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Conversion of Choline Methyl Groups through Trimethylamine into Methane in the Rumen

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1. Choline methyl groups were rapidly metabolized to trimethylamine by rumen micro-organisms. 2. Trimethylamine was further metabolized to methane, but this system was more easily saturated by an excess of substrate, so that trimethylamine accumulated in the rumen of the fed animal. 3. Although trimethylamine was the only intermediate isolated in the conversion of the methyl groups of choline into methane, methylamine also served as a substrate for methane production. 4. The methyl group of methionine was also converted into methane by rumen fluid, but the methyl groups of carnitine were not.

The main dietary source of choline in ruminants is phosphatidylcholine occurring as a component of the plant membrane material ingested by the animal. This phospholipid is rapidly degraded by the micro-organisms present in the rumen to liberate free choline (Dawson & Hemington, 1974). During an investigation into the growth requirement for choline of the rumen protozoan *Entodinium caudatum* (Broad & Dawson, 1976) it was observed that when [*Me*-^{14}C]choline was introduced into the rumen there was a rapid clearance of radioactivity from the soluble fraction. Only a small percentage of this loss could be accounted for by microbial incorporation, and consequently the possibility of rapid absorption of choline from the rumen was considered.

In the present study it has been shown that the rapid loss in the rumen of the labelled methyl groups from choline is due to their conversion through the intermediary of trimethylamine into methane, which is lost in the gaseous mixture resulting from rumen fermentation. Methylamine, a major component of the soluble nitrogen present in rumen fluid (Hill & Mangan, 1964), can also be converted by rumen micro-organisms into methane, and so can the methylmercapto group of methionine.

Experimental

Animals

Two adult Clun Forest wether sheep (*Ovis aries*) fitted with rumen fistulae were used to study the metabolism of labelled substrates in the rumen *in vivo* and to obtain rumen-fluid samples for equivalent studies *in vitro*. They were housed indoors in individual pens and fed on 1000g of lucerne/hay chaff and 100g of oats once daily. Feed was normally consumed within 1h. Water was given *ad lib.* Experiments were conducted with animals fed at least 1h before or immediately after addition of substrate to the rumen, or after 24h without food.

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Radioactive substances

[*Me*-^{14}C]Choline chloride (sp. radioactivity 52mCi/mmol), [^{14}C]methylamine hydrochloride (sp. radioactivity 55.5mCi/mmol), DL-[*Me*-^{14}C]carnitine hydrochloride (sp. radioactivity 54.3mCi/mmol) and L-[*Me*-^{14}C]methionine (sp. radioactivity 53mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. [1,2-^{14}C]Choline chloride (sp. radioactivity 6.3mCi/mmol) was purchased from N.E.N. Chemicals G.m.b.H., Frankfurt, West Germany. [*Me*-^{14}C]Trimethylamine was prepared chemically as described by Engel *et al.* (1954).

[^{14}C]Trimethylamine was also prepared by incubating *in vitro* 25μCi of [*Me*-^{14}C]choline chloride with 50ml of rumen fluid from a sheep fed 1h previously, as described below. N_2 was then bubbled through the fluid made alkaline with saturated $Ba(OH)_2$ solution, and the volatile base carried over in the gas stream was trapped in 10ml of 0.2M-HCl. The purity of the [^{14}C]trimethylamine was established by paper ionophoresis (Dawson, 1960) at pH3.6, the radioactive yield being 90%.

Metabolism in vivo

The non-metabolized marker poly(ethylene glycol) (PEG 4000; Hopkin and Williams, Chadwell Heath, Essex, U.K.; 12.5g in 100ml of water) was introduced into the rumen via the rumen fistula. After 30min, 200ml of rumen contents were removed and strained through one layer of butter muslin. The fluid was mixed with 20μCi of [^{14}C]choline chloride (17.9μmol or 0.72mmol) in 10ml of water and the mixture returned to the rumen. Serial samples of rumen

contents (20ml) were withdrawn over a 6h period and strained through one layer of muslin. Alternatively, when it was desired to observe strict anaerobiosis, the poly(ethylene glycol) and ^{14}C-labelled substrate in water were introduced into, and rumen fluid was sampled from, the rumen by means of a 50ml syringe fitted with a large-guage needle and inserted through a rubber septum sealing the rumen fistula. The rumen fluid was chilled on ice and centrifuged for 15min at 37000*g* in a 4°C room. The supernatant fluid was assayed for poly(ethylene glycol) (Hyden, 1955) and the values were extrapolated back to the addition time to assess rumen volume. Radioactivity in rumen-fluid supernatant and the pellet fraction recovered after centrifugation was determined as described below. When expired gases from sheep were analysed, the animals were enclosed in a gas-flow metabolism unit basically the same as that described by Young *et al.* (1975) and developed in this Department by Dr. D. B. Lindsay and Mr. A. Northrop. The expired gases were continuously monitored for CO_2 and methane content by i.r. analysis and passed through a Cary Talbert ionization sphere (Cary Instruments, Monrovia, CA, U.S.A.) to monitor radioactivity, and through saturated $Ba(OH)_2$ solution to absorb CO_2. Radioactive substrate alone was injected into the rumen, which remained sealed throughout the experiment.

Metabolism in vitro

Incubation was carried out in a two-necked 250ml flask fitted on each neck with a stopcock. Up to 100ml of whole rumen contents or strained rumen fluid was incubated with 0.5μCi of *Me*-^{14}C-labelled substrate in 0.5ml of water at 39°C under an enclosed atmosphere of CO_2/N_2 (19:1). Additional unlabelled substrates were added in a minimal volume of water (<1ml), except when hay was added as a degassed homogenate (Ultra-Turrex) in artificial saliva (10%, w/v; McDougall, 1948) in a volume equivalent to the rumen fluid used. After 1h, the gas phase was removed and passed over $Mg(ClO_4)_2$ to the evacuated ionization sphere to measure its radioactivity. The rumen fluid was chilled in ice and centrifuged as above before radioactive analysis.

Separation of volatile bases

Samples of rumen-fluid supernatant were made alkaline with saturated $Ba(OH)_2$ solution and immediately frozen in liquid N_2 for storage. The volatile bases and water were then transferred by vacuum sublimation at 18°C and trapped in 0.1ml of 0.2M-HCl. The bases were separated by ion-exchange chromatography in an amino acid analyser (Locarte Co., London S.W.7, U.K.) (Locarte lithium buffer systems; 8% cross-linked divinylbenzene resin; 23cm×1cm column) and the radioactivity continuously recorded as the effluent passed through scintillation-grade anthracene (Ward & Huskisson, 1972).

Bases were also separated by paper ionophoresis by using the technique essentially as described by Dawson (1960). Standard bases were used as markers and detected after spraying the chromatograms with 0.05% Bromocresol Green in 95% (v/v) ethanol. Areas of radioactivity were identified by radioautography. When [1,2-^{14}C]choline was used as substrate, volatile bases were removed from rumen fluid by steam distillation after adjustment to an alkaline pH.

Radioactivity determinations

Aqueous samples (0.1–0.5ml) were adjusted to 1.0ml with water and mixed with 10ml of Unisolve (Koch–Light, Colnbrook, Bucks., U.K.) for scintillation counting. Absolute radioactivities were obtained by using both internal and external standards. Sublimation residues were dissolved in a known volume of water, and the resulting alkaline solution was gassed with CO_2 and centrifuged to remove the precipitate, and the radioactivity in the supernatant fluid assessed by scintillation counting as above. Acidic solutions were neutralized with dilute NaOH solution before scintillation counting. The pellet material recovered after centrifugation of the strained rumen fluid was extracted by the procedure of Folch *et al.* (1957); radioactivities in the aqueous phase were determined as described above, and those in the organic phase, after removal of solvent, by scintillation counting in Unisolve. Bound radioactivity in the feed residues from strained rumen contents and the pellet materials was determined after oxidation and recovery of the $^{14}CO_2$ (Ward & Crompton, 1969).

Gas analysis

Gases were analysed by radio-g.l.c. (Ward, 1974) on silica gel (80–100 mesh) at 32°C by using standard methane, ethane and ethylene as internal markers. Radioactive gases collected by the ionization sphere were measured by using a Cary 401 vibrating-reed electrometer. Some gas effluents were passed through saturated $Ba(OH)_2$ solution to remove CO_2 and samples of the dried $BaCO_3$ precipitate were suspended in Cabosil (Koch–Light) for scintillation counting. Results from studies of [*Me*-^{14}C]choline with rumen fluid are the means of three to six experiments. Results *in vivo* have been adjusted to represent the absolute addition of 20μCi of ^{14}C-labelled substrate to a constant average rumen volume of 5.435 litres.

Results

Loss of ^{14}C from rumen fluid after intraruminal administration of [Me-^{14}C]choline

When [*Me*-^{14}C]choline at two dosages (17.9 and

720 μmol) was added to the rumen of sheep through a rumen fistula, there was a rapid loss of radioactivity from the rumen-fluid supernatant within the first 60 min. The extent of this loss depended on the time for which the choline was administered in relation to the feeding of the animal and the dose of choline. If the animal had been starved for the previous 24 h virtually all the radioactivity disappeared within 1 h, the rate of loss being somewhat less when 720 μmol of choline was given than with 17.9 μmol (Fig. 1). If the animal was fed immediately after the administration of 17.9 μmol of choline, about 80% of the radioactivity disappeared in 1 h, but the subsequent loss was very much slower (Fig. 1). With the higher dose, the extent of the initial loss was decreased to about 50%; the subsequent rate of loss showed a biphasic character (Fig. 1). Similar curves to the latter were seen if the animals were fed 1 h before the administration of either dose of choline (Fig. 1). These observations remained unchanged irrespective of precautions taken against the introduction of air during the addition of materials to the rumen or the sampling of rumen contents.

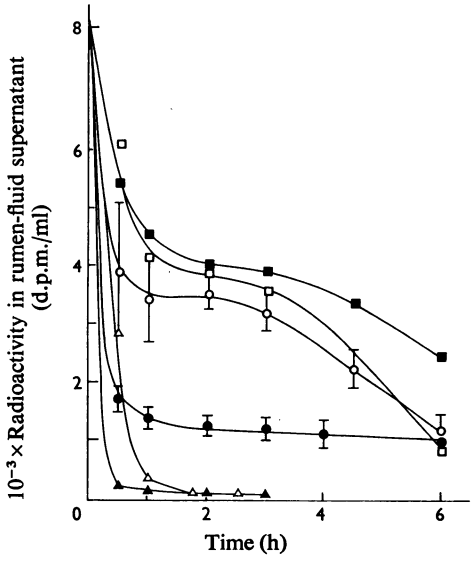

Fig. 1. *Clearance of radioactivity from rumen-fluid supernatant in vivo after adding [Me-^{14}C]choline chloride or [^{14}C]trimethylamine to the rumen*

Sheep starved 24h before adding choline and throughout experimental period: ▲, 17.9 μmol of choline; △, 720 μmol of choline. Sheep starved 24h and fed immediately after adding choline: ●, 17.9 μmol of choline (±S.D., $n = 3$); ■, 720 μmol of choline. Sheep fed 1 h before adding choline or trimethylamine: ○, 17.9 or 720 μmol of choline (±S.D., $n = 4$); □, trimethylamine.

It became apparent later that although in the starved sheep a high proportion of the initial rapid loss of radioactivity from the supernatant fraction could be accounted for by the formation of gaseous methane, this was not so with the animals that had been fed before the administration of the [*Me*-^{14}C]-choline. When the pellets obtained by the centrifugation of the rumen fluid were extracted with solvents, there was little radioactivity recovered, suggesting that the loss could not be accounted for by a rapid formation of protozoal phosphatidylcholine (Broad & Dawson, 1975). With samples withdrawn from the rumen at periods shorter than 30 min, the radioactivity present in the supernatant was very variable, presumably because the rumen contents had not had time to mix adequately with the radioisotope. However, if the contents were strained through muslin, the radioactivities in the residual material were often much higher than could be accounted for by the supernatant trapped in the residue as assessed by dry-weight determinations. These high activities suggest that there was an affinity between the radioactivity present and the solid matter in the rumen (largely undigested foodstuffs). It proved impossible to do any balance studies of radioactivity with the present techniques of rumen fistulation and sample withdrawal, which undoubtedly preferentially select the more fluid part of the rumen contents.

Trimethylamine in rumen contents after the administration of ^{14}C-choline to a fed sheep

The radioactivity remaining in the rumen-fluid supernatant of a sheep fed 1 h before the administration of [*Me*-^{14}C]choline proved to be removable by steam distillation under alkaline conditions. At 30 min after the choline had been given, 96.2% (S.D. ±1.06) of the radioactivity, and at 2 h 89.0% (S.D. ±3.15), was volatile under alkaline conditions. In contrast, only 4.4% of the original [*Me*-^{14}C]-choline substrate radioactivity proved volatile under the same distillation conditions.

On ion-exchange chromatography the volatile radioactive component emerged as a discrete basic compound 12 min after standard monomethylamine and 18 min before standard lysine. It could not be separated from an internally added marker of chemically prepared [^{14}C]trimethylamine. On paper ionophoresis the radioactive base migrated to the same position as trimethylamine (mobility relative to choline 1.24). The mobilities of methylamine and dimethylamine relative to choline were 1.53 and 1.39 respectively in the same system.

When radioactive trimethylamine was introduced into the rumen of a sheep the clearance of radioactivity from the rumen-fluid supernatant closely mimicked that after the addition of [*Me*-^{14}C]choline under the same dietary conditions (Fig. 1).

If [1,2-^{14}C]choline was administered intraruminally, none of the radioactivity became volatile under alkaline conditions, although radioactivity disappeared from the rumen-fluid supernatant (Fig. 2).

Formation of [^{14}C]methane and [^{14}C]trimethylamine when rumen fluid is incubated with [Me-^{14}C]choline in vitro

Rumen fluid was collected from 24h-starved sheep and incubated, either intact or after the removal of coarse food particles, by filtration with [*Me*-^{14}C]-choline *in vitro*. After 1h, most of the radioactivity was recovered in the gas phase (Table 1). This radioactivity remained in the gas phase when the latter was passed through saturated $Ba(OH)_2$ or 0.2M-mercury perchlorate in 2M-$HClO_4$ solution (CO_2 and ethylene absorbants respectively). On g.l.c., it exhibited a retention time of 1.0 relative to methane. Ethane and ethylene had relative retentions of 5.2 and 11.0 respectively under the same conditions.

In contrast, when rumen fluid from fed sheep was incubated with [*Me*-^{14}C]choline *in vitro* very little radioactivity was recovered in the gas phase after 1h (Table 1). Similar observations were made in incubations with rumen fluid from unfed sheep to which an excess of unlabelled choline or trimethylamine had been added, but not when an excess of methylamine was present (Table 1). Addition of homogenized hay 10min before the choline addition almost totally inhibited the release of methane from choline incubated with rumen fluid from a 24-h starved animal. However, longer periods of preincubation with the hay lessened this suppression of methane production, so that after 18h preincubation methane production was similar to that by rumen fluid from starved sheep alone. Results varied little whether whole rumen contents or strained rumen fluid was used, but no metabolism occurred if the microorganisms were removed from the medium by high-speed centrifugation (100000*g* for 30min) before incubation.

Rumen fluid removed from a fed sheep was incubated with [*Me*-^{14}C]choline *in vitro* and sampled at intervals over a 1h incubation period: under such conditions little [^{14}C]methane would be produced. After centrifugation (37000*g* for 15min) of the incubation mixture, the distribution of radioactivity between the base, volatile under alkaline conditions, and residual components of the supernatant fluid was examined (Fig. 3). [^{14}C]Trimethylamine rapidly appeared in rumen fluid and was the only intermediate detected by ion-exchange chromatography and ionophoresis during the metabolism of the methyl groups of choline by rumen fluid under such dietary conditions.

Production of [^{14}C]methane from [Me-^{14}C]choline by the sheep in vivo

One-third of the radioactivity from [*Me*-^{14}C]-choline introduced into the rumen of a 24h-starved sheep was recovered in the expired gases within 1h,

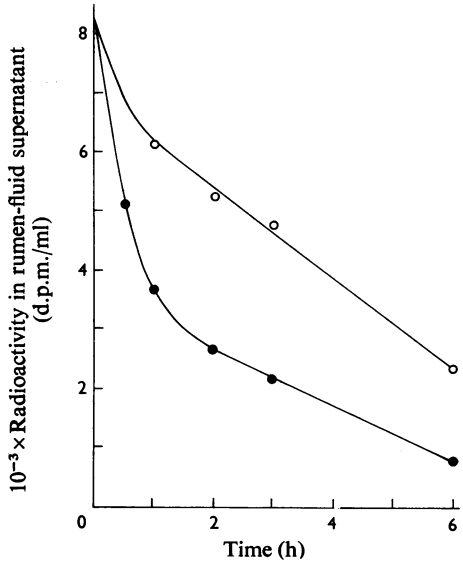

Fig. 2. *Clearance of radioactivity from rumen-fluid supernatant in vivo after adding [1,2-^{14}C]choline chloride to the rumen*

Sheep were fed 1h before (○) or immediately after (●) adding 17.9μmol of choline to the rumen.

Table 1. *Production of [^{14}C]methane from [Me-^{14}C]choline chloride in rumen fluid in vitro*

Whole rumen contents or a filtrate separated after passing the contents through one layer of muslin were incubated anaerobically at 39°C for 1h with [*Me*-^{14}C]choline chloride (3.6μM). The radioactivity released as a gas was measured. Values are single observations, unless given ±S.D. (numbers of experiments in parentheses).

Medium	^{14}C in gas phase (%)
Sheep starved for 24h before sampling	
Whole contents or filtrate	66.7±0.6 (3)
Filtrate+0.2mM-choline	5.7
Filtrate+0.17mM-trimethylamine	1.3
Filtrate+5g of homogenized hay	
(*a*) Preincubated for 10min	1.0
(*b*) Preincubated for 1.5h	5.7
(*c*) Preincubated for 18h	68.5
Filtrate+0.37mM-methylamine	64.7
Sheep fed for 1.0–3.5h before sampling	
Whole contents	1.6±0.8 (6)

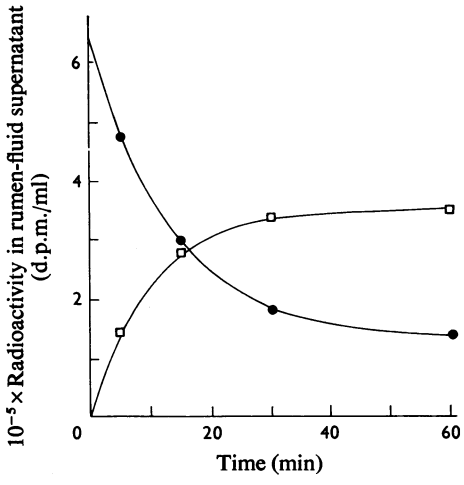

Fig. 3. *Metabolism of choline chloride to trimethylamine in rumen fluid in vitro*
Rumen fluid from a fed sheep was incubated anaerobically at 39°C with [*Me*-^{14}C]choline chloride (3.6μM; ●). The trimethylamine (□) was recovered by vacuum sublimation under alkaline conditions.

Table 2. *Radioactive methane in expired gases from a sheep after adding [Me-^{14}C]choline chloride to the rumen*
The sheep received [*Me*-^{14}C]choline chloride (17.9μmol) in water injected directly into the rumen either after 24h starvation or after feeding 1h beforehand. The expired gases were continuously analysed for radioactivity. Results for radioactivity present in the expired gas have been integrated with an accuracy estimated at ±10%. Total $^{14}CO_2$ (recovered as $Ba^{14}CO_3$) was: starved, 0.25μCi; fed, 0.12μCi.

Dietary state	[*Me*-^{14}C]Choline added (μCi)	Total [^{14}C]methane expired (μCi) after: 1h	4h	6h
Starved	17.0	5.4	18.0	
Fed	15.4	0.9	5.1	7.2

and all of the radioactivity was expired after 4h (Table 2). When the same animal was fed 1h before addition of the same substrate, radioactivity appeared more slowly in the gas phase, but about 50% of the added activity had been recovered as methane after 6h. When the expired gases were passed through saturated $Ba(OH)_2$ solution, less than 1.5% of the initial radioactivity was recovered in the precipitated $BaCO_3$ in both experiments. Actual $^{14}CO_2$ production was probably much less, since the latter results were calculated from samples having radioactivity less than 20d.p.m. above background.

Table 3. *Production of radioactive methane from other Me-^{14}C-labelled substrates incubated with rumen fluid in vitro*
Conditions were as in Table 1 except that other *Me*-^{14}C-labelled substrates (3.6μM) were used.

Dietary state of animal on taking rumen fluid	*Me*-^{14}C-labelled substrate	Percentage of added radioactivity in methane after 1h
Starved 24h	Methylamine	88.3
	Trimethylamine	76.0
	Carnitine	3.1
	Methionine	74.6
Fed		
60min before	Methylamine	1.6
60min before	Carnitine	0.0
80min before	Methionine	13.7
320min before	Methionine	35.1

Formation of methane from other Me-^{14}C-labelled substrates

Me-^{14}C-labelled methylamine, trimethylamine and methionine all produced ^{14}C-labelled gas in high yield when incubated with rumen fluid from a starved sheep (Table 3). The gas was identified as methane by co-chromatography with authentic methane. The release of this methane was suppressed by feeding, although perhaps less so when methionine was the substrate. With carnitine as substrate, ^{14}C-labelled gas production was very low in both fed and starved states.

Discussion

There seems little doubt that the methyl groups present in any free choline liberated from plant phospholipids by rumen fermentation are rapidly converted into trimethylamine by rumen micro-organisms. The bacterial production of trimethylamine is normally associated with the degradation of trimethylamine oxide in food products (Frazier, 1958), but it can also be formed from choline and other related substrates by several species of bacteria (Wood & Keeping, 1944; Campbell & Williams, 1951; Eddy, 1953; Hayward & Stadtman, 1959; Baker *et al.*, 1962). Whereas trimethylamine has been shown to be produced from choline by the action of the microflora isolated from the intestine of pigs (Michel, 1956), little attention has been directed towards rumen bacteria in this regard, even though they have long been implicated in the appearance of trimethylamine oxide in the milk of cattle fed on diets high in betaine (Baldwin, 1953). It seems unlikely that the rumen protozoa would play any part in the metabolism of choline to trimethylamine, although certain species (*Entodinium caudatum*) can rapidly take up free choline and incorporate it into phosphocholine and phosphatidylcholine (Bygrave & Dawson, 1976).

Other products of the anaerobic metabolism of choline by bacteria have been identified as acetate and ethanol (Hayward & Stadtman, 1959; Baker *et al.*, 1962) and ethylene glycol (Cohen *et al.*, 1947). It would appear from the present results that ethanol may not be a final product of choline metabolism in the rumen, since [1,2-^{14}C]choline does not produce a radioactive metabolite volatile under alkaline conditions.

Since the trimethylamine produced from choline accumulates in the rumen fluid taken from a fed animal, the subsequent demethylation to produce methane is presumably independent of the cleavage of choline and is likely to be carried out by different bacterial species. The apparent disparity in [^{14}C]-methane production between the fed and starved states appears to be related to the substrate saturation of the methanogenic system in the fed state. In contrast, although the meal fed to sheep before the administration of labelled choline contained approx. 1 g of choline, this was insufficient to prevent the rapid conversion of choline into trimethylamine by rumen bacteria, a factor further supported by the negligible concentration of free choline found in rumen fluid after feeding (Broad & Dawson, 1976). This rapid metabolism of choline questions the efficiency of oral choline supplementation to ruminants as discussed by Church (1971), and raises doubts as to the mechanism of the benefit of rumen bacterial growth produced by choline (Swingle & Dyer, 1970).

In the fed state, the only nitrogen-containing metabolite of choline detected in the rumen fluid was trimethylamine. Although methylamine accumulates in the rumen after feeding (Hill & Mangan, 1964), this presumably is formed from a dietary component other than choline. In the present investigation it has been shown that methylamine can rapidly be converted into methane by rumen fluid from a starved animal. However, the observation that an excess of methylamine does not dilute the formation of [^{14}C]methane from [*Me*-^{14}C]choline in the same system would suggest that either methylamine cannot be an intermediate or alternatively that if methylamine is an intermediate, it is further metabolized within the particular bacteria metabolizing choline or trimethylamine without ever coming into equilibrium with the general pool. Perhaps trimethylamine and methylamine are converted into methane by different bacteria or enzyme systems. Certainly the bacterial oxidation of trimethylamine and methylamine to formaldehyde appears to be carried out by independent dehydrogenases (Eady & Large, 1968; Steenkamp & Mallinson, 1976).

It has been postulated that CO_2 may be the immediate carbon precursor of the large amounts of methane produced in the rumen (Czerkawski, 1969). Since $^{14}CO_2$ was not isolated in significant amounts in the present experiments, this would indicate that the methyl groups of choline are reduced to methane possibly via a carrier such as cobalamin or tetrahydrofolate, which both abound in methanogenic bacteria. If so, this represents a novel process in methanogenesis, but it may be akin to the suggested formation of methane from methanol or acetate (Stadtman & Barker, 1951). That this type of methanogenesis is not limited to the three methyl-substituted ammonias is perhaps indicated by the formation of methane from the methylmercapto group of methionine. It has been known for some time that methionine is rapidly metabolized in the rumen and that methylmercaptide was present among the products (Zikakis & Salsbury, 1969; Bird & Moir, 1972). It is likely therefore that this methylmercaptide can subsequently be reduced to methane and H_2S. The lack of carnitine metabolism in rumen fluid indicates the high specificity of the bacterial system responsible for the cleavage of trimethylamine from choline; it is consistent with the inability of carnitine to replace choline in biological processes such as methyl-group metabolism.

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