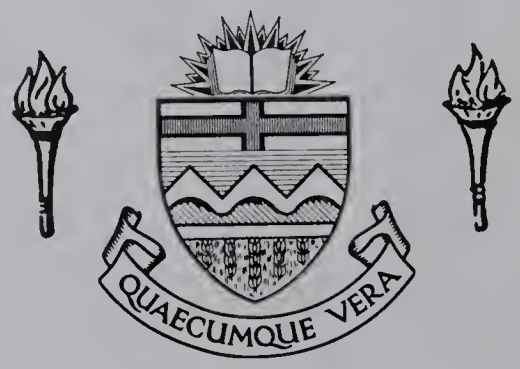

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THE UNIVERSITY OF ALBERTA

TRACER STUDIES OF UREA RECYCLING AND METABOLISM IN THE SHEEP

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Tracer Studies of Urea Recycling and Metabolism in the Sheep" submitted by Alan Lee Ford, B.S.A., in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The amount of urea transferred from the plasma to the digestive tract of fed sheep was quantitated after conditions of metabolic steady state were established. Urea-C^{14} was injected intravenously; the proportion of the injected dose not recovered in the urine represented the proportion of blood urea that was recycled. The quantity of urea recycled was considered to be (proportion of dose collected in urine ÷ urinary urea output) x proportion of dose recycled. Plasma urea concentrations were manipulated by intravenous infusion of unlabelled urea. At elevated plasma urea levels urea excretion was increased. Recycled urea, within the physiological range of plasma urea concentrations, was related linearly to plasma urea concentration. There was no indication of a point of marked change in the disposition of the flux between recycled and excreted urea. Recycling of urea in different animals appeared to follow the same relationship between recycled urea and blood urea concentration. Difference of ration had little effect on this relationship other than through an influence on the plasma urea level, in spite of a marked change in rumen urease specific activity. The linear regression equation of urea recycled (g/d) on plasma urea concentration (mg/100 ml) was $\hat{Y} = .117 + .37\ X$. The standard error of estimate was 2.27.

It was proposed that plasma urea is recycled to the digestive tract of the sheep by diffusion, and that at a threshold of about 45 mg urea/100ml of plasma, additional plasma urea exists in a bound form. As such it does not effectively contribute to a diffusion gradient and is not filtered in the kidney. Therefore plasma urea above a concentration of 45 mg/100 ml of plasma does not effect a linear increase in either recycled or excreted urea in the sheep.

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INTRODUCTION

The concept of an endogenous urea cycle entailing transfer of blood urea to the rumen, and thereby, after hydrolysis, providing a source of ammonia for the rumen population, is relatively recent. Not until the work of Schmidt-Nielsen et al. (1957) was blood urea regarded to have a role other than that of excretion of metabolic nitrogen. This belief had been based primarily on the chemical and physical indifference of the mammalian body to urea (Addis et al., 1947). The work of Schmidt-Nielsen et al. (1957) however, gave evidence indicating that camels, considered to be pseudo-ruminants by Moir (1965), were able to withhold urea, a normal urinary waste product, from urine under varying conditions of protein intake and growth. They indicated that the regulation of urea excretion could be independent of blood urea and thus depend only on the animal's need for protein. The ability to predict the amount of rumen ammonia nitrogen derived from endogenous recycling of urea under varying conditions of protein intake would have value in quantitating the nitrogen interconversions of that animal, especially if recycled urea nitrogen constitutes a significant portion of the nitrogen flux in the animal.

The experiments reported herein were undertaken to develop a method for the estimation of recycled urea and to study some of the factors which might control this recycling.

REVIEW OF LITERATURE

A. Kidney Urea Retention

A review of urea retention by the kidney is appropriate since observation of this phenomenon led to investigation of endogenous urea recycling. In preliminary research with camels (Schmidt-Nielsen et al., 1957), low protein diets were found to reduce the fraction of filtered urea that was excreted. In 1958 Schmidt-Nielsen and Osaki demonstrated the same effect in sheep. They concluded that the regulation of urea excretion was probably independent of blood urea concentration. Schmidt-Nielsen (1958) surmised that since urea clearance varied independently of the glomerular filtration rate, then regulation of urea excretion must be at the tubular level. She further suggested that since the amount of urea formed in the body would be proportional to the nitrogen intake, then kidney urea excretion was also proportional to nitrogen intake. Most important of Schmidt-Nielsen's suggestions (1958) on regulation of kidney urea excretion, was her postulate of a possible counter-current mechanism based on the urea gradient in the kidney tissue water. She proposed that active concentration of urea over a low gradient was possible, followed by a subsequent multiplication by the proposed counter-current multiplier. After some controversy and interspecies comparison (Atherton et al., 1967; Clapp, 1966; Gans, 1966; Lassiter, Mylle, and Gottschalk, 1966; Schmidt-Nielsen, 1962), Morgan and Berliner (1968) presented a currently

accepted concept of the mechanism which does not differ greatly from that originally submitted by Schmidt-Nielsen (1958). The kidney medulla permeability properties fit those required for a counter-current multiplier in the loop of Henle, the active force being a sodium extrusion pump in the ascending limb, with the vasa recta appearing to be the counter-current exchangers. The collecting ducts, although playing no active role in the counter-current system, allow for the change in permeability induced by antidiuretic hormone, which is of importance in allowing fluid within the collecting ducts to equilibrate with the interstitial fluid of the kidney. A change in membrane permeability would hence have an influence on urea reabsorption. The observation that protein depletion may increase collecting duct permeability to urea (Clapp, 1966) may in fact link the above mechanism to the observed apparent active absorption of urea in cases of low protein intake (Berliner and Bennett, 1967; Clapp, 1966; Gans, 1966; Lassiter et al., 1966; Levinsky and Berliner, 1959; Schmidt-Nielsen, 1962; Schmidt-Nielsen and Osaki, 1958; Truninger and Schmidt-Nielsen, 1964).

It would appear that urinary urea excretion is related to protein intake (Waldo, 1968), and that urine flow rate is capable of exerting control over the amount of urea excreted, at least in animals on low protein rations (Gans, 1966; Lassiter et al., 1966; Schmidt-Nielsen, 1958). These parameters, then, should not be overlooked while investigating

endogenous urea recycling.

B. Recycling of Urea to the Rumen

Although Schmidt-Nielsen et al. (1957) indicated retention of urinary urea, it was Houpt, in 1959, who suggested and quantitated the transfer of blood urea nitrogen to the rumen. Following an intravenously injected urea load, he measured the excess over basal urea in the urine. The difference between the injected load and the excess over basal in the urine represented the amount retained by the body. Measurement of accumulated ammonia in a sheep rumen, the contents of which were replaced with saline, together with measurements of ammonia resorption from the rumen gave an estimated rate of urea recycling to the rumen. In 1961, Gartner, Decker and Hill reported that increased blood urea levels did not affect the rate of urea transport into the rumen. Their conclusions formed the basis of the concept of active urea transport into the rumen. Converse to Gartner et al. (1961), Engelhardt and Nickel (1965) considered urea passage into the rumen of sheep and goats to be chiefly by means of passive diffusion. Their conclusions were drawn from observations of urea passage according to a concentration gradient *in vitro*, and to the concentration gradient of body fluids *in vivo*.

Packett and Groves (1965) demonstrated a decrease in the body urea pool of sheep during feeding. Their suggested explanation was that an energy source allows the rumen microbes to utilize the available ammonia, resulting in stimu-

lated ammonia fixation and a net transfer of endogenous urea nitrogen to the rumen. This idea had been alluded to previously by Annison et al. (1954) who suggested that the decrease in rumen ammonia observed when the starch level in a ration is increased had the direct effect of conserving protein. Houpt (1959) also presented evidence which fits this concept. His results show recycling of a lowered fraction of an intravenous (i.v.) urea load when no carbohydrate supplement was added to the ration. Blackburn (1965) proposed that greater nitrogen retention occurred when sheep were fed a carbohydrate supplement due to the lowering of rumen pH, which decreased the rate of ammonia absorption and thereby provided the rumen microflora a greater opportunity to fix it into microbial protein. Gillette (1967) demonstrated similar trends in nitrogen retention when carbohydrate supplementation was supplied. It has not been established conclusively, however, whether the effect of carbohydrates is only to reduce pH and thereby slows ammonia loss from the rumen, or whether an increase in net transfer of endogenous nitrogen occurs due to stimulation of urea transfer to the rumen. Both may well occur, with the two actions being inseparable.

In this regard, Varady et al. (1967), working with i.v. urea loads in starved sheep, presented evidence to suggest that the amount of urea nitrogen entering the rumen from the blood depended on the blood urea level as long as the rumen ammonia concentration was sufficiently low. Above a certain

as yet undetermined rumen ammonia concentration, urea transfer to the rumen was presumably inhibited. Weston and Hogan (1967) also suggested a maximal rumen ammonia concentration, and gave a quantitative measure to it, above which elevated blood urea levels failed to effect a subsequent increase in rumen ammonia concentration. Cocimano and Leng (1967) using varying protein levels and different rations suggested a linear relationship between plasma urea concentrations and urea entry rates into the body pool. Houpt and Houpt (1968) supported this linear relationship in proposing that urea nitrogen is transferred by passive diffusion across the rumen wall according to the concentration gradient. They indicated that the greatest barrier to urea transfer is the outer cornified epithelial layer of the rumen wall. Since bacterial urease could penetrate this layer (Rahman and Decker, 1966) the enhanced urea transfer when rumen fluid was present, compared to saline, could be explained by hydrolysis in the rumen epithelium with subsequent rapid diffusion of the formed carbon dioxide and ammonia.

The current thinking in regards to urea transfer to the rumen is, accordingly, somewhat inconclusive. Some workers (Cocimano and Leng, 1967; Engelhardt and Nickel, 1965; Houpt and Houpt, 1968) advance a simple diffusion mechanism dependent on a blood to rumen urea gradient for urea recycling. Others (Gartner et al., 1961) propose active urea transport independent of blood urea concentration. Still

others (Varady et al., 1967; Weston and Hogan, 1967) support the idea that rumen ammonia concentration plays a role in the control of urea transport to the rumen. Other factors possibly affecting urea recycling include the effect of dietary carbohydrate (Gillette, 1967; Houpt, 1959; Packett and Groves, 1965) and different rumen ureolytic activities (Caffrey et al., 1967; Houpt and Houpt, 1968). The evidence related to the control of urea recycling has been, however, somewhat confused by the wide variety of ration compositions and feeding regimes employed in these studies.

Although urea recycling by direct transfer from the blood to the rumen is quantitatively of most importance, other mechanisms do exist. Even though McDonald in 1952 described the importance of saliva as a vehicle for nitrogen transfer to the rumen, it was Hirose et al. (1960) who gave a quantitative estimate of the amount of nitrogen per day entering the rumen by means of saliva in young steers. Kay (1960) worked with sheep parotid glands and gave a wide range of estimated saliva flow rates. Somers' extensive research (1961a, b, c, d) with ruminant salivary glands demonstrated similar composition patterns for mixed salivaand for parotid saliva. As well as presenting salivary urea and nitrogen concentrations and flow rates, he showed that increased blood urea concentrations increased the salivary urea concentration. Because the relationship was not linear, he suggested that the mechanism of transfer of blood urea to saliva was other than passive diffusion.

Somers did not intend however to suggest an active transport system in the salivary glands, since salivary urea concentration never exceeds that in the blood. Packett and Groves (1965) reported that salivary urea levels reflected those of the blood, thus leaving doubt as to the exact nature of the transfer of urea to saliva from the blood. The fact that saliva does provide a vehicle whereby urea is recycled from blood to the gastro-intestinal tract does, however, remain uncontended. Quantitatively, however, salivary nitrogen appears less important than urea transported directly from the blood across the wall of the digestive tract. A comparison of Somers' estimate (1961a) of salivary nitrogen contribution with Houpt's estimates (Houpt, 1959; Houpt and Houpt, 1968) of direct transport of blood urea to the rumen indicates that direct transport could supply two to three times as much endogenous nitrogen to the digestive tract as does the saliva. Waldo (1968), however, suggests that probably four to six times as much urea nitrogen is transported to the rumen by direct transfer as by the salivary route.

C. Rumen Urease Activity

Rumen urease activity is commonly accepted to be of microbial origin, although investigators presently cannot conclusively identify the microbe, or microbes, responsible for its presence. Of the obligate anaerobic bacteria, conspicuously few display urease activity. Gibbsons and Doetsch (1959) were able to isolate Lactobacillus bifidus from bovine rumen

contents and identify it as a constitutive producer of urease. However, due to its presence in such low numbers, it was not thought capable of manifesting the urease activity displayed by the rumen microflora. In 1962 Blackburn and Hobson succeeded in isolating the same anaerobic bacterium, Lactobacillus bifidus, from rumen contents, while Abou Akkada and Blackburn (1963) were unable to isolate any bacteria with significant urease activity. These latter workers summarized by stating that although urea is rapidly hydrolyzed in the rumen, urease activity cannot yet be associated with the more common rumen micro-organisms. Blackburn (1964) and Jones et al. (1964a) were also able to isolate only the one strain of ureolytic bacteria. Jones et al. (1964a), as had Pearson and Smith (1943) previously, ascertained that bacterial urease was intracellular. Clifford, Bourdette and Tillman (1968) indirectly contradicted these results by finding that centrifugation, even at speeds that would certainly precipitate bacteria, had relatively little effect on the ureolytic activity of strained rumen fluid. These findings intuitively suggest that the urease enzyme is extracellular. Rahman and Decker (1966) and Seneca, Peer and Nally (1962) were, however, unable to obtain enzyme preparations free from bacteria, hence leaving the question of the existence of intracellular or extracellular urease unanswered.

The validity of the concept of the bacterial origin of urease is not above challenge. Rahman and Decker in 1966

were intrigued with the observation that ruminal muscosal tissue appeared to have higher urease activity than could be conveniently explained by rumen bacterial contamination. Their evaluation of the kinetics of the enzyme activity in this tissue did, however, support the proposal of urease activity originating from bacterial sources. This research agrees with that of Levenson et al. (1959) and Kornberg, Davies and Wood (1954) indicating that in the rat and cat gastric urease is of bacterial origin. Kornberg and Davies (1955) demonstrated an absence of urease activity in mammalian tissues other than the gut.

In investigating the constitutive or inducible nature of bacterial urease in the rumen, Caffrey et al. (1967) found ruminal ureolytic activity to be higher in lambs adapted to a non-urea diet than in those fed rations containing urea. Clifford et al. (1968), on the other hand, despite a discrepancy in their data suggested the urease activity in rumen contents might well be independent of the feeding regime. The evidence, nevertheless, substantiates the idea of the potential for bacterial degradation of urea in the rumen, though it is not at all clear whether this ureolytic potential is constant, or whether it fluctuates with changes in feeding regimes and microbial populations.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

A. General Outline

The experiments reported in this thesis were conducted through 1968 and 1969. Experiment 1 involved determination of the amount of urea being recycled from the blood to the digestive tract of sheep in a steady fed state. In Experiment 2, the urease activity of the rumen fluid of a sheep fed two different rations was measured.

1. Automatic Continual Feeder

a) Description

In order to achieve a steady-state nutritional status, a continual feeder was designed and constructed as shown in Figure 1. The feeder was constructed to attach to the top of a 30 by 24 by 54 inches feeding crate elevated 14 inches above the floor. The framework of the feeder was constructed of 1 3/8 by 1 3/8 by .070 inches Dexion 140 slotted angle iron[1] and wood. A light weight canvas, 160 inches long by 9 3/4 inches wide and sewn into a continuous band, served as the delivery conveyor belt. Two rubber ringer washing machine rollers, 1 5/8 inches in diameter and 11 1/2 inches wide, were secured by Oilite pillow blocks[2] at either end of the belt, keeping it taut. At the delivery end of the conveyor belt, a large diameter funnel was attached to the frame and

[1] Redirack Industries Limited, Weston, Ont.

[2] Commercial Bearing Service Ltd., Edmonton, Alta.

positioned to receive the feed as it left the belt. Flexible tubing, 4 inches in diameter, connected the funnel to the manger of the crate. A 19 inch diameter light weight compressed metal pulley was fastened to the rubber drive roller. A "jack-shaft", secured in place by pillow blocks, had a 19 inch diameter pulley and a 1 5/8 inch diameter steel pulley affixed. A rotisserie motor (120 v, 0.3 amp., 6 rpm) from an electric range served to drive the apparatus. The drive shaft, with one end secured by a pillow block, also had a 1 5/8 inch diameter pulley attached. A 65 inch, A type, V belt connected the drive shaft pulley to the larger "jack-shaft" pulley. Another 65 inch V belt connected the smaller "jack-shaft" pulley to the drive roller pulley. A percentage timer clock-switch[3] (Paragon type PW 10) was wired into the electrical circuit of the rotisserie motor.

b) Operation

The PW 10 type timer operates on a 10 minute time cycle and can be adjusted to operate the drive motor for any percentage of the 10 minute cycle. A setting of 22% ON, 78% OFF rotated the conveyor belt one-half revolution in 24 hours. With this scheme it was possible to load the feeder by evenly distributing the daily ration over the length of the conveyor belt and have it delivered continually during the following 24 hour interval.

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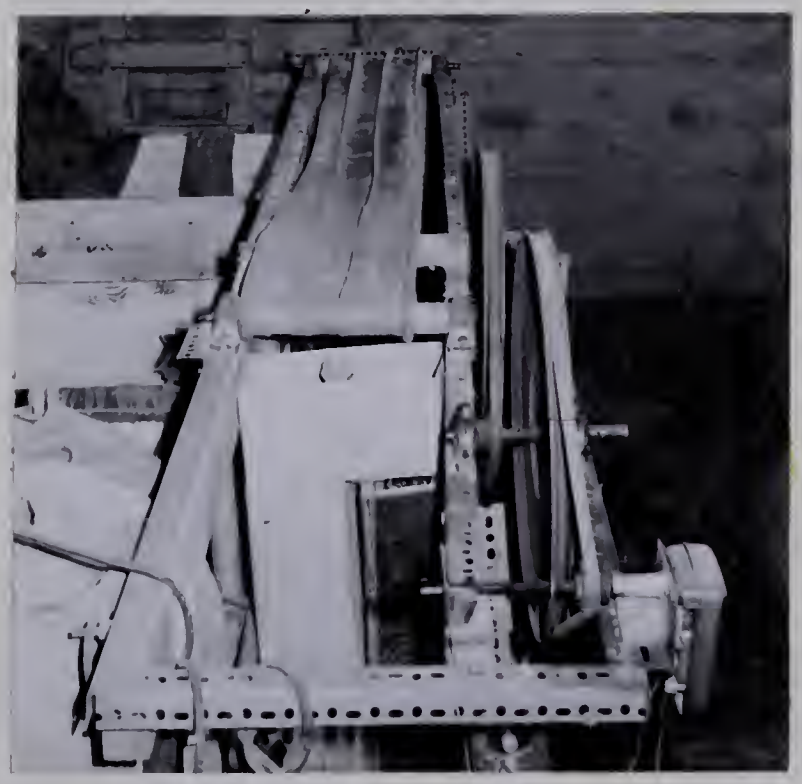
[3] Alberta Electrical Supplies Ltd., Edmonton, Alta.

Figure 1a. Continual Feeder Showing Delivery Belt, Delivery Funnel and Tube, and Pulley Drive System

Figure 1b. Rear View of Continual Feeder

Figure 1c. Continual Feeder Adapted to Deliver Chopped Roughage.

Figure 1d. Modified Continual Feeder Designed to Feed Three Animals Simultaneously



Both the pelleted hay and the rolled barley rations were fed routinely in these experiments with this type of unit. The feeding of coarsely chopped roughage rations on another unit required the modification of the delivery funnel to form a chute, thus allowing the roughage to fall directly into the manger (Figure 1c). A third continual feeder fed three sheep simultaneously when the canvas conveyor belt was sewn with four folds along its length, forming three longitudinal channels (Figure 1d). The ration for each sheep was placed on the belt, and with appropriate modification of the delivery funnel, the sheep were continually fed their separate rations.

2. Water Infusion

In order to achieve a steady state fluid balance, water was infused continuously via a rumen fistula using a Harvard[4] reciprocal infusion/withdrawal pump, model 950.

3. Statistical Analysis

The statistical analysis used in the interpretation of the data are described by Steel and Torrie (1960).

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[4] Harvard Apparatus Co. Inc., Willis, Mass.

B. Experiment 1. Urea Recycling in the Sheep.

1. Objectives and Introduction

The concept of nitrogen recycling to the rumen via blood urea is well established (Cocimano and Leng, 1967; Engelhardt and Nickel, 1965; Gartner, Decker and Hill, 1961; Houpt, 1959; Houpt and Houpt, 1968; Packett and Groves, 1965; Varady et al., 1967). Less well established, however, are the factors involved in the control of urea recycling. At present, it is difficult to attempt to define the factors controlling urea recycling because of the variety of conditions that were existant in experiments reported in the literature, ranging from extreme starvation, low protein diets, and continual feeding, to anesthetized animals and *in vitro* environment. The object in this experiment, after standardizing conditions as much as possible, was to determine the extent to which plasma urea concentration influences the amount of urea recycled from the blood to the digestive tract, and to measure the influence of a drastic change in ration on urea recycling independently of influences on the plasma urea concentration.

The original thesis of this investigation was to calculate the amount of urea recycled under standard conditions using three different procedures. Firstly, the body urea pool of a sheep was labelled with a single trace dose of urea-C^{14}. With the urea pool labelled, the urea recycled to the digestive tract and hydrolyzed by bacterial urease would

give rise to $C^{14}O_2$. It was assumed that the label so released would then mix with the body carbonate pool. If relatively little CO_2 were fixed in the rumen or in body tissues, the labelled CO_2 would emerge, after a time lag, in the respired air. A CO_2 trapping system was adapted making use of a ventilated hood, as described by Webster and Hicks (1968), and the continual feeder described previously in the General Outline. The respired air was pumped through wet gas meters to determine the air flow rate and an aliquot was drawn at a known rate through a dry ice moisture trap and then bubbled through an ethanolamine: ethylene glycol monomethyl ether (1:2v/v) mixture (Jeffay and Alvarez, 1961) to trap the CO_2 as ethanolamine carbonate. Using the procedure of Jeffay and Alvarez (1961), the total amount of label in the aliquot could be measured, thereby allowing estimation of total respired C^{14}. The proportion of injected C^{14} appearing in the respired air would then indicate the proportion of blood urea hydrolyzed in the digestive tract.

In a preliminary trial 13% of the injected C^{14} was recovered in the respired air, indicating recycling of a similar fraction of the body urea pool. Since previous reports suggest from 22% to 90% of the body urea pool is recycled to the alimentary tract of sheep (Cocimano and Leng, 1967; Houpt, 1959), the procedure was regarded to contain either serious technical problems, or a faulty theoretical approach. Invalidation of the approach was indicated by the report of

Farlin, Brown and Garrigus (1968) when they suggested that in the rumen the carbon from urea does not equilibrate with the CO_2 pool. These investigators recovered essentially 100% of the C^{14} from labelled bicarbonate added to the rumen, but less than 50% of the C^{14} from urea-C^{14}. Estimation of the amount of recycled urea by means of quantitative recovery of respired $C^{14}O_2$ was therefore abandoned.

The second method engaged to measure urea recycling to the digestive tract from blood urea followed the concept of Rittenberg and Foster (1940) and was similar in theory to that of Cocimano and Leng (1967) and of Regoeczi et al. (1965). A tracer dose of urea-C^{14} was injected to uniformly label the body urea pool. Urine samples were collected at intervals and the specific activity of the urinary urea was determined in order to estimate pool size, turnover rate and turnover time according to first order kinetic principles (Cook, 1966; Zilversmit, 1960). It was presumed that since urinary urea is derived from plasma urea, urinary urea specific activity would reflect, following a time lag for kidney filtration, that of the body urea pool.

Problems inherent in the single injection isotope dilution technique were, however, encountered. The body urea turnover constant is obtained as the slope of the line relating natural logarithm of urinary specific activity to time. The body urea production rate is estimated as the product of this slope times the pool size, pool size having

been found by extrapolation to time zero. By subtracting the rate of urinary excretion from the production rate, it is possible to obtain the rate of urea metabolism, a measure of urea recycling in the animal. It was found that small differences in fitting of the best line, although not greatly altering natural logarithm of specific activity at time zero, would change the actual specific activity by appreciable amounts (Cocimano and Leng, 1967). Hence the estimate of the pool size would have large fluctuations as consequences of rather small changes in the slope of the line. Preliminary trials indicated up to three-fold differences in pool size for the same sheep under identical conditions. Since this magnitude of fluctuation was not considered reasonable, the technique was discontinued.

The method eventually accepted involved labelling the body urea pool with a single injection of urea-C^{14} and total collection of the urine. The fraction of the injected label collected as urinary urea was equated to the portion of blood urea excreted. It was assumed that the portion of blood urea not leaving by way of the urine was recycled to the digestive tract. A wider treatment of the method is given in the next section.

2. Experimental

a) General procedure

A standard set of conditions approaching steady fed state under continuous lighting and a constant temperature of

20 C was established by means of the continuous feeder and water infusion equipment described in the General Outline. Once steady state was achieved, the body urea pool must also be in a steady state, where urea influx to the body pool equals efflux. Therefore two major possible exits from the urea pool would be urea excretion in the urine and urea transfer from the plasma into the digestive tract. Urea losses from the body pool could also occur via other routes, such as sweat and nasal mucus, but these pathways will be quantitatively insignificant.

It was reasoned that under steady state conditions, if the urea pool was labelled, and all of the label that was excreted in the urine was collected, then the difference between the amount of label injected and the amount of label collected in the urine would equal the amount of label that left the plasma and entered the digestive tract. If the amount of urea that was excreted over the same period of time was also measured, by equating the amount of urinary urea collected to the percentage of injected label recovered in the urine, the amount of urea entering the digestive tract could be estimated. The equation so used was:

$$R = \frac{1 - L}{L} \times U$$

where R is the g urea recycled per day, U is g urea excreted in the urine per day, and L is the fraction of injected label

recovered in the urine.

The trials of Experiment 1 were designed using the above model. Trace amounts of urea-C^{14} [5] were injected intravenously into sheep maintained in a steady state with respect to ration intake, water infusion, lighting and ambient temperature. It was found that 85% of the label recovered in the urine was excreted in the first 12 hours following injection of the tracer, and that 98% was excreted in the first 24 hours. In order to be completely confident of total collection of the label and also to provide a constant time period on which to base urea excretion rates, a 48 hour trial period was chosen. Total urine collection was made during each trial period. Venous blood samples were collected intermittently throughout each period. Blood urea concentrations were depressed by increasing the amount of water infused intraruminally, and were elevated by unlabelled i.v. urea infusions.

Trials were conducted using two mature Southdown sheep. The ewe received 725 g per day of a pelleted 9.52% protein hay (dry matter basis). The ram received 920 g per day of the pelleted hay for the first trial and 1000 g per day for the rest of the trials, excepting two. In these two trials results were obtained from the ram while being maintained on 500 g per day of a rolled 11.3% protein (dry matter basis) barley ration.

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[5] The Radiochemical Center, Amersham, England.

Block salt (0.015% iodine, 0.01% cobalt) was provided ad libitum. Each animal was provided with mineral mix (steamed bone meal and dicalcium phosphate, 1:3). Periodically 2 cc of vitamin suspension[6] (Duravite injectable: Vit. A 500,000 IU per cc, Vit. D 75,000 IU per cc, Vit. E 50 IU per cc) was injected intramuscularly.

The Kjeldahl method (AOAC, 1965) was followed for determination of crude protein in feed samples and for estimation of total urinary nitrogen.

b) Surgical treatment

Rumen fistulae were prepared under Nembutal anesthesia (Pentobarbital Sodium, USP, 60 mg per ml) in both sheep to allow infusion of water directly into the rumen.

Jugular catheters were prepared by means of external catheterization for Trials 10 to 26 inclusive, using a 13 gauge hypodermic needle and Intramedic[7] Polyethylene tubing (PE-90/S 36"). In Trials 28 and 29 and 30 to 32 it was necessary to surgically prepare a semi-permanent catheter in the jugular vein while the sheep was under Nembutal anesthesia. In Trials 33 and 34 the right hind femoral vein was catheterized by means of a lateral incision between the Vastus lateralis and the Biceps femoris muscles. Again, the anesthetic employed was Nembutal. Surgical placement of fistulae and

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[6] Ayerst Laboratories, Montreal, Que.

[7] Clay-Adams Inc., New York, U.S.A.

catheters were routinely followed by a 5 cc injection of penicillin (200,000 IU per cc Procaine Penicillin G; 0.25 g Dihydrostreptomycin as sulfate). The incision was dusted with sulfanilamide powder and sutures were removed six to eight days following the operation.

c) Intravenous urea infusion

In Trials 21 to 26 and 28 to 31 inclusive, plasma urea levels were elevated by infusion of 25.7 g per 100 ml and 12.9 g per 100 ml urea solutions respectively. A Harvard reciprocal infusion/withdrawal pump, model 600-950 v, with variable speed adjustment, infusing at a rate of .076 ml per minute was employed. The daily volume of solution infused was 109 ml, or 28.2 g of urea per day for Trials 21 to 26 inclusive, and 14.1 g of urea per day for Trials 28 to 31 inclusive.

d) Injection of label

The tracer solution of urea-C^{14} was made up in 0.15 M NaCl such that the activity was between 100,000 and 1,000,000 count/min per ml. A volume of 10.00 ml was injected through the jugular catheter over a period of less than 30 seconds and flushed immediately with 10 to 20 ml of 0.15 M NaCl. Exceptions were Trial 11 in which 9.50 ml was injected through the front femoral vein, and Trial 26 in which 9.90 ml was injected through the jugular catheter. In Trials 33 and 34 the tracer dose was injected through the femoral catheter.

e) Collection of blood

Five ml blood samples were obtained from the jugular catheter after first flushing the catheter and discarding the first 3 to 4 ml of blood obtained. The blood sample was collected in a glass syringe which had previously been rinsed with heparin (heparin sodium injection, 1000 USP units per cc). In Trial 10 the ewe was prepared with two jugular catheters. This procedure was abandoned when it became impossible to catheterize one of the jugular veins. In Trials 21, 22 and 23 the ram was prepared with a catheter in each jugular vein; one for infusion of urea solution, the other for collection of blood samples. This practice was also discontinued for the same reason as above. In Trials 24 to 31 inclusive, blood was taken from the catheter through which the urea solution was infused. Consequently, extreme care was exercised while taking blood, and samples were obtained after first withdrawing and injecting blood and heparinized 0.15 M NaCl six to ten times to ensure that the blood sample was not contaminated by residual urea in the catheter.

The final blood sample of Trial 34 was taken from the front right brachial vein because the femoral catheter had become non-functional.

f) Collection of urine

i) female

A urine collection device, shown in Figure 2a, was designed and constructed so that continuous urine collection

could be achieved on a female sheep. The framework was made from a Nalgene 5 oz funnel, cut and molded while hot to form the appropriate shape. The lining was a rubber condom modified by attaching the bottom to rubber tubing with rubber cement. The collection device was fixed into place by wiring the Nalgene frame to the wool. The rubber condom expanded during urination, thereby providing a reservoir for the urine until gravity flow carried the urine to the collection flask.

ii) male

The male urine collection device is shown in Figure 2b. It was prepared by molding two sheet metal templates in the shapes of shallow funnels and heating uncured rubber[8], 3 1/16 inch thick, between the two clamped templates at 150 C for one hour. A metal spout was heated into the base of the funnel such that rubber tubing could be attached. A wire rim was heated into the funnel to give it rigidity and at the same time pliancy. The device was fixed into position by means of two elastic girth straps. The under side of the abdomen was kept shorn and clean, as was the sheath, to prevent contamination of the urine sample.

Throughout Trials 10 to 19 the urine was collected by gravity flow. During Trials 20 to 34 an electric vacuum pump[9] (115 v, 0.7 amp, 1700 rpm) was employed to collect the urine under aspiration. In Trials 10 to 17 inclusive, ice

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[8] Continental Rubber Co., Edmonton, Alta.

[9] Fisher Scientific Co., Edmonton, Alta.

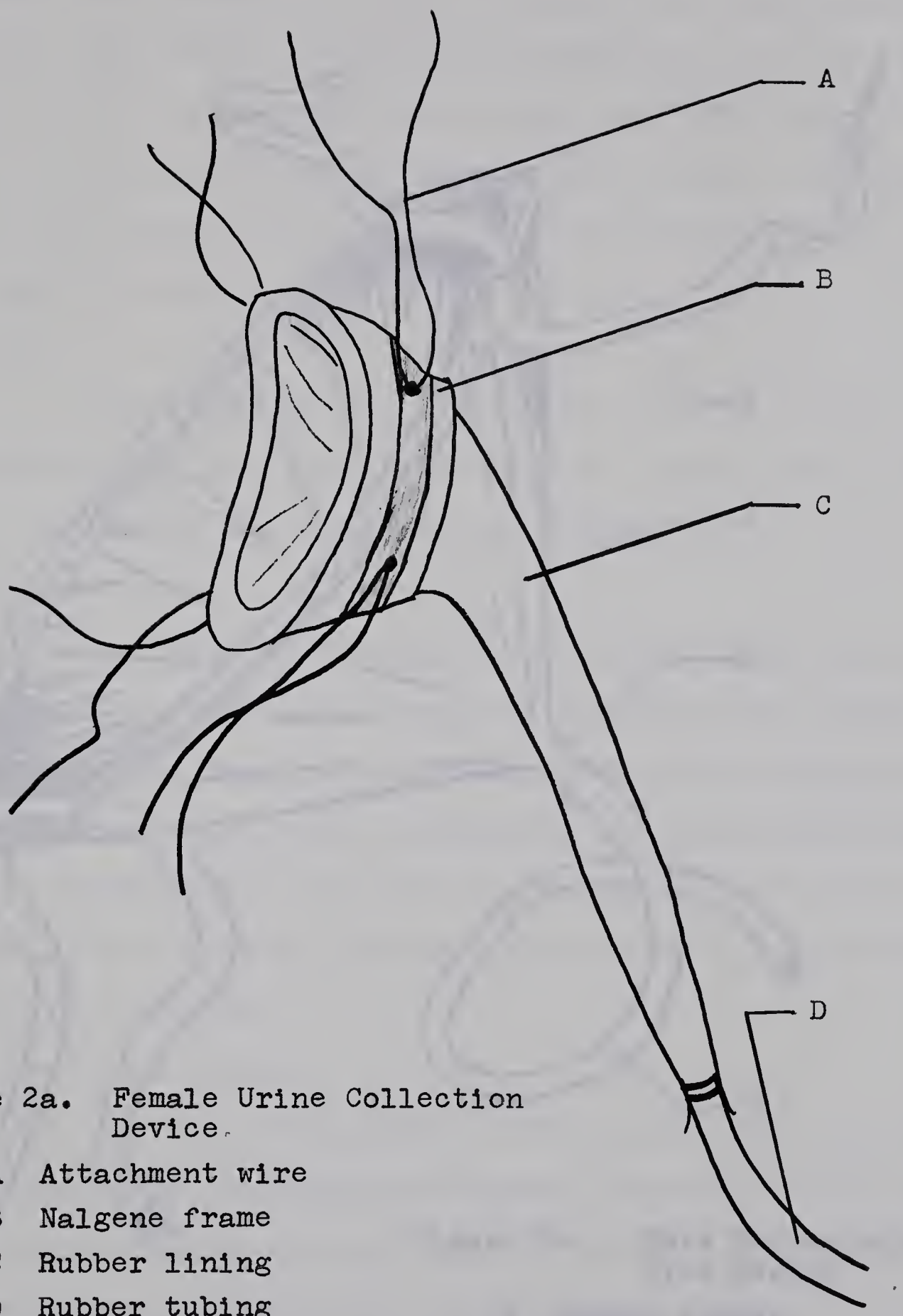

Figure 2a. Female Urine Collection Device.

A Attachment wire

B Nalgene frame

C Rubber lining

D Rubber tubing

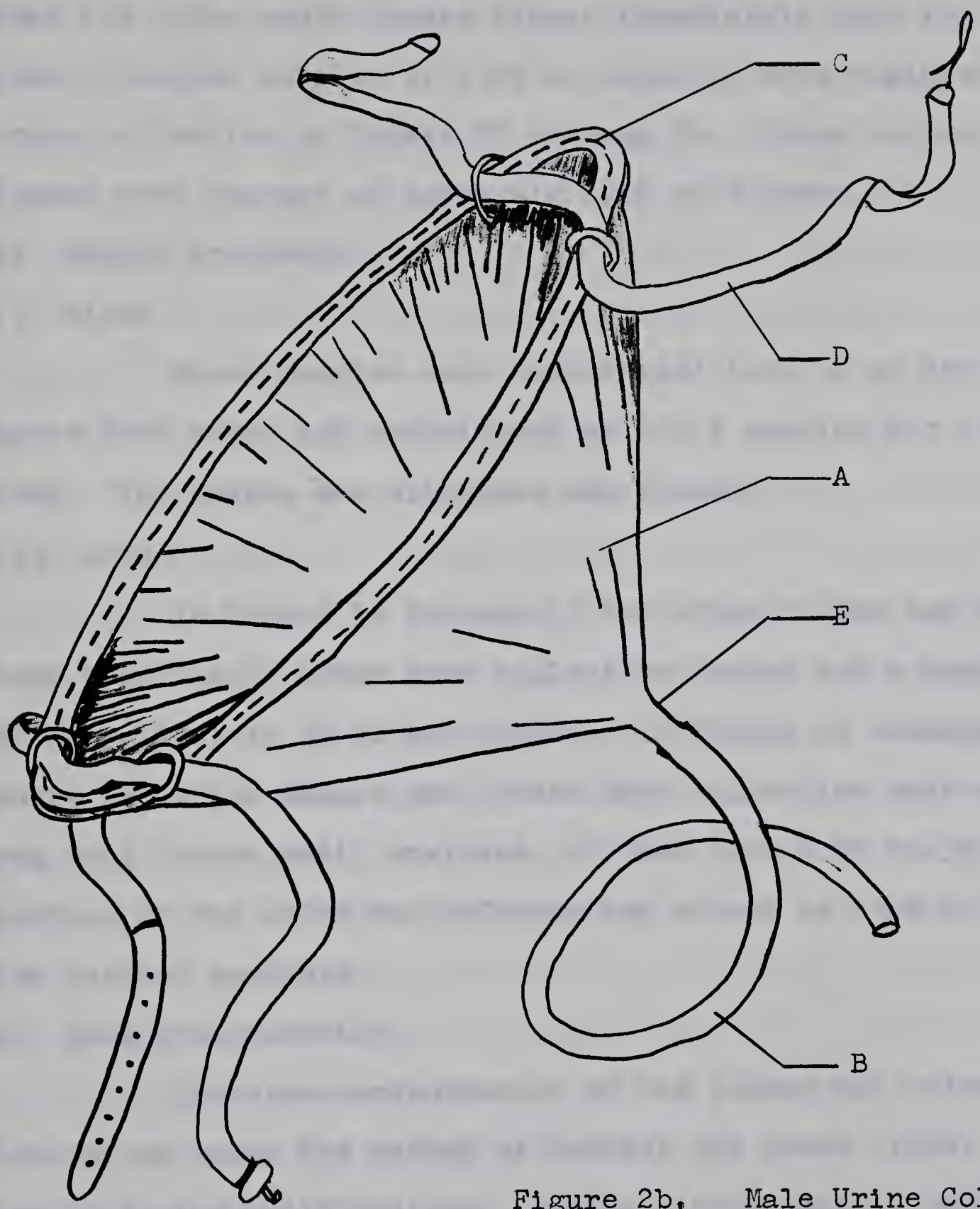

Figure 2b. Male Urine Collection Device

A Rubber Funnel

B Rubber Tubing

C Wire rim enclosed in rubber

D Girth Straps

E Metal Spout

was used to cool the Erlenmeyer urine collections flasks. For Trials 19 through 34 a dry ice bucket was constructed so that the urine would freeze almost immediately upon its collection. Nalgene bottles of 1500 ml capacity were employed for urine collection in Trials 20 through 34. Urine collection flasks were changed at intervals of 4 to 6 hours.

g) Sample treatment

i) blood

Blood samples were transferred into 12 by 200 mm pyrex test tubes and centrifuged at 900 x gravity for 20 minutes. The plasma was withdrawn and frozen.

ii) urine

In Trials 10 through 17 the urine volume was measured immediately after each collection period and a sample of approximately 50 ml was frozen. In Trials 19 through 34 where the urine sample was frozen upon collection each sample was kept frozen until analyzed; at that time a 20 to 30 ml portion of the urine was refrozen and stored in case of need for further analysis.

h) Urea determination

The urea concentration of the plasma and urine was determined using the method of Fawcett and Scott (1960) with the following modifications. Sodium nitroprusside, NaOCl, and buffered urease solution were freshly prepared prior to each urea determination sequence. The urease preparation used was Worthington precrystalline urease[10] (URPC, 120 units/mg).

[10] Worthington Biochemical Corporation, Freehold, New Jersey.

Urease incubation was carried out at room temperature for at least 20 minutes. All water used in the preparation of reagents, in dilutions and in the washing of glassware was demineralized by passing distilled water through a Bantam[11] Demineralizer column (standard cartridge). Plasma was diluted 1:100 and 1:200 depending on urea concentration, while urine was diluted 1:2000 and 1:4000, as required. Standards were included in each determination sequence. All samples were analyzed in duplicate.

i) C^{14} Measurement

The scintillation medium of Jeffay and Alvarez (1961) was employed. A urine volume of 0.20 ml was added to 15 ml of the medium in a scintillation vial. Preliminary investigations revealed that a negligible portion of the C^{14} in the urine, following urea-C^{14} administration, was present as bicarbonate or carbonate. After mixing, the samples were counted in a Nuclear Chicago Mark I Liquid Scintillation System at 10 C for four minutes; each sample was counted four times. All samples were analyzed in duplicate. A 0.10 ml volume of tracer solution from each trial was counted in 15 ml of scintillation medium. Tracer solution specific activity was determined in triplicate within two hours of the beginning of each trial. Counting efficiency was determined by the channels ratio method (Bruno and Christian, 1961; Hendler, 1964).

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[11] Barnstead Still and Sterilizer Co., Boston, Mass.

3. Results and Discussion

Body weights of the sheep remained constant at 34 and 42 kg, respectively, for the ewe and the ram during the course of the experiment. Table I provides an outline of the treatments involved in each trial. Several trials are not reported because of technical difficulties encountered during their conduct. In Trials 11, 18 and 27, complete urine collection was not achieved. The rumen fistula came out during Trial 22 and the resulting loss of rumen contents upset the steady state. An irregularity was discovered in the specific activity of the injected urea-C^{14} in Trial 24. The sheep became sick during Trial 14, and Trial 15 was started before the animal had been fully trained. A typical pattern of C^{14} recovery in the urine is demonstrated in Figure 3, where the specific activity of urinary urea for Trial 19 is plotted against time. This indicates the completeness of C^{14} recovery at 48 hours.

Table II displays a summary of the experimental results. Data listed under the column headed Urea Flux is the sum of urea excreted plus urea recycled. This represents that amount of urea passing daily through the body pool. Plasma urea concentration was constant within treatment groups. Although Cocimano and Leng (1967) have suggested that the rate of urea excretion is governed by the urine flow rate, it can be seen in Table II that urine flow was fairly uniform while urea excretion varied. It would appear

TABLE I. Trial Treatments for Urea Recycling Experiment

Trial No.	Sheep	Ration	Quantity Eaten g/day	Water Infused ml/day	Urea Infused g/day
10, 12, 13	ewe	hay	725	1570	--
16	ram	hay	920	1570	--
17, 19, 20	ram	hay	1000	1570	--
21, 23, 26	ram	hay	1000	1570	28.2
28, 29, 30, 31	ram	hay	1000	1570	14.1
32	ram	hay	1000	3100	--
33, 34	ram	barley	500	1570	--

TABLE II. Summary of Urea Recycling Data

Trial No.	Plasma Urea mg/100 ml	Urine Urea mg/100 ml	Urine Flow ml/d	Urea Excretion g/d	% Label Recovered	Urea Recycled g/d	Urea Flux g/d
10	21.6	1495	620	9.27	56.7	7.07	16.34
12	18.6	1017	1101	11.2	49.9	11.25	22.45
13	18.3	945	966	9.04	58.6	6.41	15.44
16	33.6	1520	932	14.2	52.4	12.9	27.1
17	30.9	1558	814	11.75	56.9	8.91	20.67
19	33.7	1475	642	10.10	48.4	10.77	20.88
20	25.3	1425	949	13.52	58.3	9.66	23.18
21	62	3880	838	32.51	60.3	21.38	53.89
23	62.2	4087	806	32.92	63.1	19.28	52.2
26	60.2	5544	571	31.63	61.2	20.09	51.73
28	40.6	2659	1005	26.71	65.3	14.22	40.93
29	39	2890	892	25.78	59.6	17.5	43.29
30	39.1	2658	873	23.19	57.4	17.22	40.42
31	42.9	3363	804	27.03	62.7	16.09	43.12
32	15.8	426	2619	11.16	57.2	8.33	19.49
33	21.7	570	1882	10.73	65.3	5.70	16.43
34	21.8	1400	1116	15.62	71.6	6.19	21.81

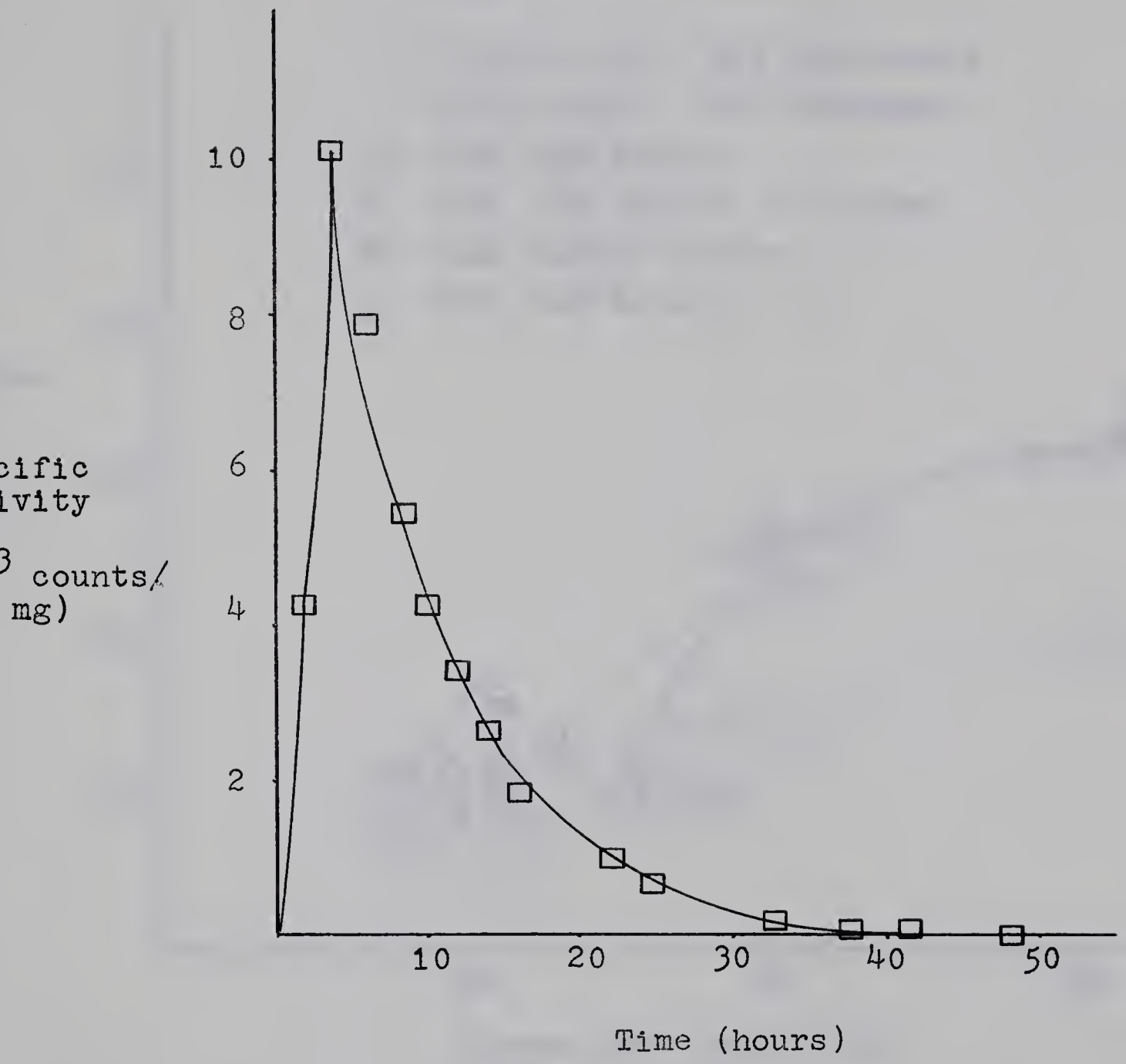


Figure 3. Label Recovery Pattern in Urine-Plot of Urinary Urea Specific Activity versus Time.

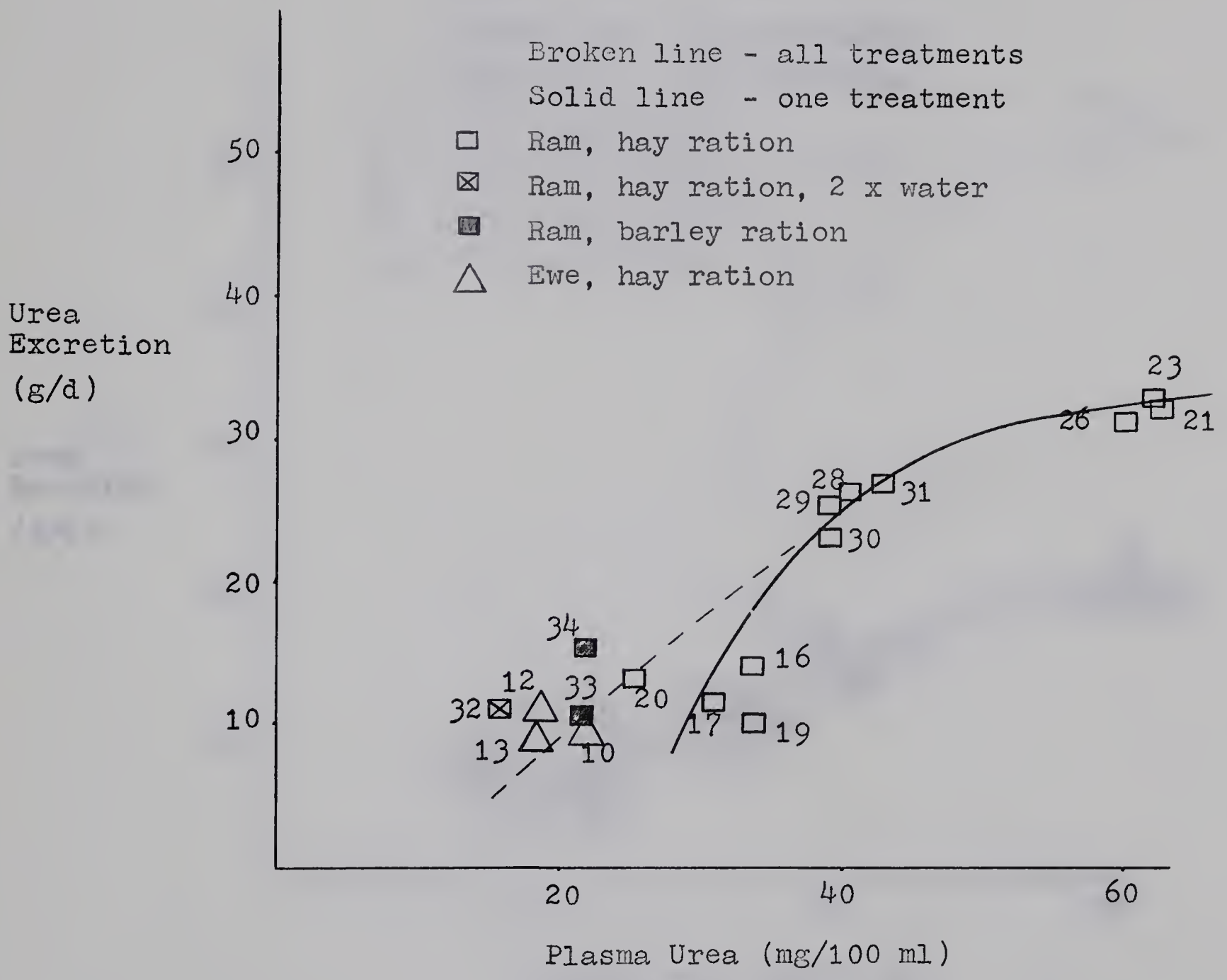
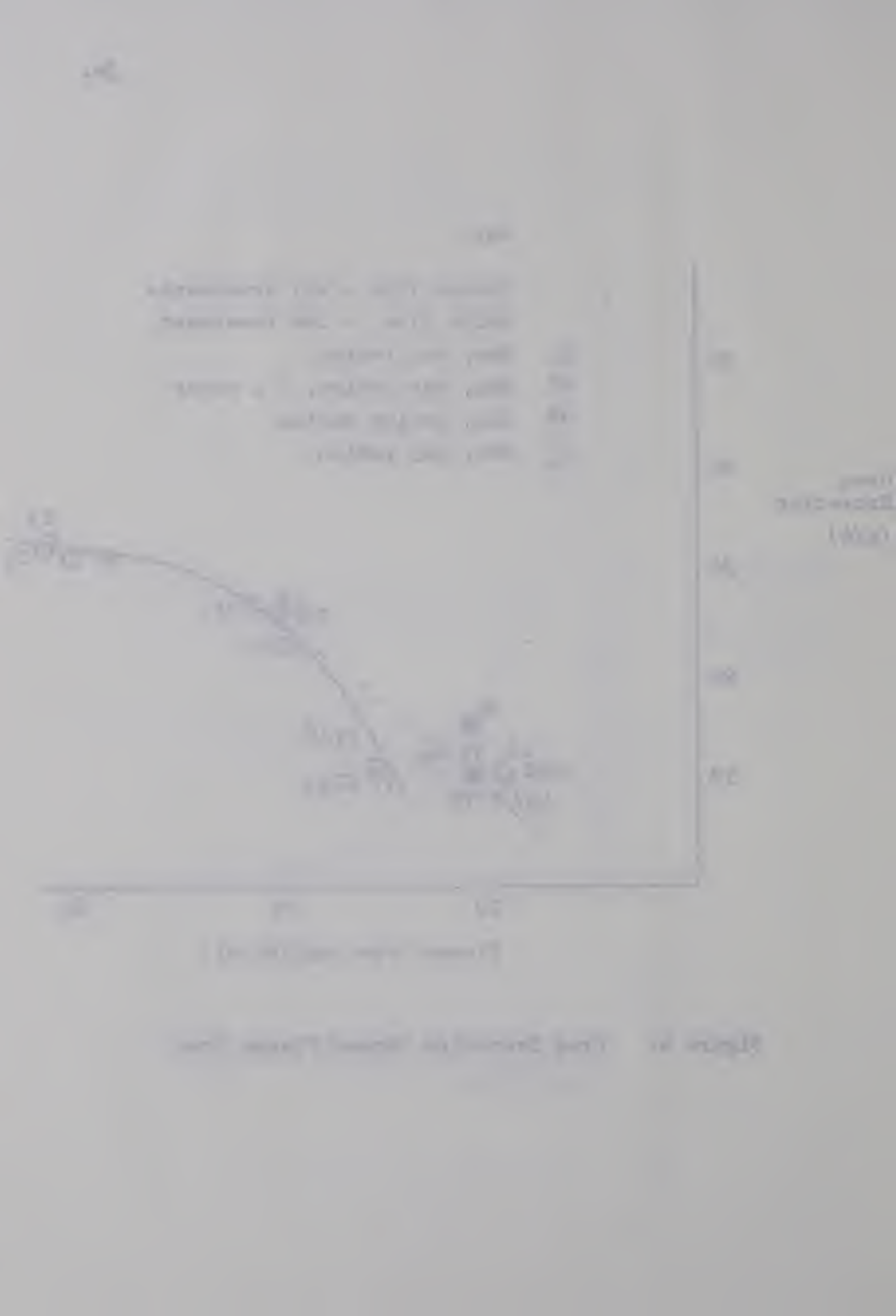


Figure 4. Urea Excretion versus Plasma Urea



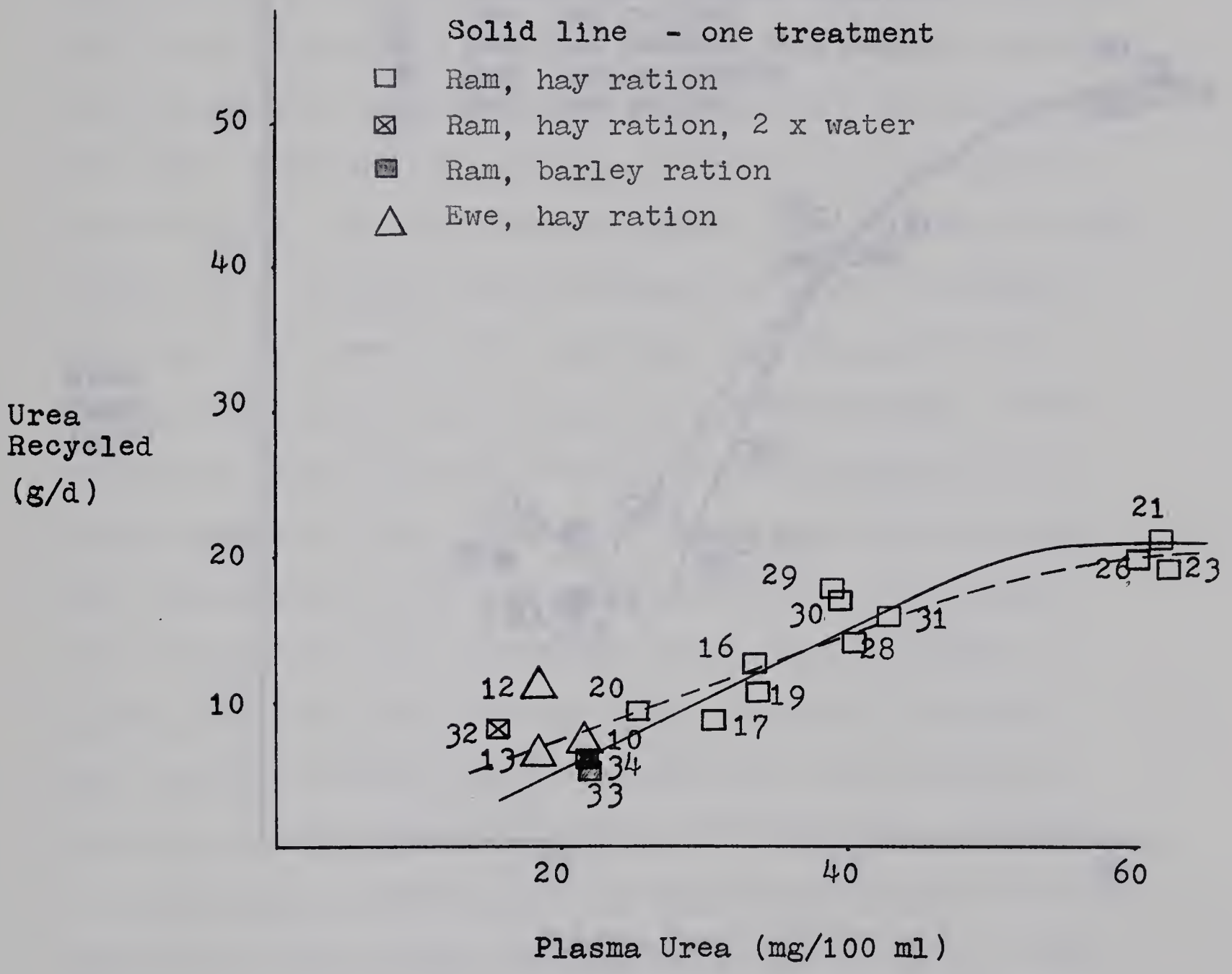
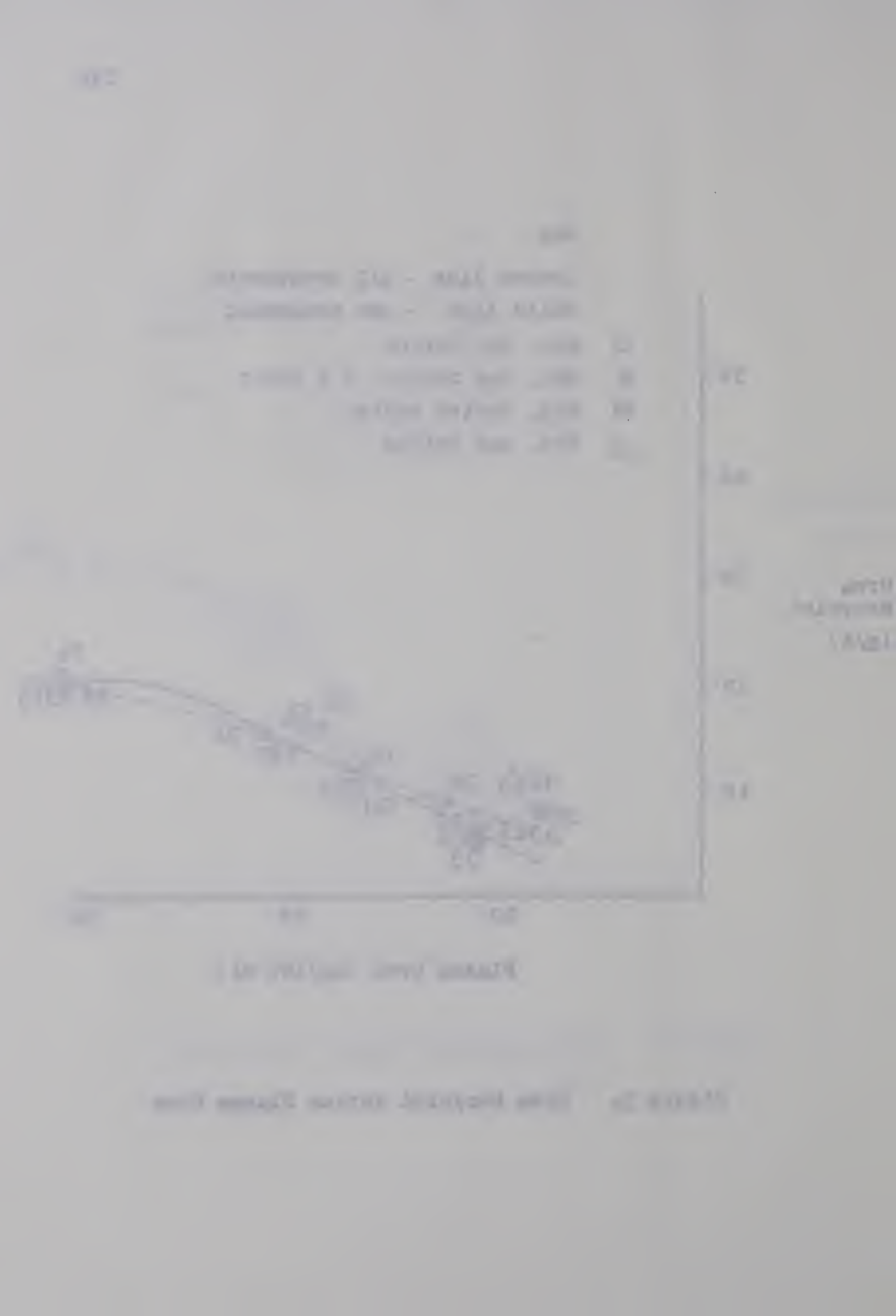


Figure 5. Urea Recycled versus Plasma Urea



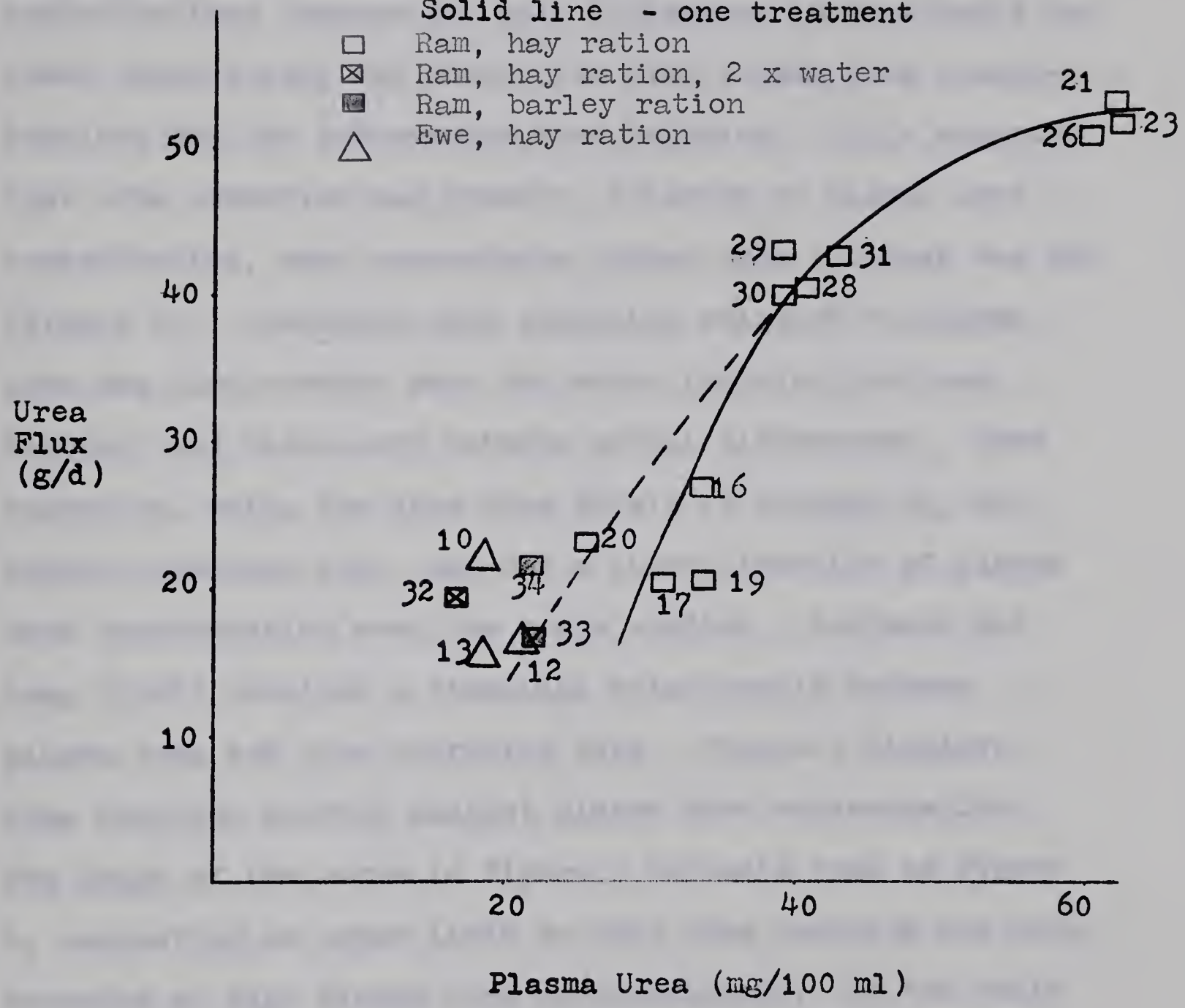
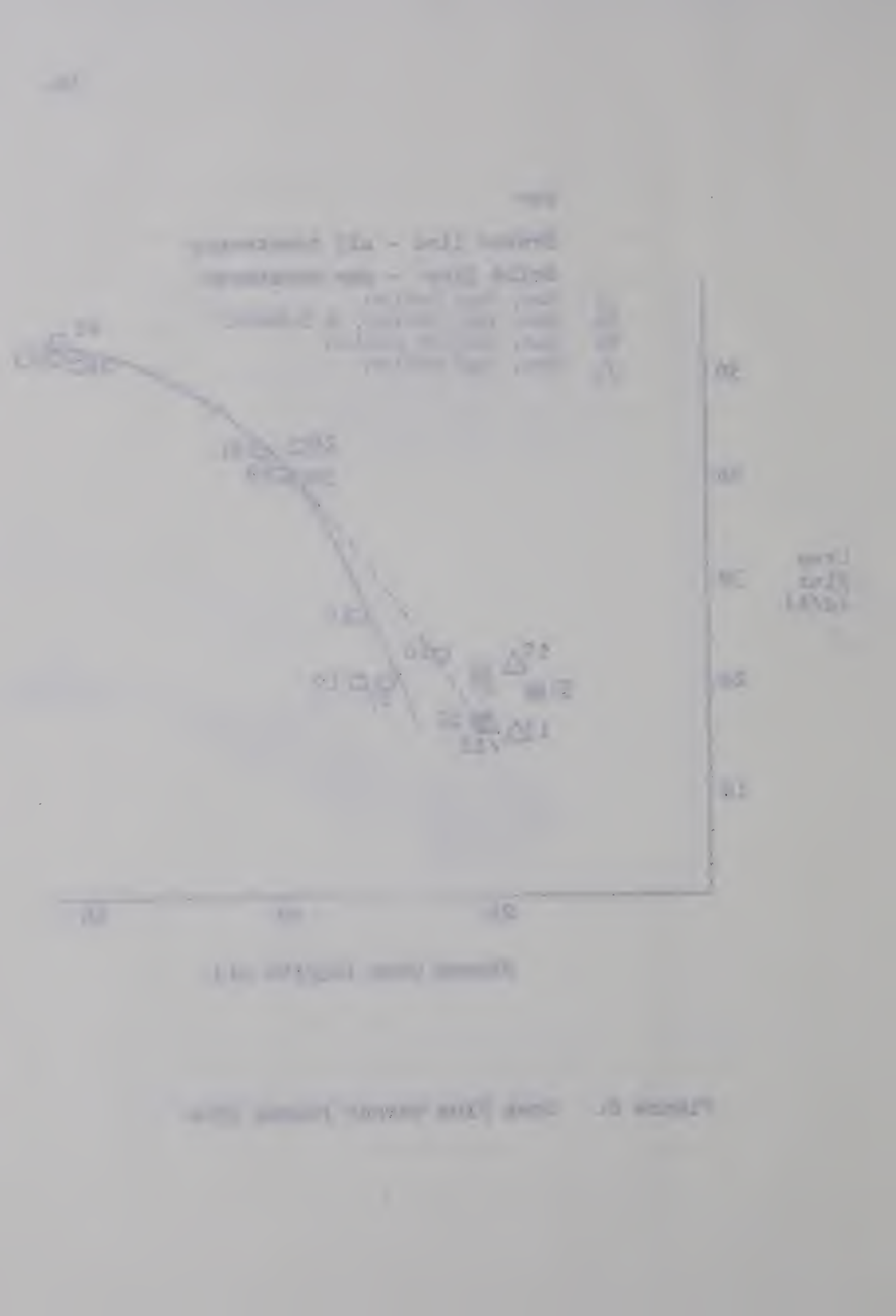


Figure 6. Urea Flux versus Plasma Urea



that urea excretion is related to plasma urea rather than urine flow rate (Figure 4).

Trials 16, 17, 19 and 20 are considered basal for comparing with those trials with higher observed plasma urea concentrations because the only difference in treatments between these trials and those with high plasma urea concentrations was the intravenous urea infusion. It is apparent that urea excretion was greater, relative to plasma urea concentration, when concentrate rather than roughage was fed (Figure 4). Increased urea excretion relative to plasma urea was also noticed when the water infusion rate was doubled, and there were between animal differences. Urea excretion, using the data from Trials 16 through 31, although dependent upon, was not a linear function of plasma urea concentration over the range studied. Cocimano and Leng (1967) obtained a sigmoidal relationship between plasma urea and urea excretion rate. Figure 5 displays urea recycled plotted against plasma urea concentration. The shape of the curve in Figure 5 reflects that of Figure 4, suggesting an upper limit to both urea recycled and urea excreted at high plasma urea concentrations. On the basis of these two curves it appears that the urea flux through the body approaches a maximum at plasma urea concentrations above 45 mg/100 ml (Figure 6).

Treatment effects are evident in urea flux (Figure 6), as in urea excretion, where the three treatments of different animal (Trials 10, 12, 13), different ration

(Trials 33 and 34), and different water influx (Trial 32) exhibited increased urea flux relative to plasma urea over that for the ram fed hay.

The effect of the different treatments is also observed in the data of Table III where the percent of flux recycled is given for each trial. Low values were observed for Trials 33 and 34. It is noted that urea recycled was about 32% of urea flux for Trials 33 and 34, while it was approximately 46% of urea flux when roughage diets were fed. There appears to be a trend toward decreasing percent of flux recycled for those trials in which plasma urea was elevated. Figures 7 and 8 show that recycled and excreted urea were linear functions of the urea flux. It is of interest that there was no point at which partitioning of increased urea flux into either the recycled or excreted routes of exit from the pool was markedly changed. However, because the plot of urea recycled as a function of urea flux would not pass through the origin, the proportion of urea flux that was recycled would tend to be reduced at elevated fluxes.

Since there was a less than proportionate increase in urea excreted at high plasma urea concentration, it was questioned whether the animal had reached its physiological maximum capacity for urea excretion and had adapted to the situation by excreting nitrogen in a form other than urea. Table II shows a wide variation between urinary urea concentrations in Trials 21, 23, and 26. Yet the daily urea excre-

TABLE III. Percent of Flux Recycled

Trial No.	Plasma Urea mg/100 ml	% Flux Recycled
32	15.8	42.8
13	18.3	41.4
12	18.6	50.1
10	21.6	43.3
33	21.7	34.7
34	21.8	28.4
20	25.3	41.7
17	30.9	43.1
16	33.6	47.6
19	33.7	51.6
29	39.0	40.4
30	39.1	42.6
28	40.6	34.7
31	42.9	37.3
26	60.2	38.8
21	62.0	39.7
23	62.2	36.9

Key

□ Ram, hay ration

⊠ Ram, hay ration, 2 x water

■ Ram, barley ration

△ Ewe, hay ration

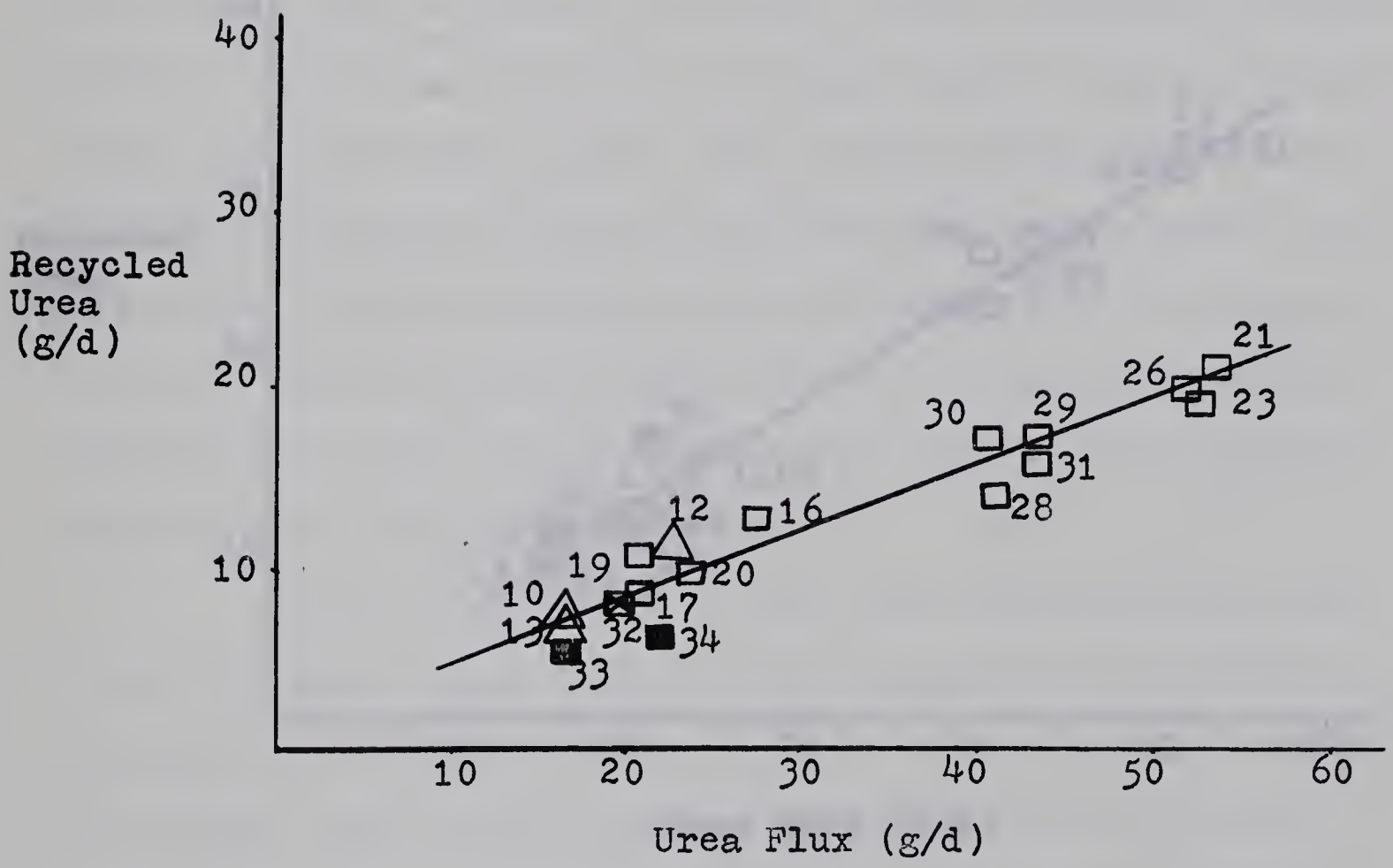
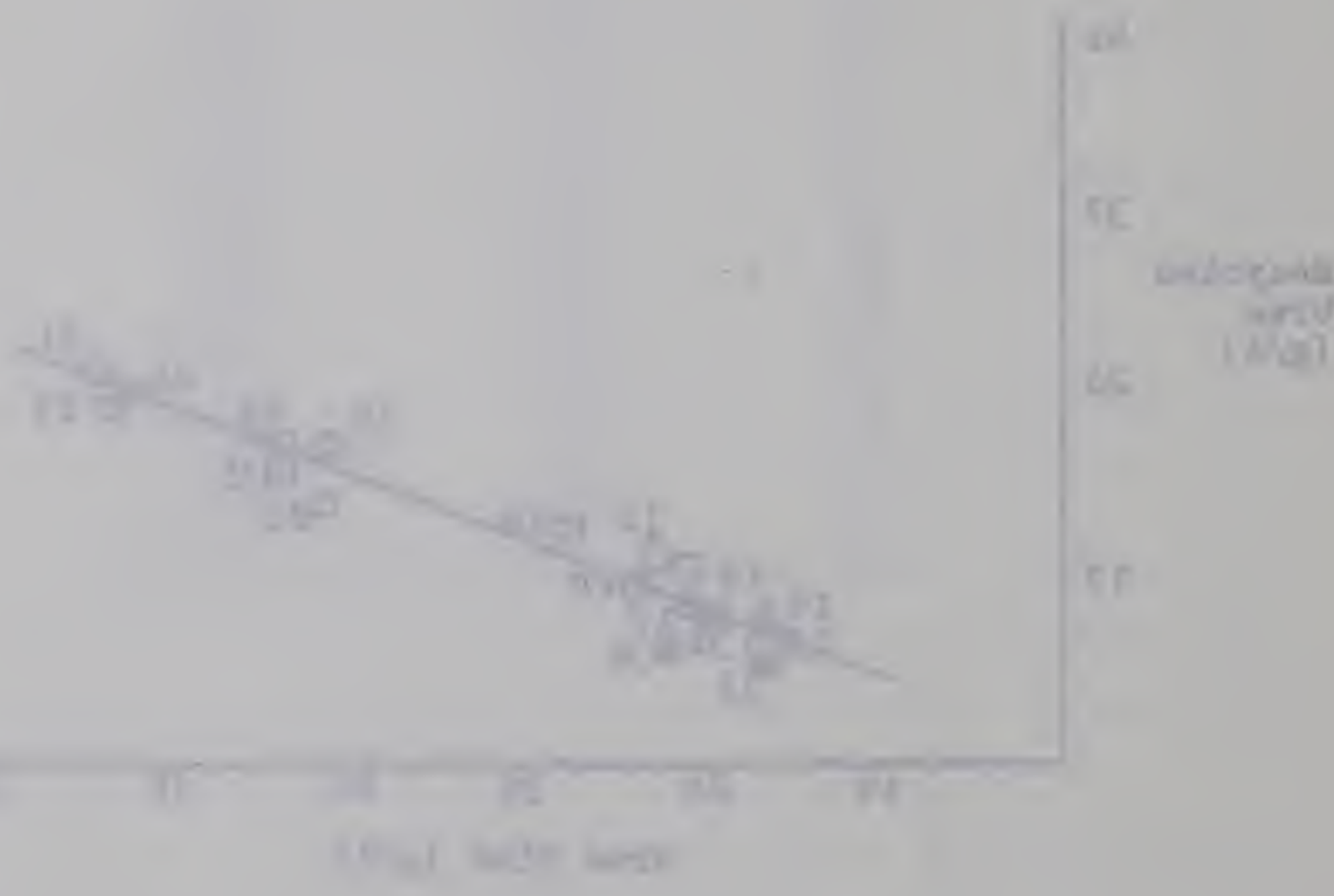


Figure 7. Recycled Urea versus Urea Flux



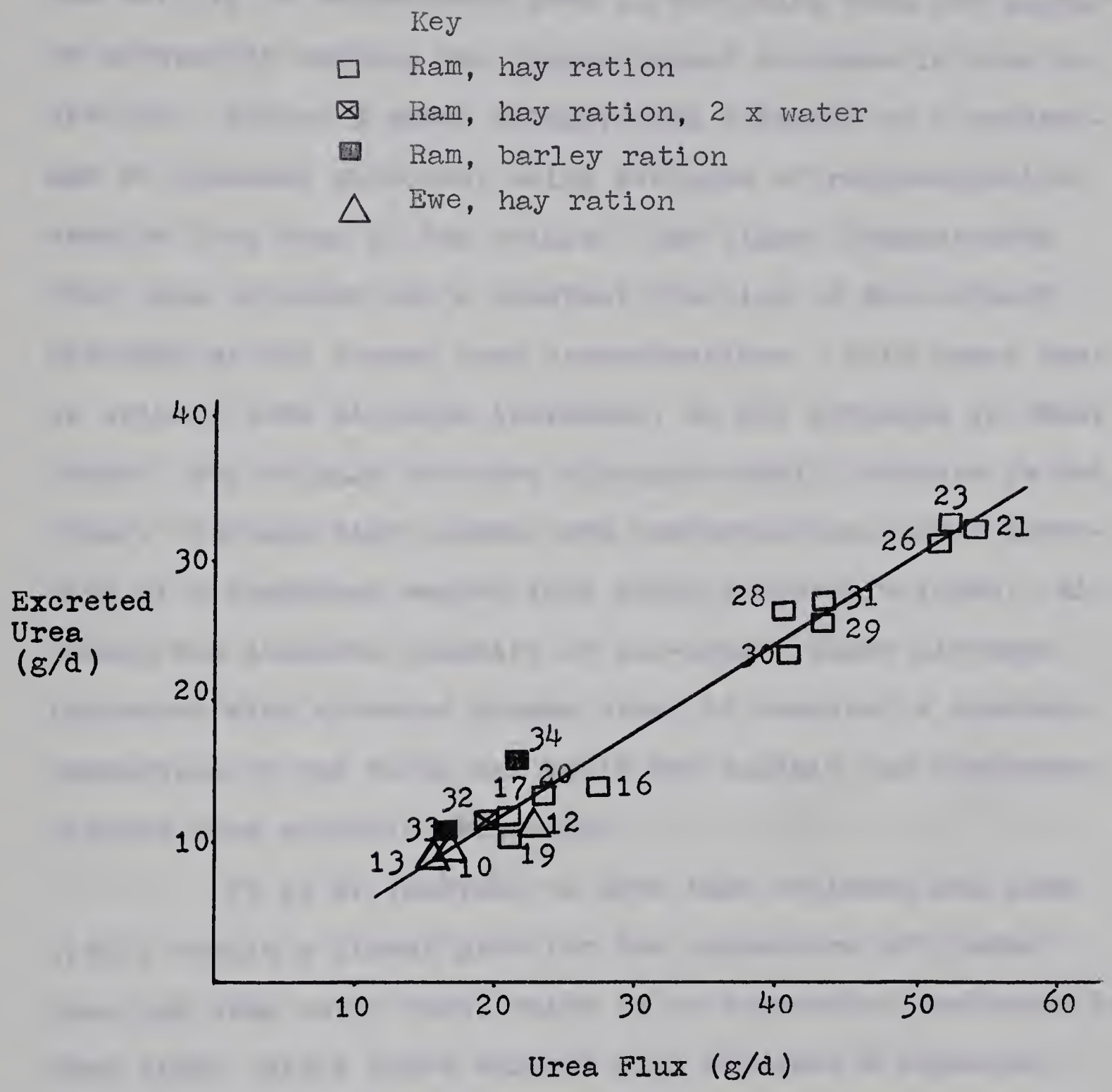
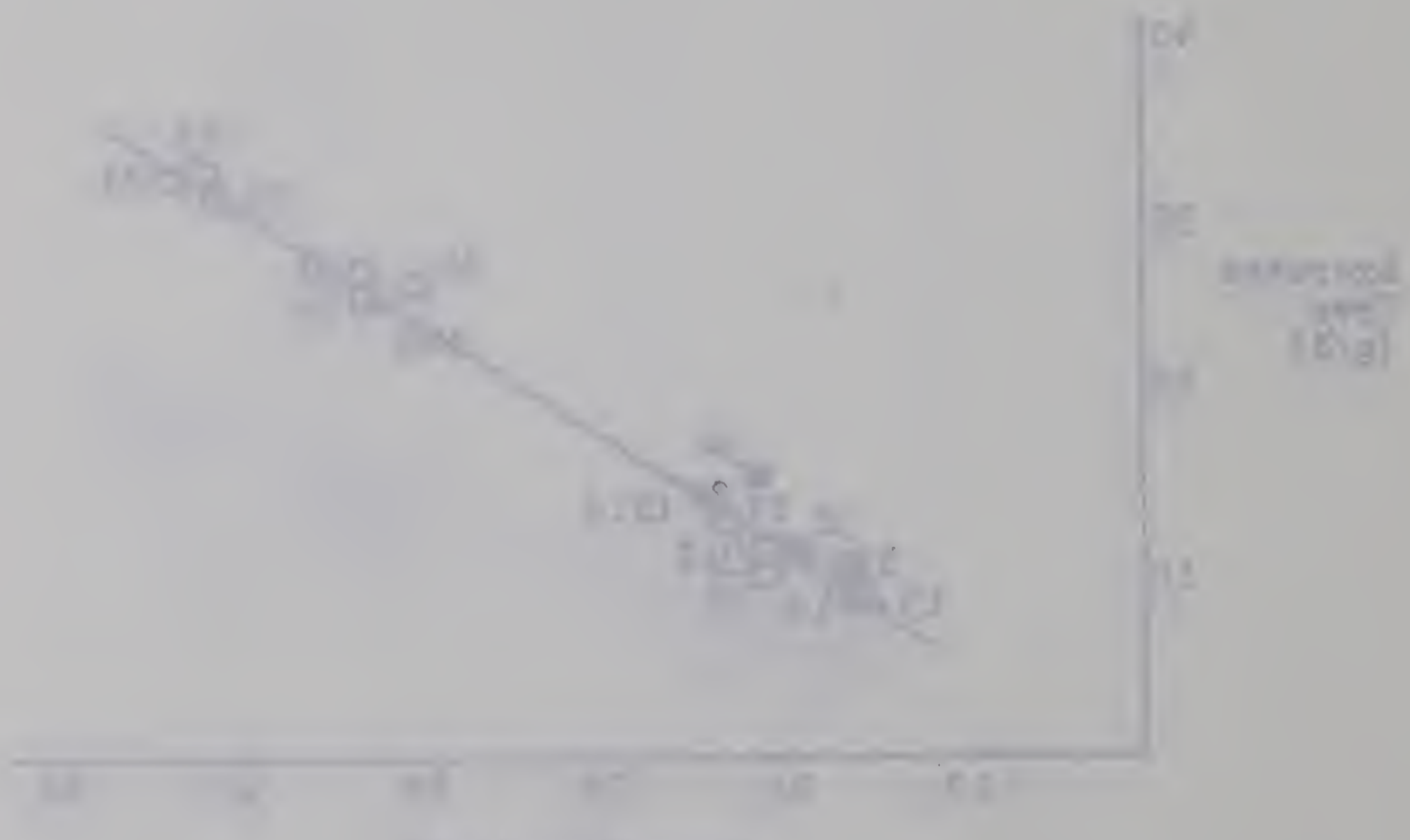


Figure 8. Excreted Urea versus Urea Flux



tions are very similar for these trials. Hence a maximum to the ability to concentrate urea in the urine does not appear to adequately explain the proportionate decrease in urea excretion. Figure 9 gives urinary urea nitrogen as a percentage of Kjeldahl nitrogen, using averages of representative samples from some of the trials. The figure demonstrates that urea nitrogen was a constant fraction of the urinary nitrogen at all plasma urea concentrations. This means that as urinary urea nitrogen increased, so did nitrogen in other forms. Why urinary non-urea nitrogen should increase is not clear. Perhaps high plasma urea concentrations cause diversion of nitrogenous wastes into other excretable forms. Although the absolute quantity of non-urea urinary nitrogen increased with elevated plasma urea, it remained a constant proportion of the total and would not explain the disproportionate urea excretion observed.

It is of interest to note that Cocimano and Leng (1967) obtain a linear plot for the parameters of plasma urea and urea entry rate, which is an expression analogous to urea flux. Since these workers also obtained a sigmoidal curve for urea excretion, it follows from their definition of urea entry as being equal to urea excreted plus urea degraded, that urea degradation must proportionately increase over that range of urea concentrations where urea excretion proportionately decreased, that is, at high plasma urea concentrations. However, when urea degradation from Cocimano

and Leng's work is plotted against plasma urea concentration (Figure 10), the data fits the relationship found in the present investigation between plasma urea concentration and urea recycled. The plot in Figure 10 suggests a discrepancy in Cocimano and Leng's results (1967) in that increased urea recycling is not evident as is required from their linear entry rate and decreased excretion rate, at high plasma urea concentrations.

Recently, Houpt and Houpt (1968) reported net urea nitrogen transfer across the wall of unrinsed rumen pouches. They have illustrated (Houpt and Houpt, 1968: Figure 5) that below a urea concentration difference between the blood and pouch of approximately 45 mg urea per 100 ml there is a rapid transfer of urea into the pouch, with a point of inflexion occurring at this urea gradient level. Above this concentration there was a very marked decrease in the urea transfer rate, as a function of urea concentration gradient. It is suggested from the blood urea concentrations observed in the present experiment on two divergent rations, and from those reported by Cocimano and Leng (1967), that much of the work of Houpt and Houpt was done in a plasma urea concentration range greater than would be physiologically normal, i.e., 45 to 300 mg urea per 100 ml as compared to 16 to 62 mg per 100 ml in the present study, where the higher urea concentrations were achieved by i.v. urea infusion. The fact remains, however, that an inflexion point is indicated in

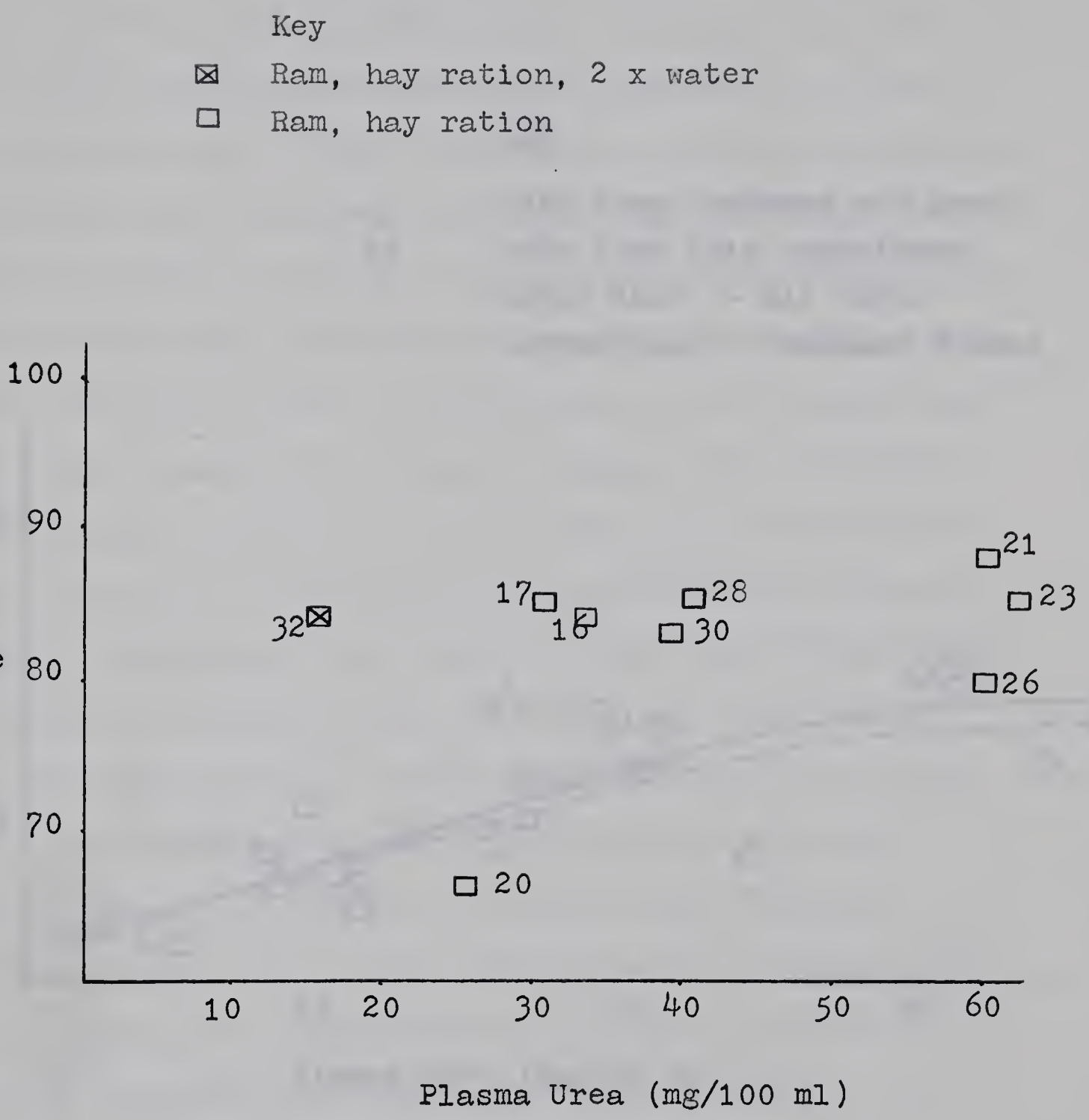


Figure 9. % Urea Nitrogen in Urine versus Plasma Urea

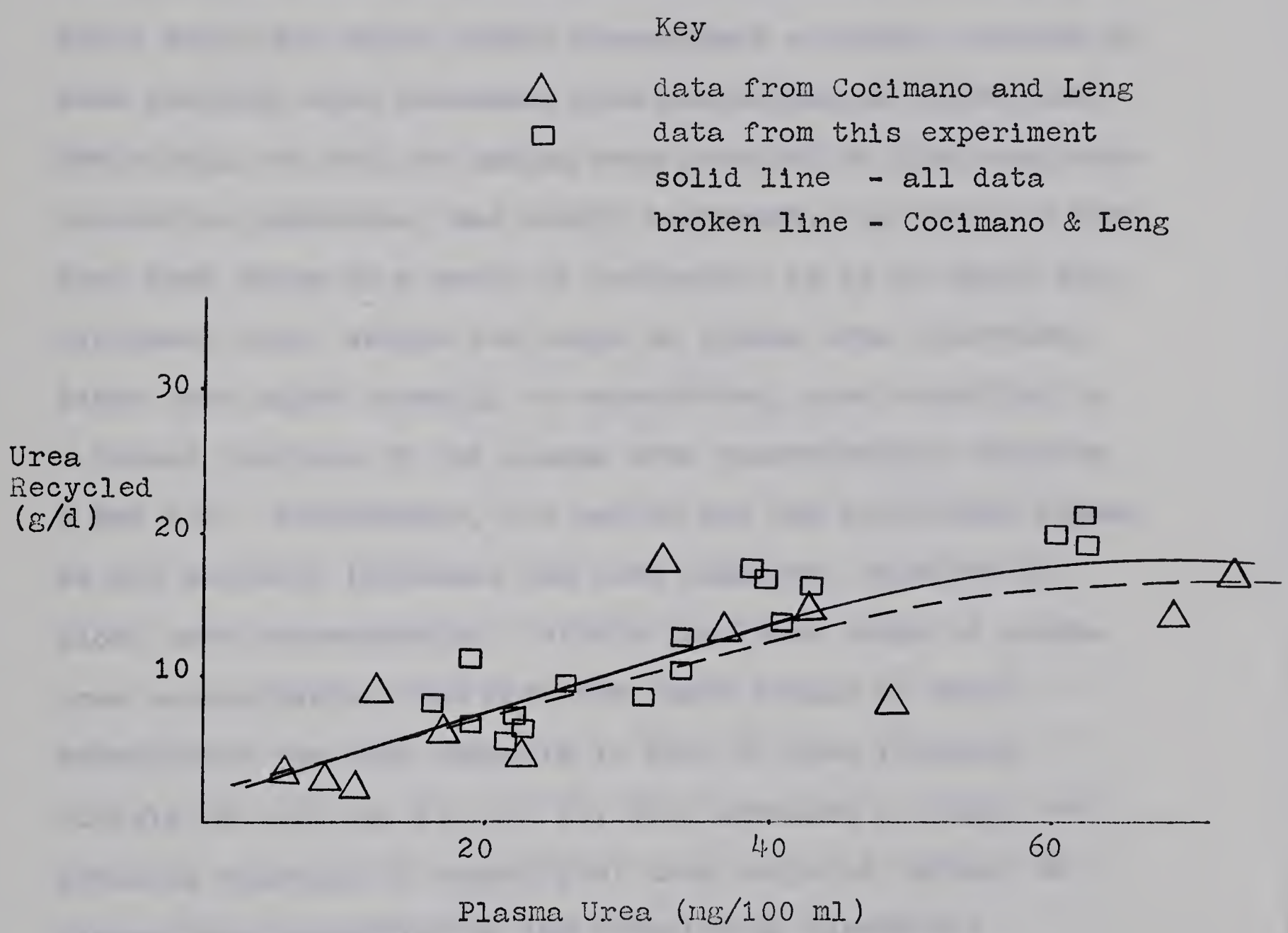


Figure 10. Urea Recycled versus Plasma Urea Including the Data of Cocimano and Leng

their paper, and that it occurs at a blood urea concentration similar to that at which inflexions in recycling and excretion were found in this investigation. It should be noted in Figure 5 that urea recycling reaches a maximum of 20 g/day, while Houpt and Houpt (1968) demonstrate a linear increase in urea transfer with increased urea concentration difference. Their data, as well as having been recorded at high urea concentration gradients, was widely scattered. In spite of the fact that there is a point of inflexion, it is of major significance that, within the range of plasma urea concentrations that might normally be encountered, urea recycling is a linear function of the plasma urea concentration (Figures 5 and 10). Furthermore, the ration and the individual animal do not markedly influence the urea transfer, relative to blood urea concentration. Within the lower range of plasma urea concentration, the data for those trials in which essentially the only variable is rate of urea infusion (Trials 16, 17, 19, 20, 21, 23, 26), produced a linear regression equation of quantity of urea recycled (g/day) on plasma urea concentration (mg urea/100 ml plasma) of:

$$\hat{Y} = -4.07 + 0.49\ X$$

(S.E. = 1.98, r = 0.84)

This equation compares with the one including all data in the range up to 45 mg urea per 100 ml plasma:

$$\hat{Y} = .117 + .37\ X$$
$$(S.E. = 2.27,\ r = 0.85)$$

The latter equation is perhaps more important because it gives an expression for urea recycling based on data collected from more than one animal, ration and water influx rate. A third, more general, equation can be calculated using the data of Cocimano and Leng (1967) together with that collected in these experiments:

$$\hat{Y} = 1.61 + 0.31\ X$$
$$(S.E. = 3.20,\ r = 0.77)$$

This latter equation would be useful for the general prediction of the quantity of urea recycled to the digestive tract, from the blood urea concentration of sheep.

Various workers have suggested a simple diffusion mechanism for urea transfer to the digestive tract (Engelhardt and Nickel, 1965; Houpt and Houpt, 1968; Juhasz, 1965). Diffusion is defined by Trumbore (1966) as "the movement of dissolved substances from an area of higher concentration to an area of lower concentration until an equilibrium is reached where there is no concentration difference". Fick's first law of diffusion (Fick, 1855, cited by Stein, 1967) is expressed as

$$\frac{dn}{dt} = D\ A\ \frac{dc}{dx}$$

where D is the diffusion coefficient and dn/dt is the number of molecules dn crossing area A of the interface in time dt when a concentration difference dc is applied over a distance dx. Therefore if D, A and x are constant, transfer by diffusion would be a linear function of the concentration difference, as was found for plasma urea concentrations up to 45 mg/100 ml. At plasma urea concentrations above 45 mg per 100 ml, a further increase in the concentration gradient, i.e., plasma urea concentration, does not lead to a linear increase in recycling. This may or may not be compatible with transfer of urea to the digestive tract by diffusion. It is possible that the mechanism entails facilitated diffusion, where a carrier, or mediator, is involved, with saturation of the carrier occurring at plasma urea concentration above 45 mg/100 ml. Urea, being a small molecule however, is usually considered to diffuse readily through the biological membrane and hence would not require a carrier. Accordingly, urea transfer by diffusion would exhibit the type of concentration relationship observed (Figure 5) if the diffusion coefficient, D, the interface area, A, or the transfer distance, x, changed at the point of inflexion.

One mechanism suggested for control of urea recycling at high plasma urea concentrations involves the alteration of the alimentary tract membrane permeability, thus limiting the amount of urea passing through it. This would be, effectively, a change of D (see above). It is

difficult to visualize how such a mechanism would work, although it could involve ammonia. Varady et al. (1967) suggested that the transfer of blood urea to the rumen is controlled by the ammonia concentration in the rumen. Weston and Hogan (1967) infused urea into sheep and demonstrated a maximum rumen ammonia level of 14-17 mg NH_3-N per 100 ml at a plasma urea concentration of 34-39 mg urea per 100 ml. At higher concentrations of blood urea, rumen ammonia concentrations were not increased. Houpt in 1959 similarly showed a maximum rumen ammonia accumulation in four out of five trials. The work of these researchers could suggest ammonia as the mechanism through which control of urea recycling is exerted. One way in which ammonia was implicated in urea recycling involves Houpt and Houpt's hypothesis (1968) for ruminal hydrolysis of blood urea. They speculate that under normal rumen conditions, blood urea that is transferred to the rumen is hydrolyzed in the rumen epithelium before all epithelial diffusion barriers are crossed. The newly formed NH_3, being a smaller molecule than urea, would then diffuse more rapidly than urea through the remaining epithelial layers. The presence of bacteria, and particularly bacterial urease thus acts to enhance urea transfer. In order for an ammonia control mechanism to be operative in this system, ammonia would have to either influence rumen membrane permeability, as discussed above, or it would have to elicit an inhibition of the urease hydrolysis reaction. Since such an

inhibition has not been demonstrated, this concept of an ammonia control mechanism appears remote. A further drawback to a proposed ammonia control concept of urea recycling is the low ammonia concentrations found in the rumen in these trials. Portugal (cited by Hungate, 1966), reported that the average rumen concentration of ammonia is less when sheep are fed continuously. Results of Mathison and Milligan (unpublished) show rumen ammonia concentrations of less than 30% those recorded by Weston and Hogan (1967) for animals with comparable plasma urea concentrations but which were continually fed.

The feasibility of alteration of x the transfer distance, or A the interface area, is questionable. If either of these parameters changed, one might expect a change in partitioning of flux between recycling and excretion. Figures 7 and 8 demonstrate that no change in partitioning of the flux occurs over the range observed. It was also noted that urea excretion, as a function of plasma urea concentration, exhibited an inflexion at the same point as urea recycling. If a change in A, or x, was responsible for the inflexion in urea recycling, it is difficult to visualize how such a change would influence urea excretion, unless the change occurred in the kidneys as well as the digestive tract membranes.

Another mechanism can be hypothesized, however, which, as well as accounting for decreased urea recycling,

also provides an adequate explanation for the apparent impediment to urea excretion at high plasma urea concentrations, while still describing the linear function of urea recycling at lower plasma urea concentrations. The protein denaturing properties of high urea concentrations have been well documented (Kauzmann, 1959; Haurowitz, 1963; Joly, 1965). The effects of high urea concentration on dilute protein solutions have been reported (Neurath and Bailey, 1953) to induce large viscosity increases coupled with a lowered rate of diffusion. This is apparently the result of the "smaller polar urea molecules being driven by their thermal movements as wedges between peptide chains, cleaving the hydrogen bonds and being bound to the liberated peptide linkages by the formation of new hydrogen bonds" (Haurowitz, 1950). At high urea concentrations this action results in the unravelling of the tertiary structure of the protein molecule. It is conceivable at the higher urea concentrations obtained in the blood, that the urea does form hydrogen bonds with plasma proteins, but does so without causing protein denaturation because plasma urea concentration does not approach that required for denaturation. That is, due to the ability of urea to readily form hydrogen bonds, it is possible to suppose that urea could form loosely bound reversible complexes with plasma proteins. Ralls (1943) indicated that urea does bind to the protein of erythrocytes. By the measurements used, plasma urea concentration was increased but because of bind-

ing, the concentration difference for diffusion (dx) would not increase correspondingly. Thus, transfer to the digestive tract by diffusion would not increase in accordance with the increase in measured plasma urea concentration. Similarly, the bound urea would not be filtered in the kidney and would not enhance excretion. Proposal of such a urea-protein complex would explain the less than proportional increase in urea flux, urea recycling, and urea excretion that was observed at higher plasma urea concentrations. This explanation would entail a threshold for increased urea binding at about 45 mg urea per 100 ml plasma. It is therefore not necessary to invoke transfer mechanisms other than diffusion to elucidate the mechanism of urea recycling in sheep.

4. Summary

Urea recycling to the alimentary tract is proportional to plasma urea concentrations below 45 mg urea per 100 ml. The linear regression line for the urea recycling data within this plasma urea range is $\hat{Y} = 0.117 + 0.37\ X$, S.E. = 2.27, r = 0.85. When the observations of Cocimano and Leng (1967) are included for the same plasma urea concentrations, the regression line is $\hat{Y} = 1.61 + 0.31\ X$, S.E. = 3.20, r = 0.77. Recycling of urea appeared to reach a maximum level of 20 g urea per day at a plasma urea concentration above about 45 mg urea per 100 ml. A mechanism was proposed which would allow urea transfer to the digestive tract to be explained by simple diffusion.

C. Experiment 2. Rumen Urease Activity

1. Objectives and Introduction

The microbial urease activity of rumen contents is well known, although investigators have had little success in establishing the microorganisms responsible (Abou Akkada and Blackburn, 1963; Blackburn, 1965; Blackburn and Hobson, 1962; Gibbsons and Doetsch, 1959; Jones, MacLeod and Blackwood, 1964a, b). Even though the urease enzyme appears to be constitutive, there has been a report suggesting differences in urease activity in rumen contents of animals fed different rations (Caffrey et al., 1967). Although Clifford, Bourdette and Tillman (1968) suggested independence of rumen urease activity from ration composition, their results could indicate a dependence of ureolytic activity on the type of ration. Since some researchers have implied a relationship of urease activity to nitrogen retention (Caffrey et al., 1967) and to recycled urea (Houpt and Houpt, 1968), it was decided to investigate the possible relationship of rumen ureolytic activity of sheep fed two very different rations to the urea recycling data gathered in Experiment 1.

This experiment, then, was designed to measure and compare the urease activity of the rumen contents of sheep offered a concentrated ration, or a roughage ration under conditions of steady state.

2. Experimental

a) General procedure

The sheep used was the same fistulated ram employed in Experiment 1. The animal was fed a maintenance ration using the continual feeder and continuous water infusion apparatus as described in Experiment 1. The rations fed were 1000 g per day of 9.52% protein (dry matter basis) hay pellets or 500 g per day of 11.3% protein (dry matter basis) rolled barley.

b) Sampling technique

The rumen sample was obtained using a portable vacuum pump (115 v, 4.0 amp, 1725 rmp) connected to two 500 ml Erlenmeyer flasks, one for collection of the rumen sample and one to serve as a trap. A sample of 75 to 150 ml was collected in the preheated collection flask through a Nalgene tube inserted into the rumen fistula. The sample was filtered through four layers of double-mesh cheese cloth into a preheated thermos.

c) Urease assay

The urease assay was started within 45 minutes of sampling. The procedure followed was a modification of the one used by Jones et al., (1964a, b). In a 25 ml stoppered Erlenmeyer flask 2.0 ml of 0.06 M sodium maleate buffer (pH 6.9), 2.0 ml of 0.2% w/v urea and 1.0 ml of water were preheated at 39 C for 5 minutes. A 1.0 ml sample of the strained rumen fluid was then added and the flask was incu-

bated at 39 C for 30 minutes in a shaker water bath. For the blank, water replaced the urea portion of the assay mixture. At the end of the assay period 2.0 ml of the assay mixture was added to 0.98 ml of 1.8% w/v $Ba(OH)_2.8H_2O$ in a 10 by 100 mm Nalgene centrifuge tube. After mixing, 1 ml of 2% w/v $ZnSO_4.7H_2O$ was added, and the tube was again mixed. The tube was then centrifuged at 500 x gravity for 10 minutes. A 0.2 ml portion of the resulting supernatant was then mixed with 0.8 ml of water in a 20 by 150 mm glass test tube. Ammonia was estimated according to the modified method of Fawcett and Scott described in Experiment 1. Ammonium chloride was used as the standard. Standards were included in each determination sequence. Demineralized water was used for all dilutions and reagents. All glassware was routinely rinsed with demineralized water.

d) Determination of trichloroacetic acid-insoluble nitrogen

A portion of strained rumen fluid was frozen at the time of the urease assay for future determination of the trichloroacetic acid-insoluble nitrogen content. One ml of warmed 80% w/v CCl_3COOH was added to 7 ml of strained rumen fluid. The suspension was mixed and chilled in ice. After 10 minutes, the suspension was centrifuged at 3000 x gravity for 10 minutes. The precipitate was analyzed for nitrogen by the Kjeldahl method (AOAC, 1965).

TABLE IV. Summary of Rumen Fluid Urease Activity

Treatment	Ration	Trichloroacetic Acid Nitrogen mg/ml	Urease Activity IUB*/ml	Specific Activity IUB/mg N
I	hay	1.13	.156	.138
II	barley	3.07	.191	.063

* Units recommended by the International Union of Biochemists where one unit (IUB) is the amount of urease which catalyzes the hydrolysis of 0.5 micromoles of urea and liberates 1 micromole of ammonia in 1 minute.

3. Results and Discussion

The results obtained in Experiment 2 are presented in Table IV. It can be seen that the greatest difference between the samples for the two rations was in bacterial nitrogen content, as measured by trichloroacetic acid insoluble nitrogen (TCA nitrogen). Ureolytic capacity per ml of the rumen contents changed only slightly on rations that are at the extremes of composition. If rumen volumes were assumed constant for the two treatments, total ureolytic activity would be similar. Although water was infused at the same rate for each treatment, 1000 g per day of hay pellets were fed in Treatment I, whereas 500 g per day of rolled barley was offered in Treatment II. Since less feed and therefore less fermentable substrate was consumed in Treatment II, one might expect a lower bacterial nitrogen value and consequently a lower urease activity. On the contrary, a higher bacterial nitrogen concentration was observed, even though urease activity per ml increased only slightly.

It appears from the greater TCA nitrogen concentration on the barley ration the rumen bacteria were more prolific, perhaps as a consequence of the barley being more readily fermented. Since the urease activity did not change greatly, it is possible in this case that the shift in microbial population associated with change in ration does not greatly affect those strains of bacteria responsible for

ureolysis.

Values of 0.156 and 0.191 umole ammonia produced per ml of rumen fluid per minute were obtained compared to those of Jones et al., (1964a, 1967) who reported a value of 0.190 umole nitrogen per ml per minute. Bloomfield, Garner and Muhrer (1960) recorded a value of 0.440 umoles nitrogen/ml/min for the urease activity of sheep rumen contents.

It is noted that the assay procedure followed in this investigation was similar to the one used by Jones et al., (1964a, b) and that the values obtained for urease activity per ml of rumen fluid do compare favourably.

Houpt (1968) has suggested that urease activity in the rumen could play an important role in urea transfer. In Experiments 1 and 2, it is apparent that extremes in ration composition did not change urease activity per ml and did not alter urea recycling independently of plasma urea concentration. It is therefore not possible from these trials to draw conclusions concerning the effect of rumen urease activity on endogenous urea recycling.

4. Summary

It was found that a change of the ration of a sheep maintained in a steady fed state from hay pellets to rolled barley increased the concentration of microbial nitrogen in the rumen. Little change was evident in urease activity per ml of rumen fluid while urease specific activity

decreased. Conclusions as to the effect of rumen urease activity on endogenous urea recycling are not possible.

GENERAL DISCUSSION

This study indicates a definite relationship of recycled urea to plasma urea concentration for urea concentrations usually found in sheep fed normal rations. A maximum to the amount of urea recycled to the digestive tract was displayed at a plasma concentration above 45 mg urea per 100 ml. The data from Trials 33 and 34 indicated that the amount of recycled urea was probably not affected by a marked change in ration. The results of Experiment 2, although indicating a twofold decrease in urease specific activity, demonstrated that ureolytic activity per ml of rumen fluid does not change markedly upon changing the ration from roughage to concentrate. Since only one trial with increased water infusion rate was recorded, it is difficult to assess the effect of water intake rate on urea recycling. Between animal variation is expected, and, considering this, there is good agreement between the two animals employed in this study. The simple regression equation obtained for the combined data of Cocimano and Leng (1967) and that of this study agrees closely with the equation for data from this study alone. Thus, a means is provided whereby the urea recycled to the digestive tract may be predicted from the plasma urea concentration within the physiological range. The importance of such a predictive value is evident when the amount of recycled urea

nitrogen is compared to total nitrogen intake. For the ram, at feed intake levels of 1000 g hay pellets per day, approximately 8.7 g of digestible nitrogen was consumed. The recycled urea nitrogen represented an addition of 4.95 g nitrogen per day constituting 36% of the total digestible nitrogen entering the digestive tract. On the barley ration, 6.5 g digestible nitrogen was ingested, and 2.78 g urea nitrogen was recycled constituting 30% of the digestible nitrogen flux. Although the experimental design does not allow for the estimation of the fraction of recycled urea that reaches the rumen, as opposed to other parts of the digestive tract, unpublished data of Mathison and Milligan indicate that this constitutes a large portion of recycled urea.

Therefore, it has been established in this study that about one third of the daily nitrogen flux available for metabolism in the rumen could be contributed by recycled urea.

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