METABOLISM OF [^{14}C]FORMALDEHYDE WHEN FED TO RUMINANTS AS AN ALDEHYDE–CASEIN–OIL COMPLEX

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Abstract

Formaldehyde has been used to protect proteins and lipids from metabolism in the rumen, and the present studies were designed to investigate the metabolism of [^{14}C]formaldehyde when given to ruminants as an aldehyde–casein–oil complex. Approximately 60–80% of the consumed [^{14}C]formaldehyde was metabolized to carbon dioxide and methane, a further 11–27% was excreted in the faeces, and 5–6% was accounted for in the urine. The amount of radioactivity excreted either in the expired air or faeces appeared to be dependent on the length of the reaction time between the aldehyde and casein prior to feeding. Small amounts of ^{14}C radioactivity were detected in body tissues and milk, but this was not present as formaldehyde. It is concluded that ruminants effectively metabolize formaldehyde and there is no accumulation of this compound in the carcass or milk.

I. Introduction

Formaldehyde (HCHO) has been used to protect dietary protein from microbial proteolysis in the rumen in an effort to increase the efficiency of utilization of amino acids for wool growth and body growth (Ferguson, Hemsley, and Reis 1967; Ferguson 1970; Faichney 1970). Formaldehyde combines with amino acids (Fraenkel-Conrat and Olcott 1946) forming a complex which is stable under the neutral conditions of the rumen, but is hydrolysed by the secretions of the abomasum and small intestine, thus enabling the protein to be digested and absorbed. Formaldehyde-treated proteins have also been used to protect polyunsaturated fatty acids from ruminal biohydrogenation and thus alter the proportion of these acids in ruminant milk and tissue lipids (Scott *et al.* 1970; Cook *et al.* 1970; Scott, Cook, and Mills 1971).

Since no information is available on the metabolism of formaldehyde by ruminants, the present studies were undertaken to examine the metabolic fate of this compound when fed to sheep and goats in the form of formaldehyde-treated casein–safflower oil particles. A preliminary account of this work was recently published (Mills *et al.* 1971).

II. Materials and Methods

(*a*) *Preparation of Formaldehyde-treated Casein–Safflower Oil Supplements*

Particles (200 kg) containing equal parts of safflower oil and casein were prepared by homogenization and spray-drying procedures as previously described (Scott *et al.* 1971). A portion

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(150 kg) was treated with formalin [37% HCHO (aq.)] (Scott *et al.* 1971) and the final concentration of formaldehyde in the particles was 1·5% by weight. Two smaller batches (each 1·5 kg) were sprayed with formalin containing [^{14}C]HCHO; the formaldehyde content was 1·5% (by weight of particles) and the specific radioactivities of batch I and batch II were 0·30 and 0·24 μCi/g particles respectively.

(*b*) *Animals and Diet*

Four Merino wethers (4 years of age) were fed once daily a mixture of lucerne chaff–crushed oats (1 : 1 w/w; 600 g), and formaldehyde-treated casein–safflower oil particles (250 g) for periods of at least 3 weeks prior to feeding the [^{14}C]HCHO-treated supplement.

Two lactating goats were fed once daily 1500 g of lucerne chaff and crushed oats (1 : 1 w/w) and 300 g of formaldehyde-treated casein–safflower oil for periods of 4 weeks prior to feeding the [^{14}C]HCHO-treated supplement. The radioactive supplements were fed to sheep and goats for a period of 1–3 days. All animals were housed in individual metabolism cages.

To examine the long-term effects of feeding formaldehyde-treated diets two Merino wethers (4 years of age) were fed 750 g of lucerne, wheat, and casein (25 : 37 : 38 by weight) once daily for 18 months; the entire diet was treated with formaldehyde using procedures described by Ferguson (1970). In other experiments sheep and lactating goats were fed a diet containing the formaldehyde-treated casein–safflower oil supplement (see above) for periods of 2 and 6 months. Tissues and goat's milk were analysed for formaldehyde using the method described below.

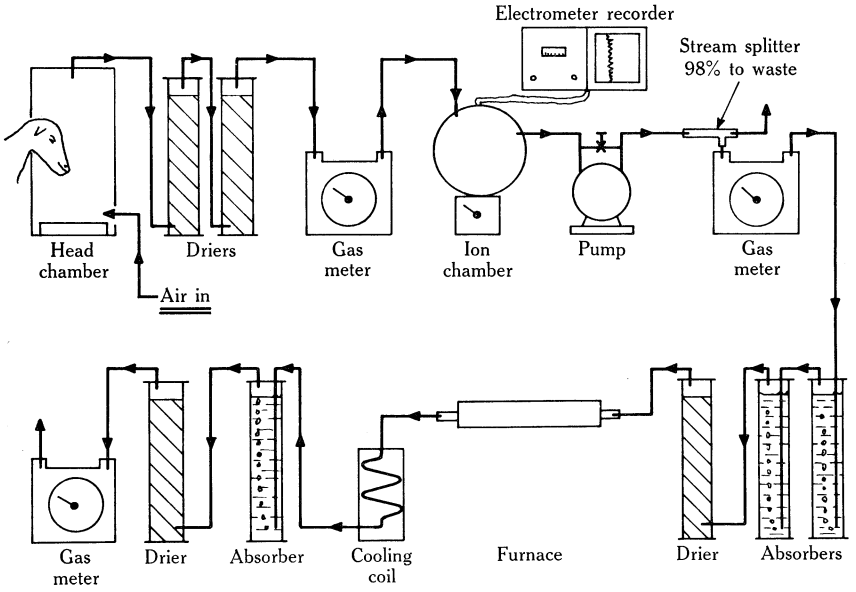

Fig. 1.—Flow sheet of apparatus used for monitoring radioactivity in expired air from sheep fed [^{14}C]HCHO-treated casein–safflower oil supplements.

(*c*) *Experimental Apparatus for Measuring ^{14}C Radioactivity in Expired Gases*

Figure 1 is a flow-sheet summarizing the method of monitoring the amount of ^{14}C radioactivity in expired air. The animal stood with its head in a steel chamber (86 by 62 by 38 cm), which was fitted on to the front of the metabolism cage. This chamber contained a front door, windows made of Perspex sheeting, and an oval opening (54 by 26 cm axes) with a neoprene cuff at the rear. The cuff was placed around the neck of the sheep and the animal was further restrained by a halter attached to a short chain of sufficient length to enable it to have access to

feed and water troughs within the chamber. Air was drawn through the chamber at the rate of 25 l/min by means of a carbon vane pump and passed through $CaCl_2$ drying towers. The effluent gases were then assayed for ^{14}C using an ion chamber and a Cary vibrating reed electrometer (Fig. 1). After passing through the ion chamber the air stream was fractionated by means of a stream splitter and 2% was bubbled through two measuring cylinders (2 litres) each containing 1800 ml of a solution of methyl cellosolve–ethanolamine (8 : 2 v/v) to absorb CO_2. The effluent from the cylinder was then passed through a copper oxide furnace (53·0 by 2·0 cm int. diam.) to combust methane (Lugg 1938). The oxidized gas was cooled and passed through another measuring cylinder containing 1800 ml of the CO_2-absorbing solution.

Samples (10 ml) from the CO_2 absorbers were mixed with 9 ml of toluene scintillation solution containing 0·01% (w/v) 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP)+0·3% (w/v) *p*-terphenyl (PTP) and assayed for ^{14}C radioactivity using a Tri-Carb liquid scintillation spectrometer (Packard Instruments, model 3375, La Grange, Illinois, U.S.A.). The entire apparatus was calibrated using radioactive carbon dioxide generated within the head chamber (Fig. 1) by acidification of $[^{14}C]Na_2CO_3$. The efficiency of methane combustion by the furnance was checked using an infrared gas analysis (Grubb Parsons Ltd., England).

(*d*) *Assay of ^{14}C Radioactivity in Body Tissues, Urine, Milk, Faeces, and Feed*

Two of the sheep were slaughtered at 3 and 4 days after the $[^{14}C]$HCHO casein–safflower oil was fed. At slaughter, blood was collected into vessels containing heparin, the entire gastrointestinal tract was removed, and each organ and its contents were weighed individually. Samples of the contents and tissue from the rumen, omasum, abomasum, small intestine, caecum, and portions (1–2 g) of liver, kidney, omental fat, muscle, and blood (5 ml) were vacuum-dried at room temperature. Small amounts (30–50 mg) of the dried samples were combusted in oxygen flasks and the CO_2 absorbed into cellosolve–ethanolamine (9 : 1 v/v) and assayed in a liquid scintillation spectrometer using the procedures described by Downes *et al.* (1970).

Urine was collected daily and fractions (1 ml) were assayed in toluene–Triton X-100 (7 : 6 v/v) scintillation fluid as described by Downes *et al.* (1970).

Faeces were collected daily and subsamples (50 g) were taken and macerated in a Waring Blendor. Small samples were dried over P_2O_5 in a vacuum desiccator, portions of these (30–50 mg) were combusted using oxygen flasks, and the ^{14}C assayed as described above.

The amount of ^{14}C radioactivity in the feed and in the feed refusals was also determined using the above combustion procedures.

Lactating goats were milked once daily and portions of milk (1 ml) were pipetted into a scintillation vial containing 6·5 ml water. Toluene–Triton X-100 scintillation fluid (10 ml) was added and radioactivity assayed.

All radioactivity measurements were corrected for counting efficiency using automatic external standardization. Some samples were also corrected by adding internal standard ($[^{14}C]$n-hexadecane).

(*e*) *Estimation of Formaldehyde*

Formaldehyde was distilled from feed, faeces, tissues, and milk after adding phosphoric acid using the technique of Nitschman, Hadorn, and Lauener (1943). Antifoam R.D. (Albright and Wilson, Lane Cove, N.S.W.) was added to prevent frothing during distillation. Formaldehyde concentration was estimated by reaction with chromotropic acid (Bricker and Vail 1950). The dimedone derivative of the $[^{14}C]$HCHO was prepared from the distillates of feed and faeces using conditions described by Yoe and Reid (1941). These products were recrystallized to constant specific radioactivity and weighed portions were dissolved in toluene scintillation fluid and assayed for ^{14}C radioactivity.

(*f*) *Radioactive Compounds*

$[^{14}C]$HCHO (20·7 mCi/mM) and $[^{14}C]Na_2CO_3$ (57 mCi/mM) were obtained from the Radiochemical Centre (Amersham, England).

III. Results

(a) *Metabolism of [^{14}C]Formaldehyde-treated Casein–Safflower Oil Particles by Sheep and Goats*

(i) ^{14}C *in Expired Air*

A large proportion of the consumed ^{14}C was accounted for in the expired air (Table 1) and the value obtained in experiment 1 (84%) was higher than in experiments 2, 3, and 4 (66, 68, and 57% respectively). This difference appeared to be related to the length of the reaction time between formaldehyde and casein prior to

Table 1

Metabolism by sheep of [^{14}C]HCHO*-treated casein–safflower oil particles

	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Period of [^{14}C]HCHO feeding (days)	2	1	3	1
[^{14}C]HCHO consumed (μCi)	135	70	208	48·9
Distribution of consumed ^{14}C (%)				
Expired air†	83·5	66·1	68·0	57·3
Digestive tract	4·4	—	5·2	—
Carcass	4·2	—	6·5	—
Faeces‡	11·4	20·7	22·7	26·6
Urine‡	4·1	5·5	5·0	5·7
^{14}C recovery (%)	107·6	92·3	107·4	89·6

* [^{14}C]HCHO was complexed to casein–safflower oil particles as described in the text. In experiments 1–3, batch I of the [^{14}C]HCHO-treated casein–safflower oil (specific activity 0·3 μCi/g product) was fed at 10, 40, and 84 days after treatment; in experiment 4 batch II of the [^{14}C]HCHO-treated casein–safflower oil (specific activity 0·24 μCi/g product) was fed 50 days after spraying with [^{14}C)HCHO. The supplement (200 g) was mixed with 600 g lucerne chaff : oats (1 : 1 w/w) and fed once daily to each of four sheep. In experiments 1 and 3 the animals were slaughtered 3 and 4 days, respectively, after the last period of ^{14}C feeding.

† Radioactivity was measured throughout the period of feeding the [^{14}C]HCHO-treated supplement and for a further 48 hr thereafter.

‡ Faeces and urine were collected throughout the period of feeding the ^{14}C supplement and for at least 3 succeeding days.

the feeding of the radioactive supplement (Table 1); during this period a change in the nature of the bonding may have occurred. Evidence for this was obtained by incubating [^{14}C]HCHO-treated particles (300 mg) in water (6 ml) at 37°C for 1 hr and measuring the amount of radioactivity leached from the particles during the incubation. Thus at 10 days after treatment approximately 40% of the total radioactivity appeared in the aqueous phase and after 40 and 84 days post-treatment this had decreased to 20% and 17% respectively.

The rate of appearance of ^{14}C in CO_2 and methane for experiments 2 and 4 is shown in Figure 2. During the first 6–8 hr after feeding the [^{14}C]HCHO-treated supplement, the amount of radioactivity (expressed as a percentage of that consumed) in CO_2 was similar to that in methane, but subsequently the amount of ^{14}C in CO_2 was greater than that in methane (Fig. 2).

(ii) *^{14}C in Digestive Tract*

The results in Table 1 (experiments 1 and 3) show that 4–6% of the radioactivity consumed was present in the alimentary tract. Further analyses of contents and tissues from the various segments of the tract are shown in Table 2. Most of the

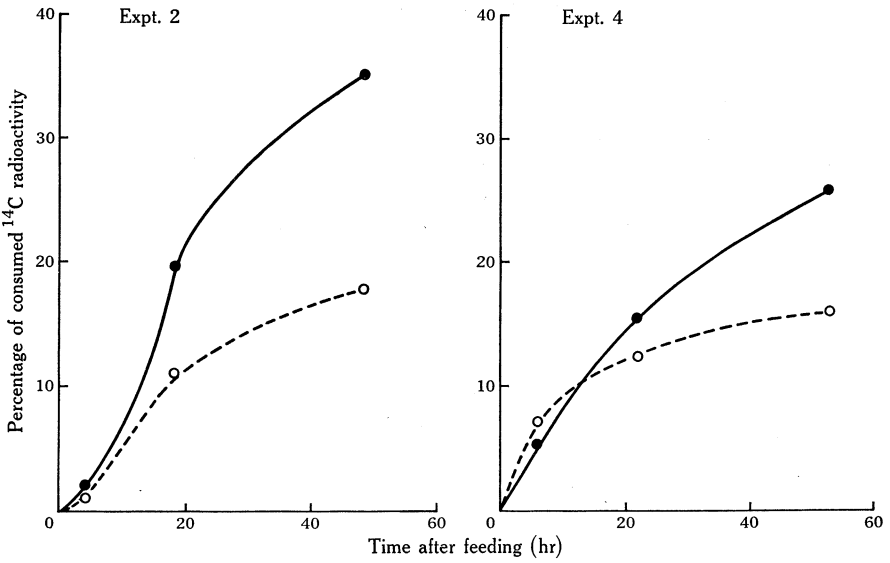

Fig. 2.—Appearance of [^{14}C]CO_2 (●) and [^{14}C]CH_4 (○) in expired air of sheep given [^{14}C]HCHO-treated casein–safflower oil supplements.

radioactivity was present in the contents from the rumen and caecum; however, there were smaller amounts in other tissues and contents (Table 2).

TABLE 2

DISTRIBUTION OF RADIOACTIVITY (% OF CONSUMED ^{14}C) IN THE ALIMENTARY TRACT OF SHEEP GIVEN [^{14}C]HCHO-TREATED CASEIN–OIL SUPPLEMENTS

Organ	Experiment 1*		Experiment 3*	
	Tissues	Contents	Tissues	Contents
Rumen	0·43	1·31	0·57	2·61
Omasum	0·06	0·02	0·08	0·03
Abomasum	0·03	0·06	—	0·14
Small intestine	0·26	0·11	0·34	0·38
Caecum	0·30	1·78	0·34	0·71

* Further details of supplements, diet, and slaughter of animals are given in Table 1.

(iii) *^{14}C in Carcass*

Approximately 3 and 6% of the consumed radioactivity appeared in the carcass of sheep slaughtered in experiments 1 and 3 respectively (Table 1). The distribution

of ^{14}C in blood, liver, kidney, fat, and muscle is shown in Table 3. Further examination of the nature of the radioactivity incorporated into fat and muscle revealed that

TABLE 3

DISTRIBUTION OF RADIOACTIVITY (% OF CONSUMED ^{14}C) IN TISSUES* OF SHEEP GIVEN [^{14}C]HCHO-TREATED CASEIN–OIL SUPPLEMENTS

The total amount of radioactivity in the tissues was calculated using the body composition data of Searle (1970)

	Blood	Liver	Kidney	Fat	Muscle
Experiment 1	0·4	0·8	0·1	1·4	1·5
Experiment 3	0·6	0·4	0·1	1·1	4·3

* Details of the supplement and diets are given in Table 1.

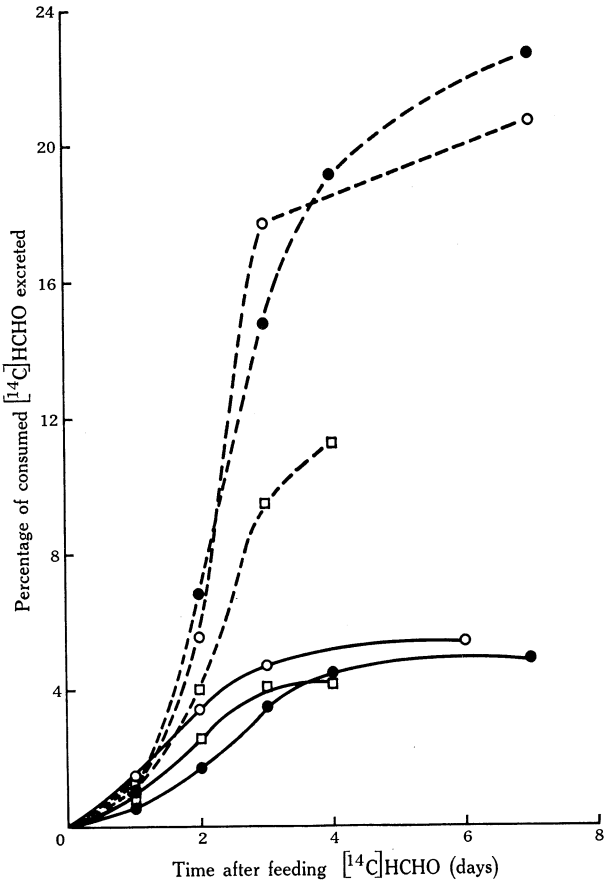

Fig. 3.—Rate of excretion of radioactivity in faeces (– – –) and urine (——) of sheep fed [^{14}C]HCHO-treated casein–safflower oil supplements. The [^{14}C]HCHO had reacted with the casein for 10 (□), 40 (○), or 84 (●) days and the supplement was fed for 1 (○), 2 (□), or 3 (●) days (see Table 1 for further details).

less than 4% was present in phosphoric acid distillates. These distillates would contain any free formaldehyde and other volatile components (e.g. acetate, formate).

(iv) *^{14}C in the Faeces and Urine*

A variable proportion of the consumed ^{14}C appeared in the faeces and the value obtained in experiment 1 (11·4%) was considerably lower than in experiments 2, 3, and 4 (20–27%, Table 1). This difference again appeared to be related to the length of reaction time between formaldehyde and casein (cf. expired air—Table 1). This is further illustrated in Figure 3, which shows the rate of excretion of ^{14}C in faeces from sheep fed the [^{14}C]HCHO-treated supplement at different time intervals after treatment.

The specific radioactivity (μCi/g) of formaldehyde in the faeces was determined in phosphoric acid distillates and it was the same as that present in the supplement (Table 4), demonstrating that there was no dilution from endogenous sources and that some of the dietary formaldehyde was excreted unchanged.

TABLE 4

CHARACTERIZATION OF [^{14}C]HCHO IN FEED AND FAECES

(*a*) Acid distillation and colorimetric measurements of formaldehyde:

	Dry weight of sample (g)	Total ^{14}C in distillate (disintegrations/min)	Formaldehyde* in distillate (mg)	Specific radioactivity of formaldehyde (μCi/g)
[^{14}C]HCHO-treated casein–oil particles	3·1	1,228,000	36·2	15·2
Sheep faeces	4·93	93,270	3·01	14·0

(*b*) Formation of dimedone derivatives

	^{14}C radioactivity (disintegrations/min)	Weight of dimedone (mg)	Weight of formaldehyde† (mg)	Specific radioactivity of formaldehyde (μCi/g)
[^{14}C]HCHO-treated casein–oil particles	2840	0·84	0·086	14·8
Sheep faeces	3710	1·05	0·108	15·4

* Formaldehyde was estimated colorimetrically with chromotropic acid as described in the text.

† Formaldehyde content was estimated from the weight of dimedone derivative on a molecular basis.

Approximately 5% of the consumed radioactivity was excreted in the urine and this proportion remained relatively constant in the different experiments (Table 1, Fig. 3).

(v) *^{14}C in Milk*

Very little radioactivity was detected in goat's milk after feeding [^{14}C]HCHO-treated supplement (Table 5). Characterization of the ^{14}C present in milk revealed that it was equally distributed between acid-soluble and acid-precipitable material and no radioactivity was detected in phosphoric acid distillates (Table 5).

(*b*) *Formaldehyde Content of Tissues and Milk*

Colorimetric estimation showed that the formaldehyde content of milk and tissue (1–9 p.p.m.) was the same as that in samples taken from animals which had not

TABLE 5

APPEARANCE OF RADIOACTIVITY IN MILK FROM LACTATING GOATS GIVEN [^{14}C]HCHO-TREATED CASEIN–SAFFLOWER OIL SUPPLEMENTS

	Goat 1	Goat 2
Period of [^{14}C]HCHO feeding (days)	1	2
[^{14}C]HCHO consumed (μCi)	65·9	136·6
^{14}C in milk* (% of consumed ^{14}C)	3·4	3·8
Distribution of ^{14}C in milk (% of consumed ^{14}C):		
Acid distillate†	<0·1	<0·1
Acid soluble	1·9	1·9
Acid precipitable	1·5	1·9

* Total ^{14}C radioactivity in milk over a 5-day collection period. Lactating goats (producing approximately 2 litres milk per day) were fed once daily a diet of [^{14}C]HCHO-treated casein–safflower oil (300 g) and lucerne chaff : crushed oats (1 : 1 w/w, 1500 g).

† Milk was acidified with H_3PO_4 (85% w/w) and distilled using procedures described in the text.

TABLE 6

CONTENT OF FORMALDEHYDE IN RUMINANT TISSUES AND MILK

	Formaldehyde-treated			Control* formaldehyde concentration (p.p.m.)
	Period of feeding (months)	Dietary formaldehyde (g/day)	Formaldehyde concentration (p.p.m.)	
		Sheep tissues		
Muscle	18†	1·8	4	3
Fat	18†	1·8	9	11
Liver	2‡	2·5	4	8
Kidney	2‡	2·5	3	4
		Goats' milk		
	2‡	5·0	1	1
	6‡	5·0	2	

* Control samples were obtained from animals that had not been given formaldehyde.

† Sheep were fed daily 750 g of a diet containing lucerne, wheat, and casein (25 : 37 : 38 by weight); the entire diet was treated with formaldehyde.

‡ Animals were fed daily supplements of 250 g (sheep) and 500 g (goats) of formaldehyde-treated casein–safflower oil (1 : 1 w/w) supplements.

been fed formaldehyde-treated diets (Table 6). Furthermore, when formaldehyde-treated proteins were fed for longer periods there was no accumulation of formaldehyde in the tissues or milk (Table 6).

IV. Discussion

The data clearly demonstrate that sheep effectively metabolize formaldehyde when this compound is fed in the form of an aldehyde–casein complex. The pattern of metabolism appears to be influenced by the length of the reaction time between the aldehyde and protein prior to feeding the supplement. Thus, with longer time intervals (e.g. 40, 84 days after treatment of the particles with [^{14}C]HCHO) more radioactivity was excreted in the faeces and less was accounted for in expired air (see Table 1). This difference may be due to the fact that with time, more of [^{14}C]HCHO becomes irreversibly linked to the protein, in such a manner, that it is not degraded within the alimentary tract.

In all experiments, a large proportion of the ingested formaldehyde is metabolized to methane and carbon dioxide. The appearance of [^{14}C]methane implies that a certain proportion of the formaldehyde is desorbed from the casein–safflower oil particles and converted to methane by methanogenic bacteria in the rumen (Czerkawski 1969). This is supported by the results of Downes and Sharry (personal communication) who showed that negligible [^{14}C]methane was produced when [^{14}C]HCHO was administered into the abomasum or blood of sheep.

The pathways whereby formaldehyde is converted to methane are obscure, but at least three mechanisms may be proposed:

(1) Conversion of formaldehyde to formate and its subsequent metabolism to carbon dioxide and methane (Koivusalo 1957; Barker 1967).
(2) Successive reduction of formaldehyde to methanol and methane (Czerkawski 1969).
(3) Acyloin condensation of formaldehyde with ribose 5-phosphate to form allulose 6-phosphate, which would be further metabolized via the glycolytic sequences to produce methane and carbon dioxide (Ribbons, Harrison, and Wadzinski 1970).

The [^{14}C]CO_2 in the expired gases may originate from reactions in the rumen (Czerkawski 1969) or alternatively from tissue metabolism (Koivusalo 1957; Strittmatter and Ball 1955) of [^{14}C]HCHO that is released by the secretions of the abomasum and small intestine. The metabolism of formaldehyde to form carbon dioxide in animal tissues has previously been demonstrated in a number of species (Koivusalo 1957; Neely 1964). This oxidation of formaldehyde accounts for the very low content of ^{14}C radioactivity in the tissues and milk (Tables 2, 3, and 5). Furthermore, the virtual absence of radioactivity in the phosphoric acid distillates of the tissues and milk strongly suggests that the incorporated ^{14}C arises from carbon dioxide fixation or transfer of one-carbon compounds to amino acids via the folic acid cycle (Koivusalo 1957). However, the possibility of adduct formation between [^{14}C]HCHO and other compounds cannot be excluded (Neely 1964).

The studies described here show that the use of formaldehyde-treated protein in ruminant nutrition does not result in the accumulation of formaldehyde in tissues or milk.

V. Acknowledgments

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VI. References

Barker, H. A. (1967).—*Biochem. J.* **105**, 1.

Bricker, C. E., and Vail, W. A. (1950).—*Analyt. Chem.* **22**, 720.

Cook, L. J., Scott, T. W., Ferguson, K. A., and McDonald, I. W. (1970).—*Nature, Lond.* **228**, 178.

Czerkawski, J. W. (1969).—*Wld Rev. Nutr. Dietetics* **11**, 240.

Downes, A. M., Reis, P. J., Sharry, L. F., and Tunks, D. A. (1970).—*Br. J. Nutr.* **24**, 1083.

Faichney, G. J. (1970).—In "Feeding Protected Protein to Sheep and Cattle". (Ed. D. W. Horwood.) p. 25. (Proc. Aust. Soc. Anim. Prod. Meeting, Sydney.)

Ferguson, K. A. (1970).—In "Feeding Protected Protein to Sheep and Cattle". (Ed. D. W. Horwood.) p. 9. (Proc. Aust. Soc. Anim. Prod. Meeting, Sydney.)

Ferguson, K. A., Hemsley, J. A., and Reis, P. J. (1967).—*Aust. J. Sci.* **30**, 215.

Fraenkel-Conrat, H., and Olcott, H. W. (1946).—*J. Am. Chem. Soc.* **68**, 34.

Koivusalo, M. (1957).—*Acta Physiol. Scand.* **39** (Suppl.), 131.

Lugg, J. W. H. (1938).—*J. agric. Sci., Camb.* **28**, 688.

Mills, S. C., Sharry, L. F., Scott, T. W., and Cook, L. J. (1971).—*Proc. Aust. Biochem. Soc.* **4**, 70.

Neely, W. B. (1964).—*Biochem. Pharmacol.* **13**, 1137.

Nitschman, H., Hadorn, H., and Lauener, H. (1943).—*Helv. Chim. Acta* **26**, 1069.

Ribbons, D. W., Harrison, J. E., and Wadzinski, A. M. (1970).—*A. Rev. Microbiol.* **24**, 135.

Scott, T. W., Cook, L. J., Ferguson, K. A., McDonald, I. W., Buchanan, R. A., and Loftus Hills, G. (1970).—*Aust. J. Sci.* **32**, 291.

Scott, T. W., Cook, L. J., and Mills, S. C. (1971).—*J. Am. Oil. Chem. Soc.* **48**, 358.

Searle, T. W. (1970).—*J. agric. Sci., Camb.* **74**, 357.

Strittmatter, P., and Ball, E. J. (1955).—*J. Biol. Chem.* **213**, 445.

Yoe, J. H., and Reid, L. C. (1941).—*Ind. Engng Chem. analyt. Edn* **3**, 365.