BIOSYNTHESIS AND METABOLISM OF TRYPTOPHAN IN THE RUMINANT AND IN REED CANARY GRASS

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ABSTRACT

Candlish, Violet Elizabeth, PhD., The University of Manitoba, October 1970. Biosynthesis and metabolism of tryptophan in the ruminant and in reed canary grass. Major Professors: Lucien J. LaCroix, Department of Plant Science; Thomas J. Devlin, Department of Animal Science.

A continuous flow artificial rumen apparatus was constructed to investigate the utilization of DL-tryptophan-^{14}C (U) -benzene ring labelled material by the rumen microorganisms. Half of the labelled material left the rumen sac in the first six hours. After a 24 hour period, less than 4 % of the label was associated with either protozoal or bacterial fraction of the rumen fluid.

Biosynthesis of tryptophan by rumen microorganisms was investigated using the in vitro apparatus. L-serine-^{14}C (U) was incubated with rumen microorganisms. Half of the labelled material diffused into the saline before 4.5 hours had elapsed. The maximum amount of label associated with protozoa was 2.1 %; the similar value for bacteria was 1.5 %. These values were obtained only after the 42 hours of incubation. Serine was a much better substrate than tryptophan for volatile fatty acid production by the microorganisms. The synthesis of tryptophan was indicated by appearance of label in the tryptophan area of the thin layer chromatograms. In all but one sample, the percent of label appearing in the tryptophan was less than one.

Labelled compounds appeared in the urine within 10 minutes after injecting DL-tryptophan -^{14}C (U) -benzene ring labelled into the rumen of 2 sheep. Label appeared in portal blood prior to its appearance in jugular blood. Most of the label in the blood samples occurred in tryptophan. Urine samples were examined for label appearing in tryptamine, indoleacetic acid and tryptophan. Less than 20 % of the label in the urine was in the analysed compounds with tryptamine showing negligible activity.

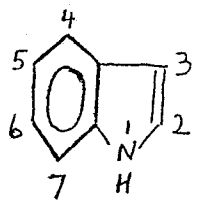
Rapid absorption of tryptophan from the rumen and reticulum of two other sheep was shown. After injection of labelled tryptophan into the rumen, 26.7 % was absorbed using the washed rumen technique, and 68.8 % was absorbed when the whole rumen technique was employed. When labelled sodium acetate was injected into the rumen and the washed rumen technique used, 49.0 % was absorbed.

Reed canary grass was injected with L-serine -^{14}C (U) and DL-tryptophan -^{14}C (U) -benzene ring labelled, prior to flowering. After seed ripening (33 days postinjection) plants were harvested and analysed. An average of 2.17 % of injected radioactivity from serine treated plants, and 13.67 % of injected radioactivity from tryptophan treated plants, appeared in the ethanol extract. The average percent of injected label which was recovered in tryptophan was 0.119 % of the labelled serine and 0.412 % of the labelled tryptophan. The alkaloid fraction of high alkaloid level plants contained 0.7 % of injected tryptophan label and 0.1 % of injected serine label.

INTRODUCTION

The importance of tryptophan to living cells was realized when proteins were shown to contain this amino acid, as a primary structural unit. The nutritional significance of tryptophan was evident when it was characterized as an essential amino acid for monogastric animals, including man.

Interest in tryptophan increased when it was established that the chemical structure of this amino acid was different and more complex than any of the other twenty amino acid building blocks involved in protein synthesis. Although phenylalanine and tyrosine are also aromatic amino acids, tryptophan reacts distinctly as a result of the indole ring.



The 2 and 3 positions of the indole ring have reactive characteristics which permit condensation with other compounds to yield intensely coloured complexes. This reactivity has been used in colour tests for identifying proteins and to study enzyme action.

Research involving amino acids has in many instances neglected the study of tryptophan due to the necessity of separate analysis as tryptophan is destroyed during the normal acid hydrolysis procedure. Nevertheless researchers working

in the area of tryptophan biosynthesis and metabolism have been rewarded for their efforts by the association of tryptophan and tryptophan metabolites with an increasing number of living processes.

Prior to the 1950's, ruminant nutritionists had accepted the view that microorganisms in the rumen provided all the amino acids necessary for the ruminant animal and microorganisms could synthesize amino acids from a simple carbon source and non-protein nitrogen. Subsequently several research reports indicated that the "quality" of dietary nitrogen had an effect on animal performance. The rates at which various proteins and amino acids were attacked by rumen microorganisms differed considerably. The assumption that amino acids were not absorbed through the rumen wall but passed to the abomasum before moving into the body blood stream was questioned.

The sparse literature available concerning tryptophan metabolism in ruminants indicated that tryptophan might be attacked slowly by rumen microorganisms. The fact that this amino acid was essential to most nonruminant mammals led to the question of whether or not tryptophan was essential to ruminants.

In addition to nutritional studies with tryptophan in animals, chemical studies of tryptophan and related indole compounds were carried out with plants. Indole alkaloids present in Phalaris tuberosa L. were thought to

be the toxic agent involved in Phalaris staggers, a disease first described in sheep in Australia. Reed canary grass (Phalaris arundinacea L.) is not palatable to ruminants but grows extensively in pasture areas. If the reason of the unpalatiblity and toxic nature of this plant could be located, the use of reed canary grass for ruminant feed might be possible. Tryptophan is also considered a precursor of the plant hormone, indoleacetic acid. Knowledge of the interrelationships of tryptophan and these important plant metabolites may solve the problems associated with the use of these plant materials as animal feeds.

The research reported in this thesis was concerned with examination of the biosynthesis and metabolism of tryptophan by rumen microorganisms using a continuous flow in vitro apparatus. The results obtained were compared with results of in vivo studies using labelled tryptophan injected into the rumen of sheep. Biosynthesis and metabolism of tryptophan were also studied in maturing reed canary grass. The possibility of incubating labelled reed canary grass with rumen microorganisms in the continuous flow in vitro apparatus was also considered.

TRYPTOPHAN BIOSYNTHESIS AND METABOLISM IN RUMINANTS.

LITERATURE REVIEW

Importance of tryptophan to animals

Tryptophan has been classified as an essential amino acid for the monogastric animal. Recent research has suggested a role for tryptophan in the protein synthesizing machinery of the body in addition to its function as a protein constituent. Munro (1968) reported studies with rats and mice fed amino acid mixtures deficient in one essential amino acid. Only the amino acid mixture lacking tryptophan gave rise to a reduction in liver polysome aggregates and accumulation of oligosomes. Sidransky et al (1968) tube-fed fasted mice a complete amino acid mixture and a mixture devoid of tryptophan They found that only the mice fed the amino acid mixture lacking tryptophan behaved like the fasted controls in causing polysome disaggregation. Tryptophan supplementation produced a response similar to that induced by the complete amino acid mixture. Methionine, threonine and isoleucine were also tested in a similar manner and did not shift the hepatic polysomes from lighter to heavier units. Freedman et al (1968) incubated rabbit reticulocytes under conditions of tryptophan deficiency and obtained results confirming Sidransky's findings. They speculated that polysome disaggregation was due to the location of

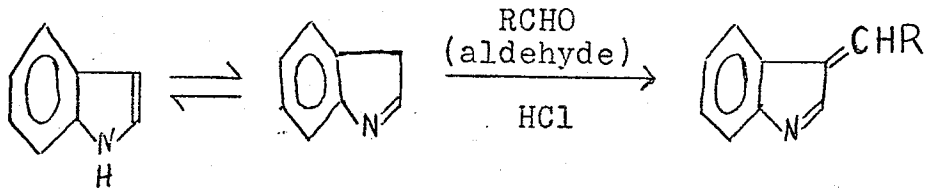
tryptophan near the amino terminal ends of rabbit hemoglobin. Since growth of the peptide chain normally proceeds from the amino terminal end, during tryptophan deficiency, the rate of translation of the messenger RNA was retarded near the proximal end, at the sites of the tryptophan codons. Beyond the tryptophan codon sites, the rate of translation was normal. The failure of the system to maintain a steady state number of ribosomes on messenger RNA resulted in polyribosome disaggregation.

According to Munro (1968), most proteins contain less tryptophan than other amino acids and therefore would contain considerable stretches of peptide chain not including tryptophan. Rate of translation would not be inhibited in non-tryptophan containing areas. Tryptophan could then readily become the limiting factor for protein synthesis. Munro also suggested that a low level of tryptophanyl-sRNA was in the cell due to tryptophan being the least abundant amino acid. Protein synthesis would be limited because insertion of tryptophan at the correct site in the peptide chain would involve the greatest delay in translation of the message.

Supporting evidence for the importance of tryptophan in protein synthesis was published by Yamashita et al (1969). Supplementation of a 20% casein diet with tryptophan, increased rat liver threonine dehydratase activity. Puromycin, an inhibitor of protein synthesis, inhibited tryptophan

stimulated threonine dehydratase activity, whereas actinomycin, an inhibitor of RNA synthesis, had no inhibitory effect. These authors concluded that the enzyme induction by tryptophan was based on de novo synthesis of protein at the translation step. Other amino acids did not increase the enzyme activity.

Chan and Schellenberg (1968) concluded from observations made on pig heart mitochondrial malate dehydrogenase that tryptophan was present at the active site of the enzyme. The tryptophanyl residues of some dehydrogenases were thought to participate in the hydrogen transfer through an intermediary indolenine structure.



The synthesis and metabolism of tryptophan has been found to be under a close metabolic control which differs from one species to another. Leklem et al (1969) found induction of tryptophan oxygenase in rats with less response in cats. Several urinary metabolites of tryptophan were known to be bladder carcinogens in mice. The urinary excretion of tryptophan metabolites was greater in rats than in cats and less spontaneous incidence of bladder cancer was observed in cats.

The above findings illustrate the diverse nature of tryptophan research and diseases which interfere with tryptophan metabolism further indicate the importance of

this compound to the body. Disturbance of tryptophan metabolism appears as a secondary effect in hyperhistinemia, phenylketonuria and maple syrup urine disease. Few direct tryptophan deficiency diseases are known in man, probably because a deficiency leads to general protein deficiency. Hartnup disease is a hereditary pellagra believed to be caused by defective absorption of tryptophan from the upper gut. In this disease there is increased excretion of indole derivatives and decreased excretion of metabolites of the nicotinic acid pathway.

An oral dose of tryptophan was found effective in producing interstitial pulmonary emphysema in experiments reported by Carlson et al (1968). The concentration of tryptophan in the plasma was not directly related to the development of the syndrome in cattle. Evidence indicated that some product of ruminal metabolism of tryptophan might be the causative factor. As sheep were not susceptible to tryptophan-induced interstitial pulmonary emphysema and the naturally occurring acute bovine pulmonary emphysema had not been reported in sheep, the authors concluded that the tryptophan-induced syndrome was species specific and that a difference in tryptophan metabolism might exist between cattle and sheep.

Impaired metabolism of tryptophan was shown in bovine tympanites or bloat (Johnson and Dyer 1968). The tympanitic bovine tended to excrete greater quantities of most of the tryptophan metabolites measured. The monoamines, 5-hydroxy-tryptamine (serotonin) and tryptamine, were thought to be

the most likely metabolites involved in the tympanitic syndrome. Previously Dyer et al (1964) had shown a difference in the metabolism of tryptophan and tyrosine by the normal and bloater bovine. More tryptophan was excreted in the urine by the bloater and it was excreted more rapidly than by the normal bovine.

The different metabolism of tryptophan in bloat is believed to have its effect through the tryptophan metabolite serotonin. Serotonin is a hormone involved in smooth muscle contraction, antidiuresis, depression of central ganglionic transmission and vasoconstriction. Barnes and Lowman (1968) showed that serotonin also protected mice against x-ray lethality.

Kuhn et al (1968) showed that sleep deprivation in humans led to a disturbance of tryptophan metabolism. An increased 5-hydroxy indoleacetic acid excretion in urine was explained by serotonin being bound in blood cells by ATP. Sleep deprivation led to a drop in level of ATP and subsequent release of serotonin from blood cells in the brain. On release, the serotonin was deaminated and then excreted as 5-hydroxy indoleacetic acid, considered to be a stress manifestation of sleep deprivation.

Two other tryptophan metabolites, 3-hydroxykynurenine and kynurenine, were found to be precursors of pigment in insect eyes (Fruton and Simmonds 1958). Mutagenic effects were produced by 3-hydroxykynurenine, along with 3-hydroxy anthranilic acid, which induced chromosomal rearrangements

in human primary embryonic tissue (Kuznetsova 1969).

The most thoroughly investigated metabolite of tryptophan in the animal body is nicotinamide. This B vitamin is an essential part of the enzyme system concerned with hydrogen transport in the living cell. Nicotinamide is the functional group of two coenzymes which act with flavoprotein enzymes in the electron transport chain.

General amino acid studies of ruminants

General reviews on the subject of nitrogen metabolism in the ruminant have been published by Moir (1957); Weller et al (1962) and Smith (1967). Less information is available concerning amino acid nutrition of the ruminant than the accumulated knowledge concerning other animals.

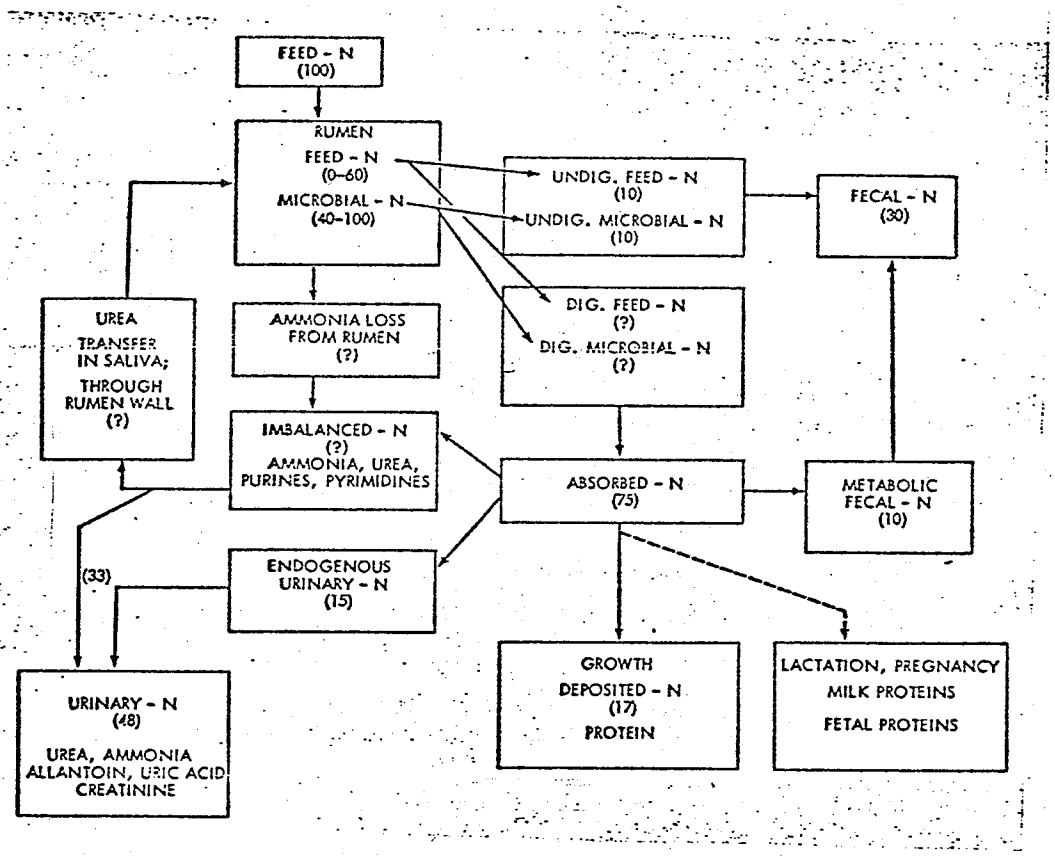
In the past, ruminants were thought to be independent of dietary sources of "essential" amino acids through the symbiotic relationship between the ruminant and rumen microorganisms. The assumption was made that much of the food nitrogen, whether of protein or non-protein nature, was synthesized by the bacteria into their own cellular protein; the protozoa utilized bacterial protein for growth; the ruminant then digested the protozoal and remaining bacterial protein. Black et al (1952 and 1955) provided evidence that the tissues of the cow synthesized only non essential (to the rat) amino acids. Therefore, the balance of amino acids absorbed from the gastrointestinal

tract may determine the relative importance of pathways (Fig. 1) as suggested by Jacobson et al (1970).

Since the recognition of the possible importance of amino acids to ruminants various approaches have been undertaken---research into the limiting amino acid, amino acid infusion into the abomasum, amino acid patterns available for absorption, alteration in microbial populations as affecting metabolism within the rumen and possibilities of coating amino acids and by-passing rumen microbial degradation.

Microbial degradation of dietary nitrogen sources in the rumen may be acceptable or desirable if microbial protein quality exceeds dietary protein quality. Feeding high levels of protein encourages greater voluntary consumption of net energy and therefore supports increased performance and increased efficiency of conversion of feed to milk. Reductions in milk production are associated with reductions in energy intake (Jacobson et al 1970).

The biological value of rumen microorganisms has been calculated at about 80 and the protozoa are considered slightly more digestible than bacteria (Annison and Lewis 1959). The amino acid composition of ruminal bacteria remains very constant and similar to that of grass protein, irrespective of the medium in which they grow (Moir 1957). When dietary protein is converted to microbial protein, a 10% loss of nitrogen occurs because of polynucleotide formation. In addition to the consideration of synthesis


FEED - N
(100)
RUMEN
FEED - N
(0–60)
MICROBIAL - N
(40–100)
UNDIG. FEED - N
(10)
UNDIG. MICROBIAL - N
(10)
FECAL - N
(30)
DIG. FEED - N
(?)
DIG. MICROBIAL - N
(?)
UREA
TRANSFER
IN SALIVA;
THROUGH
RUMEN WALL
(?)
AMMONIA LOSS
FROM RUMEN
(?)
IMBALANCED - N
(?)
AMMONIA, UREA,
PURINES, PYRIMIDINES
ABSORBED - N
(75)
METABOLIC
FECAL - N
(10)
(33)
ENDOGENOUS
URINARY - N
(15)
URINARY - N
(48)
UREA, AMMONIA
ALLANTOIN, URIC ACID
CREATININE
GROWTH
DEPOSITED - N
(17)
PROTEIN
LACTATION, PREGNANCY
MILK PROTEINS
FETAL PROTEINS

of tissue protein and replacement of endogenous nitrogen losses common to all animals, ruminant nutritionists must consider the effect of alteration of rumen microflora on nitrogen metabolism. Most strains of bacteria synthesize amino acids de novo (Wright and Hungate 1967 b). Evidence exists that certain strains of bacteria have a need for amino acids in addition to ammonia (Hatfield 1970), while others utilize peptide carbon more efficiently than amino acid carbon (Wright 1967).

El-Shazly (1952) observed deamination of amino acids in the rumen and Lewis and Emery (1962a) have calculated the relative deamination rates by rumen microorganisms. Tryptophan was in the group of amino acids where deamination was least pronounced and both D and L forms of tryptophan were catabolized by rumen microorganisms. Sirotnak et al (1953) tested L forms of 22 amino acids (including tryptophan) for in vitro degradation reactions and degradation occurred with only six---aspartic acid, glutamic acid, serine, arginine, cysteine and cystine. The other 16 amino acids were not degraded in vitro.

Moir (1957) reported the presence of low concentrations of free amino acids in the rumen. However, Leibholz (1965 and 1969) found that the free amino acids in rumen liquor increased up to 100 times the initial prefeeding values between 1 and 3 hours after feeding. Although tryptophan was not determined in the experiments reported, the phenylalanine and tyrosine results showed an increase in concentration of

11 and 3.4 times at the end of one hour. The amino acid content of rumen fluid was found to differ with dietary treatment (Richardson and Tsien 1963). Twin steers fed a urea diet had 55.99 and 45.78 mg/l tryptophan in rumen fluid and when the diet was changed to soybean meal the values rose to 78.20 and 129.23 mg/l respectively. Similar changes were found for all other amino acids.

Growth and feed efficiency were reduced by 35% with steers fed a urea diet as compared to a protein containing diet (Oltjen 1969). Attempts to improve performance of ruminants with amino acid supplementation have been largely unsuccessful. McLaren et al (1965) observed retention of absorbed nitrogen by lambs fed rations containing either supplemental methionine or tryptophan or both amino acids, was 15% greater than lambs fed a urea-basal ration. The authors suggested the results might reflect the inability of microorganisms of lambs fed urea diets to synthesize tryptophan and methionine at a rate permitting maximal rumen microbial protein synthesis. Poley (1965) described tryptophan as the second limiting amino acid in abomasal ingesta when high levels of corn gluten meal or corn protein were fed as these feeds were low in tryptophan and microorganisms were marginal in tryptophan content. He concluded that the limiting amino acids for ruminants were methionine, tryptophan and lysine. Bunn et al (1968) compared amino acid and alfalfa supplementation of purified diets for growth of lambs. The basal urea diet did not support growth as well as the

supplemented diets. There was a marked difference in rumen flora with the amino acid supplemented diet and many large, curved, rod-shaped gram negative organisms were apparent. Growth increased with abomasal amino acid administration to lambs in experiments by Schelling and Hatfield (1968) and the voluntary feed intake was increased by administration of amino acids in protein form.

No extracellular free proteolytic enzyme has been found in the rumen (Blackburn 1965). Protozoa and large bacteria play the most active part in proteolysis (Blackburn and Hobson 1960 a) and only 1% of bacteria in the rumen were proteolytic (Blackburn and Hobson 1960 b).

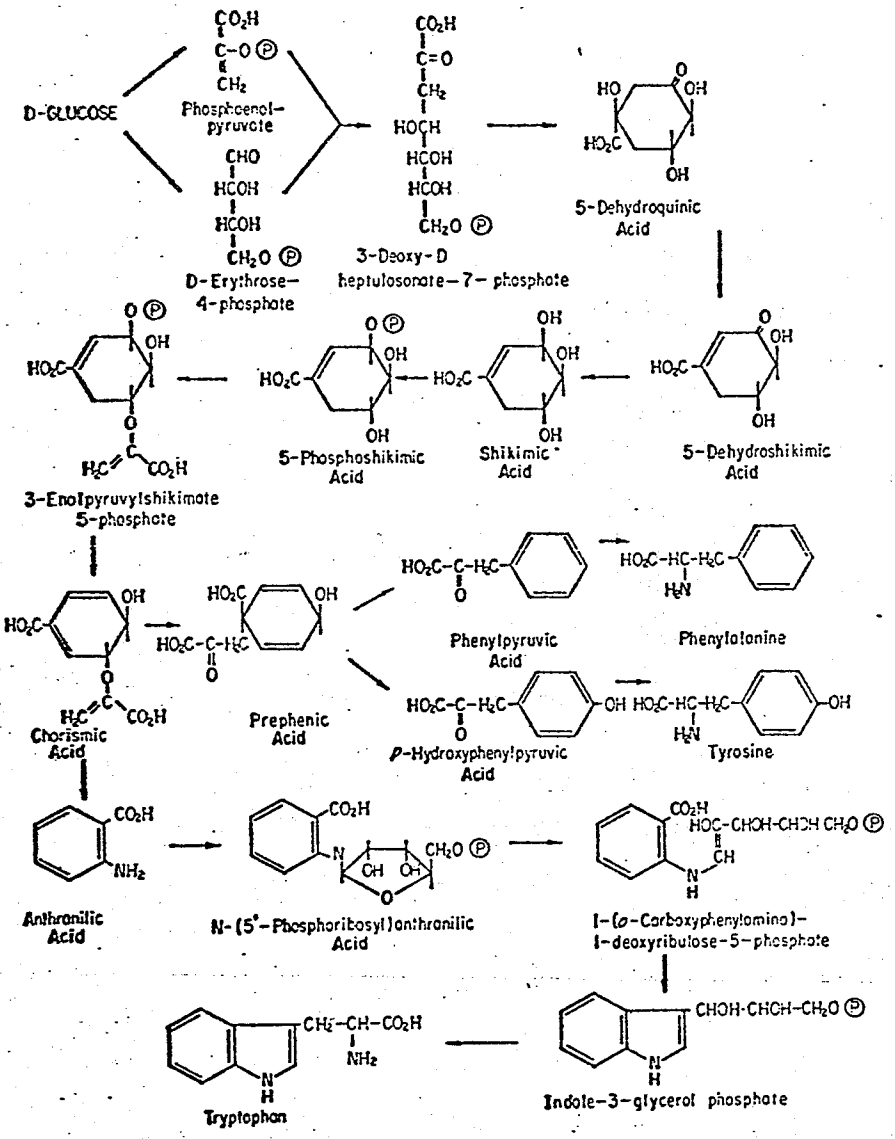
Evidence obtained by Cook et al (1961) suggested that the competition between amino acids for absorption sites which occurs in intestinal tissues of other species, may influence amino acid absorption from the rumen. Ruminants fed protein-free diets had depressed free blood plasma concentrations of the essential amino acids (Oltjen 1969). The significance of plasma amino acid pattern was not clear, but data showed that lambs fed nitrogen from different sources have different plasma amino acid patterns which could influence tissue metabolism and subsequent animal performance (Theurer et al 1968). Purser (1970) suggested that elevated levels of certain amino acids in plasma would more closely reflect catabolism rather than absorption. Poley (1965) found a rise in free tryptophan of plasma shortly after abomasal supplementation of tryptophan. A reduction in total free

amino acids of plasma was recorded when tryptophan was supplemented to the abomasum which Poley indicated might possibly mean an increase in tissue protein synthesis.

Biosynthesis of tryptophan

The biosynthesis of tryptophan in E. coli (Fig. 2) was outlined by Greenberg (1967). Recent reviews (Gibson and Pittard 1968; Doy 1968; Lingens 1968) have indicated a shift in research emphasis from tryptophan pathway studies to the control of tryptophan biosynthesis. Differences in the pathway of tryptophan synthesis in related organisms have been discussed by Hütter and De Moss (1967). The first enzyme in the pathway, 3-deoxy-D-arabino-heptulosonate-7-phospate (DAHP) synthetase, was found to be three isoenzymes, each repressed or inhibited by a different product (Umbarger 1969).

Although tryptophan has been classified as essential for monogastric animals the status with regard to ruminants is unresolved. Initial work carried out by Loosli et al (1949) indicated net synthesis of all amino acids by rumen micro-organisms. Retention of tryptophan by the animal was the poorest of the amino acids tested. Allison and Robinson (1967) demonstrated carboxylation and amination of indoleacetic acid by anaerobic bacteria from the rumen. The microbial cells incorporated 5% of ^{14}C from indoleacetic acid-1-^{14}C in 2 hours. Virtually all the label migrated with tryptophan. A number of pure


D-GLUCOSE
Phosphoenol-pyruvate
D-Erythrose-4-phosphate
3-Deoxy-D heptulosonate-7-phosphate
5-Dehydroquinic Acid
5-Dehydroshikimic Acid
Shikimic Acid
5-Phosphoshikimic Acid
3-Enolpyruvylshikimate 5-phosphate
Chorismic Acid
Prephenic Acid
Phenylpyruvic Acid
Phenylalanine
p-Hydroxyphenylpyruvic Acid
Tyrosine
Anthranilic Acid
N-(5'-Phosphoribosyl)anthranilic Acid
1-(o-Carboxyphenylamino)-1-deoxyribulose-5-phosphate
Indole-3-glycerol phosphate
Tryptophan

cultures of important anaerobic bacteria from the rumen have been grown in media lacking tryptophan and containing indoleacetic acid-1-^{14}C. Ruminococcus albus strains 7 and 20 were the only organisms found to incorporate appreciable radioactivity. Piana and Piva (1968) incubated $(^{15}NH_4)_2HPO_4$ with rumen contents and observed the label appeared in all amino acids except tryptophan.

Tryptophan metabolism

There are many metabolites of tryptophan (Fig. 3). The carbon catabolism of tryptophan in microorganisms takes place primarily through the aromatic and quinoline pathways. The quinoline pathway is incomplete in vertebrate animals and the kynurenine-anthranilic acid aromatic sequence (Fig. 4) predominates with the serotonin pathway providing an alternative metabolic pathway (Meister 1965; Greenberg 1967; Umbreit 1960; Munro and Allison 1964).

A comparative study carried out by Lan and Gholson (1965) indicated a good correlation between the ability of organisms to oxidize the benzene ring of tryptophan in vivo and the presence of glutarate pathway enzymes whereas arthropods did not have these enzymes. Most, if not all, oxidative reactions of tryptophan metabolism involved oxygen fixation (Hayaishi and Nozaki 1969). An indication that liver tryptophan pyrrolase activity was lower in ruminants than in rats was reported recently by Carlson and Dyer (1970).

Figure 3

Summary scheme for tryptophan metabolism

Ref. Meister, A. 1965.

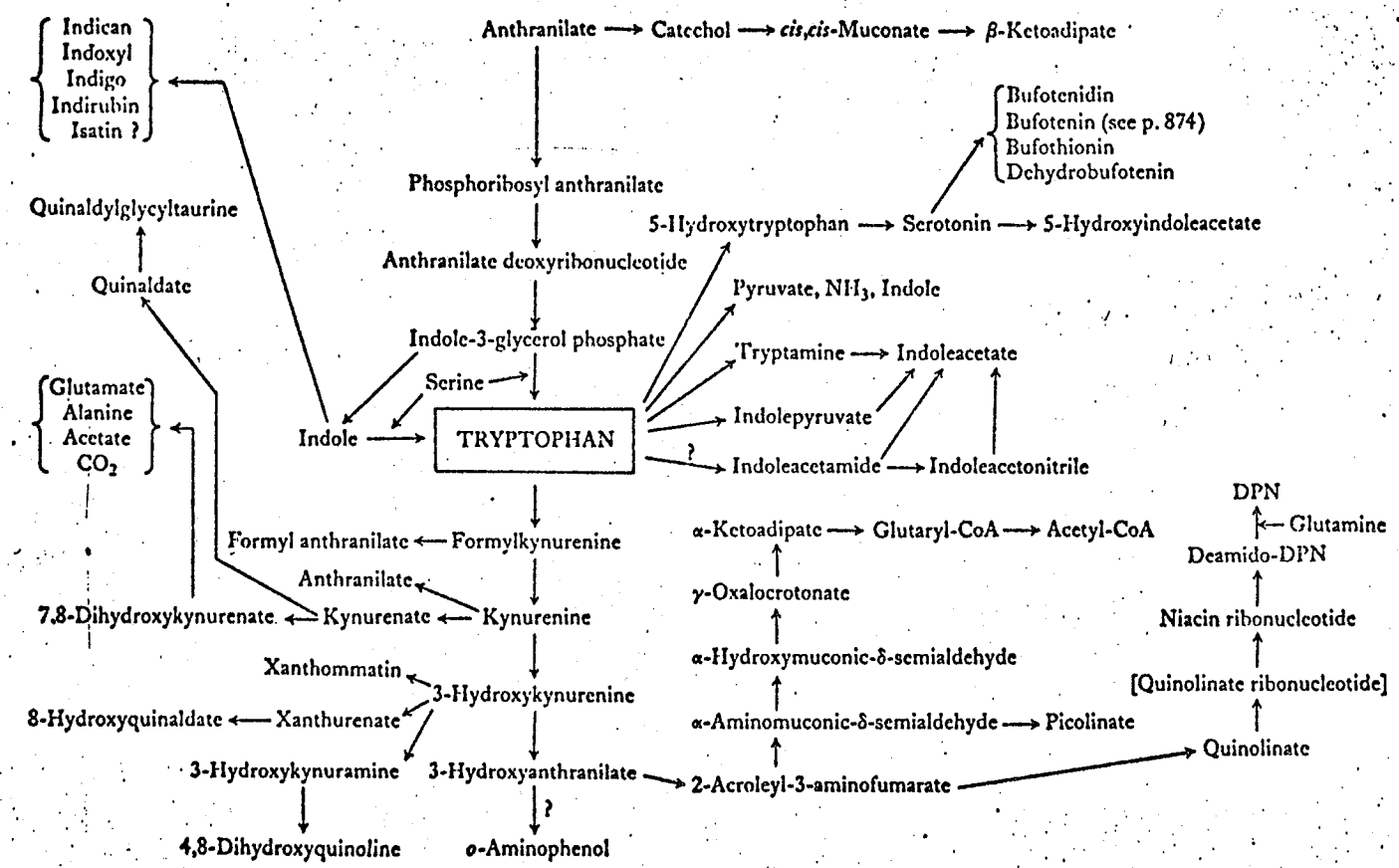
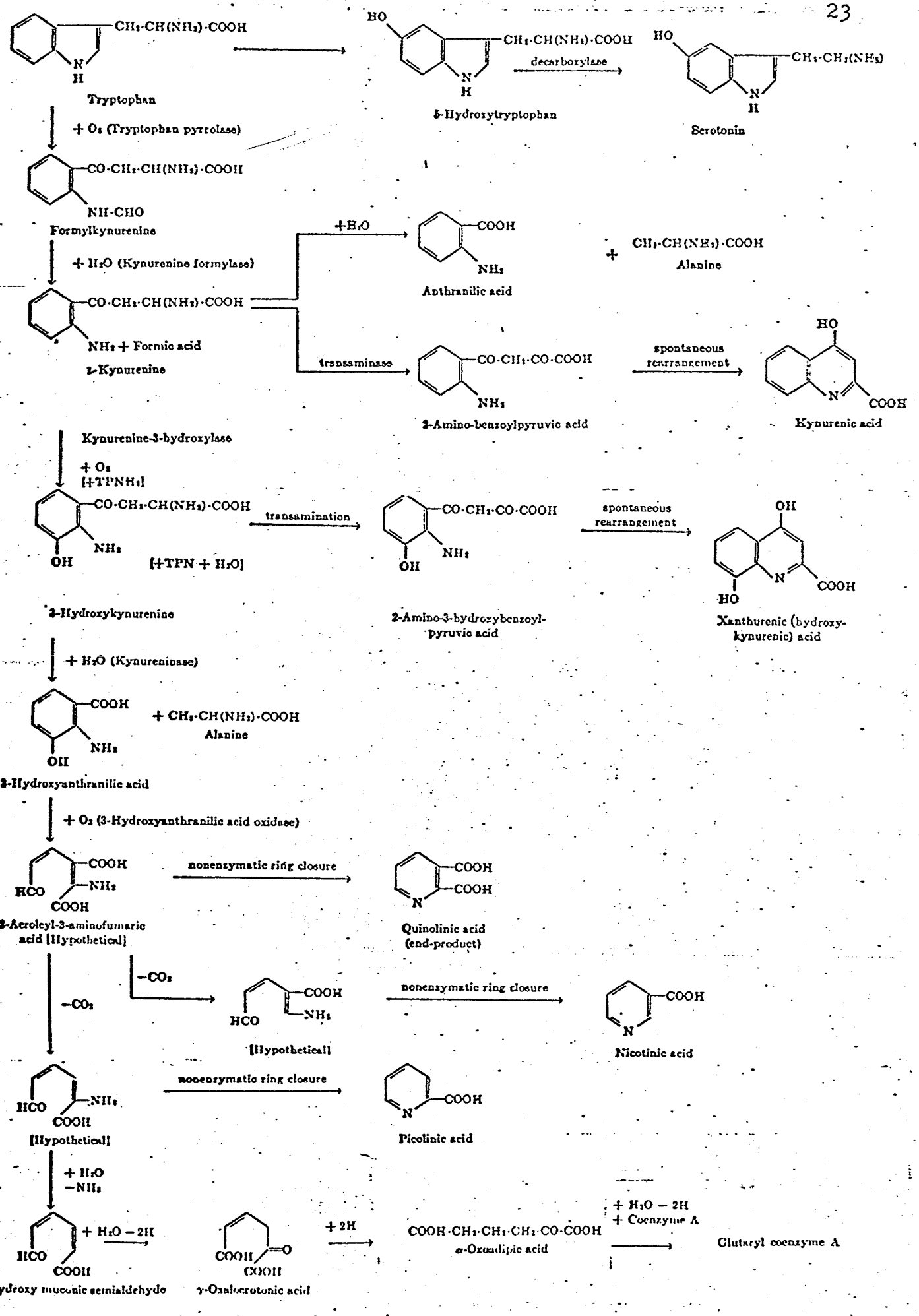

Indican
Indoxyl
Indigo
Indirubin
Isatin ?
Anthranilate
Catechol
cis,cis-Muconate
β-Ketoadipate
Bufotenidin
Bufotenin (see p. 874)
Bufothionin
Dehydrobufotenin
Phosphoribosyl anthranilate
Quinaldylglycyltaurine
5-Hydroxytryptophan
Serotonin
5-Hydroxyindoleacetate
Anthranilate deoxyribonucleotide
Quinaldate
Pyruvate, NH3, Indole
Indole-3-glycerol phosphate
Tryptamine
Indoleacetate
Glutamate
Alanine
Acetate
CO2
Serine
Indole
TRYPTOPHAN
Indolepyruvate
Indoleacetamide
Indoleacetonitrile
DPN
Glutamine
Deamido-DPN
Formyl anthranilate
Formylkynurenine
α-Ketoadipate
Glutaryl-CoA
Acetyl-CoA
Anthranilate
γ-Oxalocrotonate
Niacin ribonucleotide
7,8-Dihydroxykynurenate
Kynurenate
Kynurenine
α-Hydroxymuconic-δ-semialdehyde
Xanthommatin
3-Hydroxykynurenine
[Quinolinate ribonucleotide]
8-Hydroxyquinaldate
Xanthurenate
α-Aminomuconic-δ-semialdehyde
Picolinate
3-Hydroxykynuramine
3-Hydroxyanthranilate
2-Acroleyl-3-aminofumarate
Quinolinate
4,8-Dihydroxyquinoline
o-Aminophenol

Figure 4

Kynurenine-anthranilic acid aromatic pathway (glutarate pathway)

The main pathway of complete degradation of tryptophan leads to glutaryl coenzyme A which can be degraded to acetyl coenzyme A in a manner similar to lysine.

Ref. Munro, H. N. and Allison, J. B. 1964


Tryptophan
+ O₂ (Tryptophan pyrrolase)
Formylkynurenine
+ H₂O (Kynurenine formylase)
NH₂ + Formic acid
L-Kynurenine
5-Hydroxytryptophan
decarboxylase
Serotonin
+H₂O
Anthranilic acid
Alanine
transaminase
2-Amino-benzoylpyruvic acid
spontaneous rearrangement
Kynurenic acid
Kynurenine-3-hydroxylase
+ O₂
[+TPNH₂]
[+TPN + H₂O]
3-Hydroxykynurenine
transamination
2-Amino-3-hydroxybenzoyl-pyruvic acid
spontaneous rearrangement
Xanthurenic (hydroxy-kynurenic) acid
+ H₂O (Kynureninase)
3-Hydroxyanthranilic acid
Alanine
+ O₂ (3-Hydroxyanthranilic acid oxidase)
2-Acroleyl-3-aminofumaric acid [Hypothetical]
nonenzymatic ring closure
Quinolinic acid (end-product)
−CO₂
[Hypothetical]
nonenzymatic ring closure
Nicotinic acid
nonenzymatic ring closure
[Hypothetical]
Picolinic acid
+ H₂O −NH₃
+ H₂O − 2H
α-Hydroxy muconic semialdehyde
γ-Oxalocrotonic acid
+2H
α-Oxoadipic acid
+ H₂O − 2H
+ Coenzyme A
Glutaryl coenzyme A

The mechanism for enzyme regulation seemed different in the ruminant than mice. Augmented incidence of bladder carcinomas in mice exposed to such tryptophan metabolites as 3-hydroxy-L-kynurenine, 3-hydroxy-anthranilic acid and xanthurenic acid (Bryan 1969) showed the importance of regulation of tryptophan metabolism.

Scott et al (1964) found there was conversion of ^{14}C labelled tryptophan to volatile fatty acids by rumen microorganisms but the conversion was small when compared to tyrosine and phenylalanine. Decarboxylation of tryptophan to yield tryptamine seemed to be a factor influencing bloat (Johnson and Dyer 1968). Lewis and Emery (1962 b) did not detect amine production in rumen fluid when tryptophan, phenylalanine, histidine and arginine were incubated. The slow metabolism of tryptophan by rumen microorganisms was noted by Lacoste (1961) and Lewis and Emery (1962 c). The products of any metabolism in the rumen were indoleacetic acid, indole propionic acid, indole and skatole.

The importance of tryptophan to animals stimulated research into the biosynthetic and metabolic pathways followed in various species. The lack of definite information with regard to the ruminant animal is probably due to the complicating factor of the rumen microorganisms. In vitro techniques (Johnson 1966) facilitated the study of the activity of the microorganisms away from the control and influence imposed by the host animal. However, in vivo experiments provided more practical information about

tryptophan utilization, metabolism and absorption from the rumen (White et al 1969). The conflicting and sparse results reported to date have made postive statements about tryptophan synthesis and metabolism in the ruminant impossible. The research reported in the present thesis was initiated to expand the knowledge of tryptophan synthesis and utilization in the ruminant.

MATERIALS AND METHODS

In vitro technique

A continuous flow artificial rumen apparatus similar to the Davey et al (1960) design was constructed (Fig. 5-8). The artificial rumen sac, hereafter referred to as "rumen sac", was prepared by soaking a 16" length of 2" diameter dialysing tubing in formaldehyde solution for at least 24 hours. The dialysing tubing was then tied at one end and the open end of the sac was fitted into the apparatus. A Magni-whirl water bath was used to maintain a temperature of 39°C. A Radiometer pH meter with a combination electrode GK 2025c (Canlab 43-9141) was used to monitor the pH of the medium. The pH was maintained between 6.9 and 7.0 during each experiment. If the pH was below 6.9, Na_2CO_3 (106 g/l of water) was added at the rate of 2.5 ml for every 0.1 unit drop. If the pH was above 7.0, H_3PO_4 (concentrated) was added. The saline solution was dialysed against a sample of rumen fluid for 48 hours before the start of each experiment. The rumen sac contents were agitated by a continuous flow of 5% CO_2 in N_2 (Liquid Carbonic, Winnipeg), through a sparger.

Rumen samples were collected by inserting a stomach tube with strainer into the rumen of a fistulated

Figures 5-8

The continuous flow *in vitro* apparatus.

Legend for diagram

A container for saline solution

B saline collection container

C artificial rumen sample flask

D fermentation vessel

K column of liquid

S_1 and S_2 screw clamps for equalizing flow rates

F leads from pH meter to combination electrode

1 tube to manometer and liquid column

2 thermometer

3 tube for removing artificial rumen sample

4 gas inlet tube for sparger

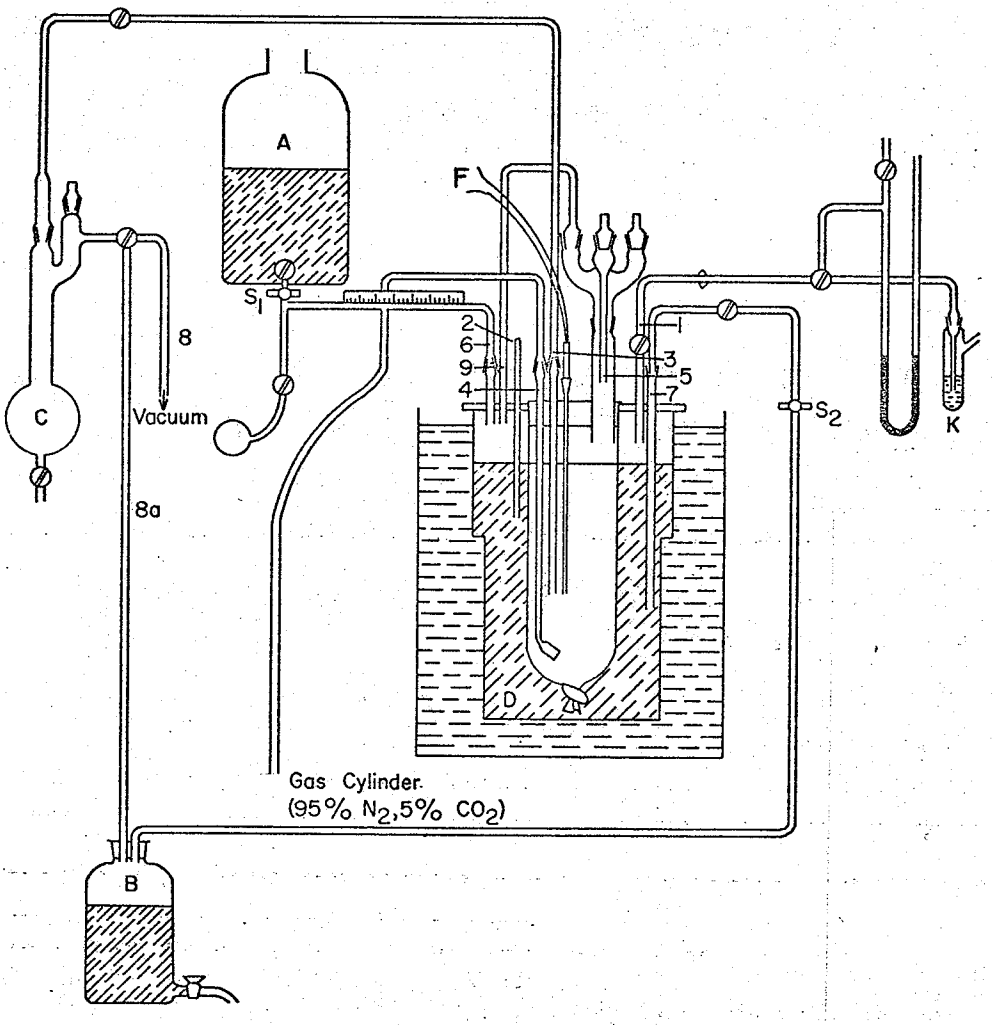
5 tube for "feeding" artificial rumen

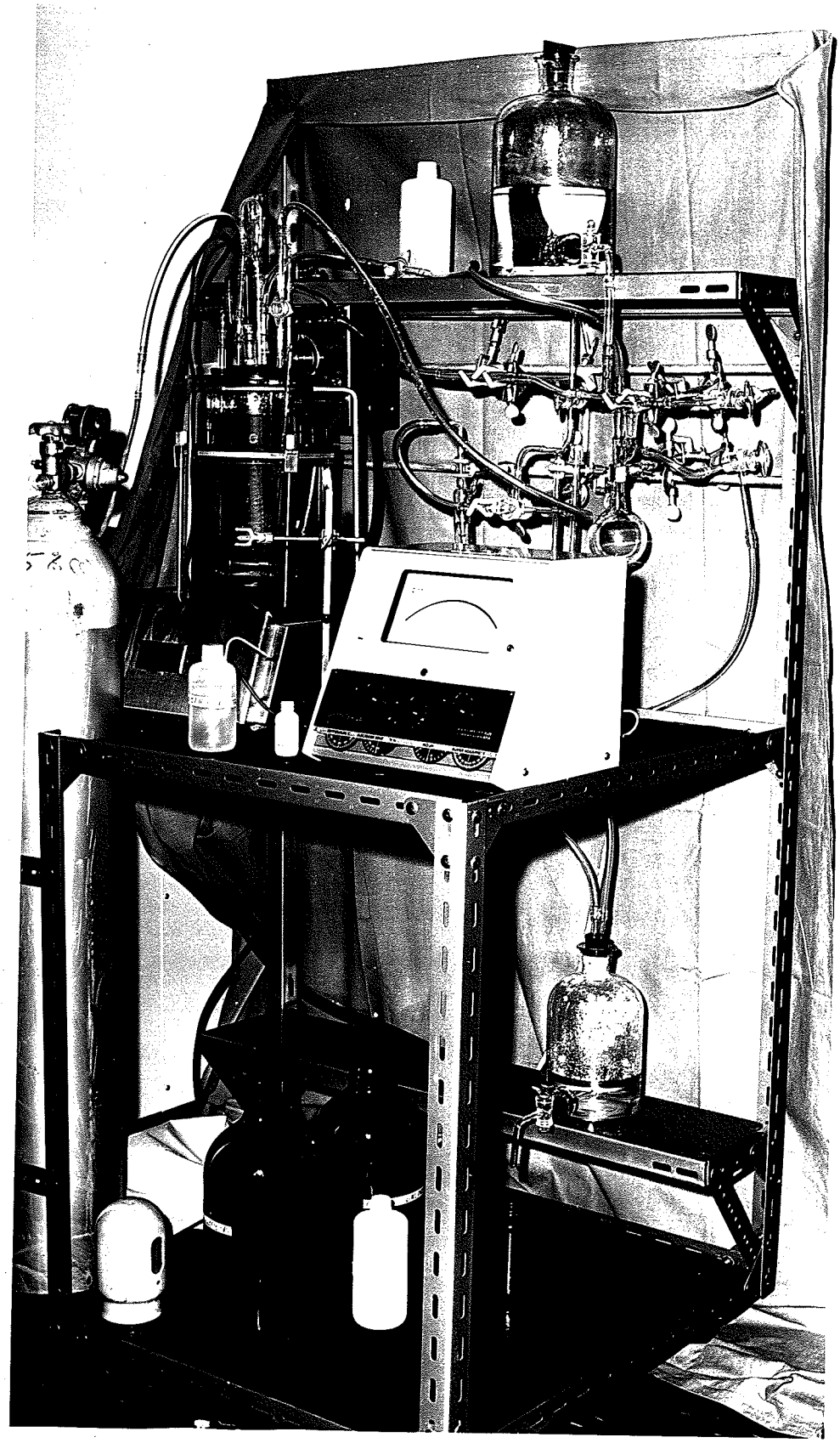
6 inlet tube from saline reservoir

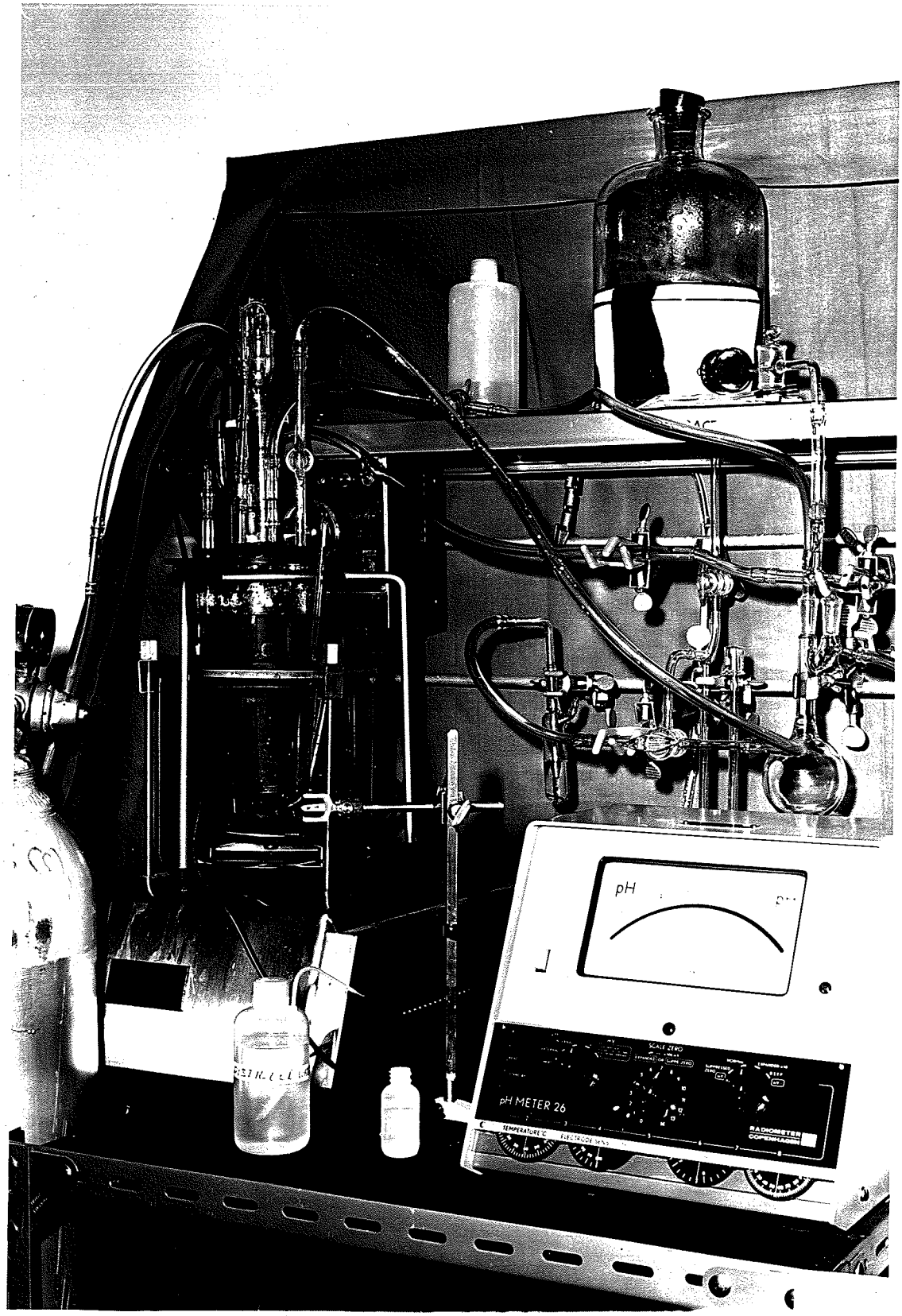
7 outlet tube from saline solution surrounding artificial rumen membrane

8 and 8a vacuum line for removing samples

9 tube for equilibrating gas inside artificial rumen and in saline compartment


A
F
S1
8
C
Vacuum
8a
2
6
9
4
3
5
1
7
S2
K
D
Gas Cylinder.
(95% N2,5% CO2)
B




pH
pH METER 26
RADIOMETER


FULL-VISIBILITY
JAR BATH
MICROTROL

Holstein cow prior to the daily feeding of grass alfalfa hay mixture. Volatile fatty acid (VFA) analysis (Davey et al 1960), micro-kjeldahl nitrogen determinations (Albanese 1963) and microscopic examination of the extracted rumen sac contents were conducted on all in vitro experiments to assess protozoa viability.

In each experiment, the experimental compounds plus McDougall's solution (McDougall 1948) were added to the rumen fluid sample in the rumen sac and the sac was surrounded by 2400 ml saline (0.9% NaCl in water).

Experiment 1: 100 mg of DL-tryptophan and 0.5 uCi DL-tryptophan-^{14}C (U)-benzene ring labelled (Nuclear-Chicago, DesPlaines, Illinois) in 20 ml of McDougall's solution were added to the rumen sac at "0" time.

Experiment 2: 0.5 uCi DL-tryptophan-^{14}C (U)-benzene ring labelled in 20 ml of McDougall's solution was added to the rumen sac at "0" time.

Experiment 3: 10 uCi DL-tryptophan-^{14}C (U)-benzene ring labelled in 20 ml of McDougall's solution was added to the rumen sac at "0" time.

Experiment 4: 63 mg L-serine, 160 ug pyridoxine. HCl, 963 ug indole, 1 g sodium acetate and 10 uCi L-serine-^{14}C (U) (Radiochemical Center, Amersham) in 20 ml of McDougall's solution were added to the rumen sac at "0" time and again at "23 hours" time (Hoch et al 1969).

Experiment 5: The same compounds as experiment 4 except 17 uCi of L-serine-^{14}C (U) was added to the rumen sac at only "0" time.

A Nuclear-Chicago Model 720 liquid scintillation counter was used to count samples. Saline solution (0.1 ml) was counted in Bray's solution (Bray 1960). Rumen sac samples (0.1 ml) were incubated overnight with 1ml 1M hyamine hydroxide in methanol (Amersham/Searle, DesPlaines Illinois 60018) at 80°C and counted in Bray's solution. The bacterial cells were digested with formamide (Moore et al 1962) for 2.5 hours at 60°C in glass stoppered test tubes (1 ml of formamide per 5-20 mg of cells). Half of the digest was counted in Bray's solution.

The rumen sac sample was separated by centrifugation into protozoal, bacterial and supernatant fractions (Moore et al 1962) and analysed for tryptophan content (Inglis and Leaver 1964; Kupfer and Atkinson 1964), labelled material and total nitrogen. Determinations were completed at all time periods, provided that sample quantities were adequate.

In addition, for experiments 4 and 5, saline, supernatant, bacterial and protozoal fractions were analysed for tryptophan using ion exchange and thin layer chromatography. To prepare bacterial and protozoal fractions for the ion exchange procedure, samples were hydrolysed in 5N NaOH for 20 hours before neutralizing with 5M H_2SO_4 and making up to 25 ml with water (Sjoerdoma 1962). An ion exchange column was prepared using Rexyn 101 (Na^+) for the column material. The sample was adjusted to acidic pH and added to the column.

The column (1.2 X 16 cm) was run with 25 ml pH 2.2 buffer[1], 10 ml water, 10 ml 2N NaOH, 30 ml water and an additional 20 ml pH 2.2 buffer. The tryptophan was in the fraction collected following the NaOH addition. The tryptophan containing fraction was dried, using a flash evaporator, taken up in about 3 ml water and streaked on thin layer plates. The adsorbent used was cellulose MN 300 (10.0 g): silicia gel H (4.0 g): water (80 ml) (Bieleski and Turner 1966). Separation was effected by n-butanol: water: acetic acid (120:50:30 ml). After separation was completed (3 hours), the plates were dried and the areas containing serine (R_F=0.2) and tryptophan (R_F=0.5) as well as the area between serine and tryptophan were lifted and placed in counting vials. One ml 95% ethanol was added to the adsorbent in the vial, and the vial was then filled with Bray's solution and labelled material counted.

In vivo tryptophan absorption--intact rumen

Portal vein catheters were established in two mature Rambouillet ewes using a surgical procedure similar to the method described by Conner and Fries (1960).

[1]19.6 g sodium citrate.$2H_2O$; 16.5 ml concentrated HCl; 5ml thioglycol; 2 ml Brij solution; 0.1 ml pentachlorophenol (50 mg in 10 ml 95% ethanol) final volume is 1 l.

Heparinized electrolyte[1] was used to clean the portal vein catheters daily to maintain blood flow through the catheter. The ewes were maintained in a metabolism crate for the duration of the experiment. Jugular vein catheters were inserted immediately before the labelled material was injected into the rumen. A urinary catheter was used for collection of urine.

Experiment 6: Sheep 1-10 uCi DL-tryptophan-^{14}C (U)-benzene ring labelled material in water was injected directly into the rumen one hour after the ewe had consumed a grass alfalfa hay mixture.

Experiment 7: Sheep 2-20 uCi DL-tryptophan-^{14}C (U)-benzene ring labelled material in water was injected directly into the rumen one hour after the ewe had consumed a grass alfalfa hay mixture. Subsequently, blood samples were taken from jugular and portal catheters at ten minute intervals during the first hour; 1.5, 2, 3, 4, 5, 6, 8, and 12 hr and then once daily for four days. Urine and feces samples, when available were collected at the same time as the blood samples. Sheep 1 was slaughtered 3 weeks after the injection of the labelled tryptophan while sheep 2 was slaughtered 1 week after the injection.

Portal and jugular blood was collected in tubes with heparin added and centrifuged at 2000 x g for ten

[1] NaCl (5.5 g); $CaCl_2$ (0.3 g); $MgCl_2.6H_2O$ (0.3 g); CH_3COONa (5.0 g); CH_3COOK (1.0 g); sodium citrate (0.8 g) and 0.5 ml heparin solution (containing 100 mg/ml); in 1 l. of water and whole solution sterilized.

minutes. Plasma was drawn off and prepared for counting by heating 1 ml plasma with 3 ml of 1M hyamine hydroxide in methanol overnight at 80°C. The sample was decolourized with H_2O_2 before adding 15 ml Bray's scintillation solution. Feces samples were extracted with water in a steam bath overnight and 1 ml aliquot of the liquid extract was counted in a manner similar to plasma. An ethanol extraction of feces was also performed and a fraction counted. Urine samples were counted after heat treatment of 1 ml sample with H_2O_2 at 80°C overnight.

Urine samples were prepared for thin layer chromatography by drying 15 ml of urine and adding water to a total volume of 1 ml. The adsorbent used was cellulose MN 300 (10.0 g): silica gel H (4.0 g): water (80 ml). Separation was effected by n-butanol: water: acetic acid (120:50:30 ml). After development of 1 ul spots, compounds were located by using Ehrlich's reagent (1 g p-dimethylaminobenzaldehyde in 10 ml concentrated HCl and 90 ml acetone) and Ninhydrin spray (0.3 g ninhydrin dissolved in 100 ml n-butanol and 3 ml acetic acid) in conjunction with ultraviolet mapping. Labelled components were located by streaking a 20 ul sample on the adsorbent, followed by development and counting of the lifted layer material. Twenty sections (fractions) were lifted per plate and counted.

Urine samples were also partitioned in benzene to separate tryptamine, indoleacetic acid and tryptophan (Sjoerdoma 1962). The fraction containing the tryptophan

was further separated by ion exchange chromatography using Rexyn 101 (Na^+) for the column material. Elution of tryptophan was monitored using a flow through fluorimeter (Turner-model 111). The tryptophan containing fraction was dried, made up to 1 ml with water and spotted on thin layer plates. The area containing tryptophan was lifted and counted. The tryptamine and indoleacetic acid fractions were also spotted on thin layer and the specific areas counted to identify the proportion of the label in each of these compounds.

Blood samples were pooled in an attempt to obtain sufficient radioactivity to be assayed in a manner similar to the urine samples. Three portal plasma samples from each sheep were analysed. These samples represented early (0 to 2 hr); mid (3 to 5 hr); and late (after 5 hr) time periods. Only one sample of jugular plasma was obtained from sheep 1 and it corresponded to the late time period. Two samples of jugular plasma (an early and mid sample) were analysed from sheep 2.

Tissue samples of the kidney, mammary gland, lung, spleen, liver, brain and muscle were taken at the time of slaughter for examination of label content.

In vivo tryptophan absorption--isolated rumen

Three mature Rambouillet ewes were surgically fitted with rumen cannula for the absorption experiments. In the initial absorption experiment (sheep 3) the subsequent

surgical operations followed immediately, whereas with sheep 4 and 5, three complete days passed to allow the ewe to recover from the cannula operation. After anaesthetizing each sheep, the reticulo-omasal orifice was tied off, urinary and jugular catheters inserted, and labelled material introduced into the rumen.

Experiment 8: Sheep 3 received 1.5 uCi sodium acetate-^{14}C in the rumen.

Experiment 9: Sheep 4 received 5 uCi of DL-tryptophan-^{14}C (U)-benzene ring labelled material into the rumen.

Experiment 10: Sheep 5 received 5 uCi of DL-tryptophan-^{14}C (U)-benzene ring labelled material into the rumen. Labelled compounds were added with McDougall's solution, sodium acetate and polyethylene glycol marker.

The washed rumen technique (Masson and Phillipson 1951) was used with sheep 3 and 4 and the whole rumen contents were left intact in sheep 5. Jugular blood, urine and rumen samples were taken at 0,15,30 min and 1,2 and 3 hr after introduction of the labelled compound. These experiments were terminated at 3 hr. Counting procedures for these samples were the same as those described earlier.

RESULTS AND DISCUSSION

In vitro technique

Rumen samples were taken from the cow just prior to feeding and contained 36 ug tryptophan per ml in the supernatant. This amount was assumed to be the minimum value of tryptophan since the level would be expected to rise after feeding the grass alfalfa hay mixture.

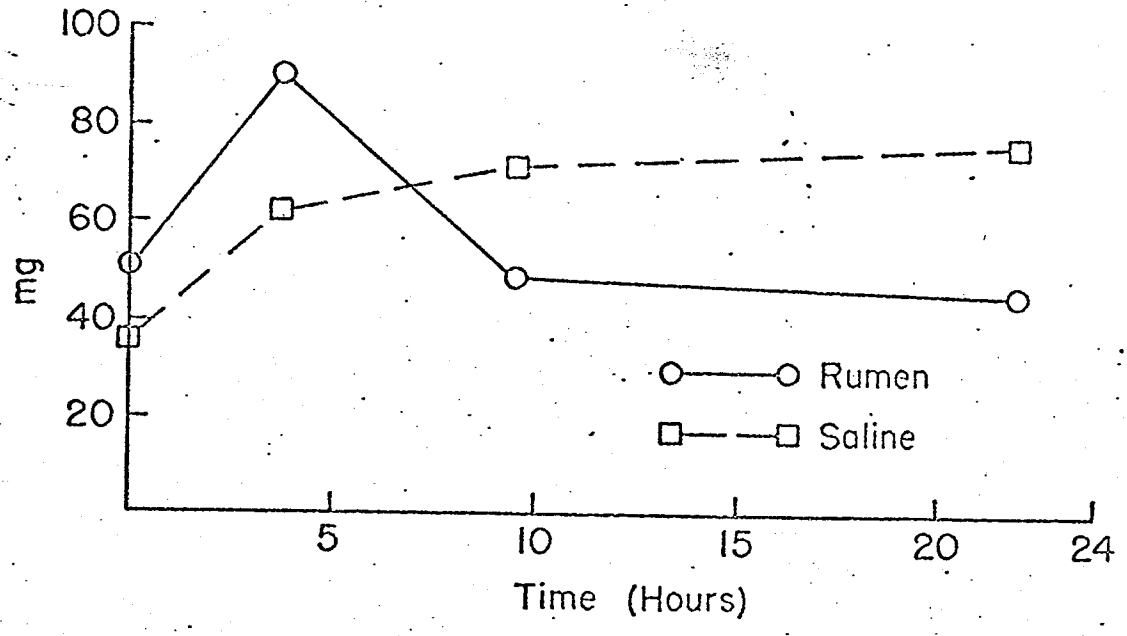
The values at time zero (Fig. 9) represent the tryptophan initially present in the system as the 100 mg tryptophan was added to the rumen sac contents after the zero sample was obtained. Total tryptophan in the rumen sac after addition (zero time)should have been 190 mg (Table 25). The total tryptophan determined by colorimetric analysis in the system when the first sample was taken was 152 mg. This error in colorimetric determination of tryptophan appeared throughout the determinations. As the determinations of radioactivity showed good agreement between the calculated and observed values in Experiment 1 (Table 26), the error must have been introduced by way of the colorimetric procedure. The standard curve for tryptophan when the Kupfer and Atkinson (1964) method was used only cut the y-axis at zero when optical density values below .1 were disregarded.

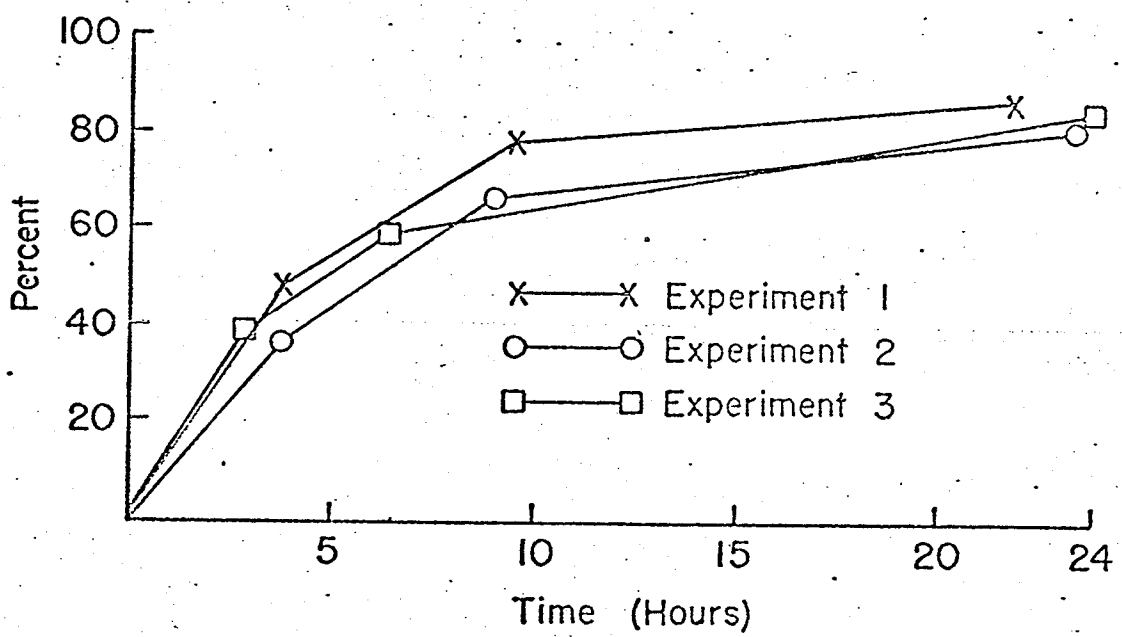
Figure 9

Total quantities of tryptophan in rumen sac and saline solution during 24 hour period as determined by colorimetric analysis (Experiment 1). DL-tryptophan was added immediately following "0" sampling time. (See table 25).

Figure 10

Labelled compounds appearing in the saline solution expressed as a percent of the labelled tryptophan added to the rumen sac. (See table 27).


100
80
60
40
20
mg
Rumen
Saline
5
10
15
20
24
Time (Hours)


100
80
60
40
20
Percent
Experiment 1
Experiment 2
Experiment 3
5
10
15
20
24
Time (Hours)

Half of the labelled material left the rumen sac in the first six hours of incubation (Fig. 10). The presence of unlabelled tryptophan in experiment 1 did not seem to markedly influence the rate of appearance of labelled material in the saline but experiment 1 values were higher at all time periods than in experiments 2 and 3.

The specific activity of tryptophan in the rumen contents (Table 1) was initially ("0" time) 7.55 dpm/ug tryptophan. The specific activity of the labelled material dropped to 2.93 dpm/ug tryptophan. As most of the labelled tryptophan was initially located in the fluid portion of the rumen sac, the drop in specific activity of the labelled material in the rumen sac showed a rapid rate of transfer across the rumen sac into the saline solution. More definite quantitative results would have been obtained if the specific activity of tryptophan could have been calculated throughout the experiment. However, the general conclusion could only have been further verified as the drop in dpm/ug recorded for the rumen sac contents would have been more drastic if some of the label was in compounds other than tryptophan.

The slow metabolism of tryptophan by rumen microorganisms was suggested previously (Lacoste 1961). Tryptophan pyrrolase, the initial degradative enzyme in tryptophan metabolism, is an oxygen requiring enzyme (Hayaishi and Nozaki 1969). As the rumen supports anaerobic growth, the reducing atmosphere would not provide the ideal medium for tryptophan metabolism. Less than 4% of the labelled

Table I

Labelled material compared to tryptophan content in the rumen sac and saline solution (Experiment 1)

Time Period[1]	Rumen sac Tryptophan content ug/ml	Rumen sac dpm/ug	Saline solution Tryptophan content ug/ml	Saline solution dpm/ug
0	514	7.55	15	0.00
1	280	6.27	26	7.13
2	200	4.65	32	11.70
3	150	2.93	32	11.50

[1]Time periods used were:
Experiment 1: 0; 3 hr 45 min.; 9 hr 30 min.; 22 hr.
Experiment 2: 0; 3 hr; 9 hr 15 min.; 23½ hr.
Experiment 3: 0; 3 hr; 6 hr 30 min.; 24 hr.

material was associated with bacterial or protozoal fractions (Table 2). The higher level of radioactivity associated with protozoal and bacterial fractions in time period 1 of experiment 1 than at other time periods might be attributed to contamination with supernatant trapped in the sediment. To verify the percentage of label associated with the microbial fraction, twenty times the amount of labelled tryptophan was added in the third experiment as compared with the first two experiments. When the values in brackets (Table 2) were compared, the experiment 3 values for labelled material in each fraction, expressed as a percent of the total label in the rumen sac, were lower than corresponding values for experiment 1 and 2. When only experiment 3 values were observed, less than 2% of the labelled material was associated with bacterial or protozoal fractions. The results (Table 2) indicated more thorough washing of the protozoal and bacterial fractions in experiment 3 than in earlier experiments. The amount of labelled material associated with microbial fractions increased with time in experiment 3 but was comparatively small.

In order to decrease the diffusion of VFA from the rumen sac into the saline, dialysis was performed before the experiment proceeded (Davey et al 1960). The initial saline values were much lower than initial values for rumen sac contents, however saline volume was 6 times the rumen sac volume. Diffusion across the membrane from rumen sac into saline resulted in a decrease in rumen sac VFA values with time (Table 3). The activity of the microorganisms did not compensate for losses of VFA to the

Table 2

Amount of label occurring in rumen sac fractions
(dpm per ml rumen sac contents)

Time Period[1]	Fraction Examined	Experiment 1	Experiment 2	Experiment 3
0	amount added	3,640	4,100	85,500
1	rumen contents	1,757	1,958	51,499
	protozoa	61 (3.5)[2]	30 (1.45)	304 (0.59)
	bacteria[3]	73 (4.0)	-	556 (1.08)
2	rumen contents	930	1,000	35,058
	protozoa	-	33 (3.3)	215 (0.61)
	bacteria	38 (4.0)	34 (3.4)	408 (1.16)
3	rumen contents	439	505	13,531
	protozoa	-	-	171 (1.26)
	bacteria	0 (0.0)	18 (3.5)	249 (1.84)

[1] Time periods used were:

Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr .
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

[2] Values in brackets are labelled material expressed as percent of total label.

[3] Analysis data were not obtained where no values are given.

Table 3

Volatile fatty acid (VFA) analysis in saline solution and rumen sac of the *in vitro* apparatus during experiments 1 and 3 [1]

Time Period[2]	0	1	2	3
Saline - meq/100 ml				
Experiment 1	0.7	1.2	1.4	1.3
Experiment 3[3]	-	2.1	1.4	1.4
Rumen sac - meq/100 ml				
Experiment 1	8.6	3.5	2.05	1.5
Experiment 3	-	3.8	2.4	2.0
Radioactivity in VFA obtained from rumen sac				
Experiment 3				
dpm/ml		352	212	142
dpm/meq		9,250	8,840	7,100

[1] VFA determinations were not conducted for experiment 2.

[2] Time periods used were:
Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr .
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

[3] Analysis data were not obtained where no values are given.

saline. During these first three experiments, no substrate was available to the microorganisms except the labelled material. Breakdown of tryptophan by the microorganisms was suggested by the higher percentage of the available label appearing in the VFA fraction as the experiment proceeded. In the third experiment, the label occurring in the VFA fraction of the rumen sac (dpm/ml values of rumen sac contents-Table 3) expressed as a percent of the total label present in the rumen sac (dpm/ml values of rumen sac contents-Table 2) rose from 0.6% to 1.05% at the end of one day. The decrease of radioactivity in VFA obtained from the rumen sac (Table 3), expressed as dpm/meq VFA produced, was due to the much lower amount of labelled material available in the rumen sac of the in vitro apparatus as diffusion into the saline proceeded.

The decrease of radioactivity per meq VFA produced was used by Gray et al (1962) to test the artificial rumen for VFA production by the microorganisms. The rates of formation of volatile fatty acids were reflected in the rates of decline of the specific activities. VFA production was indicated (Table 3) by the decrease in dpm/meq values with time.

Total nitrogen in the rumen sac contents decreased (Tables 4 and 5) during the time periods examined, possibly indicating a reduction in the microbial population as the microorganisms contain most of the nitrogen of the rumen contents (Hungate 1966). In the two samples examined for nitrogen in rumen sac fractions (Table 5) the supernatant contained 50%

Table 4

Total nitrogen determined for rumen sac contents
(mg N/ml rumen sac contents)

Time period[1]	Experiment 1	2	3	% N as tryptophan[2]
0[3]	-	.682	-	-
1	.280	.444	.280	2.74
2	.210	.364	.280	2.74
3	.182	.252	.252	2.40

1 Time periods used were:

Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr .
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

2 Values are for experiment 3 only.

3 Analysis data were not obtained where no values are given.

Table 5

Total nitrogen determined for rumen sac fractions[1]
(mg N/ml rumen sac contents)

Experiment 3	Time period 2 (3 hr)	Time period 3 (6 hr. 30 min)
Protozoa	.042	.040
Bacteria	.090	.085
Supernatant	.140	.084
Total	.272	.219
Total (from Table 4)	.280	.252

[1] Due to insufficient sample quantities, determinations were not made for time periods 0 and 1.

and 38% of the total nitrogen which indicated some destruction of microorganisms and the nitrogen containing compounds released into the supernatant.

Serine appeared to move across the membrane into the saline solution slightly faster than tryptophan (Fig.11). The serine molecule is of smaller size as compared to tryptophan, and as a result faster diffusion would be expected in a system where active transport was not possible. Based on the observed results, half of the serine diffused into the saline before 4.5 hours had elapsed as compared to the 6 hours required for half of the tryptophan to diffuse (Fig. 9). The calculated values (percent) for labelled compounds appearing in the saline solution (Fig. 11) were determined using as denominator the total label remaining in the in vitro system. The calculated value (Table 28) was consistently higher than the observed values.

The amount of label associated with bacterial and protozoal fractions (Table 6) were of a similar order of magnitude as those determined for tryptophan (Table 2)[1]. Higher amounts of label were associated with the bacterial fraction than with the protozoal fraction when tryptophan was the labelled material added to the rumen sac. The situation was reversed when serine was the labelled compound under examination.

[1]Experiment 3 values for amount of label in the rumen sac compares more favourably with the serine experimental values.

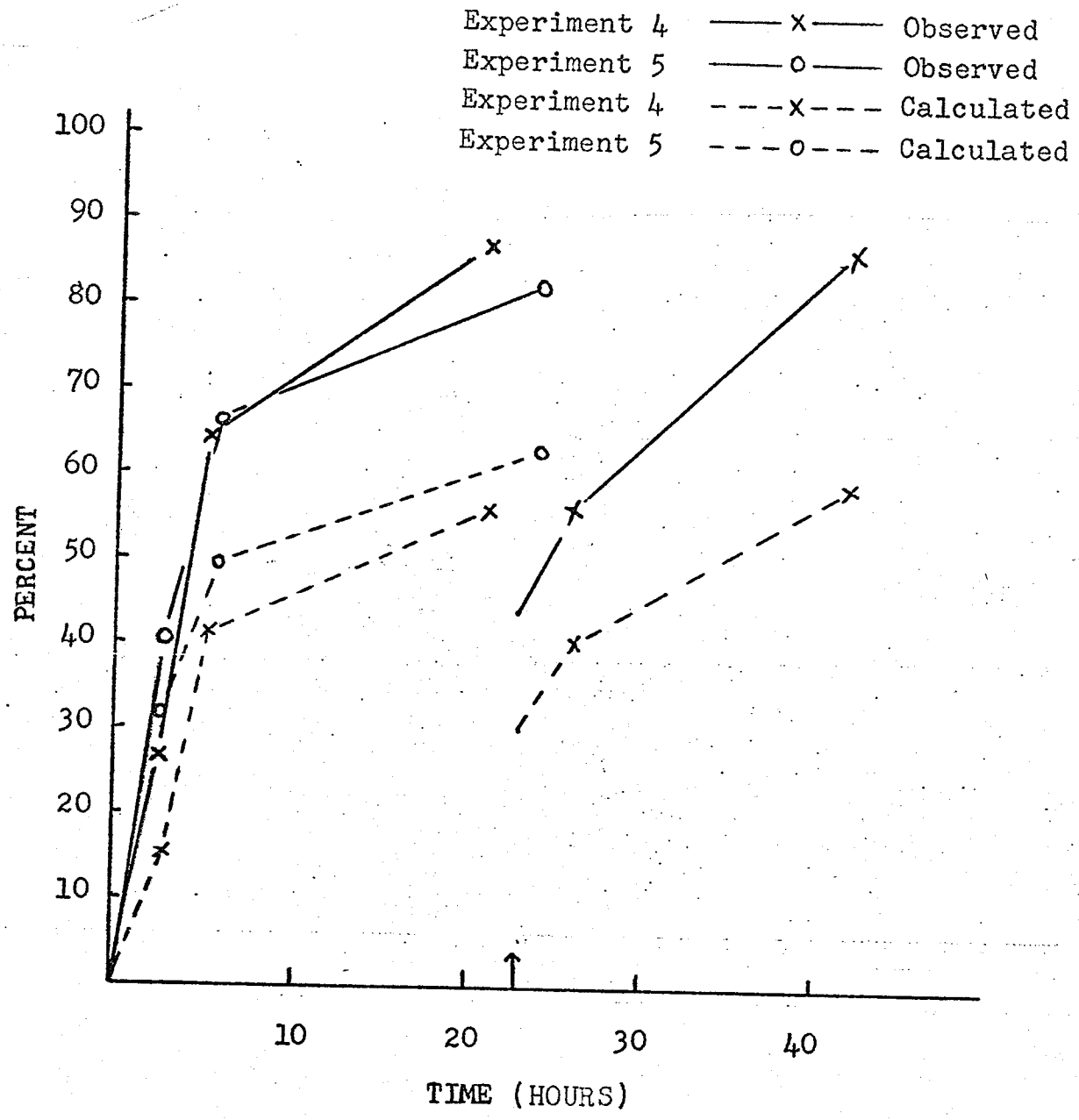

Experiment 4 —x— Observed
Experiment 5 —o— Observed
Experiment 4 - - -x- - - Calculated
Experiment 5 - - -o- - - Calculated
100
90
80
70
60
50
40
30
20
10
PERCENT
10
20
30
40
TIME (HOURS)

Table 6

Amount of label added as serine[1] occurring in rumen sac fractions (dpm per ml rumen sac contents)

Time Period[2]	Fraction Examined	Experiment 4	Experiment 5
1	rumen contents	26,300	45,000
	protozoa	130 (0.50)[3]	191 (0.42)
	bacteria	100 (0.38)	123 (0.27)
2	rumen contents	12,700	22,700
	protozoa	155 (1.22)	172 (0.76)
	bacteria	87 (0.69)	92 (0.41)
3	rumen contents	4,070	11,220
	protozoa	84 (2.06)	70 (0.63)
	bacteria	63 (1.55)	57 (0.51)
4	rumen contents	31,000	
	protozoa	196 (0.63)	
	bacteria	60 (0.19)	
5	rumen contents	8,600	
	protozoa	180 (2.10)	
	bacteria	130 (1.51)	

[1] Serine added (dpm/ml): Experiment 4 - 58,500 (0 hours), 58,500 (23 hours, between time periods 3 and 4).
Experiment 5 - 98,200 (0 hours).

[2] Time periods used were:

Experiment 4: 2.5 hr ; 5 hr ; 21 hr ; 26 hr ; 42 hr.
Experiment 5: 2.5 hr ; 5.5 hr ; 24 hr.

[3] Values in brackets are labelled material expressed as percent of total label.

Addition of sodium acetate to medium for the microorganisms in experiments 4 and 5 probably accounted for the higher values obtained for volatile fatty acid analysis of the saline solution and rumen sac (Table 7) as compared to the values reported for the first three tryptophan experiments (Table 3).

The specific activity of the VFA fraction taken from the rumen sac (Table 8), indicated production of volatile fatty acids by the microorganisms. A control experiment was conducted using rumen liquor from which bacteria and protozoa were removed by centrifugation. Under these conditions no label appeared in the VFA fraction.

The percentage of total radioactivity appearing in VFA increased over time (Table 9). Serine was a better substrate for VFA production by the microorganisms than tryptophan as most of the label from serine eventually appeared in VFA whereas the maximum value obtained for tryptophan was 1% during a similar time period. The VFA values (dpm/ml) for the rumen sac contents obtained for experiment 5 were lower than the values for experiment 4. Volatile fatty acid production in the total system (Table 10) showed less VFA production in experiment 5 than in experiment 4. The initial decrease in total amounts of VFA in the system appeared in all experiments (bracketed figures-table 10-in time period 1 of experiments 1, 4 and 5 and time period 2 of experiment 3). Sodium acetate was added as substrate for the microorganisms in the serine experiments in addition to the

Table 7

In vitro production of volatile fatty acids (VFA) with addition of sodium acetate and labelled serine to the media. (Experiments 4 and 5)

(meq / 100 ml)

Time period[1]	0	1	2	3	4	5
Saline						
Experiment 4[2]	1.11		1.80	2.00	2.41	2.30
Experiment 5	1.10	1.68	1.94	2.12		
Rumen sac						
Experiment 4	12.50		3.32	2.18	2.89	2.55
Experiment 5	12.90	6.50	4.63	2.43		

[1] Time periods used were:

Experiment 4: 0; 2.5 hr ; 5 hr ; 21 hr ; 26 hr ; 42 hr.
Experiment 5: 0; 2.5 hr ; 5.5 hr ; 24 hr.

[2] Analysis data were not obtained where no values are given.

Table 8

In vitro rumen sac and saline solution radioactivity associated with volatile fatty acids (VFA) with addition of sodium acetate and labelled serine to the media
(Experiments 4 and 5)

Time period[1]	1	2	3	4	5
Saline - dpm/ml					
Experiment 4	84	421	1714	3760	5060
Experiment 5[2]	638	1960	1302		
Saline - dpm/meq					
Experiment 4	37	250	861	1675	2180
Experiment 5	380	1010	615		
Rumen sac - dpm/ml					
Experiment 4		7170	4010	5170	4950
Experiment 5	5550	4505	1790		
Rumen sac - dpm/meq					
Experiment 4		2160	1840	1760	1650
Experiment 5	855	973	736		

[1] Time periods used were:

Experiment 4: 2.5 hr; 5 hr; 21 hr; 26 hr; 42 hr.
Experiment 5: 2.5 hr; 5.5 hr; 24 hr.

[2] Analysis data were not obtained where no values are given.

Table 9

In vitro rumen sac and saline solution radioactivity associated with volatile fatty acids (VFA) as a percent of total radioactivity with addition of sodium acetate and labelled serine to the media (Experiments 4 and 5)

Time period hr from "0"	Radioactivity in rumen sac			Radioactivity in saline solution		
	Total dpm/ml	VFA dpm/ml	%	Total dpm/ml	VFA dpm/ml	%
Experiment 4[1]						
2.5[2]	26,300			1230	84	6.8
5.0	12,700	7170	56.4	3600	367	10.2
21.0	4,070	4010	98.5	3900	1572	40.4
26.0	31,100	5170	16.6	6600	3780	57.3
42.0	8,600	4490	52.0	9100	5130	56.3
Experiment 5						
2.5	45,000	5550	12.9	5070	638	12.6
5.5	22,700	4505	19.8	7180	1960	27.3
24.0	11,220	1790	16.0	8300	1302	15.7

1 10 uCi of labelled serine was added to the media at 0 hr and again at 23 hr in experiment 4.

2 Analysis data were not obtained where no values are given.

Table 10

In vitro volatile fatty acid production (meq) in the total system (saline solution plus rumen sac contents)
(Experiments 1, 3, 4 and 5)

Experiment	Time period[1] 0	1	2	3
Tryptophan				
1	42.60	40.02 (- 2.58)[2]	40.76 (+3.61)	35.79 (+ 2.19)
3[3]		62.57	40.55(-18.83)	39.30 (+ 1.00)
Serine				
4	69.20	55.82 (-13.38)	55.65 (+9.65)	65.70 (+10.47)
5	88.10	65.30 (-22.80)	64.53 (+5.33)	60.30 (+ 4.65)

1. Time periods used were:

   Experiment 1: 3 hr 45 min ; 9 hr 30 min ; 22 hr.
   Experiment 3: 3 hr ; 6 hr 30 min ; 24 hr.
   Experiment 4: 5 hr ; 21 hr ; 42 hr.
   Experiment 5: 2 hr 30 min ; 5 hr 30 min ; 24 hr.

2. Values in brackets show the difference between the VFA production in the period listed and the preceeding period, taking into account the VFA removed in sampling.

3. Analysis data were not obtained where no values are given.

labelled serine. The total VFA production was greater in the experiments where sodium acetate was added.

After ion exchange chromatography of saline solution and rumen liquor supernatant, the percentage of label occurring in the tryptophan (basic) fraction of the effluent decreased with time (Table 29). More label occurred in compounds eluted with the acidic fraction as time progressed. Serine tended to elute with both acidic and basic fractions, which made duplication difficult. However, tryptophan only eluted with the second or basic fraction. The percent recovery of radioactivity was approximately 80% for most of the saline samples and reflected quenching by the media. Fraction 1 solution quenched counts detected from radioactive disintegrations much more than fraction 2. Rumen liquor supernatant had more quenching compounds than the saline as evidenced by the lower figures for the percent recovery of radioactivity from the column.

The percentage recovery of radioactivity from the column, when the hydrolyzed bacterial and protozoal fractions of rumen liquor were chromatographed, exceeded 100 (Table 30). The value for the total radioactivity in the eluted fraction was obtained after the formamide digestion procedure. The high recovery calculated after ion exchange chromatography indicated that with the formamide digestion procedure all of the radioactive material in the bacterial and protozoal fractions was not counted. In experiment 5, the three samples of bacterial and protozoal fractions were combined before

hydrolyzing, whereas in experiment 4 each sample was hydrolyzed separately and combined before addition to the ion exchange column. The lower values obtained for the percent recovery of radioactivity from the column in experiment 5 as compared to experiment 4 would indicate hydrolysis of smaller samples was more complete.

The synthesis of tryptophan from serine was indicated by appearance of label in the tryptophan area of the thin layer chromatograms (Table 11). In all but one sample, the percent of label appearing in tryptophan was less than one. No label appeared in the tryptophan area when hydrolysed bacterial and protozoal fractions were chromatographed (between 30 and 320 dpm of labelled material were applied to the chromatograms).

In vivo tryptophan absorption

Counts of ^{14}C label in plasma samples from sheep 1 and 2 (Fig. 12) rose in portal blood during the first hour; a slight corresponding increase also occurred in counts from jugular blood. The portal and jugular blood values of ^{14}C label tended to equilibrate after two days.

Theurer et al (1966) compared portal and jugular blood plasma amino acids and concluded that jugular blood levels were representative of body blood supply. In their study, sampling started at two hours after feeding. In our experiments, labelled compounds appeared in the urine within

Table 11

Labelled tryptophan in saline solution and rumen liquor supernatant after addition of L-serine-^{14}C (U) to the *in vitro* rumen sac

Time period[1]	Saline solution		Rumen liquor supernatant	
	dpm in fraction applied to plate	dpm in area containing tryptophan	dpm in fraction applied to plate	dpm in area containing tryptophan
Experiment 4				
1[2]	1450 1100	0 0	4575	22 (0.48)
2	1503 2720	0 0	1600	5 (0.31)
3	1942 860	0 0	306	0
4	3180 4060	12 (0.38) 0	10620 8180	35 (0.33) 32 (0.39)
5	2490 1309	12 (0.48) 3 (0.23)	352	0
Experiment 5				
1	2760 1610	0 5 (0.31)	8700 5640	17 (0.19) 11 (0.19)
2	3600 1975	10 (0.28) 10 (0.51)	5640 5270	7 (0.13) 24 (0.46)
3	520 3200	10 (1.92) 33 (1.03)	1270 795	0 3 (0.38)

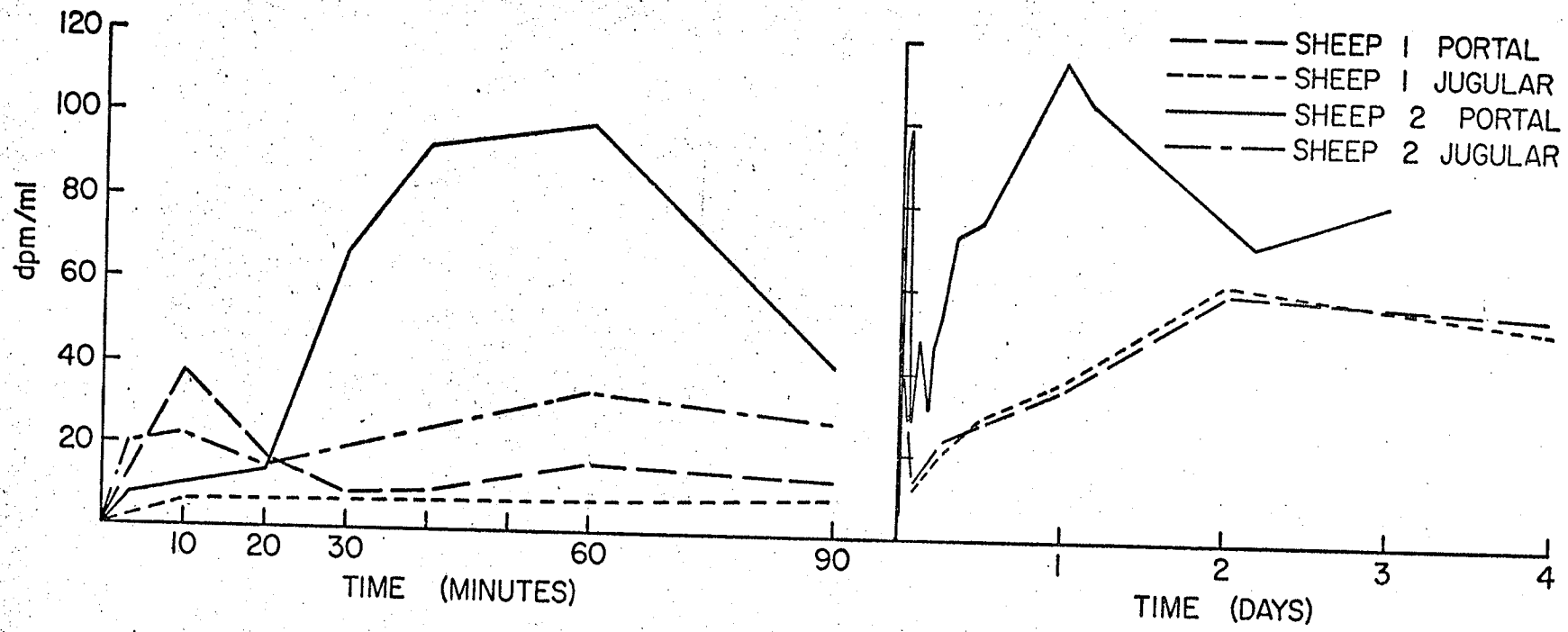
[1] Time periods used were:

Experiment 4: 2.5 hr ; 5.0 hr ; 21.0 hr ; 26.0 hr ; 42.0 hr.
Experiment 5: 2.5 hr ; 5.5 hr ; 24.0 hr.

[2] Where a value for tryptophan occurs, the bracketed figure is the percent of the dpm applied to the plate which occurs in tryptophan.

Figure 12

The appearance of labelled compounds in jugular and portal blood of sheep 1 and 2 after injection of labelled tryptophan into the rumen.


120
100
80
60
40
20
dpm/ml
10
20
30
60
90
TIME (MINUTES)
1
2
3
4
TIME (DAYS)
SHEEP 1 PORTAL
SHEEP 1 JUGULAR
SHEEP 2 PORTAL
SHEEP 2 JUGULAR

ten minutes after introducing the labelled tryptophan, indicating that early sampling is essential in plasma amino acid studies. When radioactive methionine was introduced into the rumen (McCarthy et al 1970) radioactivity appeared in blood at the 30 min. sampling time indicating absorption of free amino acids. Significant absorption occurred as indicated by blood levels of amino acids 9 hours post-injection which could represent release of amino acids by digestion in the small intestine. Amino acids might be able to reach the host animal by direct absorption from the rumen. When tryptophan was injected into the rumen of sheep (results reported later in thesis) 26.7% and 68.8% of the free tryptophan in the rumen was absorbed from the rumen and reticulum in 3 hours. Tryptophan in portal blood might be removed by the liver, metabolized, and excreted or transferred to other locations by way of the jugular blood.

Labelled compounds appearing in the urine (Fig. 13) expressed as dpm/ml, increased for the first two hours following dosing with tryptophan. The urinary excretion of label then fluctuated and subsequently fell after one day. Less labelled material appeared in the urine of sheep 2 than in sheep 1 during the time samples were collected. The experiment with sheep 1 was undertaken before normal feed consumption had resumed following surgery. The higher excretion rate of labelled compounds in urine observed in sheep 1 as compared with sheep 2 may have reflected poorer conversion to body protein and certainly indicated lower utilization of tryptophan.

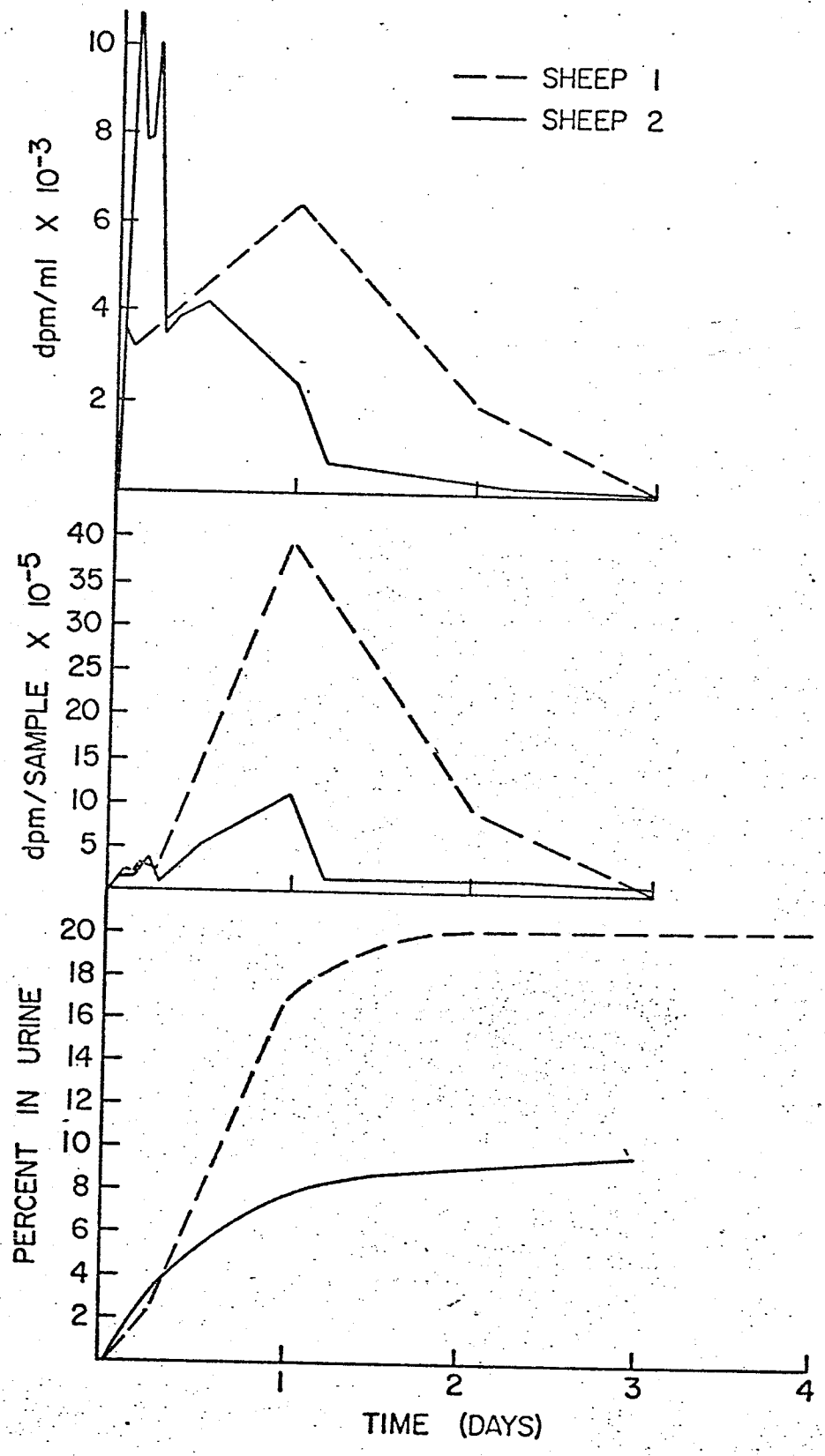
Figure 13

The appearance of labelled compounds in urine of sheep 1 and 2 after injection of labelled tryptophan into the rumen.

Top: Radioactivity in 1 ml. of urine.

Middle: Radioactivity in the total urine sample collected over the time period examined. (dpm/ml x ml urine collected).

Bottom: Percentage of the injected radioactivity which appeared in the urine.


SHEEP 1
SHEEP 2
dpm/ml X 10⁻³
dpm/SAMPLE X 10⁻⁵
PERCENT IN URINE
TIME (DAYS)

The small amount of radioactivity detected in the feces (sheep 1) indicated absorption of labelled tryptophan was high (Table 12). The actual amount of label excreted in the urine might have been considerably higher than recorded in these experiments as there is considerable quenching of radioactivity with the urine samples.

Compounds analysed in the urine (tryptophan, tryptamine and indoleacetic acid) contained less than 20% of the label. Most of the radioactivity in urine was either in the first fraction of the ion exchange eluant or accompanied tryptophan in the second fraction. The compounds involved in the kynurenic acid breakdown pathway of tryptophan occurred in the two fractions where most of the urinary label was located. Presumably most of the label was associated with the tryptophan metabolites of this pathway.

Thin layer chromatography indicated there was a change in the type of labelled compounds excreted in the urine as time after feeding progressed (Fig.14). Unidentified peaks of activity were associated with fractions 10, 13 and 16. Of the three compounds examined (tryptophan, tryptamine and indoleacetic acid), tryptophan was initially present in the urine in the highest quantities (Table 13). Indoleacetic acid contained more than three percent of the label present in the urine for most of the samples examined until the twelve hour mark, after which a decrease occurred. Negligible amounts of labelled tryptamine were found at all sampling times.

Table 12

The appearance of labelled compounds in feces of sheep 1 after injection of tryptophan into the rumen

Time	Total radioactivity in feces dpm	Radioactivity injected which appeared in feces (% of total)
10 min	control	
2 hr	2,835	0.013
1 day	26,600	0.097
2 day	104,800	0.384
3 day	57,250	0.265
4 day	24,600	0.210
total	216,080	0.790

Figure 14

Labelled material recovered from thin layer chromatograms of urine samples.

(A) and (E) sheep 2

(B) (C) and (D) sheep 1

Analysis of samples obtained between 0 and 2 hours after the labelled tryptophan was injected are shown in (A) and (B). (C) (D) and (E) show the analysis of samples obtained later than 2 hours after the labelled tryptophan was injected.

Fraction 2: Corresponded to a R_F value of 0.85-0.9 and included any indoleacetic acid present in the sample.

Fraction 7: Corresponded to a R_F value of 0.55-0.65 and includes tryptamine.

Fraction 8: Corresponded to a R_F value of 0.5-0.55 and includes most of the tryptophan.

Fraction 10, 13 and 16 contained unidentified peaks of activity.

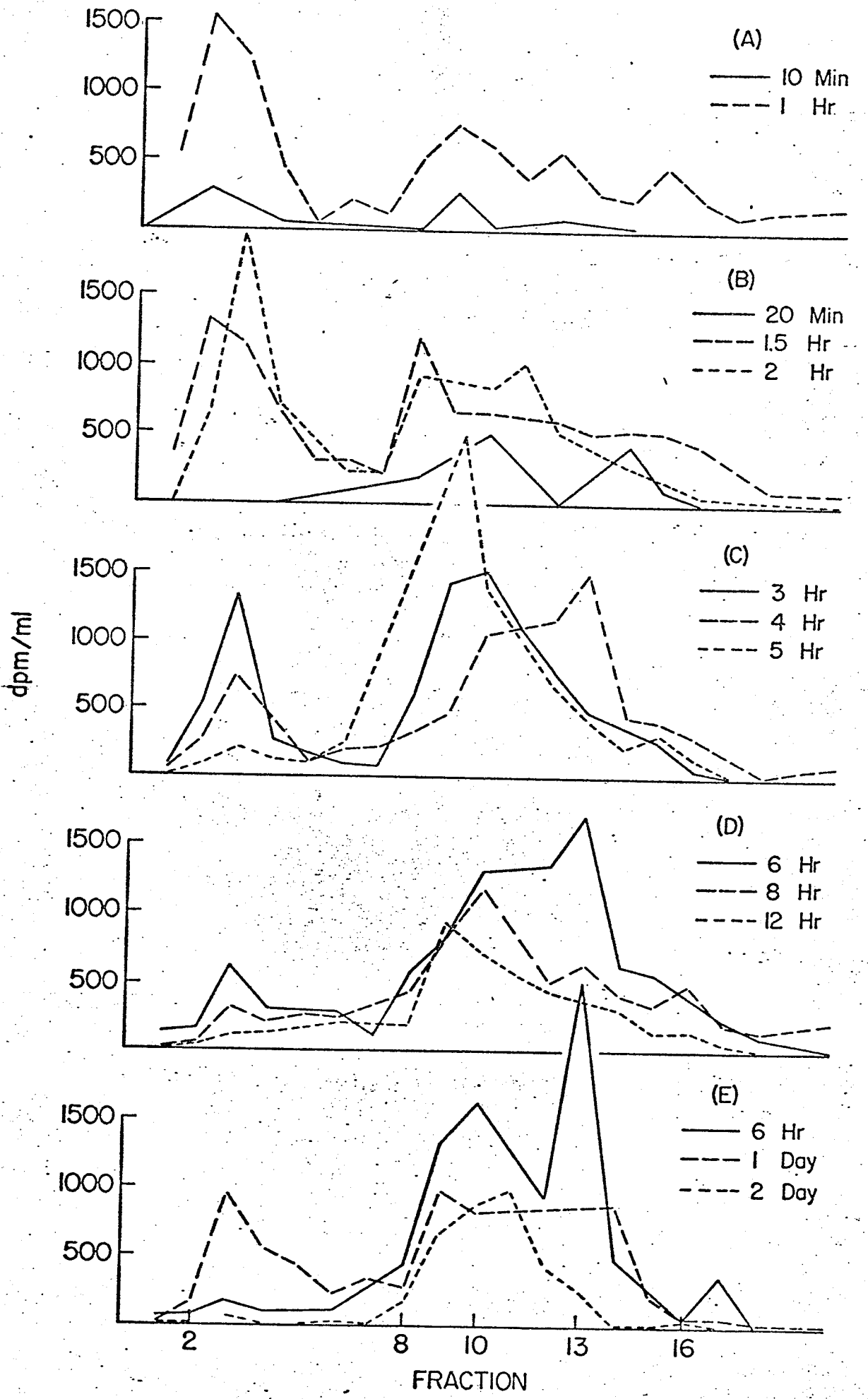

1500
1000
500
(A)
10 Min
1 Hr
(B)
20 Min
1.5 Hr
2 Hr
(C)
3 Hr
4 Hr
5 Hr
(D)
6 Hr
8 Hr
12 Hr
(E)
6 Hr
1 Day
2 Day
dpm/ml
2
8
10
13
16
FRACTION

Table 13

Percentage of total label in urine samples collected from sheep 1 and 2 which was associated with tryptamine, indole-acetic acid and tryptophan

Time of Urine Collection	Sheep	Tryptamine	Indoleacetic Acid	Tryptophan
10 min	1	0.210	4.20	5.72
20 min	2	6.000	2.30	14.20
1 hr	1	0.011	4.50	1.83
1.5 "	2	0.081	3.50	4.00
2 "	2	0.122	4.20	1.92
4 "	2	0.090	3.25	1.59
6 "	1	0.000	0.89	0.89
6 "	2	——[1]	——	1.50
8 "	2	——	5.66	——
12 "	2	0.016	2.24	1.08
1 day	1	——	0.16	1.73
3 "	1	0.000	0.30	5.80

[1] Where a blank occurs, no reading made.

Plasma sample analysis for radioactivity (Table 14) indicated that most of the label in the blood was associated with tryptophan. The quantity of label in blood occurring in other compounds increased with time.

Only the liver (sheep 2) of the tissues examined had a significant amount of label (71 dpm/g) while kidney (10 dpm/g) and muscle (4 dpm/g) contained a lesser amount. The time interval between introduction of the labelled tryptophan and slaughter (3 weeks) could account for the lack of detectable label in tissues from sheep 1.

In experiments 8, 9 and 10, the rumen and reticulum were isolated from the remainder of the digestive tract and thus absorption must have occurred from the rumen-reticulum. The percentage absorption of labelled tryptophan was 68.8 and 26.7 for sheep 5 and 4 respectively. Sheep 3 administered with sodium acetate had an absorption value of 49.0% (Table 15). Excretion of labelled material in urine was higher (dpm/ml) for sheep 5 when compared to sheep 4, for the first two hours (Table 16) indicative of a more rapid rate of absorption and subsequent excretion. The concentration of labelled material in urine was still increasing in the 3 hr samples in both sheep.

Absorption rates observed in the first hour were higher with sheep 5 (whole rumen contents), compared to sheep 4 (washed rumen technique), and could have been due to a higher concentration of labelled tryptophan near the absorption sites. It was also possible that sheep 5 was not

Table 14

Labelled compounds appearing in pooled blood plasma samples obtained from sheep 1 and 2
(dpm/sample)

	Sheep	Period	Tryptamine	Indoleacetic Acid	Tryptophan[1]
Jugular					
	2	early[2]	0	0	690 (740)
	2	mid	0	7	960 (1015)
	1	late	0	7	540 (336)
Portal					
	1	early	0	0	330 (192)
	1	mid	0	0	210 (224)
	1	late	0	12	1650 (342)
	2	early	0	0	450 (585)
	2	mid	0	6	2015 (1050)
	2	late	0	9	1320 (273)

[1] The first value was obtained from the solution containing the tryptophan after extracting tryptamine and indoleacetic acid, the value in parenthesis was obtained after column chromatography. When the value in brackets is much lower than the first value, the difference represents compounds other than tryptophan.

[2] Time periods for the pooled samples were: early, 0 - 2 hr; mid, 3-5 hr; late, 6 hr to completion.

Table 15

Absorption of tryptophan -^{14}C (U)-benzene ring labelled from the rumen and reticulum[1]

Sheep	Rumen volume (ml) initial	Rumen volume final	Radioactivity (ml) in rumen (dpm) initial	Radioactivity in rumen (dpm) final	Radioactivity absorbed[2] (dpm)	% of Total radioactivity absorbed after 3 hours
			Sodium acetate			
3	4,015	4,015	3.84×10^6	1.90×10^6	1.88×10^6	49.0
			Tryptophan			
4	4,400	5,040	11.60×10^6	8.35×10^6	3.05×10^6	26.7
5	8,500	8,500	12.70×10^6	3.92×10^6	8.69×10^6	68.8

1 The washed rumen technique was used with sheep 3 and 4; the whole rumen technique was used with sheep 5.

2 Radioactivity absorbed (dpm) = initial radioactivity in rumen (dpm) - radioactivity removed in samples (dpm) - final radioactivity in rumen (dpm).

Table 16

Excretion of labelled compounds in urine during tryptophan absorption (Experiments 9 and 10 - sheep 4 and 5)

Time (hr)	Radioactivity (dpm/ml urine)	Volume (ml) of urine	Total radioactive compounds excreted (dpm)
	Sheep 4		
0.25	3	3	9
0.50	100	10	1,000
1.00	370	20	7,400
2.00	832	40	33,280
3.00[1]	1,445	-	----
	Sheep 5		
0.25	0	14	0
0.50	234	8	1,872
1.00	690	12	8,280
2.00	860	30	25,800
3.00	1,000	35	35,000

[1] The total volume of urine collected for this time period was not recorded.

as heavily anaesthetized as sheep 4 and thus had a higher metabolic rate.

No label was detected in the jugular blood samples taken from sheep 4. Less labelled tryptophan was injected into the rumen of sheep 4 and 5 than sheep 1 and 2. Therefore, detectable label in blood would not be expected in the case of sheep 4 and 5 as values obtained in initial experiments for sheep 1 and 2 were low.

CONCLUSIONS

Tryptophan appears to be absorbed from the rumen and reticulum at significant rates and the only apparent limitation to absorption is the concentration of tryptophan in the rumen fluid. The labelled material travelled against a concentration gradient when the washed rumen technique was used in the absorption experiments which would indicate the possibility of active transport of tryptophan across the rumen epithelium.

The early excretion of labelled compounds in urine and rapid metabolism of tryptophan indicate that the body may have a very small circulating pool of tryptophan and rapidly excretes any excess. The results of the in vitro experiments show utilization of serine for synthesis of tryptophan by the rumen microorganisms. The rate at which tryptophan is synthesized may not be sufficient to account for the amount necessary for microorganism growth and multiplication.

The average division time of the rumen population was found to be 8.6 hours (Hungate 1966). Bacteria have a variable division time but usually much less than 8.6 hours, whereas 16 hours was an approximate value obtained for protozoa division time. A slow rate of utilization of tryptophan would allow time for the free amino acid to be absorbed.

Feeding of tryptophan to the ruminant might be beneficial if tryptophan was limiting in the feed.

Supplementation of the ruminant ration, to allow a release of tryptophan at a time when the maximum amount of amino acids supplied by the feed are available for absorption, would be the most satisfactory. Tryptophan is not generally required in large amounts, therefore it might also be feasible to supplement feeds with higher than necessary levels of tryptophan to be certain sufficient quantities would be available at the most opportune time. This last suggestion would not be valid, however, if as has been suggested by several investigators (Carlson et al 1968; Johnson and Dyer 1968), tryptophan is toxic when present in higher than normal quantities.

TRYPTOPHAN SYNTHESIS AND METABOLISM IN REED CANARY GRASS

LITERATURE REVIEW

Importance of tryptophan to plants

In addition to plant protein synthesis, tryptophan is metabolized to the plant hormone, auxin or indoleacetic acid (Galston and Davies 1969). Auxin facilitates cell extension and increases the plasticity of the cell wall which is then stretched by water uptake resulting from the osmotic potential of the vacuolar sap. One possible way to accomplish the plasticity would be by breaking the cross-links between the cellulose microfibrils of the cell wall. Auxin does not act directly on growth because there is a lag phase which may indicate qualitative changes in protein synthesis or an action on a preformed system. Apical buds produce auxin which is transported down the stem where it inhibits growth of lateral buds, perhaps by production of ethylene. Kinetin applied to the lateral buds overcomes the inhibitory effect of native or applied auxin and enables buds to commence development.

A change in sex-expression in plants is accomplished by the plant hormones; with auxin and cytokinins shifting the sex balance toward femaleness, and gibberellins favouring male expression (Letham 1969). There is some evidence that phytohormones may function at, or very near, the level of the gene and may act through a gene repressor system.

Tryptamine alkaloids, formed from tryptophan, produce known physiological effects in animals. There is a strong action on the nervous system and Phalaris tuberosa alkaloids interfere with the effects of serotonin. Culvenor et al (1964) indicated that tryptamine alkaloids might be partly responsible for the toxic effects of Phalaris species. Grasses and grains parasitized by fungi of Claviceps and Fusarium genera are recognized sources of livestock toxicosis (Keeler 1969). It is the fungi themselves from which alkaloids are derived and parasite hosts include wheat, rye, barley, wild wheat, ryegrasses, various bromes, reed, fescue and bluegrasses.

Ergot alkaloids are also derived from tryptophan. When animals ingest small daily doses of ergot alkaloids, a gangrenous ergotism results and the animal becomes lame. Reduced circulation ultimately causes gangrene in effected extremeties. On ingestion of large amounts of ergot alkaloids, a convulsive type of ergotism results in hyperexcitability, belligerency, tumbling, incoordination and subsequently death within a few days.

Poisonous mushrooms (genus Amanita) contain a toxic peptide, tryptathionine, synthesized from L-tryptophan and L-cysteine. Some phallotoxins (cyclic heptapeptides) also contain tryptophan. Another plant metabolite of tryptophan which has enjoyed importance is the dye, indigo, produced by oxidation of a glucoside obtained from Indigofera species.

Biosynthesis of tryptophan in plants

Plant synthesis of tryptophan is believed to be similar to that in microorganisms (Yoshida 1969). Chen and Boll (1968) detected tryptophan synthetase in the shoot and root tissue of pea seedlings. A high serine to indole molar ratio was necessary to demonstrate the synthetase action. Similar activity has been demonstrated in seedlings of wheat and maize (Bonner and Varner 1965). Tryptophan can be synthesized by transamination of the α-keto acid with the α-amino group of glutamic acid (Fowden 1967).

Tryptophan metabolism in plants

The importance of indoleacetic acid to plants stimulated research into the biosynthesis of this plant hormone. Tryptophan was believed to be the precursor of indoleacetic acid in plants (Stowe 1959; Mahedevan 1964; Moore and Shaner 1967). Reasons to doubt the tryptophan origin of indoleacetic acid were presented by Thimann (1963); Libbert et al (1966) and Winter (1966). Bacteria were known to degrade tryptophan to indoleacetic acid (Montuelle 1966; Montuelle and Beerens 1964). When experiments were conducted under sterile conditions, Winter (1966) found no conversion of tryptophan to indoleacetic acid and confirmed earlier proposals that microorganisms were responsible for any conversion noticed previously by other workers. Tryptamine appeared to occupy a central role in biosynthesis of indoleacetic acid and was formed from indole, not tryptophan. Higher plants were known to have an active monoamine

oxidase (Mann 1955).

Under cell free conditions, Valdovinos and Sastry (1968) found that gibberellin enhances cell elongation in avena coleoptile by directly increasing auxin biosynthesis. Gibberellic acid enhanced tryptophan decarboxylation. The enzyme system which synthesized indoleacetic acid was isolated from oats by Lantican and Muir (1967) who found that gibberellic acid doubled the conversion rate of tryptophan to auxin.

The conversion of tryptophan to indoleacetic acid in the absence of contaminating epiphytic microorganisms, known to inhabit the interior of healthy plant tissue, was indicated by Sherwin and Purves (1969). Tryptophan - 3 -^{14}C was incorporated into indoleacetic acid -^{14}C to a greater extent than tryptamine - 2 -^{14}C, by cucumber hypocotyls and the incorporation remained similar in aseptic conditions. Normal cell expansion was accompanied by protein synthesis which Sherwin and Purves contend would account for much more of the disappearance of free tryptophan than would conversion to indoleacetic acid. A much greater proportion of the drain on the tryptamine pool would result from indoleacetic acid biosynthesis. Therefore, application of tryptamine to tissue segments would be expected to cause greater growth promotion than would application of tryptophan.

Indole alkaloid biosynthesis from tryptophan was shown by Bowden and Marion (1951 a; 1951 b), Leete and Marion (1953), and O'Donovan and Leete (1963). Alkaloid

biosynthesis was reviewed by Leete (1967). Agurell and Nilsson (1968) incorporated tryptophan -^{14}C into psilocybin, a toxic compound of poisonous mushrooms. Between 0.86 and 1.49 % of the labelled tryptophan was located in psilocybin.

Biosynthesis and metabolism of tryptophan in plants have been studied using cell free extracts, seedlings and mature plants. The alkaloid level of reed canary grass varied and alkaloids were associated with toxicity problems encountered in animal feeding.

MATERIALS AND METHODS

Reed canary grass (Phalaris arundinacea L.) tillers were injected with either 0.0820 uCi L-serine -^{14}C (U) or 0.0955 uCi DL-tryptophan -^{14}C (U) - benzene ring labelled in 1 ml water. Five tillers in each of three different clones were injected with labelled serine and a similar number were injected with labelled tryptophan. Injections were made on June 19, prior to flowering of the plants, with injection between the first and second node of the stem, as close as possible to the first node. Plants were harvested on July 22 (33 days after injection) after seed ripening. The three different clones used in the experiment had high, detectable (low), and undetectable (none) amounts of alkaloid, as determined by thin layer chromatography (Woods 1969). After harvesting the plants, the five tillers of the same clone were dried together at 60°C for 24 hours. Heads were separated from the stem and leaf portion of the plants and ground in a Waring blender before storing.

Extraction of alkaloid substances

Between 5 and 10 g ground leaf and stem plant material was extracted by Soxhlet extraction with 95 % ethanol. Only 1 g of ground material from plant heads was extracted due to limited sample quantities. Subsequent

extraction was carried out on a pooled sample in order to obtain a greater quantity of material. Ethanol extract was evaporated to dryness at 60-65°C using a flash evaporator and the dried extract was dissolved in 5.6 % H_2SO_4 and chloroform. The chloroform and sulfuric acid solutions were mixed, shaken in a separatory funnel until the acid layer was nearly colourless. The acid layer on top was reddish brown whereas the chloroform layer was green. Several extractions with chloroform were necessary to free the acid layer of the green chlorophyll colour. The acid layer was saturated with NaCl and neutralized to pH 9 with 19 % NH_4OH. The neutralized layer was extracted with chloroform until the chloroform layer was colourless. The chloroform layers were collected, dried by adding K_2CO_3 and filtered before evaporating to dryness using a flash evaporator. The resultant residue contained mostly bases.

At each stage in the procedure, aliquots were taken for counting in the liquid scintillation counter. The alkaloid fraction was dissolved in as little chloroform as possible and spotted on two sets of thin layer plates. The adsorbent used was silica gel G and the chromatogram was developed using methanol:ammonia (7:1). One set of plates was examined with Van Urk's reagent (1 g p-dimethylamino-benzaldehyde dissolved in 50 ml concentrated HCl and 50 ml 95 % ethanol). Specific areas of the second set of plates were lifted and counted in the liquid scintillation counter.

Tryptophan extraction

The neutral aqueous layer from the alkaloid extraction was acidified before separation by ion exchange chromatography using Rexyn 101 (Na^+) for the column material. Thin layer chromatography using silica gel H: cellulose MN 300 plates and n-butanol: water: acetic acid for development, as described earlier, was used to locate tryptophan. The area containing tryptophan was counted.

RESULTS AND DISCUSSION

Initial samples of ground leaf and stem material, marked 1 in tables, were thought to be too large for efficient ethanol extraction and the second samples, marked 2 in tables, were smaller and extracted for a longer period of time. Radioactivity appearing in the first ethanol extract of the serine treated plants averaged 1.53 % of the total and 2.17 % of the total in the second extraction (Table 17). Radioactivity appearing in the first ethanol extract of the tryptophan treated plants averaged 7.82 % of the total and 13.67 % of the total in the second extraction (Table 18).

The head portion of the plants injected with serine incorporated a higher percentage of the radioactivity, based on dpm/g dry weight of sample, than the leaf and stem portion (Table 17) whereas the reverse was the case with the tryptophan injected plants.

More than five times as much radioactive tryptophan than serine remained in the plants after the 33 days. The head portion of the plants contained approximately the same percentage of label whether injected with serine or tryptophan. The leaf and stem portion showed greater incorporation when tryptophan was injected. Thus serine was apparently more mobile or more rapidly metabolized than tryptophan. The

Table 17

Incorporation of L-serine -^{14}C (U) into reed canary grass as measured in the ethanol extracts

Alkaloid level	Sample[1]	Amount extracted dry wt (g)	Total sample[2] dry wt (g)	Ethanol extraction dpm/g	Ethanol extract as[3] % of total
Leaf and stem portion					
None	1	9.30	20.6595	725	1.77
	2	5.00	20.6595	1030	2.51
Low	1	10.25	22.2915	420	1.15
	2	5.00	22.2915	872	2.39
High	1	10.00	19.8264	682	1.66
	2	5.00	19.8264	660	1.61
Head portion					
None		1.00	1.3002	1190	2.90
Low		1.00	2.3555	1560	4.28
High		1.00	2.1600	1260	3.07
Combined		2.30	7.8157	2060	5.30

1 Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

2 As 5 tillers were combined for the analysis, the total amount of L-serine-^{14}C (U) injected was 9 X 10^5 dpm (0.40 uCi).

3 Injected label was calculated on a dpm/g basis.

Table 18

Incorporation of DL-tryptophan -^{14}C (U) -benzene ring labelled into reed canary grass as measured in the ethanol extracts

Alkaloid level	Sample[1]	Amount extracted dry wt (g)	Total[2] sample dry wt (g)	Ethanol extraction dpm/g	Ethanol extract as % of total[3]
Leaf and stem portion					
None	1	7.60	24.5780	4690	11.60
	2	5.00	24.5780	5640	13.90
Low	1	9.70	25.0473	1517	4.15
	2	5.00	25.0473	4240	11.60
High	1	9.35	21.5720	3500	7.70
	2	5.00	21.5720	6260	13.70
Head portion					
None		1.00	1.3325	2940	7.25
Low		1.00	3.6280	770	2.10
High		1.00	1.6073	1210	2.66
Combined		3.50	6.5678	3020	7.45

1 Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

2 As 5 tillers were combined for the analysis, the total amount of DL-tryptophan -^{14}C (U) -benzene ring labelled injected was 10.5 X 10^5 dpm (0.47 uCi).

3 Injected label was calculated on a dpm/g basis.

amount of alkaloid present in the plants did not relate to the proportion of radioactivity appearing in the ethanol extract in either the serine or tryptophan injected plants.

The amount of label appearing in the basic chloroform extract (Table 19), which contained the alkaloids, increased in the tryptophan injected plants from the none to high alkaloid level rating given the clones based on thin layer chromatographic determinations (Woods 1969). The acidic chloroform extract contained most of the indoleacetic acid which would be present in the plants. The higher values obtained for the acidic chloroform extract of the tryptophan injected plants compared to the values for the serine injected plants probably indicated incorporation of label from tryptophan into indoleacetic acid. The more complete extraction obtained with the second samples was again evident.

The head portion of the plants was analysed but no radioactivity was detected in the 1 g samples. However, values for the combined head samples of the serine injected plants were 295 dpm/g in the acidic chloroform fraction, 6 dpm/g in the basic chloroform fraction and 783 dpm/g in the aqueous extract. Comparable values for the tryptophan injected plants were 442 dpm/g in the acidic chloroform fraction, 73 dpm/g in the basic chloroform fraction and 1280 dpm/g in the aqueous extract. The values obtained when larger samples were extracted indicated that lack of sufficient head portion sample might be the reason no radioactivity was detected.

Table 19

Amount of label appearing in acidic and basic chloroform extracts and in the aqueous extract of the leaf and stem portion of the plants
(dpm/g dry weight of plant material)

Alkaloid level	Acidic chloroform extract		Basic chloroform extract		Aqueous extract	
	sample 1[1]	sample 2	sample 1	sample 2	sample 1	sample 2
Serine injected plants						
None	0	480	0	58	460	1088
Low	0	354	2	47	181	1064
High	0	275	14	41	216	786
Tryptophan injected plants						
None	950	845	6	101	4300	5880
Low	0	430	27	270	673	3780
High	0	890	34	303	1480	5160

[1] Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

The total radioactivity appearing in the acidic and basic chloroform extracts and aqueous fraction was generally higher than the amount determined in the ethanol extract (Table 31). The ethanol extract contained chlorophyll which would quench the fluorescence from the scintillator in the counting fluid resulting in a below normal percentage of the disintegrations being counted.

Serine appeared to be converted into tryptophan (Table 20). The specific activity of the injected samples was 160 mc/mM for serine and 52 mc/mM for tryptophan. The average percentage of injected label which was recovered in tryptophan (using only sample 2 values) was 0.119 % of the labelled serine injected and 0.412 % of the labelled tryptophan injected. Therefore, approximately 1 of every 10.6 tryptophan molecules were synthesized using serine.

Most of the labelled compounds extracted with ethanol were in the aqueous portion. There was an even distribution of labelled compounds between acidic and basic effluent after ion exchange chromatography of the tryptophan injected plants, whereas in the case of the serine injected plants only 25 % was in the basic effluent. Based on the observations of thin layer plates, less than 5 % of the radioactivity in the ethanol extract was present as tryptophan.

More label appeared in the alkaloid fraction of the tryptophan injected plants of low and high alkaloid level than in the plants of no alkaloid detectable by thin layer chromatography (Table 21). The values obtained for

Table 20

Recovery of label from tryptophan containing area of thin layer plates when leaf and stem portion samples were analysed

Alkaloid level	Sample[1]	Tryptophan area total dpm	Labelled tryptophan/g dry wt plant material (dpm)	Percentage of label injected recovered in tryptophan[2]
Serine injected plants				
None	1	170	18	0.044
	2	240	48	0.117
Low	1	lost	lost	lost
	2	280	27	0.074
High	1	180	18	0.044
	2	340	68	0.166
Tryptophan injected plants				
None	1	620	82	0.202
	2	620	124	0.306
Low	1	680	70	0.191
	2	1100	250	0.683
High	1	520	56	0.123
	2	560	112	0.246

1 Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

2 Injected label was calculated on a dpm/g basis.

Table 21

Percentage of total radioactivity injected into the plants which appeared in the alkaloid fraction

Alkaloid level	Sample[1]	Plant portion	Label determined in alkaloid fraction[2] dpm/g	Percentage of injected label appearing in alkaloid fraction[3]
Serine injected plants				
None	1	leaf and stem	0	0.000
	2	leaf and stem	58	0.141
Low	1	leaf and stem	2	0.006
	2	leaf and stem	47	0.129
High	1	leaf and stem	14	0.034
	2	leaf and stem	41	0.100
Combined		head	6	0.014
Tryptophan injected plants				
None	1	leaf and stem	6	0.015
	2	leaf and stem	101	0.250
Low	1	leaf and stem	27	0.075
	2	leaf and stem	270	0.737
High	1	leaf and stem	34	0.075
	2	leaf and stem	303	0.665
Combined		head	73	0.181

[1] Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

[2] No label was detected in the single head samples analysed.

[3] Injected label was calculated on a dpm/g basis.

tryptophan incorporation agreed with levels found by other investigators (Agurell and Nilsson 1968; Bowden and Marion 1951 a, 1951 b; Culvenor et al 1964; Leete and Marion 1953; O'Donovan and Leete 1963). A much higher incorporation of label occurred in the alkaloid fraction of the head portion of the tryptophan injected plants than of the serine injected plants. The sample 2 values obtained for the serine injected plants were consistent and indicated serine might be incorporated into alkaloids other than just by way of the tryptophan route.

The thin layer plate radioactivity evaluation of alkaloids in the tryptophan injected plants (Table 22) did not follow the alkaloid level except at the R_F value of approximately 0.3. The ultraviolet detected spots did follow the alkaloid level. Perhaps the tryptamine alkaloids were mainly synthesized before flowering of the reed canary grass.

Alkaline hydrolysis of the ethanol extracted plant material indicated radioactivity located in protein, and in plant material insoluble in ethanol (Table 23). Considerably more label occurred in the bound form in serine injected plants than in the ethanol soluble portion. The values for tryptophan injected plants showed a fairly equal distribution between soluble and bound forms of the labelled compounds. When poor extraction was performed, the sample 1 values showed more label in the hydrolysed ethanol extracted plant material than sample 2 values for tryptophan injected plants. The alkaline hydrolysis of standards showed 77 % recovery of

Table 22

Recovery of radioactivity from silica gel G thin layer plate when alkaloid extract was chromatographed in methanol and ammonia [1]

Plate[1] R_F	Alkaloid from tryptophan treated plants[2]			Known location of some alkaloids
	Level of alkaloid			
	none	low	high	
0.8	P	P	P	unknown
0.7	16	8 E	32 EE	hordenine and unknown colour reacting compound
0.6	0	0	11	
0.5	4	0	3	di N methylated tryptamines
0.4	2	0	6	unmethylated tryptamines
0.3	0	3	11	N methylated tryptamines
0.2	10	2	5	

[1] A plate exposed to x-ray film (radioautography) for 6 weeks showed no detectable radioactivity.

[2] P was the pigmented area; E coloured purple with Van Urk's reagent. Fluorescence under ultraviolet examination moderate ---- bright——

Table 23

Total radioactivity in hydrolysed (alkaline) samples of leaf and stem portion of the reed canary grass

Alkaloid level	Sample[1]	Ethanol extract dpm/g	Hydrolysed ethanol extracted plant material[2] dpm/g
Serine injected plants (table 20)			
None	1	725	5250
	2	1030	4750
Low	1	420	750
	2	872	3750
High	1	682	3250
	2	660	3250
Tryptophan injected plants (table 21)			
None	1	4690	5750
	2	5640	3500
Low	1	1517	4500
	2	4240	3250
High	1	3500	8000
	2	6260	4250

1 Sample 1 was extracted for 6 hr; sample 2 was extracted for 18 hr.

2 The radioactivity determined in the hydrolysed ethanol extracted head portion samples was negligible (less than 10 % of the values obtained for sample 2).

tryptophan by colorimetric determination and 81 % recovery by radioactivity measurements.

The ratio of tryptophan to serine calculated for various fractions (Table 24) indicated the variation with regard to incorporation. All tryptophan injected plant fractions had a higher proportion of the original label than did the serine injected plant fractions. The highest ratios were obtained in the ethanol extract and in the aqueous fraction of the ethanol extract after basic and acidic chloroform extraction.

Due to the low levels of label appearing in the alkaloids, a second extraction of the plant material was thought to be more beneficial in reporting the experiment than incubating the labelled reed canary grass with rumen microorganisms, as originally planned.

Table 24

Comparison of amounts of radioactivity appearing in various fractions of plant material from tryptophan and serine injected plants [1]

Description	Tryptophan injected plants	Serine injected plants	Tryptophan / Serine
Injected (uCi)	0.47	0.40	1.17
Percent injected label incorporated into ethanol extract	13.67	2.17	6.30
Acidic chloroform extract (dpm/g)	722	370	1.95
Basic chloroform extract (dpm/g)	225	49	4.60
Aqueous extract (dpm/g)	4940	979	5.05
Percent of label injected recovered in tryptophan	0.412	0.119	3.46
Percent of injected label appearing in alkaloid fraction	0.551	0.123	4.50

[1] Sample 2 values only were averaged for the results.

CONCLUSIONS

Serine was incorporated into tryptophan in the reed canary grass plant, but only accounted for approximately 10 % of the tryptophan synthesis. Less than 1 % of the injected tryptophan was found in the alkaloid fraction. Most of the label in the alkaloid fraction was recovered from the area where hordenine and an unknown compound which reacts with Van Urk's reagent were located. The labelling did not follow the concentration levels of alkaloids as determined with Van Urk's reagent and ultraviolet. Apparently most of the alkaloid portion was synthesized prior to the time of injection of the labelled compounds. The acidic chloroform extract, which contained indoleacetic acid, made up 12 % of the labelled material in the ethanol extract of the tryptophan injected plants. The maximum amount of labelled tryptophan in indoleacetic acid after 33 days would then be less than 1 %.

The higher levels of radioactivity in the tryptophan injected plants as compared to the serine injected plants, indicated a longer retention time for tryptophan and its metabolites in reed canary grass.

GENERAL CONCLUSIONS

Tryptophan metabolism differs in sheep and cattle according to Dyer et al (1964). In our experiments conducted with sheep, little tryptamine was recovered as a metabolite whereas this metabolite was apparently important in cattle.

Tryptophan was rapidly absorbed from the rumen of the sheep. On the basis of the peak value obtained of 100 dpm/ml in blood when 20 uCi was injected into the rumen, a calculation was made of the proportion of label which could be absorbed in 30 minutes, using a portal blood flow rate of 1.5 l/min. The portal blood would transport 2 uCi of labelled material to the liver in 30 minutes, or 10 % of the original amount of labelled tryptophan.

Less free tryptophan would likely be present in the rumen when a tryptophan deficient diet was fed compared to a balanced diet. If feed given ruminants was low in tryptophan, the experiments reported herein indicated that the microorganisms of the rumen might not be able to synthesize sufficient tryptophan to provide for optimum growth of the microbial population. Values for tryptophan content of various protein sources indicated the microorganisms were marginal as sources of tryptophan for tissue production or milk production (Table 32). Plants tended to have a similar tryptophan content as compared to microorganisms, or even less. The possibility of tryptophan

limiting protein synthesis or milk production seems likely.

The reed canary grass experiments showed that the main alkaloid production may take place early in the plant growth. Phalaris staggers was noticed after sheep fed on fresh pasture growth, early in the growing season. Reed canary grass grew more quickly than other pasture species and was toxic when consumed as the sole diet. If alkaloids were the cause of toxicity, they were formed early in plant growth and might indicate a protective role for these compounds in plants.

The general approach followed in the experiments described offered guidelines for future amino acid research to relate diet to function of both the ruminant microorganisms and animal body. In vitro procedures can provide initial information for in vivo work. As tracer experiments provide useful information for metabolism studies, the much smaller volume involved in the in vitro apparatus as compared to a live animal experiment makes the in vitro technique a valuable tool for preliminary studies. The in vitro technique also allows the investigator a chance to examine the effect of toxic substances on the rumen microbial population with much smaller amounts of plant material than would be needed in vivo. The ultimate test has to remain the whole animal study.

Many areas of activity in non-ruminant amino acid nutrition about which virtually nothing is known in ruminants include amino acid imbalances, interactions, timing effects on amino acid utilization, digestion, absorption, transport,

appetite interactions and other metabolic interactions.

Research into specific amino acid reactions under practical circumstances is necessary for practical application of knowledge to plant and animal nutrition.

SUMMARY

(1) Half of the DL-tryptophan -^{14}C (U) -benzene ring labelled material initially added to the rumen microorganisms in the in vitro apparatus, left the rumen sac within 6 hours. Less than 4 % of the label was associated with either protozoal or bacterial fraction.

(2) When L-serine -^{14}C (U) was incubated with rumen microorganisms in vitro, half the labelled material diffused into the saline before 4.5 hours had elapsed. As a maximum value, 2.1 % of the label appeared associated with protozoa, the similar value for bacteria was 1.5 %. Only the 24 hr sample of experiment 5 showed greater than 1 % incorporation of serine into tryptophan.

(3) Labelled compounds appeared in the urine within 10 minutes after injecting DL-tryptophan -^{14}C (U) -benzene ring labelled into the rumen of two sheep.

(4) Rapid absorption of tryptophan from the rumen and reticulum of two sheep was shown and this absorption rate compared to the rate of sodium acetate absorption.

(5) An average of 2.17 % of label from L-serine -^{14}C (U) treated reed canary grass appeared in the ethanol extract. The corresponding value for DL-tryptophan -^{14}C (U) -benzene ring labelled plants was 13.67 %. The alkaloid fraction of high alkaloid containing plants was found to contain 0.7 % of injected tryptophan label and 0.1 % of injected serine label.

APPENDIX

Table 25

Total quantities of tryptophan in rumen sac and saline solution during 24 hr period as determined by colorimetric analysis (Experiment 1).

Time hr	Rumen sac mg	Saline solution mg	Tryptophan added or removed mg	Total mg
0	54.00	36.00		90.0
			+100.0	190.0
3.75	89.60	62.40		152.0
			- 13.2	
9.50	61.20	76.80		148.0
			- 12.5	
22.00	45.90	76.80		122.7

APPENDIX

Table 26

Total radioactivity as determined in the *in vitro* experiments.
(dpm X 10^{-6})

Time period[1]	1		2		3	
	Calc.[2]	Obs.	Calc.	Obs.	Calc.	Obs.
Tryptophan experiments						
1[3]	1.16	1.01	0.92	1.19	1.10	1.01
2	1.44	1.09	0.96	0.83	0.75	0.71
3	27.40	25.50	22.50	24.10	22.30	25.50
Serine experiments						
4	22.20	12.95	39.00	28.70	36.50	25.30
5	37.70	29.50	35.00	26.00	31.40	24.10

[1] Time periods used were:

Experiment 1: 3 hr 45 min; 9 hr 30 min; 22 hr.
Experiment 2: 3 hr; 9 hr 15 min; 23 hr 30 min.
Experiment 3: 3 hr; 6 hr 30 min; 24 hr.
Experiment 4: 2 hr 30 min; 26 hr; 42 hr.
Experiment 5: 2 hr 30 min; 5 hr 30 min; 24 hr.

[2] Obs. represents observed values; Calc. represents values calculated.

[3] In experiment 1: Total tryptophan added was 100 mg and the amount calculated by colorimetric determination was 62 mg.

APPENDIX

Table 27

Labelled compounds appearing in the saline solution expressed as a percent of the labelled tryptophan added to the rumen sac.

Experiment	Time hr	Total dpm X 10^{-6}	Saline dpm X 10^{-6}	%
1	3.75	0.93	0.44	47.3
	9.50	1.16	0.90	77.6
	22.00	1.00	0.87	87.0
2	4.00	1.40	0.43	30.7
	9.00	0.80	0.52	65.0
	23.50	0.70	0.56	80.0
3	3.00	25.00	10.00	40.0
	6.50	24.00	14.00	58.3
	24.00	24.00	20.00	83.3

APPENDIX

Table 28

Labelled compounds appearing in the saline solution expressed as a percent of the labelled serine added to the rumen sac.

Experiment	Time hr	Labelled compounds (dpm X 10^{-6})			%	
		Total label[1]		Saline		
		Obs.	Calc.	Obs.	Obs.	Calc.
4	2.5	12.95	22.20	3.44	26.5	15.5
	5.0	13.46	20.65	8.64	64.0	41.8
	21.0	10.79	16.80	9.37	87.0	55.7
	26.0	28.20	39.00	15.80	56.0	40.5
	42.0	25.29	36.53	21.80	86.1	58.7
5	2.5	29.50	37.74	12.20	41.0	32.4
	5.5	26.00	34.96	17.20	66.0	49.7
	24.0	24.10	31.43	19.90	82.5	63.3

[1] Total label includes label in rumen sac + label in saline - label removed in sampling. 10 uCi of labelled serine was added to the media at 0 hr and again at 23 hr in experiment 4; 17 uCi of labelled serine was added in experiment 5.

[2] Obs. represents observed values; Calc. represents values calculated.

$$\% \text{ Obs. is } \frac{\text{saline obs.}}{\text{total label obs.}} \quad ; \quad \% \text{ Calc. is } \frac{\text{saline obs.}}{\text{total label calc.}}$$

APPENDIX

Table 29

Percentage of radioactivity in saline solution and rumen liquor supernatant appearing in the tryptophan containing fraction of the effluent from the ion exchange column.

Time period[1]	Saline solution		Rumen liquor supernatant	
	Total radioactivity	%[2]	Total radioactivity	%
Experiment 4				
1	62,100 55,000	50.5 (86.5) 44.7 (75.4)	228,500	27.4 (47.4)
2	50,100 89,700	13.9 (38.7) 25.0 (51.0)	64,200	14.5 (18.8)
3	58,900 47,800	15.1 (82.5) 12.2 (58.7)	11,200	not done
4	176,500 135,500	26.8 (81.8) 29.4 (79.4)	394,000	44.1 (72.8)
5	82,800	9.1 (80.1)	18,750	6.8 (62.6)
Experiment 5				
1	83,000 81,308	16.4 (43.5) 20.8 (39.0)	234,500	15.2 (26.8)
2	108,000 63,000	15.0 (60.7) 5.8 (35.8)	190,000	22.5 (50.5)
3	24,000 128,300	2.9 (94.4) 15.5 (55.1)	48,650	16.7 (39.2)

[1] Time periods used were:

Experiment 4: 2.5 hr; 5.0 hr; 21.0 hr; 26.0 hr; 42.0 hr.
Experiment 5: 2.5 hr; 5.5 hr; 24.0 hr.

[2] Number in brackets represents the % recovery of radioactivity both from the acidic and basic fractions applied to the ion exchange column.

APPENDIX

Table 30

Percentage of radioactivity in hydrolysed bacterial and protozoal fractions of rumen liquor appearing in the tryptophan containing fraction of the effluent from the ion exchange column.

Time period[1]	Bacterial fraction	Protozoal fraction
Experiment 4		
1	99	280
2	111	152
3	188	71
4	261	236
5	200	492
Experiment 5		
1	113	111
2	lost	95
3	523	650

[1] Time periods used were:

Experiment 4: 2.5 hr; 5.0 hr; 21.0 hr; 26.0 hr; 42.0 hr.
Experiment 5: 2.5 hr; 5.5 hr; 24.0 hr.

APPENDIX

Table 31

Percentage of radioactivity in acidic and basic chloroform extracts, and aqueous extract as compared to the total radioactivity calculated for the ethanol extract[1].
(total dpm)

Alkaloid level	Chloroform extracts		Aqueous	Ethanol extract	%
	acidic	basic			
Serine injected plants					
None	2,400	292	5,440	5,150	158.0
Low	1,700	236	5,320	4,360	168.0
High	1,375	204	3,930	3,300	167.0
Tryptophan injected plants					
None	4,230	504	34,134	28,200	158.0
Low	2,150	1,350	22,400	21,200	168.0
High	4,450	1,516	31,766	31,300	167.0

[1] Only sample values from the second extraction are tabulated.

APPENDIX

Table 32

Tryptophan content of several sources
(g tryptophan/100 g crude protein)

Source	Amount	Reference
Rumen microorganisms	1.8	Abdo et al (1964)
Whole egg	1.5	
Soybean meal	1.6	
Rumen bacteria (dry feed)	1.6	Holmes et al (1953)
Rumen bacteria (green feed)	1.4	
Whole egg	1.5	
Milk	1.4	Jacobson and Van Horn (1970)

Tryptophan Utilization by Rumen Microorganisms *in vitro*.[1]

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ABSTRACT

A continuous flow artificial rumen apparatus was used to investigate the utilization of DL-tryptophan -^{14}C (U) - benzene ring labelled material by rumen microorganisms. Half of the labelled material left the rumen sac in the first six hours. After a 24 hour period, less than 4% of the label was associated with either protozoal or bacterial fraction of the rumen fluid.

[1] Contribution No. 213 (Plant Science)

[2] Departments of Animal And Plant Science

[3] Department of Animal Science

[4] Department of Plant Science

INTRODUCTION

There is little information on ruminant metabolism of tryptophan. Lewis and Emery (7) divided amino acids into three groups with regard to their relative rates of deamination by rumen microorganisms. Tryptophan was in the group in which deamination was less pronounced. Lacoste (6) found that rumen microorganisms metabolize tryptophan slowly.

This paper reports the results of studies conducted *in vitro* to determine the utilization of tryptophan by rumen microorganisms.

MATERIALS AND METHODS

Three experiments of 24 hour duration were conducted. A continuous flow artificial rumen apparatus of the Davey *et al.* (3) design was constructed. Instead of two electrodes, a combination electrode was used to monitor the pH of the medium. The pH was maintained between 6.9 and 7.0 during each experiment. The procedure used by Davey *et al* (3) was followed in these experiments.

The sample of rumen microorganisms was collected from a fistulated Holstein cow prior to the daily feeding of a grass alfalfa hay mixture. Volatile fatty acid (VFA) analysis (3), micro-kjeldahl nitrogen determinations (1) and microscopic examination of the extracted rumen sac contents were conducted

to assess protozoa viability.

Tryptophan plus McDougall's solution (8) was added to the microorganisms in the rumen sac and the sac was surrounded by 2,400 ml saline. In the first experiment, 100 mg of DL-tryptophan and 0.5 uCi DL-tryptophan - ^{14}C(U) - benzene ring labelled (Nuclear - Chicago, Des Plaines, Illinois) in 20 ml of McDougall's solution were added to the rumen sac. In the second and third experiments only labelled tryptophan was added with McDougall's solution, the amounts were 0.5 and 10 uCi, respectively.

A Nuclear-Chicago Model 720 liquid scintillation counter was used to count samples. Saline solution (0.1 ml) was counted in Bray's solution (2). The bacterial cells were digested with formamide (9) for 2.5 hours at 60°C in glass stoppered test tubes (1 ml of formamide per 5-20 mg of cells). Half of the digest was counted in Bray's solution.

The rumen liquor sample was separated by centrifugation into protozoal, bacterial, and supernatant fractions (9) and analysed for tryptophan content (4,5), labelled material and total nitrogen. Saline samples were analysed for tryptophan (5), labelled material and total nitrogen. Determinations were completed at all time periods, provided that sample quantities were adequate.

RESULTS

The rumen liquor sample, as obtained from the cow, contained 36 ug tryptophan per ml in the supernatant which corresponded to the equilibrated zero saline value (Fig. 1). Half of the labelled material left the rumen sac in the first six hours of incubation (Fig. 2). The specific activity of tryptophan in the rumen contents (Table 1) was initially 7.55 dpm/ug tryptophan. Labelled material compared to tryptophan content in the third time period had dropped to 2.93 dpm/ug.

Less than 4% of the labelled material was associated with bacterial or protozoal fractions. (Table 2). The higher level of radioactivity associated with protozoal and bacterial fractions in time period one of experiment 1 than at other time periods might be attributed to contamination with supernatant trapped in the sediment. To verify the percentage of label associated with the microbial fraction, twenty times the amount of labelled tryptophan was added in the third experiment as compared with the first two experiments.

A decrease was observed in the rumen VFA values (Table 3). The amount of labelled material, which was associated with each millequivalent of VFA, also decreased with time.

Total nitrogen in the rumen sac contents decreased (Tables 4 and 5) during the time periods examined, possibly indicating a reduction in the microbial population.

DISCUSSION

The slow metabolism of tryptophan by rumen microorganisms was suggested previously (6). The results of the *in vitro* experiments described herein indicate that less than 4% of the labelled tryptophan was associated with the protozoal or bacterial populations.

Breakdown of tryptophan for energy by the microorganisms was suggested by the higher percentage of the available label appearing in the VFA fraction as the experiment proceeded. In the third experiment, the label occurring in the VFA fraction of the rumen sac, expressed as a percent of the total label present in the rumen, rose from 0.6% to 1.05% at the end of one day. The decrease of radioactivity in VFA obtained from the rumen sac (Table 3), expressed as dpm/meq VFA produced, was due to the much lower amount of labelled material available in the rumen sac of the *in vitro* apparatus as diffusion into the saline proceeded.

The drop in specific activity of tryptophan in the rumen sac (Table 1) would be consistent with a slow uptake of label by microorganisms and rapid diffusion of labelled material out of the rumen sac into the saline solution. As most of the labelled tryptophan was initially located in the fluid portion of the rumen sac, the drop in specific activity of the tryptophan in the rumen sac showed a faster rate of transfer across the sac into the saline solution than uptake of labelled material by the microorganisms.

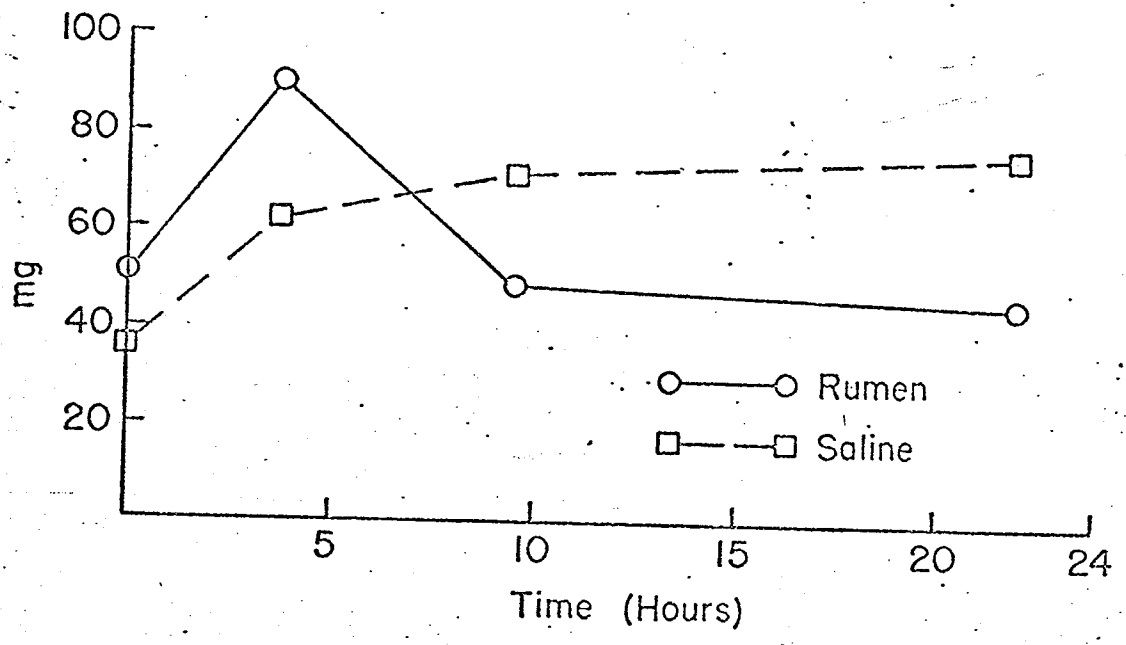
The minimum amount of free tryptophan in the rumen with the grass alfalfa hay mixture fed in the described experiments was 36 ug/ml. The amount of labelled material taken up by the microorganisms in 24 hours was comparatively small. Any tryptophan in the rumen fluid could then be available for absorption from the rumen.

Figure 1

Total quantities of tryptophan in rumen sac and saline solution during 24 hr period as determined by colorimetric analysis (Experiment 1). DL-tryptophan was added immediately following 0 sampling time.

Figure 2

Labelled compounds appearing in the saline solution expressed as a percent of the labelled tryptophan added to the rumen sac.


100
80
60
40
20
mg
Rumen
Saline
5
10
15
20
24
Time (Hours)

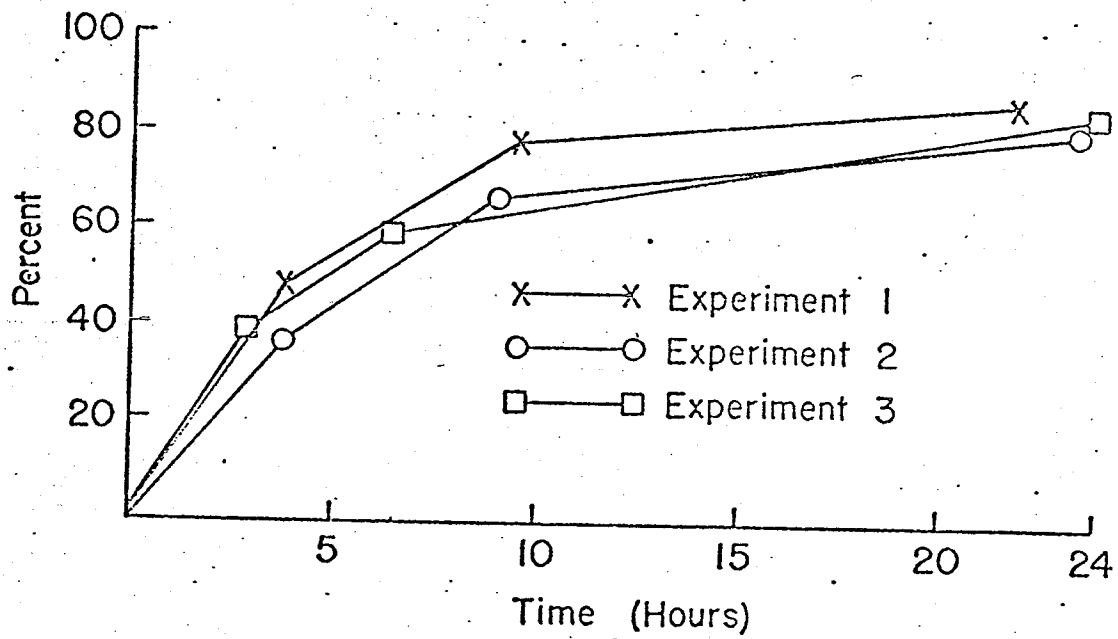

100
80
60
40
20
Percent
Experiment 1
Experiment 2
Experiment 3
5
10
15
20
24
Time (Hours)

Table I

Labelled material compared to tryptophan content in the rumen sac and saline solution, (Experiment 1.)

Time Period[1]	Rumen sac Tryptophan content ug/ml	Rumen sac dpm/ug	Saline solution Tryptophan content ug/ml	Saline solution dpm/ug
0	514	7.55	15	0.00
1	280	6.27	26	7.13
2	200	4.65	32	11.70
3	150	2.93	32	11.50

[1]Time periods used were:
Experiment 1: 0; 3 hr 45 min.; 9 hr 30 min.; 22 hr.
Experiment 2: 0; 3 hr; 9 hr 15 min.; 23½ hr.
Experiment 3: 0; 3 hr; 6 hr 30 min.; 24 hr.

Table 2

Amount of label occurring in rumen sac fractions
(dpm per ml rumen sac contents)

Time Period[1]	Fraction Examined	Experiment 1	Experiment 2	Experiment 3
0	amount added	3,640	4,100	85,500
1	rumen contents	1,757	1,958	51,499
	protozoa	61 (3.5)[2]	30 (1.45)	304 (0.59)
	bacteria[3]	73 (4.0)	-	556 (1.08)
2	rumen contents	930	1,000	35,058
	protozoa	-	33 (3.3)	215 (0.61)
	bacteria	38 (4.0)	34 (3.4)	408 (1.16)
3	rumen contents	439	505	13,531
	protozoa	-	-	171 (1.26)
	bacteria	0 (0.0)	18 (3.5)	249 (1.84)

[1] Time periods used were:

Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr .
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

[2] Values in brackets are labelled material expressed as percent of total label.

[3] Analysis data were not obtained where no values are given.

Table 3

Volatile fatty acid (VFA) analysis in saline solution and rumen sac of the *in vitro* apparatus during experiments 1 and 3.[1]

Time Period[2]	0	1	2	3
Saline - meq/100 ml				
Experiment 1	0.7	1.2	1.4	1.3
Experiment 3[3]	-	2.1	1.4	1.4
Rumen sac - meq/100 ml				
Experiment 1	8.6	3.5	2.05	1.5
Experiment 3	-	3.8	2.4	2.0
Radioactivity in VFA obtained from rumen sac				
Experiment 3				
dpm/ml		352	212	142
dpm/meq		9,250	8,840	7,100

[1] VFA determinations were not conducted for experiment 2.

[2] Time periods used were:
Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr .
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

[3] Analysis data were not obtained where no values are given.

Table 4

Total nitrogen determined for rumen sac contents
(mg N/ml rumen sac contents)

Time period[1]	Experiment 1	2	3	% N as tryptophan[2]
0[3]	-	.682	-	-
1	.280	.444	.280	2.74
2	.210	.364	.280	2.74
3	.182	.252	.252	2.40

[1] Time periods used were:

Experiment 1: 0; 3 hr .45 min.; 9 hr .30 min.; 22 hr .
Experiment 2: 0; 3 hr .; 9 hr .15 min.; 23½ hr..
Experiment 3: 0; 3 hr .; 6 hr .30 min.; 24 hr .

[2] Values are for experiment 3 only.

[3] Analysis data were not obtained where no values are given.

Table 5

Total nitrogen determined for rumen sac fractions[1]
(mg N/ml rumen sac contents)

Experiment 3	Time period 2 (3 hr .)	Time period 3 (6 hr .30 min.)
Protozoa	.042	.040
Bacteria	.090	.085
Supernatant	.140	.084
Total	.272	.219
Total (from Table 4)	.280	.252

[1] Due to insufficient sample quantities, determinations were not made for time periods 0 and 1.

Acknowledgement

Financial assistance for this work from the National Research Council of Canada is gratefully acknowledged.

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Tryptophan Absorption and Metabolism in Sheep[1]

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ABSTRACT

DL-tryptophan -^{14}C (U) benzene-ring labelled was injected into the rumen of two sheep. Label appeared in portal blood prior to its appearance in jugular blood. Most of the label in the blood samples occurred in tryptophan. Label appeared in the urine within ten minutes of introducing the labelled tryptophan into the rumen. Urine samples were examined for label appearing in tryptamine, indoleacetic acid and tryptophan. Less than 20% of the label in the urine was in the analysed compounds with tryptamine showing negligible activity.

Rapid absorption of tryptophan from the rumen and reticulum of two other sheep was shown. Absorption of 26.7% of the total labelled tryptophan injected occurred with the washed rumen technique, and 68.8% was absorbed when the whole rumen technique was employed.

1 Contribution No. 215 (Plant Science)

2 Departments of Animal and Plant Science

3,4 Department of Animal Science

5 Department of Plant Science

INTRODUCTION

Schelling and Hatfield (10) reported that when urea was the sole source of nitrogen in the feed, animal growth was favourably influenced by amino acid administration posterior to the rumen. Feed intake was also increased by the feeding of amino acids in protein form. An increased retention of absorbed nitrogen was observed by McLaren *et al* (8) when 0.8 g L-tryptophan was substituted for an isonitrogenous amount of urea in a lamb ration. Lewis (6) reported that tryptophan was in the class of amino acids slowly deaminated. Piana and Piva (9), using ^{15}N labelled $(NH_4)_2\ HPO_4$, concluded that tryptophan could only be synthesized in the rumen if nicotinic acid was fed to the microorganisms at the same time as the $(NH_4)_2HPO_4$. They concluded that tryptophan was the limiting amino acid in the utilization of non-protein nitrogen by ruminants.

In vitro experiments conducted in this laboratory (2) indicated that tryptophan fed as the free amino acid was not rapidly utilized by rumen microorganisms and if absorbed through the rumen epithelium could be available for utilization by the animal body. The experiments described here, deal with the metabolism and absorption of tryptophan in sheep.

MATERIALS AND METHODS

Portal vein catheters were established in two mature Rambouillet ewes using a surgical procedure similar to the method described by Conner and Fries (4). DL-tryptophan -^{14}C (U) benzene-ring labelled material in water (10 uCi for sheep 1 and 20 uCi for sheep 2) was injected directly into the rumen one hour after the ewe had consumed a grass alfalfa hay mixture. Subsequently, blood samples were taken from jugular and portal catheters at ten minute intervals during the first hour and at 1.5, 2, 3, 4, 5, 6, 8 and 12 hr and then once daily for four days. Urine and feces samples, when available, were collected at the same time as the blood samples. A Nuclear-Chicago, Model 720, liquid scintillation counter was used for counting the radioactivity of samples, prepared by standard procedures.

Thin layer chromatography of urine was performed by drying 15 ml of urine and adding water to a total volume of 1 ml. The adsorbent used was cellulose MN 300 (10.0 g): silica gel H (4 g): water (80 ml) (1). Separation was effected by n-butanol: water: acetic acid (120:50:30 ml). After development of 1 ul spots, Ehrlich's reagent and Ninhydrin spray were used in conjunction with ultra-violet mapping. Labelled components were located by streaking a 20 ul sample on the adsorbent, followed by development and counting of lifted layer material. Twenty sections (fractions) were lifted per plate and counted.

Urine samples were also partitioned in benzene to separate tryptamine, indoleacetic acid and tryptophan (11). The fraction containing the tryptophan was further separated by ion exchange chromatography using Rexyn 101 (Na^+) for the column material. Elution of tryptophan was monitored using a flow through fluorimeter (Turner-model III). The tryptophan containing fraction was dried, made up to 1 ml with water and spotted on thin layer. The area containing tryptophan was lifted and counted. The tryptamine and indoleacetic acid fractions were also spotted on thin layer and the specific areas counted to identify the proportion of the label in each of these compounds.

Blood samples were pooled in an attempt to obtain sufficient radioactivity to be assayed in a manner similar to the urine samples. Three portal plasma samples from each sheep were analysed. These samples represented early (0 to 2 hr); mid (3 to 5 hr); and late (after 5 hr) time periods. Only one sample of jugular plasma was obtained from sheep 1 and it corresponded to the late time period. Two samples of jugular plasma (an early and mid sample) were analysed from sheep 2.

Tissue samples of the kidney, mammary gland, lung, spleen, liver, brain and muscle were taken at the time of slaughter for examination of label content. Sheep 1 was slaughtered 3 weeks after the injection of the labelled tryptophan while sheep 2 was slaughtered 1 week after the injection.

Three mature Rambouillet ewes were surgically fitted with rumen cannula for the absorption experiments. In the initial absorption experiment (sheep 3) the subsequent surgical operations followed immediately, whereas with sheep 4 and 5, three complete days passed to allow the ewe to recover from the cannula operation. After anaesthetizing each sheep, the reticulo-omasal orifice was tied off, urinary and jugular catheters inserted, and labelled material introduced into the rumen. Sheep 3 received 1.5 uCi sodium acetate -^{14}C in the rumen while sheep 4 and 5 each had 5 uCi of D L-tryptophan -^{14}C (U)-benzene-ring labelled introduced into the rumen. The washed rumen technique (7) was used with sheep 3 and 4 and the whole rumen contents were left intact in sheep 5. Blood, urine and rumen samples were taken at 0, 15, 30 min. and 1, 2, and 3 hr after introduction of the labelled compound. These experiments were terminated at 3 hr. Counting procedures for these samples were the same as described in a previous publication (2).

RESULTS

Counts of ^{14}C label in plasma samples from sheep 1 and 2 (Fig. 1) rose in portal blood during the first hour; a slight increase also occurred in counts from jugular blood. The rise in portal and jugular blood values of ^{14}C label tended to equilibrate after two days. Labelled compounds appearing in the urine (Fig. 2) expressed as dpm/ml, increased for the first two hours following dosing. The urinary level then fluctuated and sub-

sequently fell after one day. Less labelled material appeared in the urine of sheep 2 than in sheep 1 during the time samples were collected.

Thin layer chromatography indicated a change in the type of labelled compounds excreted in the urine as time after feeding increased (Fig 3). Unidentified peaks of activity were associated with fractions 10, 13, and 16. Of the three compounds examined, tryptophan was initially present in the urine in the highest quantities (Table 1). Indoleacetic acid contained more than three percent of the labelled material present in urine for most of the samples examined until the twelve hr mark after which a decrease occurred. Negligible amounts of labelled tryptamine were found at all sampling times.

Plasma sample analysis (Table 2) indicated that most of the label in the blood was associated with tryptophan. The quantity of label occurring in other compounds increased over time, as evidenced from increasing differences between the label in the solution containing tryptophan and the tryptophan after it was extracted.

Only the liver of sheep 2 of the tissues examined had a significant amount of label (71 dpm/g) while kidney (10 dpm/g) and muscle (4 dpm/g) of sheep 2 showed a lesser amount. The time interval between introduction of the labelled tryptophan and slaughter could account for the lack of detectable label in tissues from sheep 1.

A higher percentage of the labelled tryptophan was absorbed through the rumen epithelium in sheep 5 while a lower percentage was absorbed in sheep 4 as compared to sodium acetate in sheep 3 (Table 3). Excretion of labelled material in urine showed a higher dpm/ml value for the initial samples taken from sheep 5 as compared to sheep 4 (Table 4) indicative of a more rapid rate of absorption. The final samples of urine obtained (3 hr) showed that the concentration of labelled material in urine was still increasing.

DISCUSSION

When tryptophan was injected into the rumen of sheep, 25% to 70% of the free tryptophan in the rumen was absorbed into portal blood within 3 hr. Tryptophan in portal blood might then be removed in the liver, metabolized, and excreted or recirculated to other locations by way of the jugular blood.

Theurer et al (12) compared portal and jugular blood plasma amino acids and concluded that jugular blood was representative of body blood supply. In their study sampling started at two hr after feeding. In our experiments, labelled compounds appeared in the urine within ten minutes after introducing the labelled tryptophan, indicating that early sampling is essential in plasma amino acid studies.

The experiment with sheep 1 was undertaken before normal feed consumption had resumed following surgery. The higher

excretion rate of labelled compounds in urine observed in sheep 1 as compared with sheep 2 may have reflected poorer conversion to body protein and certainly indicated lower utilization of tryptophan. Compounds analysed in urine (tryptophan, tryptamine and IAA) contained less than 20% of the labelled material. Most of the label in urine was either in the first fraction of the ion exchange eluant or accompanied tryptophan in the second fraction. The compounds involved in the kynurenic acid breakdown pathway of tryptophan occur in the two fractions where most of the urinary label was located. Presumably most of the label was associated with tryptophan metabolites.

The procedures followed in the absorption experiments isolated the rumen and reticulum of the digestive tract and any absorption must have occurred through the rumen or reticulum. The higher rate of absorption observed in the first hour with sheep 5 (whole rumen contents), compared to sheep 4 (washed rumen technique), could have been due to a higher concentration of labelled tryptophan near the absorption sites. It was also possible that sheep 5 was not as heavily anaesthetized as sheep 4 and thus had a higher metabolic rate.

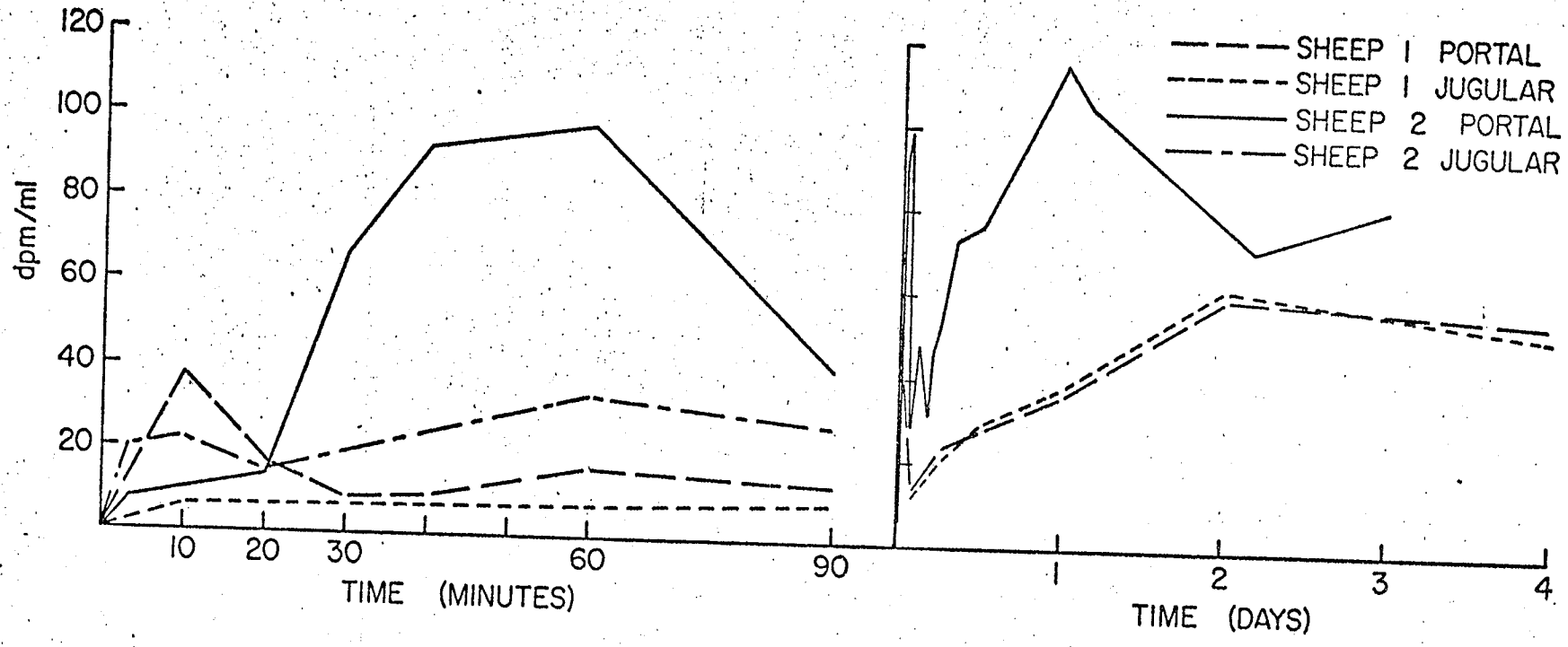
Tryptophan appears to be absorbed from the rumen and reticulum at significant rates and the only apparent limitation to absorption is the concentration of tryptophan in the rumen fluid. The early excretion of labelled compounds in urine and the rapid metabolism of tryptophan indicate that the body may have a very

small circulating pool of tryptophan and rapidly excretes any excess.

Several suggestions, based on our reported results and interpretation of the available literature, might help to insure optimum feed utilization if tryptophan is limiting. Supplementation of the ruminant ration, to allow a release of tryptophan at a time when the maximum amount of amino acids supplied by the feed are available for absorption, would be the most satisfactory. Tryptophan is not generally required in large amounts, therefore it might also be feasible to supplement feeds with higher than necessary levels of tryptophan to be certain sufficient quantities would be available at the most opportune time. This last suggestion would not be valid, however, if as has been suggested by several investigators (3,5), tryptophan is toxic when present in higher than normal quantities.

Figure 1

The appearance of labelled compounds in jugular and portal blood of sheep 1 and 2 after injection of labelled tryptophan into the rumen.



Tryptophan absorption and metabolism in sheep.
Fig. 1.
Elizabeth Cundliff, N.E. Stanger, T.J. Devlin, L.J. La Croix.

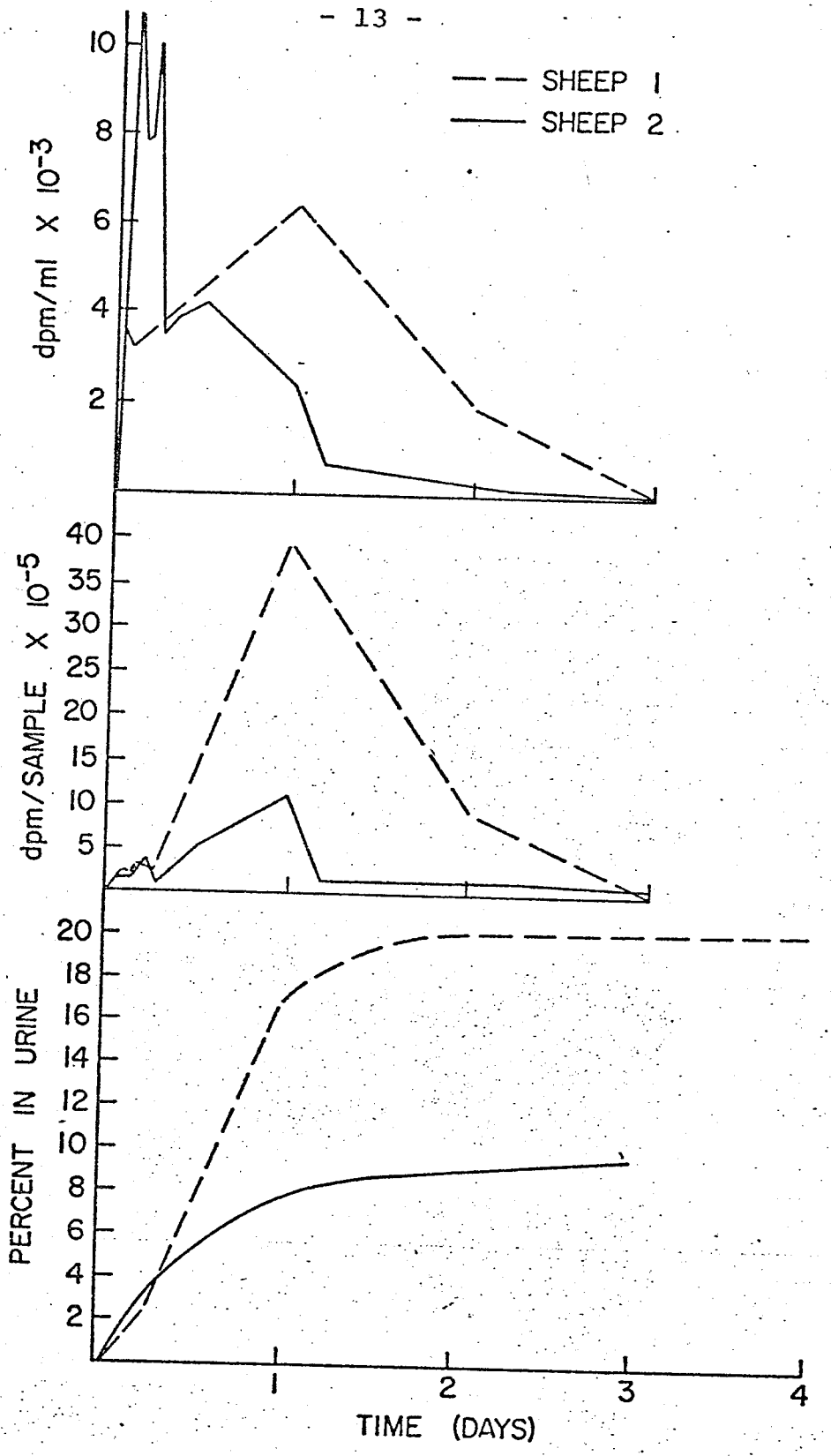
Figure 2

The appearance of labelled compounds in urine of sheep 1 and 2 after injection of labelled tryptophan into the rumen.

Top: Radioactivity in 1 ml. of urine.

Middle: Radioactivity in the total urine sample collected over the time period examined. (dpm/ml x ml urine collected).

Bottom: Percentage of the injected radioactivity which appeared in the urine.



ryptophan absorption and metabolism in sheep.
ig 2.
Gilbert Cunliffe, N.C. Stanger, T.J. Conlin, L.J. La Croix

Figure 3

Labelled material recovered from thin layer chromatograms of urine samples.

(A) and (E) sheep 2

(B) (C) and (D) sheep 1

Analysis of samples obtained between 0 and 2 hours after the labelled tryptophan was injected are shown in (A) and (B). (C) (D) and (E) show the analysis of samples obtained later than 2 hours after the labelled tryptophan was injected.

Fraction 2: Corresponded to a R_F value of 0.85-0.9 and included any indoleacetic acid present in the sample.

Fraction 7: Corresponded to a R_F value of 0.55-0.65 and includes tryptamine.

Fraction 8: Corresponded to a R_F value of 0.5-0.55 and includes most of the tryptophan.

Fraction 10, 13 and 16 contained unidentified peaks of activity.

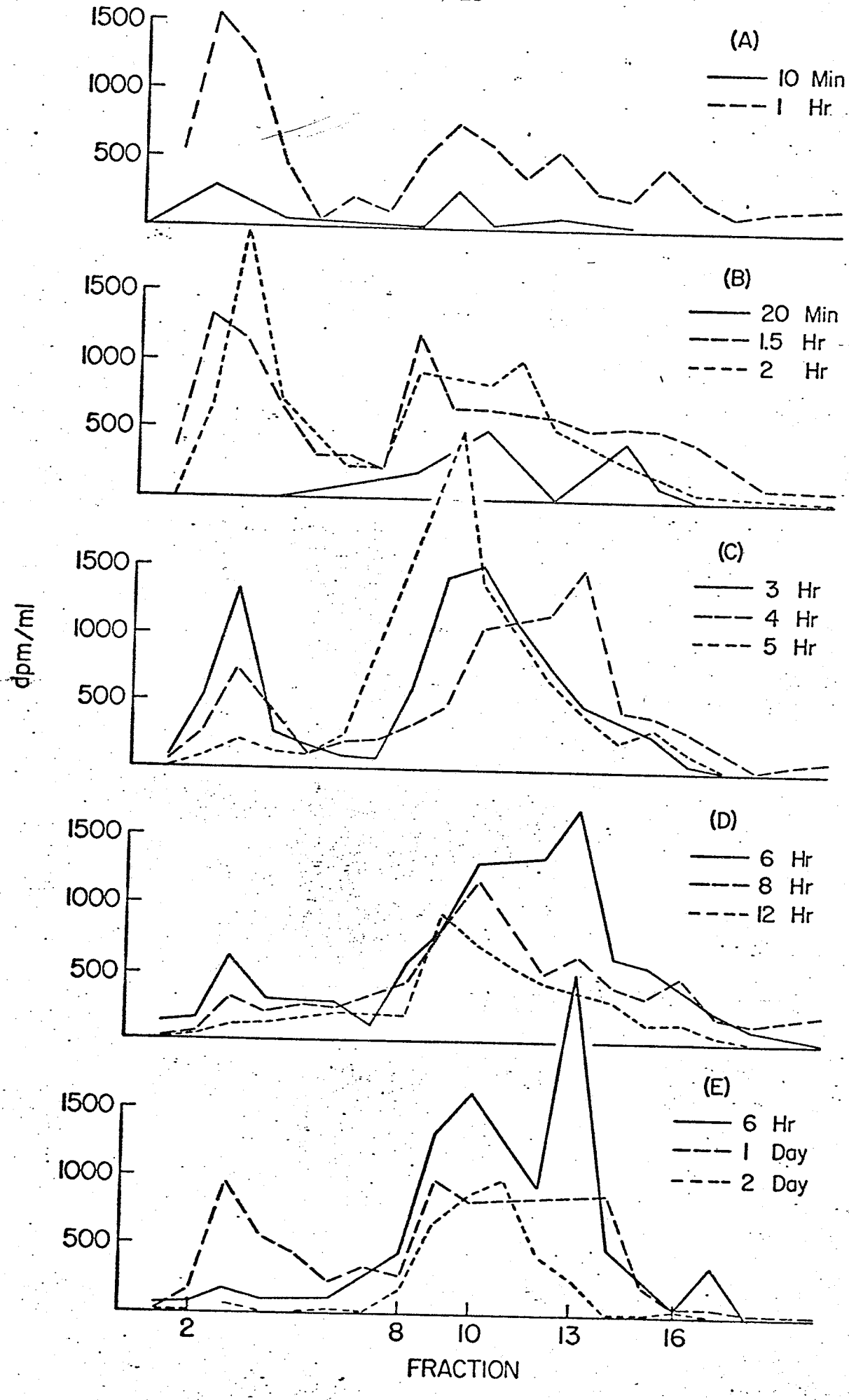

1500
1000
500
(A)
10 Min
1 Hr
(B)
20 Min
1.5 Hr
2 Hr
(C)
3 Hr
4 Hr
5 Hr
dpm/ml
(D)
6 Hr
8 Hr
12 Hr
(E)
6 Hr
1 Day
2 Day
2
8
10
13
16
FRACTION

TABLE 1

Percentage of total label in urine samples collected from sheep 1 and 2 which was associated with tryptamine, indole-acetic acid and tryptophan

Time of Urine Collection	Sheep	Tryptamine	Indoleacetic Acid	Tryptophan
10 min	1	0.210	4.20	5.72
20 min	2	0.000	2.30	14.20
1 hr	1	0.011	4.50	1.83
1.5 "	2	0.081	3.50	4.00
2 "	2	0.122	4.20	1.92
4 "	2	0.090	3.25	1.59
6 "	1	0.000	0.89	0.89
6 "	2	——— [1]	———	1.50
8 "	2	———	5.66	———
12 "	2	0.016	2.24	1.08
1 day	1	———	0.16	1.73
3 "	1	0.000	0.30	5.80

[1] Where a blank occurs, no reading made.

TABLE 2

Labelled compounds appearing in pooled blood plasma samples obtained from sheep 1 and 2 (dpm/sample)

	Sheep	Period	Tryptamine	Indoleacetic Acid	Tryptophan[1]
Jugular					
	2	early[2]	0	0	690 (740)
	2	mid	0	7	960 (1015)
	1	late	0	7	540 (336)
Portal					
	1	early	0	0	330 (192)
	1	mid	0	0	210 (224)
	1	late	0	12	1650 (342)
	2	early	0	0	450 (585)
	2	mid	0	6	2015 (1050)
	2	late	0	9	1320 (273)

1 The first value was obtained from the solution containing the tryptophan after extracting tryptamine and indoleacetic acid, the value in parenthesis was obtained after column chromatography. When the value in brackets is much lower than the first value, the difference represents compounds other than tryptophan.

2 Time periods for the pooled samples were: early, 0 - 2 hr; mid, 3-5 hr; late, 6 hr to completion.

TABLE 3

Absorption of tryptophan -^{14}C (U) benzene-ring labelled from the rumen and reticulum[1].

Sheep	Rumen volume (ml) initial	Rumen volume final	Radioactivity (ml) in rumen (dpm) initial	Radioactivity in rumen (dpm) final	Radioactivity absorbed[2] (dpm)	% of Total radioactivity absorbed after 3 hours
			Sodium acetate			
3	4,015	4,015	3.84×10^6	1.90×10^6	1.88×10^6	49.0
			Tryptophan			
4	4,400	5,040	11.60×10^6	8.35×10^6	3.05×10^6	26.7
5	8,500	8,500	12.70×10^6	3.92×10^6	8.69×10^6	68.8

1 The washed rumen technique was used with sheep 3 and 4; the whole rumen technique was used with sheep 5.

2 Radioactivity absorbed (dpm) = initial radioactivity in rumen (dpm) - radioactivity removed in samples (dpm) - final radioactivity in rumen (dpm).

TABLE 4

Excretion of labelled compounds in urine during tryptophan absorption experiments with sheep 4 and 5.

Time (hr)	Radioactivity (dpm/ml urine)	Volume (ml) of urine	Total radioactive compounds excreted (dpm)
		Sheep 4	
0.25	3	3	9
0.50	100	10	1,000
1.00	370	20	7,400
2.00	832	40	33,280
3.00[1]	1,445	-	----
		Sheep 5	
0.25	0	14	0
0.50	234	8	1,872
1.00	690	12	8,280
2.00	860	30	25,800
3.00	1,000	35	35,000

[1] The total volume of urine collected for this time period was not recorded.

ACKNOWLEDGMENTS

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