachusetts, 02131.

[2]Bellco Glass Inc., Vineland, New Jersey, 08360.

Care of Glassware

Prior to use, all glassware was washed in hot water containing Tide[3] detergent and rinsed in distilled water. Glassware was made ammonia-free by soaking for 30 minutes in 0.1 N. sodium hydroxide and was rinsed five times in ammonia-free water to remove the excess sodium hydroxide. After drying in an oven, the glassware was stored in ammonia-free containers until use.

Preparation of Resin

Blood ammonia (NH_4^+-N) was collected on ion exchange resin,[4] 30-80 mesh, by exchanging ammonium ions for sodium and potassium ions. Prior to use, the resin was rendered ammonia-free by washing with 0.1 N. sodium hydroxide, and converted to sodium-potassium form by washing with sodium-potassium phosphate buffer solution, the pH of which was adjusted to 7.4 with sodium hydroxide. The resin was stored in a dark place under 400 ml. of ammonia-free water in a glass stoppered reagent bottle.

Preparation of Standards and Reagents

A standard ammonium-nitrogen stock solution was prepared by placing 4.7166 Gm. of ammonium sulfate in a one liter volumetric flask and adding ammonia-free water to the 1-liter mark. This yielded a stock

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[3]Procter and Gamble, Cincinnati, Ohio, 45202.

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

solution containing 1 mg. of NH_4^+-N/ml. After thorough mixing, the solution was transferred to a plastic container and stored at 7 C.

Phenol color reagent was prepared by placing 10 Gm. of phenol and 50 mg. of sodium nitroprusside in a one liter volumetric flask and adding ammonia-free water to the 1-liter mark. The mixed solution was stored in a covered plastic container at 7 C.

Alkaline hypochlorite reagent was prepared by adding 6 ml. of a 6 percent sodium hypochlorite solution and 5 Gm. of sodium hydroxide to approximately 200 ml. of ammonia-free water and diluting to 1 liter. The solution was stored in a covered plastic container at 7 C.

Intoxication and Collection of Test Materials

After drawing pretreatment blood samples, the principal animals were administered 2.5 Gm. of urea/kg. of body weight in a 3.3 M. urea solution prepared with tap water. The control animals were given 12.5 ml. of tap water/kg. of body weight, which approximated the amount of water given to the principal animals in a 3.3 M. urea solution. Subsequently, blood was drawn at 30-minute intervals until death. The control animals were electrocuted two hours after administration of the water, which approximated the time of death by intoxication.

Seven-ml. blood aliquots were drawn by jugular venipuncture, using a 14-gauge bleeding needle. One ml. of the aliquot was withdrawn with a tuberculin syringe and 0.5 ml. was ejected into each of two 5 ml. glass-stoppered centrifuge tubes which contained 1 ml. of resin suspension. Each whole blood-resin mixture was immediately mixed for one

minute with a Vortex[5] Genie Mixer and refrigerated at 7 C. The remaining 6 ml. of the blood to be used for serum analysis was covered with parafilm and refrigerated.

Tissues

At the time of death, a median incision was made in the ventral abdominal wall to facilitate collection of liver and kidney tissues. After collection of tissues, the incision was closed with hemostats to minimize exposure of the viscera to the environmental temperature (21 C.). Kidney slices were made to include medullary and cortical areas. Liver slices were not confined to a specific lobe, but were taken from various areas throughout the liver. Tongue tissues were collected by clipping from the tip each time. The tissue samples, weighing approximately 5 Gm. each, were taken at death and 2, 4, 8, 16 and 24 hours later. Tissues were placed in individual Whirl Pak[6] plastic bags and immediately frozen at -20 C.

Analytical Procedure

All analyses were made in duplicate, with a reagent blank and a standard included with each group of 30 tests. Working standards (40 µg. NH_4^+-N for principal group tissue analysis and 20 µg. NH_4^+-N for whole blood, serum and control group tissue analyses) were prepared

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[5]Scientific Industries, Inc., Springfield, Massachusetts, 01103.

[6]Nasco, Fort Atkinson, Wisconsin, 53538.

daily by diluting the NH_4^+-N stock solution. A method reported by Miller and Rice (1963) was modified for NH_4^+-N analysis.

Whole Blood

Whole blood was analyzed approximately 4 hours after the blood was collected. The whole blood-resin mixtures were washed with ammonia-free water until a clear supernatent fluid was obtained. During the washing procedure, the samples were mixed for 1 minute with a Vortex Genie Mixer. The supernatent fluid was aspirated and discarded with a Pasteur pipette attached to a suction apparatus. After the washing was complete, 1 ml. of 0.1 N. sodium hydroxide was added to each tube and mixed to elute the ammonia from the resin. One-half ml. of the supernatent fluid was placed in a 10-ml. centrifuge tube and 1 ml. each of phenol color reagent and alkaline hypochlorite reagent were added. After adding ammonia-free water to the 10 ml. mark, the tubes were stoppered and mixed by inversion before placing in a water bath at 37 C. for 30 minutes to obtain color development. The optical density (O.D.) of the color mixture was determined, using 7.5 x 12.8 x 46.6 mm. cuvettes in a Beckman Model DB-G Grating Spectrophotometer operated at a 640 nm. wavelength. A corrected O.D., to eliminate contamination errors, was obtained by subtracting the O.D. of the reagent blank (read against a water blank) from the O.D.'s of the unknown and standard. The NH_4^+-N concentration of the whole blood was calculated by using the following formula

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

(20 equals the μg. of NH_4^+-N in the standard):

$$\frac{\frac{20 \text{ (corrected O.D. of unknown)}}{\text{corrected O.D. of standard}}}{0.5 \text{ ml.}} = \mu g.\ NH_4^+\text{-N/ml.}$$

Serum

The blood for serum analysis was refrigerated for 48 hours before separating serum by centrifugation. One-half ml. of serum was placed in each of two 5-ml. centrifuge tubes containing 1 ml. of resin suspension. The balance of the procedure as used for whole blood analysis was repeated.

Tissues

Approximately 2 Gm. of tissue were placed in the homogenization vessel (Fig. 1) which was resting in a dry ice-acetone bath to maintain the tissue in a frozen state. Tissues were homogenized in approximately 30 seconds of operation with the mechanized homogenizer (Fig. 2).

One ml. of resin suspension was placed in 5-ml. centrifuge tubes and weighed on a Sartorius[8] balance. Approximately 150 mg. of homogenized tissue were placed in the tared tubes which were weighed again to determine the amount of tissue. Tissues were analyzed by the same procedure as used for whole blood and serum. The concentration of NH_4^+-N was calculated according to the following formula (20 and 40 equal the amount of NH_4^+-N in the standard and 100 was used to convert the final result to a 100 mg. basis):

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[8]Brinkman Instruments, Inc., Westbury, New York, 11590.

$$\frac{\frac{20 \text{ or } 40 \text{ (corrected O.D. of unknown)} 100}{\text{corrected O.D. of standard}}}{\text{mg. of tissue}} = \frac{\mu\text{g. } NH_4^+\text{-N}/100}{\text{mg. tissue}}$$

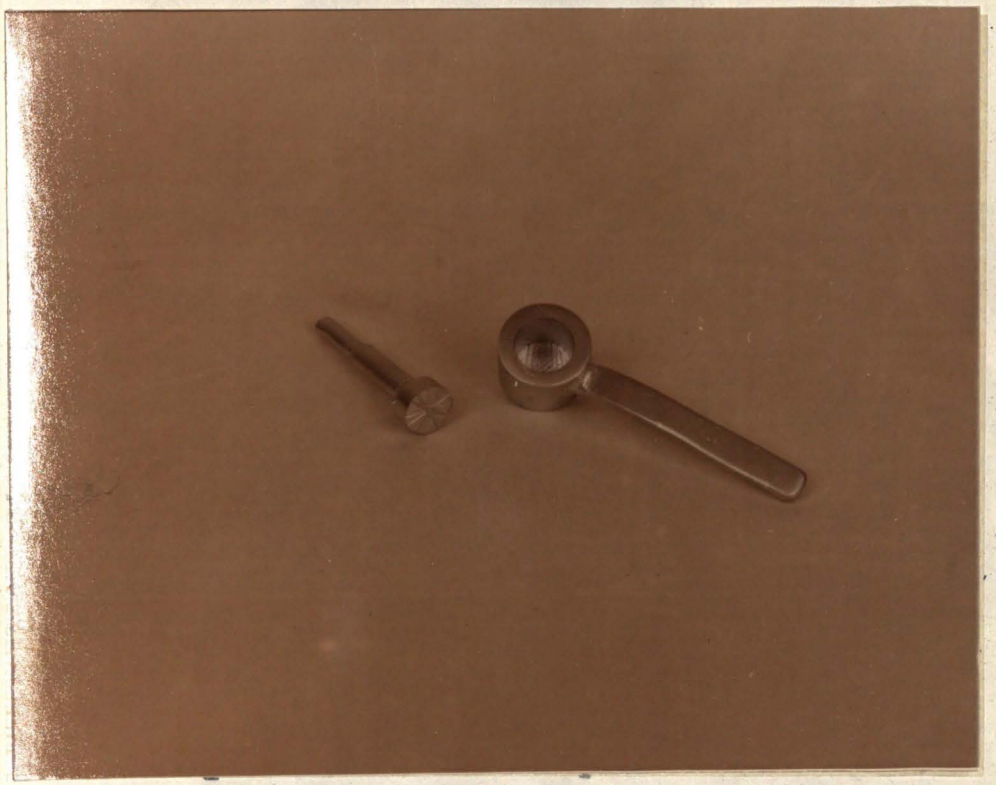

Figure 1. Close-up view of homogenization unit.

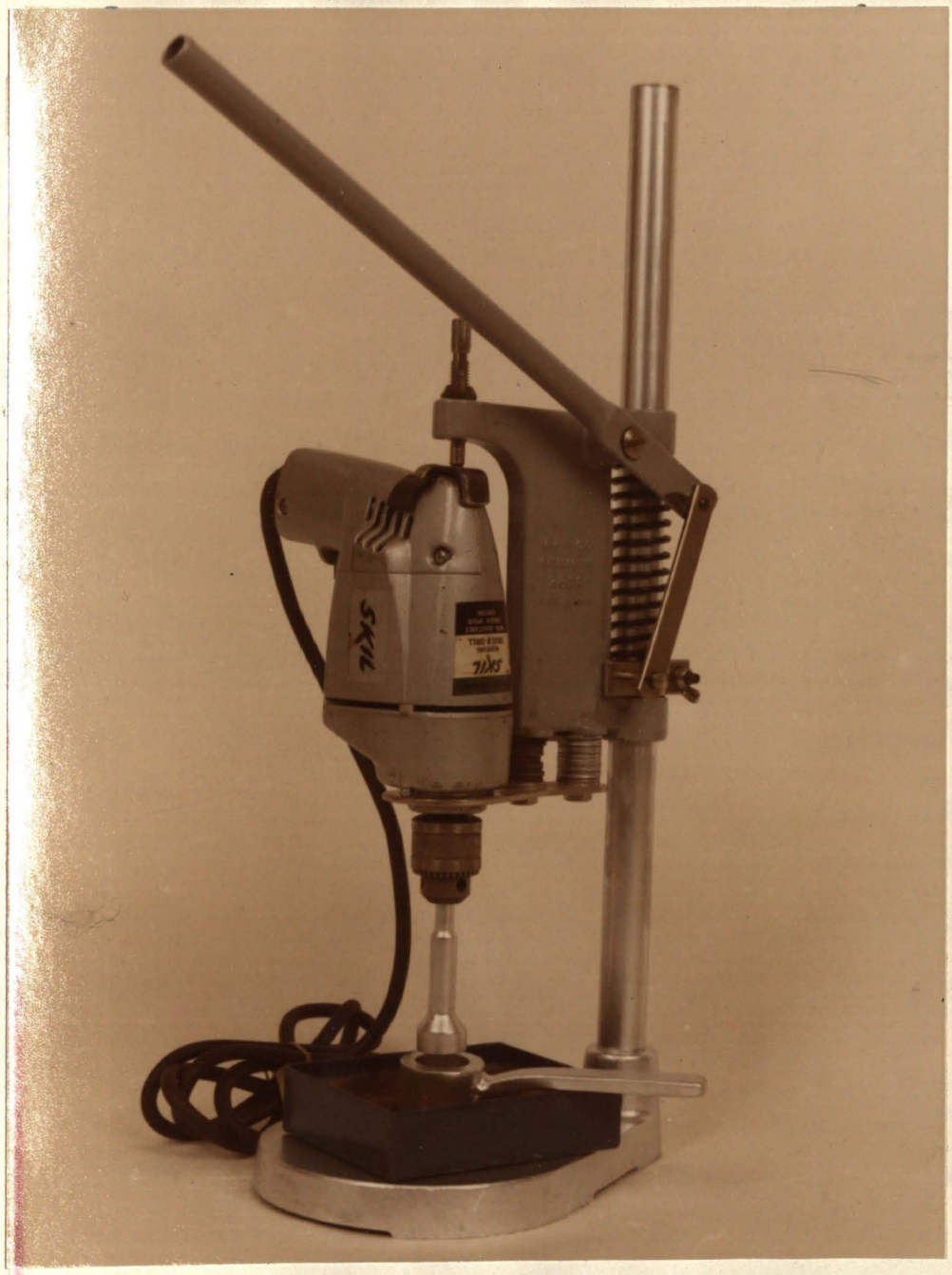

Figure 2. Variable speed equipment used to homogenize frozen tissues.

RESULTS

Data from this study indicate that ammonium nitrogen concentrations increased in principal animals after drenching with urea (Table 1, Fig. 3), and after death (Table 2, Fig. 4, 5, 6, 7).

Table 1A indicates significant differences between principal and control group whole blood ammonium nitrogen after sheep were drenched. The difference between principal and control group serum ammonium nitrogen was significant at all times except before and 90 minutes after drenching. Ammonium nitrogen was consistently higher in serum than in whole blood (Table 1B). This difference was significant only in the principal group before, 30 and 60 minutes after drenching. The difference was not significant in the control group at any time period.

Tissue ammonium nitrogen was higher for principal animals than for control animals (Table 2). A paired t test demonstrated the difference between principal and control groups to be significant ($P < 0.01$) for all collection periods. Means of ammonium nitrogen values indicate increases (principal versus control) of 117, 124, and 165 percent for tongue, liver and kidney tissues, respectively. Lines of regression with time demonstrated increases in ammonium nitrogen in principal group tongue tissues (Fig. 5) and control group kidney tissues (Fig. 7) that were significant ($P < 0.05$). Ammonium nitrogen in other tissues increased with time after death, however those increases were not significant.

Table 1. Means of ammonium nitrogen in whole blood and serum. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

A	μg. NH_4^+-N/ml.		
	Principal	Control	Difference
Whole Blood			
Drenched	1.8	1.7	0.1
30 minutes	5.6	1.5	4.1**
60 minutes	12.3	1.5	10.8**
90 minutes	19.3	1.5	17.8*
120 minutes	17.2	1.8	15.4**
Serum			
Drenched	4.2	3.7	0.5
30 minutes	11.0	3.1	7.9*
60 minutes	17.2	4.0	13.2**
90 minutes	21.7	4.3	17.4
120 minutes	18.6	3.4	15.2*

B	Serum	Whole Blood	Difference
Principal Group			
Drenched	4.2	1.8	2.4**
30 minutes	11.0	5.6	5.4**
60 minutes	17.2	12.3	4.9*
90 minutes	21.7	19.3	2.4
120 minutes	18.6	17.2	1.4
Control Group			
Drenched	3.7	1.7	2.0
30 minutes	3.1	1.5	1.6
60 minutes	4.0	1.5	2.5
90 minutes	4.3	1.5	2.8
120 minutes	3.4	1.8	1.6

* $P < 0.05$
** $P < 0.01$

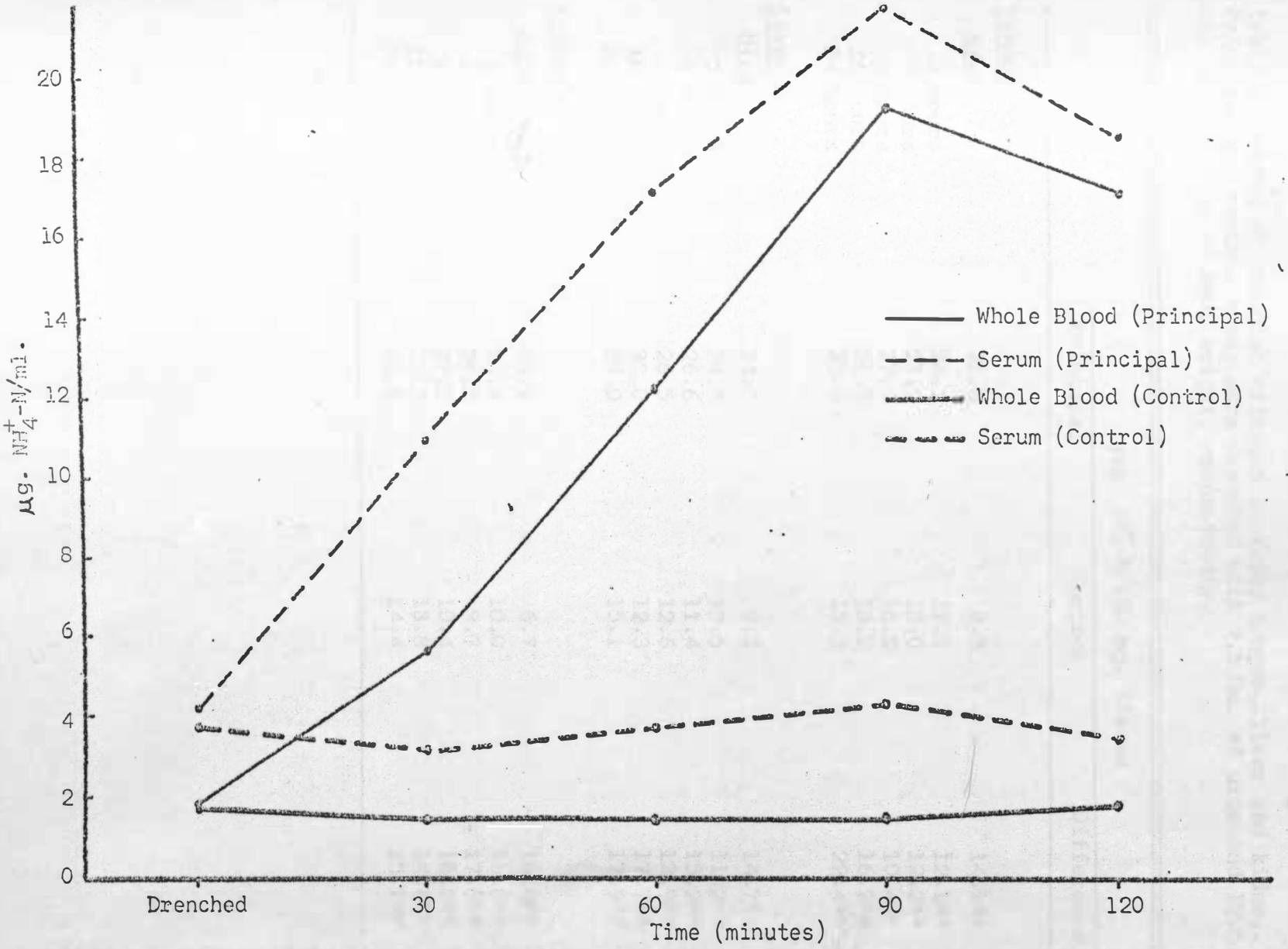

Figure 3. Sheep whole blood and serum ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

Table 2. Means of ammonium nitrogen in sheep tongue, liver and kidney. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

	μg. NH_4^+-N/100 mg. tissue		
	Principal	Control	Difference
Tongue			
Died	22.6	8.5	14.1**
2 hours	24.2	12.1	12.1**
4 hours	27.2	15.0	12.2**
8 hours	27.2	16.8	10.4**
16 hours	29.7	13.5	16.2**
24 hours	35.6	13.2	22.4**
Liver			
Died	24.0	9.3	14.7**
2 hours	24.7	13.5	11.2**
4 hours	26.6	11.4	15.2**
8 hours	26.5	12.6	13.9**
16 hours	30.0	12.3	17.7**
24 hours	33.0	15.1	17.9**
Kidney			
Died	23.1	6.7	16.4**
2 hours	25.8	10.0	15.8**
4 hours	26.3	8.7	17.6**
8 hours	27.2	10.4	16.8**
16 hours	28.2	13.8	14.4**
24 hours	30.1	14.4	15.7**

** $p < 0.01$

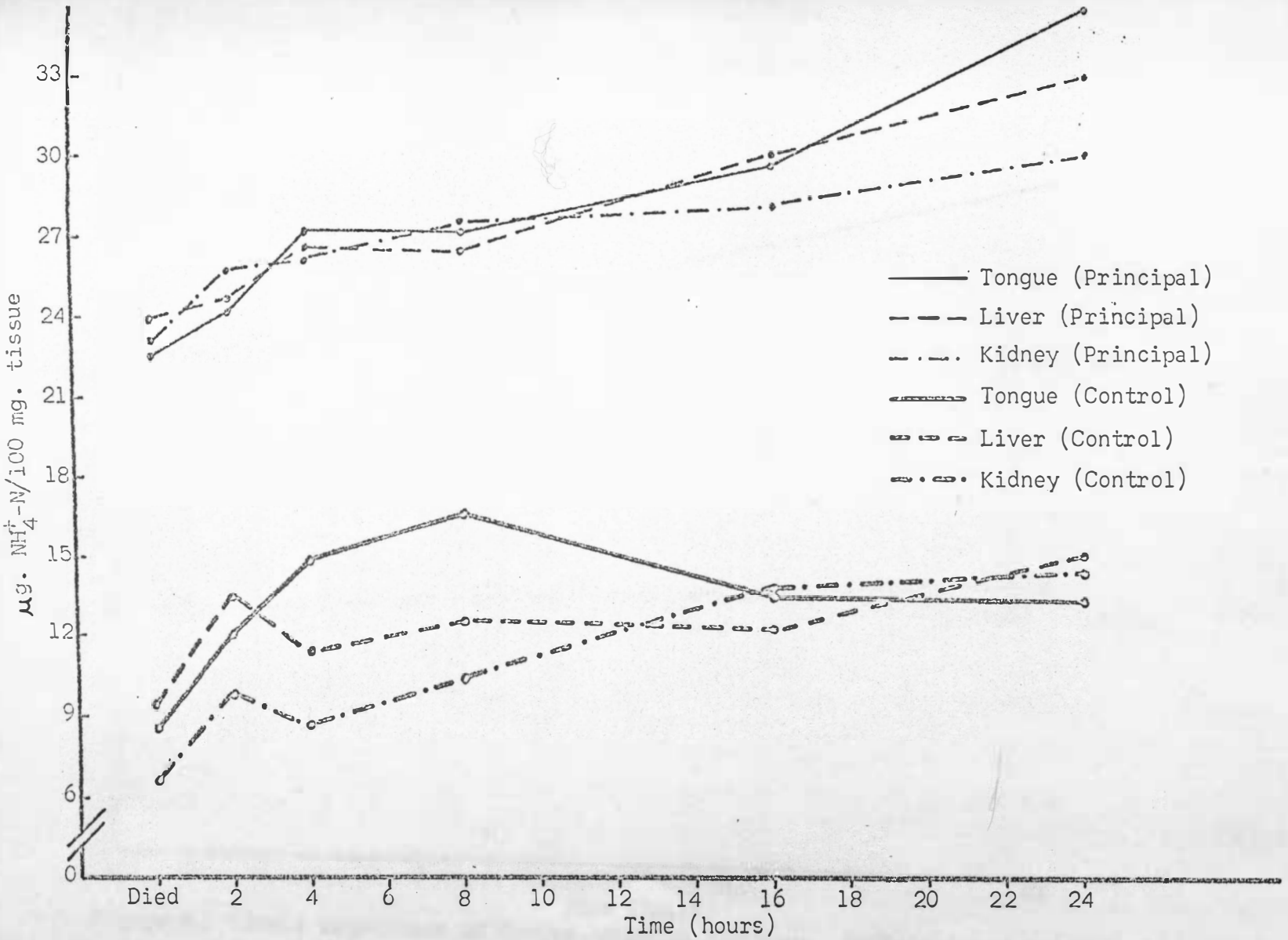

Figure 4. Sheep tissue ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

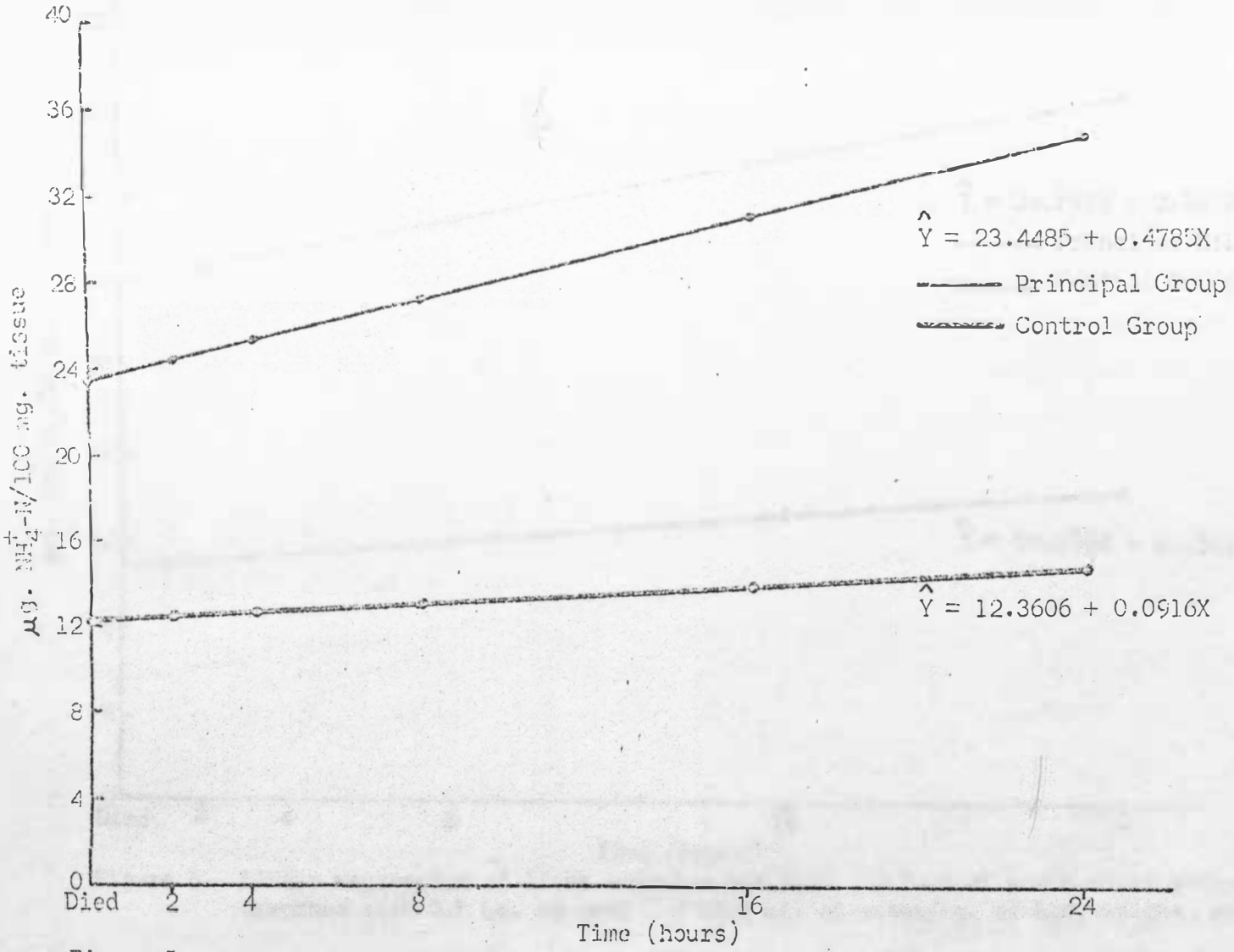

Figure 5. Linear regression of tongue ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

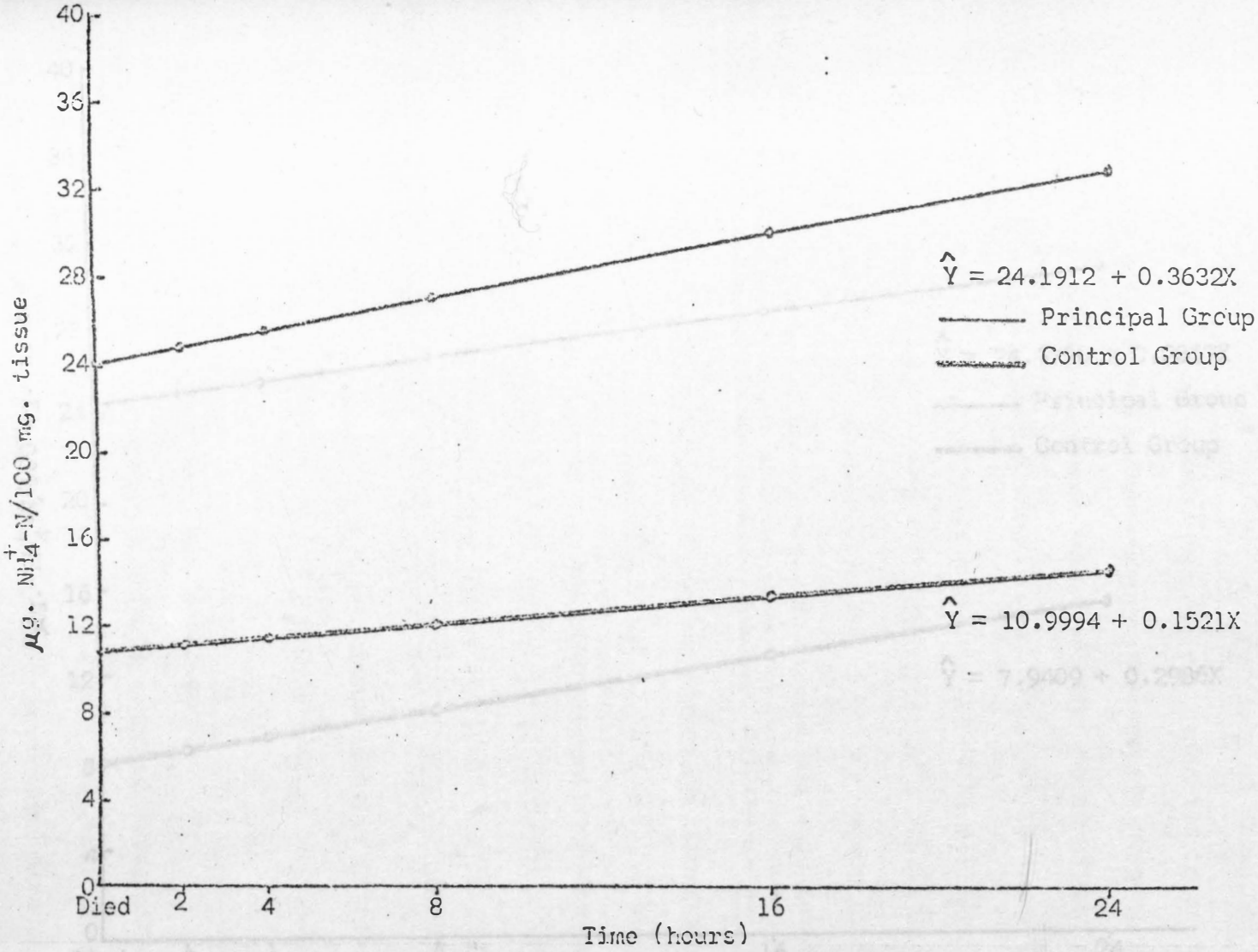

Figure 6. Linear regression of liver ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

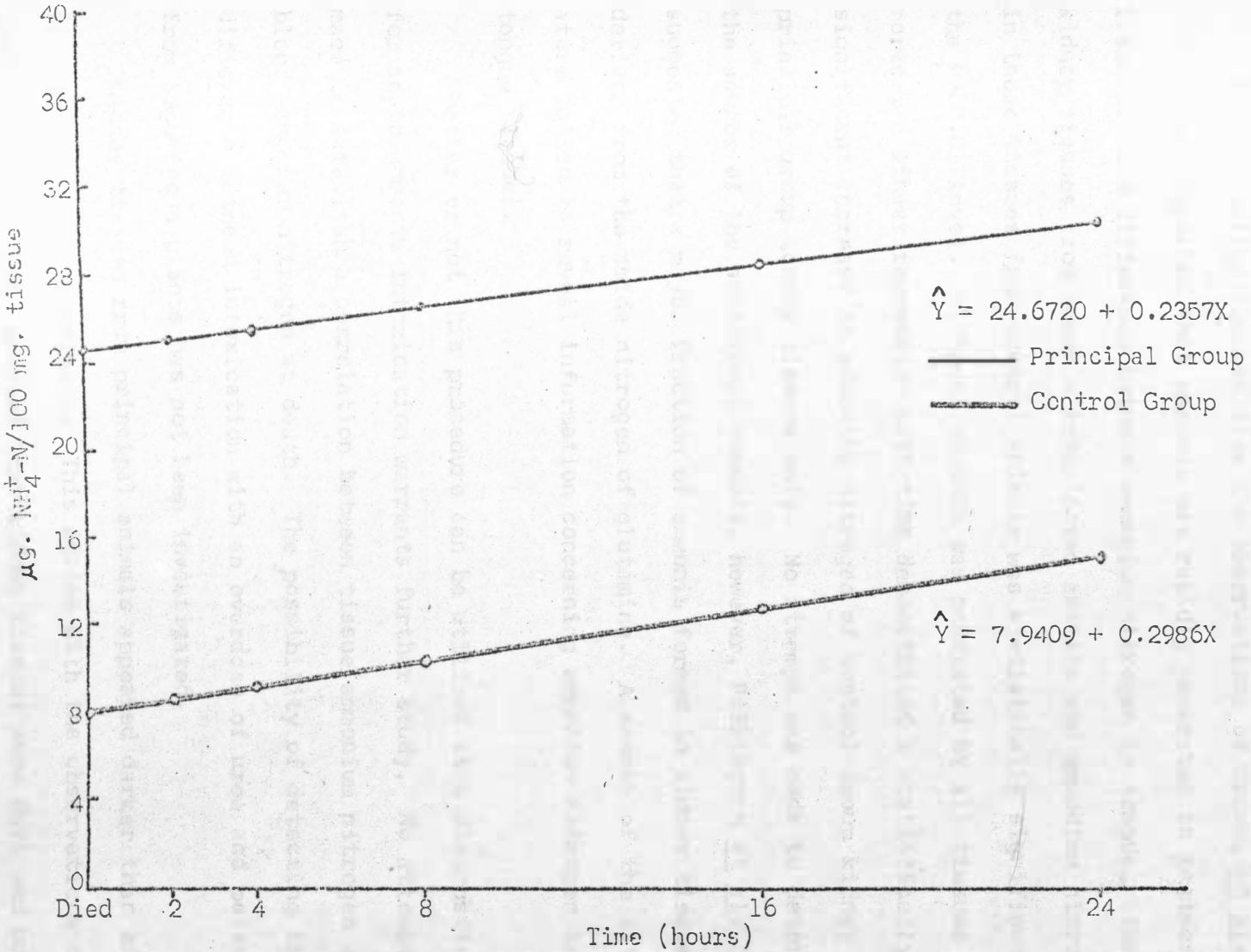

Figure 7. Linear regression of kidney ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

DISCUSSION

This investigation verifies the observations of Brown, et al. (1957), who reported that ammonia was rapidly generated in postmortem tissues. The difference between ammonium nitrogen in tongue, liver and kidney tissues from ammonia intoxicated animals and ammonium nitrogen in those tissues from control animals was statistically significant at the $P < 0.01$ level. Although ammonia was generated by all tissues postmortem, a linear regression with time demonstrated a statistically significant increase in ammonium nitrogen of control group kidney and principal group tongue tissues only. No attempt was made to determine the source of the additional ammonia, however, Pilkington et al. (1964) suggested that a major fraction of ammonia formed in kidney tissues was derived from the amide nitrogen of glutamine. A search of the literature failed to reveal information concerning ammonium nitrogen in tongue tissues.

Whether or not this procedure can be utilized as a diagnostic tool for acute ammonia intoxication warrants further study. No attempt was made to establish a correlation between tissue ammonium nitrogen and blood ammonium nitrogen at death. The possibility of detecting the difference between intoxication with an overdose of urea and poisoning from leguminous plants has not been investigated.

Kidney tissues from principal animals appeared darker than kidney tissues from control animals. This agrees with the observations of Handford (1961), who reported that kidney tissues were dark and congested

in dogs poisoned with urease. Hart, et al. (1939) found kidney degeneration in heifers fed a high urea diet.

The serum ammonium nitrogen values were slightly lower than those reported by Kirkpatrick, et al. (1972), who intoxicated adult sheep with 2 Gm. urea/kg. body weight, and employed an ion exchange method in which Nessler's reagent was used for color development.

The choice of 2.5 Gm. urea/kg. body weight as a toxic dose was based on the work of Kirkpatrick (1971), who administered 2 Gm. urea/kg. body weight to sheep that were fasted for 24 hours prior to the experiment. The increase of 0.5 Gm. urea/kg. body weight was included to compensate for the shorter period of fasting in this study.

Two animals in the principal group were alive 120 minutes after drenching with urea and were electrocuted, while the remaining five principal animals died within 90 minutes after drenching. The blood ammonium nitrogen in the two animals that survived for 120 minutes rose until death, but was lower than in other animals in the principal group at all time periods. These individual differences are expected because of variable quantities of digesta and variable concentrations of urease.

Electrocution with 110 volts proved to be a satisfactory means of killing control animals. One terminal was placed on the lip and the other on the flank. Current was not turned off until muscle relaxation occurred.

Mixing the whole blood, serum and tissue samples for one minute with a Vortex Genie Mixer with a speed control setting of six was adequate to ensure hemolysis of erythrocytes and adsorption of ammonium ions

on the resin. A nearly complete adsorption of ammonium ions was achieved due to the large exchange capacity of the resin (4 meq./Gm.) and its preferential affinity for ammonium ions over sodium and potassium ions.

The variable speed homogenizer was much easier to operate, and produced more uniform homogenates than other methods attempted. Placing the homogenization vessel in a dry ice-acetone bath kept the amount of ammonia lost due to heat of friction to a minimum.

SUMMARY

The ammonium nitrogen concentrations in whole blood and serum and tongue, liver, and kidney tissues of seven wether lambs, intoxicated with 2.5 Gm. urea/kg. of body weight were compared with concentrations in three control animals, drenched with 12.5 ml. of water/kg. of body weight. Ammonium nitrogen values were higher in serum than in whole blood in both principal and control animals, but the difference was not statistically significant at all time periods.

Tissues were collected at death and 2, 4, 8, 16, and 24 hours after death. Although ammonia in principal and control groups increased during the 24-hour collection period, the mean tissue ammonia concentration was greater in the principal group than in the control group at all times. At death, the mean ammonia concentrations (μg. NH_4^+-N/100 mg. tissue) were 8.2 in the control group and 23.2 in the principal group; twenty four hours later, the values were 14.2 and 32.9, respectively. Lines of regression with time demonstrated a significant rise in ammonium nitrogen in kidney tissues of the control group and tongue tissues of the principal group. All tissue ammonium nitrogen differences between principal group and control group were shown to be statistically significant at the $P < 0.01$ level for all time periods.

LITERATURE CITED

Acland, J. D., and Strong, R.: Comparison Between a Microdiffusion and an Ion Exchange Method for the Determination of Plasma Ammonium. J. Clin. Path., 21, (1968): 12-13.

Bessman, S. P., and Bessman, A. N.: The Cerebral and Peripheral Uptake of Ammonia in Liver Disease with an Hypothesis for the Mechanism of Hepatic Coma. J. Clin. Invest., 34, (1955): 622-628.

Bessman, S. P., and Bradley, J. E.: Uptake of Ammonia by Muscle: Its Implications in Ammoniagenic Coma. New England J. Med., 253, (1955): 1143-1147.

Bolleter, W. T., Bushman, C. J., and Tidwell, P. W.: Spectrophotometric Determination of Ammonia as Indophenol. Anal. Chem., 33, (1961): 592-594.

Brown, R. H., Duda, G. D., Korkes, S., and Handler, P.: A Colorimetric Micromethod for Determination of Ammonia; the Ammonia Content of Rat Tissues and Human Plasma. Arch. Biochem. Biophys., 66, (1957): 301-309.

Calkins, W. G.: The Blood Ammonia in Normal Persons. J. Lab. and Clin. Med., 47, (1956): 343-348.

Chaney, A. L., and Marbach, E. P.: Modified Reagents for Determination of Urea and Ammonia. Clin Chem., 8, (1962): 130-132.

Chaykin, S.: Analysis of Nicotinamide Deaminase. Determination of Ammonia by the Indophenol Reaction. Anal. Biochem., 31, (1969): 375-382.

Conn, H. O.: Studies on the Origin and Significance of the Blood Ammonia. I. Effect of Various Anticoagulants on the Blood Ammonia Determination. Yale J. Biol. and Med., 35, (1962): 171.

Conn, H. O., and Kuljian, A. A.: The Preservation of Blood by Rapid Freezing for Subsequent Determination of Blood Ammonia. J. Lab. and Clin. Med., 63, (1964): 1033-1040.

Conway, E. J.: Microdiffusion Analysis and Volumetric Error. 1st ed. Chemical Publishing Co., Inc., New York, N. Y., (1963).

Conway, E. J., and Cook, R.: Blood Ammonia. Biochem. J., 33, (1939): 457-478.

Dienst, S. G.: An Ion Exchange Method for Plasma Ammonia Concentration. J. Lab. and Clin. Med., 58, (1961): 149-155.

Dienst, S. G., and Morris, B.: Plasma Ammonia Determination by Ion Exchange. J. Lab. and Clin. Med., 64, (1964): 495-500.

Dimond, E. G.: Inhibitory Effect of Heparin on Adenylic Deaminase. J. Lab. and Clin. Med., 46, (1955): 807-808.

Faulkner, W. R.: Studies on the Concentration of Blood Ammonium, Description of a Modified Conway Technique. Clin. Chem., 4, (1958): 543-544.

Fenton, J. C., and Williams, A. H.: Improved Method for the Estimation of Plasma Ammonia by Ion Exchange. J. Clin. Path., 21, (1968): 14-8.

Folin, O., and Bell, R. D.: Applications of a New Reagent for the Separation of Ammonia. J. Biol. Chem., 29, (1917): 329-335.

Handford, S. W.: Urease Poisoning in the Dog. Am. J. Physiol., 201, (1961): 71-73.

Hart, E. B., Bohstedt, B., Deobald, H. J., and Wegner, M. I.: The Utilization of Simple Nitrogenous Compounds such as Urea and Ammonium Bicarbonate by Growing Calves. J. Dairy Sci., 22, (1939): 785-798.

Hutchinson, J. H., and Labby, D. H.: New Method for Microdetermination of Blood Ammonia by Use of Cation Exchange Resin. J. Lab. and Clin. Med., 60, (1962): 170-178.

Jacobs, M. H., and Parpart, A. K.: Osmotic Permeability of the Erythrocyte. X. On the permeability of the Erythrocyte to Ammonia and the Ammonium Ion. J. Cellular Comp. Physiol., 11, (1938): 175-192.

Jacquez, J. A., Jeltsch, R., and Hood, M.: The Measurement of Ammonia in Plasma and Blood. J. Lab. and Clin. Med., 53, (1959): 942-954.

Keynes, W. M.: The Distribution of Urea and Ammonia in the Body Fluids and Tissues of the Normal Dog and of Dogs in Renal and in Liver Failure. In: Proceedings of the Physiological Society, Dec. 14-15, 1962. J. Physiol. 166, (1963): 50P.

Kirkpatrick, W. C.: The Effects of Acute Ammonia Toxicity on Certain Hemogram Parameters in Sheep. Unpublished M. S. thesis, South Dakota State University, Brookings, South Dakota, (1971).

Kirkpatrick, W. C., Roller, M. H., and Swanson, R. N.: Serum and Tissue Ammonium Nitrogen and Tissue Water Values in Ammonia-Intoxicated Sheep. Am. J. Vet. Res., 33, (1972): 1187-1190.

Leffler, H. H.: Measurement of Ammonia in Plasma. Am. J. Clin. Path., 48, (1967): 233-236.

Merchant, A. C., Goldberger, R., and Barker, H. G.: The Preservation of Blood for Ammonia Analysis. J. Lab. and Clin. Med., 55, (1960): 790-795.

Miller, G. E., and Rice, J. D.: Determination of the Concentration of Ammonia Nitrogen in Plasma by Means of a Simple Ion Exchange Method. Am. J. Clin. Path., 39, (1963): 97-103.

Nathan, D. G., and Rodkey, F. L.: A Colorimetric Procedure for the Determination of Blood Ammonia. J. Lab. and Clin. Med., 49, (1957): 779-785.

Noble, E. D.: Determination of Trace Kjeldahl Nitrogen in Petroleum Stocks. Anal. Chem., 27, (1955): 1414-1416.

Pilkington, L. A., Preuss, H., and Pitts, R. F.: Origin of Urinary Ammonia. Fed. Proc., 23, (1964): 258.

Preston, R. L.: Comparative Blood Ammonia Procedures in Ruminants. J. Anim. Sci., 29, (1969): 51-56.

Reinhold, J. G., and Chung, C. C.: Formation of Artifactual Ammonia in Blood by Action of Alkali: Its Significance for the Measurement of Blood Ammonia. Clin. Chem., 7, (1961): 54-69.

Roller, M. H., Swanson, R. N., and Lang, M. L.: Blood and Tissue Ammonium-nitrogen Levels and Tissue Water in Pregnant Urea-treated Rabbits. Am. J. Vet. Res., 31, (1970): 327-330.

Rosado, A., Flores, G., Mora, J., and Soberon, G.: Distribution of an Ammonia Load in the Normal Rat. Am. J. Physiol., 203, (1962): 37-42.

Schwartz, A. E., Lawrence, W. L., and Roberts, E.: Elevation of Peripheral Blood Ammonia Following Muscular Exercise. Proc. Soc. Exptl. Biol. and Med., 98, (1958): 548-550.

Seligson, D., and Hirahara, K.: The Measurement of Ammonia in Whole Blood, Erythrocytes, and Plasma. J. Lab. and Clin. Med., 49, (1957): 962-974.

Tashiro, S.: Studies on Alkaligenesis in Tissues. Am. J. Physiol., 60, (1922): 519-543.

Ternberg, J. L., and Hershey, F. B.: Colorimetric Determination of Blood Ammonia. J. Lab. and Clin. Med., 56, (1960): 766-776.

Thompson, H. S.: On the Absorbent Power of Soils. J. Royal Agr. Soc. Engl., 11, (1850): 68. In Kunin, R., and Meyers, R. J.: Ion Exchange Resins. John Wiley and Sons, Inc., New York, N. Y., (1950).

Warren, K. S.: Ammonia Toxicity and pH. Nature, 195, (1962): 47-49.

Wearne, J. T.: Nonspecificity of Hypochlorite-phenol Estimation of Ammonium in Biological Material. Anal. Chem., 35, (1963): 327-329.

White, L. P., Phear, E. A., Summerskill, W. H. J., and Sherlock, S.: Ammonia Tolerance in Liver Disease: Observations based on Catheterization of the Hepatic Veins. J. Clin. Invest., 34, (1955): 158-168.

Wilder, C. E.: Changes in Ammonia Values of Protein Free Filtrates of Blood During Storage in the Frozen State. Clin. Chem., 8, (1962): 593-597.

APPENDIX

Table 3. Whole blood and serum ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

	μg. NH_4^+-N/ml.									
	sheep number									
	Control			Principal						
	1	6	10	2	3	4	5	7	8	9
Whole Blood										
Drenched	1.7	1.5	1.8	2.0	1.7	1.6	1.8	1.6	1.5	2.2
30 minutes	1.8	1.0	1.8	5.7	4.3	3.9	8.3	6.8	5.1	4.9
60 minutes	1.5	1.3	1.7	11.7	9.7	9.2	18.0[a]	16.5[a]	10.7[a]	10.6[a]
90 minutes	1.4[b]	1.2[b]	1.9[b]	35.0[a]	12.6[b]	10.2[b]	----	----	----	----
120 minutes	1.6[b]	1.3[b]	2.4[b]	----	21.2[b]	13.2[b]	----	----	----	----
Serum										
Drenched	6.1	2.9	2.1	6.0	2.5	2.3	5.1	4.7	4.6	4.6
30 minutes	5.7	1.8	1.9	12.0	4.9	4.6	14.1	11.2	15.5	14.6
60 minutes	7.0	1.8	2.3	18.9	11.2	9.6	24.7[a]	15.9[a]	18.1[a]	21.9[a]
90 minutes	7.3	3.4	2.3	37.6[a]	15.2	12.2	----	----	----	----
120 minutes	6.5[b]	2.0[b]	1.7[b]	----	24.0[b]	13.1[b]	----	----	----	----

[a]died

[b]electrocuted

Table 4. Sheep tongue, liver, and kidney ammonium nitrogen. Principal and control animals were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

	μg. NH_4^+-N/100 mg. tissue									
	Sheep number									
	Control			Principal						
	1	6	10	2	3	4	5	7	8	9
Tongue										
Died	3.6	15.4	6.6	24.6	23.4	13.6	29.2	24.1	26.2	17.2
2 hours	13.7	14.7	8.0	23.0	22.6	18.0	27.1	----	33.2	21.2
4 hours	19.8	12.6	12.5	20.7	24.1	24.8	30.2	25.0	39.5	26.2
8 hours	15.3	19.1	16.0	24.3	33.6	21.0	29.3	31.4	30.6	20.4
16 hours	13.8	13.6	13.0	18.0	40.8	28.2	----	28.4	40.4	22.6
24 hours	15.4	12.5	11.8	20.6	42.5	31.0	34.8	----	51.9	32.7
Liver										
Died	7.1	11.1	9.8	28.6	23.3	24.0	19.9	27.4	25.8	19.0
2 hours	15.3	11.6	13.6	----	24.4	25.0	17.7	28.2	29.9	23.1
4 hours	10.9	15.2	8.0	33.6	28.0	27.4	17.0	28.6	33.7	17.8
8 hours	11.4	15.2	11.1	24.6	27.2	26.3	20.9	33.4	36.0	17.0
16 hours	11.0	13.0	13.0	26.2	32.8	33.6	25.9	29.2	36.6	26.0
24 hours	18.1	16.9	10.4	28.4	33.4	42.8	28.7	39.5	33.6	24.3
Kidney										
Died	4.7	9.0	6.5	24.5	24.2	22.3	30.2	24.0	20.8	15.6
2 hours	13.7	7.3	8.6	20.3	23.8	22.9	32.8	29.4	26.6	25.0
4 hours	11.0	8.8	6.2	22.4	23.4	23.9	28.9	27.4	33.3	25.1
8 hours	9.8	12.6	8.8	23.4	----	26.4	40.2	24.2	----	22.0
16 hours	14.0	13.1	14.2	27.4	32.6	21.3	40.0	27.6	27.8	20.6
24 hours	17.2	12.9	13.0	27.6	26.3	27.7	39.9	29.2	36.2	23.8

Table 5. Values of paired t test for whole blood, serum, tongue, liver, and kidney ammonium nitrogen. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

	t values		
	Tongue	Liver	Kidney
Died	3.6592**	6.4613**	5.9741**
2 hours	3.7268**	4.6458**	5.7653**
4 hours	3.1207**	3.5907**	7.2699**
8 hours	3.2236**	3.4466**	3.7423**
16 hours	3.2173**	6.7717**	3.6253**
24 hours	3.8184**	4.3017**	4.4270**

	Principal vs Control				Whole Blood vs Serum			
	Whole Blood	df	Serum	df	Principal	df	Control	df
Drenched	0.6620	8	0.5099	8	4.7582**	12	1.6600	4
30 minutes	4.3504**	8	2.8026*	8	3.0087**	12	1.2206	4
60 minutes	5.2244**	8	3.9695**	8	1.9863*	12	1.3253	4
90 minutes	2.2492*	4	2.1255	4	0.2133	4	1.8507	4
120 minutes	5.1255**	3	3.3449*	3	0.1996	2	1.0295	4

* $P < 0.05$

** $P < 0.01$

Table 6. Analysis of variance for linear regression of ammonium nitrogen with time. Principal and control sheep were drenched with 2.5 Gm. of urea and 12.5 ml. of water/kg. of body weight, respectively.

	Source	df	SS	MS	F
Liver					
Principal	X	1	2932.7364	2932.7364	7.1112
	Residual	4	1649.6386	412.4096	
Control	X	1	587.0722	587.0722	6.7098
	Residual	4	349.9757	87.4939	
Tongue					
Principal	X	1	3171.9702	3171.9702	8.1592*
	Residual	4	1555.0361	388.7590	
Control	X	1	616.3448	616.3448	5.2927
	Residual	4	465.7991	116.4497	
Kidney					
Principal	X	1	2616.7284	2616.7284	6.0904
	Residual	4	1718.5660	429.6415	
Control	X	1	538.5475	538.5475	11.7929*
	Residual	4	182.6678	45.6669	

* $p < 0.05$