New inhibitors of methane production by rumen micro-organisms. Development and testing of inhibitors in vitro

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1. A procedure is described for assaying in vitro the activity of various inhibitors of methane production by rumen micro-organisms.

2. Methods of preparation of various inhibitors are described together with attempts to characterize these compounds by determining their physical properties (physical state, density, chromatographic behaviour), their hydrolysis by rumen contents and their relative potency as inhibitors.

3. The results of preliminary studies with trichloroethanol and its ester with pivalic acid are given.

4. The inhibitory activities of several groups of related compounds are reported. These include the polyhalogenated alcohols and their esters with pivalic acid, the esters of tri-halogenated alcohols and monobasic fatty acids from C_2 to C_{16} and the trihalogenated alcohol esters of dibasic acids. The results of experiments with esters of alcohols and polyhalogenated carboxylic and sulphonic acids are also given.

5. It is concluded that the mechanism of action of the inhibitors might be similar to that of known polyhalogenated methane analogues (e.g. chloroform). The relative activity of various compounds might be partly governed by the ease of their absorption into the microbial cells and by the extent to which the esters can be hydrolysed by rumen contents.

6. The results show that some substances are very poor inhibitors, unless they are esterified (e.g. trichloroacetic acid) but on the whole the esters in which the polyhalogen grouping is on the alcohol portion of the molecule are better inhibitors than those in which it is on the acid portion of the molecule.

Between 6 and 8 % of the energy in the food of ruminants is converted to methane, eructed and therefore lost to the animal. It is argued that if the methane production could be inhibited specifically, rumen fermentation might change towards greater efficiency. Methane is produced from carbon dioxide and metabolic hydrogen and the manipulation could be effective if the hydrogen saved from methanogenesis could be used in formation of products that might subsequently be used by the host animal (cf. Czerkawski, 1973). The processes that are likely to require hydrogen are lipid and protein synthesis, biohydrogenation of unsaturated fatty acids and production of propionic acid in preference to acetic acid (Czerkawski, 1972; Marty & Demeyer, 1973). Therefore, an important prerequisite in developing an inhibitor of methane production is that it will not interfere with those processes in particular and with normal metabolism of non-methanogenic micro-organisms in general.

It is also important that the inhibitor should be potent, not toxic to the host animal, easily incorporated in food (e.g. volatile compounds are not very useful) and not too expensive.

The inhibitors of methane production can be divided into two groups: alternative

acceptors of hydrogen, and specific inhibitors of particular steps in methanogenesis. The former include the unsaturated long-chain fatty acids (see for example, Blaxter & Czerkawski, 1966), other hydrogen acceptors such as limonene (Crane, Nelson & Brown, 1957), sulphite (Noordam, Manten & Muller, 1949), viologen dyes (Wolin, Wolfe & Wolin, 1964) or propanediol (Czerkawski & Breckenridge, 1973). Most of these inhibitors are not very potent, and in sheep doses of 40–100 g/d are required to give significant inhibition of methane production, but some have the advantage (e.g. unsaturated fatty acids) that they occur naturally in ruminant diets.

In the instance of long-chain fatty acids, the inhibition of methane production is not due to a simple competition for hydrogen, for although the inhibitory activity of the acids increases with unsaturation, saturated acids such as stearic and palmitic acids are also inhibitors (Blaxter & Czerkawski, 1966). A direct demonstration of the effect of the long-chain fatty acids on pure cultures of methane bacteria was described by Prins, Van Nevel & Demeyer (1972) and by Henderson (1973).

Bauchop (1967) found that the halogen-substituted analogues of methane, such as chloroform, are powerful inhibitors of methane production and Wood, Kennedy & Wolfe (1968) found that the activity is probably due to formation of halogen analogues of methylcyanocobalamin from reduced cyanocobalamin. Many related substances were subsequently found to be strong inhibitors of methane production. Rufener & Wolin (1968) used carbon tetrachloride, Singh & Trei (1971), Johnson, Wood, Store & Morgan (1972) and Sawyer, Hoover & Sniffen (1974) used bromochloromethane. Johnson (1972) and Trei, Scott & Parish (1972) used a hemi-acetal of chloral (trichloro-acetaldehyde) and starch. McBride & Wolfe (1971) showed that DDT was a potent inhibitor of methanogenesis.

The advantage of using compounds such as chloral (Van Nevel, Henderickx, Demeyer & Martin, 1969) or trichloroacetamide (Trei, Parish, Singh & Scott, 1971) is their non-volatility compared with chloroform or bromochloromethane.

Following the successful use of long-chain fatty acids to inhibit methane production (Czerkawski, Blaxter & Wainman, 1966; Demeyer & Henderickx, 1967) further work (Clapperton & Czerkawski, 1971) showed that some tertiary fatty acids with more than ten carbon atoms (the versatic acids) were more active as methane inhibitors than the straight-chain fatty acids. It seemed worthwhile to try and combine the properties of these acids and polyhalogen compounds in single substances. This was the primary objective, but, as will be seen subsequently, in the course of the present work many other compounds have been synthesized, tested and shown to be even more interesting.

EXPERIMENTAL

Assay of inhibitory activity in vitro

The inhibitory activity of various compounds was determined by incubation of rumen contents in a modification of a small-scale artificial rumen, essentially as described by Czerkawski & Breckenridge (1970).

The samples of rumen contents were taken before the morning feed from sheep that were provided with rumen fistulas and given 500 g molassed sugar-beet pulp at

09.00 hours and 350 g hay at 17.00 hours. The rumen contents were strained through four layers of gauze and used within 30 min.

Samples of rumen contents (20 ml) were introduced into 50 ml glass syringes provided with three-way plastic taps. To each syringe was then added 1·0 ml of a solution containing 50 mg glucose, followed by 4·0 ml of a test solution containing the inhibitor emulsified in Tween 80 (Honeywill & Stein Ltd, London W1) solution (10 g/l). Some air bubbles that inevitably collected in the syringes were expelled and 5 ml carbon dioxide–nitrogen (5:95, v/v) was introduced into each syringe. With each series of incubations at least two controls were set up with 4 ml Tween 80 solution. The contents in the syringes were mixed gently by inversion and the syringes were incubated in a water bath at 39° for 4 or 5 h, the contents being mixed intermittently throughout the incubation. At the end of incubation the gas was transferred to a 20 ml glass syringe that was wetted with water, and the volume of gas was noted. The gas was analysed chromatographically as described by Czerkawski & Clapperton (1968) and the volumes of methane, hydrogen and carbon dioxide were calculated.

In most experiments at least three levels of inhibitor were tested to obtain between 20 and 80 % inhibition, and the amount of inhibitor required to give 50 % inhibition was calculated by interpolation. In some experiments the liquid samples at the end of incubation were used for the determination of the concentration of volatile fatty acids by the method of Cottyn & Boucque (1968).

Hydrolysis of esters

The extent of hydrolysis of esters was determined using rumen contents as described in the assay of inhibitory activity, but the concentration of inhibitor was much greater. Samples of rumen contents (8 ml) were pipetted into glass-stoppered test-tubes. To each was added 1 ml water containing 25 mg glucose, and 1 ml Tween 80 solution (10 g/l) containing 0·3 mmol ester. Tween 80 solution without ester was added to control tubes. Carbon dioxide–nitrogen (5:95, v/v) was bubbled through the contents of each tube. The tubes were then stoppered loosely and incubated at 39° for 2 or 5 h, during which time they were shaken at intervals. After incubation, portions (4 ml) of reaction mixtures were treated with 1 ml precipitating agent (Cottyn & Boucque, 1968), allowed to stand for 30 min and centrifuged. The amounts of products of hydrolysis were analysed using gas–liquid chromatography (GLC) (see p. 433).

Materials

Many compounds were synthesized; the following compounds were purchased: 2-chloroethanol, pentan-1-ol, benzoyl chloride, trichloroacetyl chloride, from BDH Ltd, Poole, Dorset; trichloroacetone, oxalyl chloride, hexachloroacetone, 1,1,1-trichloro-2-propanol, 1,1,1-trichloro-2-methyl-2-propanol hydrate, 2,2-dichloroethanol, 2,2,2-trichloroethanol (TCE), 2,2,2-trifluoroethanol, 2,2,2-tribromoethanol, 4-methyl-1-pentanol, neopentyl alcohol, 1,6-hexanediol, methane sulphonyl chloride, glutaryl dichloride, isobutyryl chloride, isovaleryl chloride, from Ralph N. Emanuel Ltd, Wembley, Middx.; *p*-toluene sulphonyl chloride, trichloromethane sulphonyl

chloride, pivaloyl chloride, from Koch-Light Laboratories Ltd, Colnbrook, Bucks.; adipoyl chloride, from Hopkin and Williams, Romford, Essex. Isobutyraldehyde and 2,5-hexanediol were kindly given by Ralph N. Emanuel Ltd, Wembley, Middx.

Preparation of esters

If possible the acid chlorides were obtained commercially and used as starting materials for the preparation of esters. Occasionally the acid chlorides were prepared by treating the acids with about five times their weight of oxalyl chloride and allowing to stand for 2 d. The excess oxalyl chloride was removed at room temperature using a rotary evaporator. The alcohols were added gradually to the acid chlorides until the alcohol was 20–30 % in excess of the amount required for complete reaction (with dihydroxy alcohols, an excess of acid chloride was used). The mixtures were allowed to stand for 1–2 h, heated at 70° under reflux for 1 h and then allowed to stand overnight. The heating was not necessary with some compounds. The reaction mixtures were treated with dilute sodium hydroxide to neutralize the acid and decompose the unreacted acid chloride and then washed repeatedly with water in a separating funnel or a centrifuge tube to remove the unreacted acid and alcohol. When the ester was a solid at room temperature, after the initial treatment with sodium hydroxide solution, the ester was dissolved in n-hexane and purified in the usual manner. Occasionally, when it was difficult to remove the alcohol completely by the method described, the samples (1–2 g) were applied to columns (20 × 400 mm) of Florisil (60–100 mesh; BDH Chemicals, Poole, Dorset) and eluted with n-hexane or with increasing concentrations of diethyl ether in n-hexane, leaving the alcohol on the column. Sometimes large amounts of esters had to be prepared and the procedure had to be modified. Trichloroethyl adipate (TCE-A) was a solid at room temperature. In a typical preparation a reaction mixture of 100 ml adipoyl chloride and 200 ml TCE, after standing for 24 h, was treated with n-hexane (300–400 ml) and extracted once with water, then extracted repeatedly with 0·1 M-sodium hydroxide, until the extracts were alkaline, and then finally with water. The n-hexane was evaporated using a rotary evaporator to give about 280 g residue which was impure TCE-A, containing some TCE and some brown-coloured material. It was purified further by melting, dissolving in 2 vol. ethanol–water (95:5, v/v) (industrial spirit) at 50°, cooling to below 10°, and allowing the product to crystallize with stirring. The crystals were filtered using a Buchner funnel, dried and recrystallized twice more to give about 220 g white, odourless crystals that were chromatographically pure (about 75 % yield).

Characterization of compounds

The esters were characterized by determining their melting or boiling points, their density and their behaviour on GLC. It was assumed that a new single peak with a retention volume different from that of the constituent acid and alcohol was due to the prepared ester. This was usually confirmed by hydrolysing the ester in alkali and analysing the products.

GLC was done using a gas–liquid chromatograph (Model no. 104; Pye Unicam Ltd,

Cambridge) with 1.5×5 mm columns of 50 g Carbowax 20M TPA/kg Chromosorb G (Perkin-Elmer Ltd, Beaconsfield, Bucks.) at 100, 140 or 155°, or with 1.5×5 mm columns of 150 g ethylene glycol succinate (EGS-X)/kg Gas-chrom P (Field Instruments Co. Ltd, Richmond, Surrey) at 120 or 190°. A flame-ionization detector (Pye-Unicam Ltd) was used and the peak areas were measured with a disc integrator (Disc Instruments Ltd, Hemel Hempstead, Herts.), mounted on a Speedomax W recorder (Leeds & Northrup, Birmingham). Sometimes, when the compounds were not sufficiently volatile or when they tended to decompose on GLC, they were analysed by thin-layer chromatography (TLC) by Dr W. W. Christie of this Institute.

The relative retention times of most of the compounds tested are summarized in Table 1; the relative retention times of common compounds are also given for comparison. The TCE esters of monobasic and dibasic acids were well separated, as well as the trichloroacetic acid (TCA) esters of mono- and dihydric alcohols. The TCA ester of 2,5-hexanediol gave two peaks of approximately equal area. This compound migrated as one spot on TLC, fractionally faster than the TCA ester of 1,6-hexanediol, which gave a single peak on GLC. It was concluded that the preparation contained a mixture of stereoisomers, with a large proportion of the *meso*-compound.

Solubility in water. The solubility of some other compounds in water at room temperature was determined by adding an excess of ester at 30°, cooling to room temperature, centrifuging, concentrating a portion of the supernatant fraction and determining the amount dissolved using GLC. In some instances the ester had to be hydrolysed and the alcohol determined.

TCE pivalate (TCE-P) was slightly soluble in water (32 mg/l at 20°), whereas the solubility of tribromoethyl pivalate was much less (0·05 mg/l). The solubility of TCE succinate was 5·7 mg/l and that of TCE-A was less than 0·5 mg/l. The solubility of tribromoethyl adipate appeared to be less than 0·1 mg/l.

<div align="center">RESULTS</div>

Preliminary studies with TCE-P

Comparison of inhibitory activity of TCE-P and its constituents. Clapperton & Czerkawski (1971) have shown that pivalic acid does not inhibit methane production, whereas TCE is a strong inhibitor. An example of a dose-response curve obtained with this compound is shown in Fig. 1. Clearly the response was not linear; it was rapid with small concentrations of inhibitor. Hydrogen gas accumulated in proportion to inhibition of methane production at about 1·4 mol/mol methane. Other similar experiments showed that when the concentrations of inhibitor were increased further, the apparent methane production remained at about 25 μl/h per 20 ml rumen contents, the accumulation of hydrogen increased further and then began to decrease. We concluded that the apparent methane production in the presence of high concentrations of inhibitor was due to methane that was in solution in rumen contents at the start of incubation. The decrease in accumulation of hydrogen at high concentrations of inhibitor suggested that at these concentrations the inhibitor began to interfere with general fermentation. The accumulation of hydrogen reached a maxi-

Table 1. *Relative retention times on gas–liquid chromatography (GLC) and some physical properties of various compounds*

(The retention times of trichloroethanol (TCE) (9–11 min), n-pentanol 5 (min) and TCE-adipate (30–35 min) were each taken as 100. Values for common compounds, in parentheses, are given for comparison)

Group	GLC conditions	Compound	Relative retention time	Molecular weight	Density ($\times 10^3$) (kg/m³)
TCE pivalate and constituents	Carbowax, 155°	TCE	100	149·5	1·55
		Pivalic acid	61		
		TCE pivalate	44	233·5	1·22
		(Crotonic acid)	(139)		
Halogen-substituted ethanols	Carbowax, 155°	TCE	100	149·5	1·55
		Dichloroethanol	67	115·0	1·15
		Monochloroethanol	26	80·5	1·20
Esters of halogen-substituted ethanols and pivalic acid	Carbowax, 155°	TCE	100	149·5	1·55
		TCE pivalate	39	233·5	1·22
		Dichloroethyl pivalate	41	199·0	1·10
		Monochloroethyl pivalate	26	165·0	0·99
Esters of TCE	Carbowax, 155°	TCE	100	149·5	1·55
		TCE acetate	41	191·4	1·41
		TCE butyrate	71	219·5	1·22
		TCE valerate	107	233·5	1·21
		TCE hexanoate	158	247·5	nd
		TCE isobutyrate	54	219·5	1·25
		TCE isovalerate	78	233·5	1·21
		TCE pivalate	44	233·5	1·22
		TCE isohexanoate	124	247·5	nd
Esters of TCE (high molecular weight)	Ethylene glycol succinate, 190°	TCE versatate	8, 9	303·5	nd
		TCE palmitate	100	387·5	0·97
		TCE linolenate	338	409·8	1·08
Esters of trichloroacetic acid (TCA)	Carbowax, 140°	TCE	100	149·5	1·55
		Ethyl TCA	22	191·5	1·37
		TCE TCA	291	295·0	1·65
		n-Pentyl TCA	72	233·5	1·20
		Isopentyl TCA	57	233·5	1·18
		Neopentyl TCA	35	233·5	1·22

Table 1 (*cont.*)

| Group	GLC conditions	Compound	Relative retention time	Molecular weight	Density ($\times 10^3$) (kg/m³)
Esters of TCA	Carbowax, 100°	n-Pentanol	100	88·1	0·81
		Ethyl TCA	192	191·5	1·37
		Isobutyl TCA	298	219·4	1·26
		t-Butyl TCA	174	219·4	1·26
		(Ethanol)	(16)		
Esters of TCE and dibasic acids	Ethylene glycol succinate, 190°	TCE succinate	55	281·0	nd
		TCE glutarate	72	395·0	nd
		TCE adipate	100	409·0	nd
		(Methyl palmitate)	(5)		
Esters of TCA and dihydric alcohols	Ethylene glycol succinate, 190°	TCE adipate	100	409·0	nd
		2,5-Hexanediol TCA	27, 29	409·0	1·45
		1,6-Hexanediol TCA	86	409·0	1·38
		2,5-Hexanediol	3	118·2	0·96
		1,6-Hexanediol	10	118·2	nd
		(Isobutyl adipate)	(6)		
Tri- and hexachloro C_3 compounds	Ethylene glycol succinate, 120°	TCE	100	149·5	1·55
		Trichloroacetone	15	161·6	1·43
		Hexachloroacetone	63		
		Trichloro-2-propanol (TCP)	81	163·5	nd
		(Hexachloro-2-propanol)	(26)	267·0	nd
		TCP pivalate	32	233·5	1·22
Other compounds	Carbowax, 155°	TCE	100	149·5	1·55
		TCE benzoate	827	253·5	1·43
Non-volatile compounds or compounds that decompose on GLC	No GLC	Tribromoethyl pivalate	nd	367·0	1·52
		Methane sulphonic acid	nd	96·1	nd
		TCE methyl sulphonate	nd	227·6	1·20
		Trichloromethane sulphonic acid	nd	199·6	nd
		TCE trichloroemethane sulphonate	nd	331·1	nd
		Ethyl trichloromethane sulphonate	nd	227·6	nd
		TCE tosylate	nd	303·6	nd

nd, Not determined.
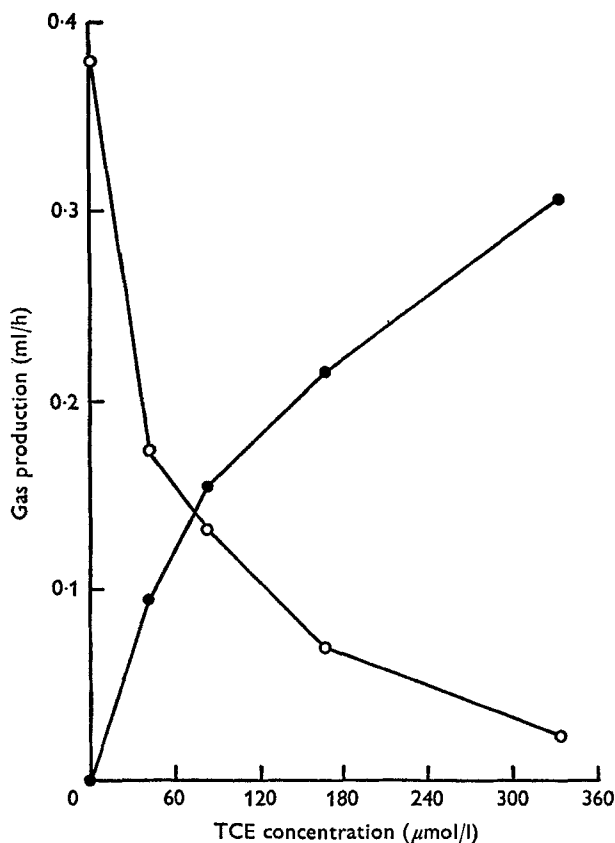
Fig. 1. Production of methane and hydrogen during incubation of sheep rumen contents in vitro with increasing amounts of trichloroethanol (TCE) under standard conditions (see pp. 430–1); O——O, methane; ●——●, hydrogen.

mum at higher concentrations of inhibitor than were necessary for nearly complete inhibition of methane production. Therefore it appears that the inhibitor is specific in reasonable concentration. Experiments like those shown in Fig. 1 showed that TCE-P was as active as TCE. It is unlikely that the ester is hydrolysed instantaneously (see p. 437) and these results indicated that the ester itself might inhibit methane production. It is possible to estimate by interpolation the amounts of inhibitor required to give 50 % inhibition. When experiments were done on different occasions the amounts of inhibitor required to give 50 % inhibition varied, although the same sheep on a constant feeding regimen were used as donors of rumen contents. This variation was unavoidable and difficult to explain and, therefore, during subsequent development and testing of various inhibitors a positive control of TCE-P was included with every series of incubations. The amounts used gave 40–60 % inhibition of methane production. The results of these control incubations for two particular sheep are shown in Fig. 2. There was no difference between sheep, and the supplementation of the rations with minerals (particularly cobalt) did not appear to have any effect. A sample of TCE-P, stored in the dark at 5°, was used in most of these assays, but after 3 and 6
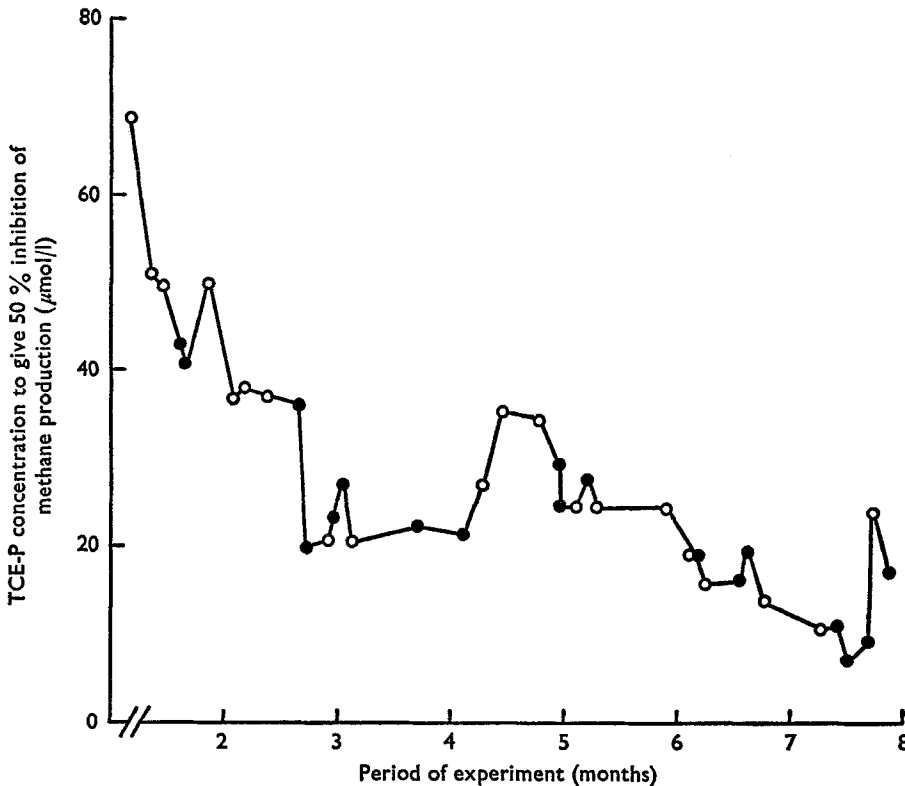
Fig. 2. Concentration of trichloroethyl pivalate (TCE-P) required to give 50 % inhibition of methane production during incubation, under standard conditions (see pp. 430–1), of samples of rumen contents taken from two sheep that were given a constant diet of molassed sugar-beet pulp and hay for a period of about 8 months. ●—●, Sheep, 564; ○—○, sheep 565.

months of storage, new samples of TCE-P were prepared and tested at the same time as the old sample. There was no difference in inhibitory activity, and it was concluded that the tenfold increase in potency of the inhibitor over 6–7 months, was not due to formation of a strong inhibitor fragment, but related to changes in the composition of rumen contents.

Hydrolysis of TCE-P during incubations with rumen contents. The inhibitory concentrations of TCE-P were very low (10–100 μmol/l) and it was impossible to measure the extent of hydrolysis at these concentrations with the analytical techniques available. However, several experiments were done in which the concentration of inhibitor was much greater (1–2 mmol/l). Under these conditions it was possible to measure the disappearance of the ester and the formation of the acid and alcohol.

Using undiluted rumen contents and 1·4 mM-TCE-P, the concentration of the ester decreased rapidly at first (0·6 mmol/l per h) and then more slowly. After incubation for 5 h about 25 % of the substrate remained. Pivalic acid and TCE were produced in approximately stoichiometric amounts. When increasing concentrations of TCE-P (1–6 mmol/l) were incubated with undiluted rumen contents for 2 h, there was only a slight increase in hydrolysis with increasing substrate concentrations, the mean

Table 2. *Effect of the number and type of halogen atoms in halogen-substituted ethanols and their pivalate esters on the inhibition of methane production during incubation of sheep rumen contents in vitro**

(Mean values; no. of incubations in parentheses)

Halogen compound	Concentration to give 50 % inhibition (μmol/l)		Maximum concentration tested (mmol/l)	
	Alcohol	Pivalate	Alcohol	Pivalate
Monochloroethanol	45 900 (2)	—† (2)	47·5	23·0
Dichloroethanol	1700 (2)	5750 (2)	2·5	11·4
Trichloroethanol	41 (4)	37 (4)	—‡	—‡
Trifluoroethanol	—† (2)	29 300 (2)	13·9	35·9
Tribromoethanol	3 (3)	13 (3)	—‡	—‡

* For details of experimental procedure, see p. 431.
† No inhibitory activity at maximum concentration tested.
‡ Maximum concentration tested was usually twice the concentration to give 50 % inhibition.

values for the whole range were (mmol/l per h): disappearance of TCE-P 0·45, production of pivalic acid 0·50, production of TCE 0·47. It was impossible to see whether this rate would decrease when the concentrations became similar to those used for inhibition studies.

When rumen contents were diluted with cell-free rumen fluid and incubated with 3 mM-TCE-P for up to 2 h, with an inoculum concentration of less than 0·5 ml/ml, the disappearance of TCE-P and the appearance of the alcohol and acid were proportional to the concentration of rumen contents. With an inoculum concentration greater than 0·5 ml/ml, the rates were independent of concentration of rumen contents and remained at about 0·55 mmol/l per h.

These results suggest that intact TCE-P might be an active inhibitor of methane production, but they do not exclude the possibility that the hydrolysis might be very rapid at low concentrations of the ester and that the active component is the free alcohol.

Halogen-substituted ethanol and its esters

Alcohols and esters of pivalic acid. Two series of experiments were done: (1) the effect of the number of chlorine atoms in the alcohol on methane production was studied, (2) the effect of the type of halogen was studied. In each instance several levels of inhibitor were tested to determine amounts that would give 40–60 % inhibition. Subsequently the esters were tested at the same time as the corresponding alcohol, and the concentrations required to give 50 % inhibition were calculated, if possible by assuming an exponential function of inhibitor concentration. The results of these experiments are summarized in Table 2. Monochloroethanol was a very weak inhibitor and its ester with pivalic acid was non-inhibitory at the levels tested. The di-substituted alcohol was moderately inhibitory, but its pivalate ester was much weaker. TCE, as found already in preliminary studies, was a potent inhibitor. TCE-P was marginally, but consistently, a stronger inhibitor than the alcohol. Trifluoroethanol did not inhibit methane production at the concentrations tested and its ester with

Table 3. *Inhibition of methane production during incubation of sheep rumen contents in vitro with trichloroethyl esters of fatty acids, and hydrolysis of esters by rumen contents**

(All values, except where indicated, are results of a single comparative experiment)

Ester of:	Concentration to give 50 % inhibition (μmol/l)	Rate of hydrolysis under standard conditions* (mmol/mol per h)
Acetic acid†	19·2 (9·7)	> 250
Butyric acid	27·5	157
Isobutyric acid	30·2	171
Valeric acid	20·7	288
Isovaleric acid	25·4	45
Pivalic acid	25·6	15
Hexanoic acid	21·3	250
Isohexanoic acid	41·4	251
Palmitic acid†	163 (45)	8
Versatic acid†‡	242 (52)	< 1
Linolenic acid†	980 (50)	nd

nd, Not determined.

* For details of experimental procedures, see p. 431.

† Separate determinations; the values in parentheses refer to trichloroethyl pivalate determined at the same time.

‡ A tertiary C_{10} fatty acid with at least one methyl group on the α-C atom.

pivalic acid was only weakly inhibitory. Tribromoethanol was the most potent inhibitor. Tribromoethyl pivalate was less active, but it was considerably more active than TCE and its ester. As will be seen later, when other inhibitors are considered, TCE was unusual in that its ester with pivalic acid was consistently as active as the free alcohol and sometimes more active.

TCE esters of monobasic fatty acids. After preparation and purification, each compound was tested at several concentrations to determine the concentration required to give 50 % inhibition of methane production under standard conditions. The amounts required were calculated by interpolation. When the complete series of compounds was available, they were tested simultaneously using the single concentrations calculated to give about 50 % inhibition, and the actual amounts required to give 50 % inhibition were calculated by assuming an exponential law. The two sets of results were similar and the mean values are given in Table 3. In general, the inhibitory activity was maximal with the C_5 fatty acids of both the straight-chain and the *iso*-series, although the differences between the straight-chain and the *iso*-series were small with C_4 and C_5 compounds and increased with C_6 fatty acids. The inhibition was less with the TCE esters of palmitic acid and versatic acid, a tertiary C_{10} carboxylic acid with at least one methyl group on the α-C atom. Linolenate, the longest-chain acid used, was a very weak inhibitor. Although pivalic acid is a heavily branched isomer of valeric acid, TCE-P did not follow the trends shown by the esters of the other acids. The results of the determination of the extent of hydrolysis of the esters are also shown in Table 3. All the straight-chain compounds were hydrolysed relatively rapidly, while TCE isovalerate was hydrolysed more slowly than the TCE esters of isobutyric and isohexanoic acids; TCE-P had the slowest rate of hydrolysis.

Table 4. *Inhibition of methane production during incubation of sheep rumen contents in vitro with esters of dibasic acids, and hydrolysis of esters by rumen contents**

(The values in parentheses are those for trichloroethyl pivalate determined at the same time)

	Concentration to give 50 % inhibition (μmol/l)			Rate of hydrolysis under standard conditions* (mmol/mol per h)
		Comparative experiments†		
Compound	Individual estimations	a	b	
Trichloroethyl succinate	22·7 (27)	18·9	20·6	80
Trichloroethyl glutarate	18·6 (20)	10·0	4·9	28
Trichloroethyl adipate	69·0 (53)	9·5	6·5	36
Tribromoethyl succinate	4·3 (27)	nd	nd	nd
Tribromoethyl adipate	10·3 (27)	nd	nd	nd
Isobutyl adipate	1930 (25)	nd	nd	nd
Trichloroethanol	45·2 (53)	33·1	nd	—
Tribromoethanol	4·5 (25)	6·1	nd	—
Trichloroethyl pivalate	—	20·0	17·0	15

nd, Not determined.

* For details of experimental procedures, see p. 431.

† Results of two experiments in which the compounds were incubated concurrently with rumen contents. The test concentrations of the compounds were chosen to give about 50 % inhibition.

It is possible that this was due to steric hindrance brought about by the three methyl groups in close proximity to the ester bond, and this supports the hypothesis that TCE-P acts in the ester form as an inhibitor of methane production.

Esters of dibasic acids. The inhibitory activity of esters of dibasic acids is given in Table 4. Since each ester of a dibasic acid has two active groups, to compare their activity with the other compounds one might have to double the values given in Table 4. The most active ester was that of tribromoethanol, but it was not as active as the alcohol itself. With the trichloro-substituted esters, the succinate ester was less active than the adipate or glutarate esters and the activity was parallelled by the extent of hydrolysis. Isobutyl adipate was a weak inhibitor.

Comparison of alcohol- and acid-substituted halogen compounds

Esters of TCA. Although the amide of TCA is a strong inhibitor of methane production (Trei *et al.* 1971), TCA itself is a very weak inhibitor. In the course of present work several TCA esters were prepared and tested. The results, together with those of some constituents of the esters, and other substances of interest, are summarized in Table 5. In general, TCA esters were more active than TCA itself; the TCA ester with TCE was a good inhibitor but the esters of TCA and the non-chlorinated alcohols were weaker. Generally the activity of the esters increased with the increased branching of the alcohol part of the ester. It is possible that the ethyl ester was losing its activity because of hydrolysis to inactive or weakly active products and that the branched compounds were less easily hydrolysed and therefore more active.

The effect of branching in the isomers of C_5 alcohols esterified with TCA can also be seen in Table 5. These esters were isomers of TCE-P but even the most active one,

Table 5. *Inhibition of methane production during incubation of sheep rumen contents in vitro with the esters of trichloroacetic acid (TCA) and related compounds and hydrolysis of esters by rumen contents**

(For individual estimations the values in parentheses are those for the trichloroethanol (TCE) ester of pivalic acid incubated with rumen contents at the same time)

	Concentration to give 50% inhibition (μmol/l)		Rate of hydrolysis under standard conditions* (mol/mol per h)
Compound	Individual estimations	Comparative experiment†	
TCA	3300 (16)	3890	—
TCE	27 (26)	nd	—
TCE TCA ester	22 (16)	nd	nd
Ethyl TCA	773 (26)	1750	7500
Isobutyl TCA	344 (16)	717	196
t-Butyl TCA	140 (16)	257	nd
n-Pentyl TCA	846 (7)	nd	136
Amyl TCA	481 (7)	nd	90
Neopentyl TCA	307 (7)	nd	56
TCA 2,6-hexanediol	695 (30)	nd	nd
TCA 1,6-hexanediol	710 (30)	nd	nd
Isobutyl isobutyrate	24100 (26)	nd	nd
Isobutyraldehyde	25700 (26)	nd	nd
TCE adipate	5 (6)	nd	26
TCE pivalate	—	21	15

nd, Not determined.
* For details of experimental procedures, see p. 431.
† All compounds were tested concurrently, using concentrations that would give about 50% inhibition.

neopentyl TCA, was about forty times weaker than TCE-P. The fact that compounds with the trichloro group on the alcohol residue are better inhibitors than those with the trichloro group on the acid residue is supported by results for two other isomeric esters given in Table 5. Clearly, TCE adipate was about 130 times more active than TCA hexanediol.

The results of earlier work indicated that isobutanol was not an inhibitor of methane production (Czerkawski & Breckenridge, 1972), and the results summarized in Table 7 show that both isobutyl isobutyrate and isobutyraldehyde are very weak inhibitors. Isobutyric acid occurs naturally in the rumen.

Normally, TCA did not inhibit methane production to any great extent, but occasionally it became more potent and this was shown subsequently to be due to some changes brought about by storage. The results of a typical experiment to illustrate this was shown in Table 6. Clearly, when solutions of the sodium salt of TCA were kept for 19 d there was an eightfold increase in inhibitory activity and this was increased further when TCA solutions were stored in the presence of Tween 80. The latter could have been due to transesterification with the detergent, but the mechanism of the former is unknown. It could have been due to slow cleavage to chloroform and sodium bicarbonate. Chloroform is a potent inhibitor and the conversion of only 2% of the TCA to chloroform would have resulted in the increase found in inhibitory activity.

Table 6. *Increase in the inhibitory activity of trichloroacetic acid (TCA) with respect to methane production during incubation of sheep rumen contents in vitro*, after storage, with or without Tween 80, in aqueous solution (2·5 mg/ml, neutralized with sodium hydroxide) in the dark at room temperature for 7 or 19 d*

(The values are expressed as relative inhibitory activity, i.e. the activity relative to that of tricholoroethyl pivalate (1000) tested at the same time; all samples were tested at a concentration to give approximately 50 % inhibition)

| | Period of storage (d) | | Relative inhibitory activity | | |
| | TCA alone | TCA + Tween 80 | Total measured | Increment due to storage of TCA and Tween 80 | Increment due to Tween 80 |
Amount used/incubation vessel (mg)					
10·0	0	0	6·9	—	—
1·25	19	0	57·1	50·2	—
10·0	0	0	7·8	—	—
1·25	19	0	42·1	34·3	—
1·25	19	7	64·4	56·6	22·3
1·25	19	19	77·8	70·0	35·7

* For details of experimental procedure, see pp. 430–1.

Esters of sulphonic acids and other trichloro-derivatives

Two main series of compounds were studied, derivatives of methane sulphonic acid and of trichloromethane sulphonic acid. The results are given in Table 7, with the results of tests with the ester of an aromatic sulphonic acid (tosylate) and the only ester of an aromatic carboxylic acid tested, benzoic acid.

Methane sulphonic acid (neutralized) was not inhibitory, but its TCE ester was a potent inhibitor. There were difficulties in characterizing this and other sulphonic acid esters, because they tended to decompose during GLC. However, the TLC showed, albeit less accurate, that contamination with alcohol was less than 5 %.

Trichloromethane sulphonic acid was a very weak inhibitor and its esters with TCE and ethanol were moderately active inhibitors. Thus, there was an analogy with the esters of TCA. TCE trichloromethane sulphonate was more active than the ethyl ester, presumably because of hydrolysis and release of active TCE, but the ethyl ester, which on hydrolysis would release an inactive alcohol and a very weakly active acid, was nevertheless active and it must be assumed that the ester itself is an active inhibitor in this instance.

The esters of both the aromatic acids were good inhibitors, but not as potent as TCE-P. The trichloro-2-propanol was more active than trichloroacetone and unlike the esters of TCE, the ester of the trichloro C_3 alcohol was less active than the constituent alcohol.

Summary of results

The relative inhibitory activities (RIA) of most of the compounds studied in these experiments are given in Table 7 where, in general, the higher the value the better the inhibitor. The values for RIA given in Table 7 were calculated from the equation,

Table 7. *Relative inhibitory activity (RIA) of various compounds with respect to methane production during incubation of sheep rumen contents in vitro**

(The concentrations of the test compounds and of the trichloroethanol (TCE) ester of pivalic acid incubated at the same time and required to give 50 % inhibition of methane production were used to calculate the RIA under standard conditions; RIA is the concentration of TCE pivalate divided by the concentration of the test compound and multiplied by 1000)

Compound	RIA	Compound	RIA
Halogen-substituted ethanols		Esters of trichoroacetic acid (TCA)	
Monochloroethanol	1	TCA	4
Dichloroethanol	22	TCE TCA	714
TCE	909	Ethyl TCA	17
Trifluoroethanol	0	Isobutyl TCA	21
Tribromoethanol (TBE)	10000	t-Butyl TCA	96
Esters of halogen-substituted ethanols and pivalic acid		Isobutyl isobutyrate	1
		Isobutyraldehyde	1
Monochloroethyl pivalate	0	n-Pentyl TCA	8
Dichloroethyl pivalate	6	Amyl TCA	14
TCE pivalate	1000	Neopentyl TCA	23
Trifluoroethyl pivalate	1	2,5-Hexanediol TCA	43
TBE pivalate	2500	1,6-Hexanediol TCA	8
Esters of TCE and fatty acids		Esters of sulphonic acids	
TCE acetate	500	Methane sulphonic acid	0
TCE butyrate	909	TCE methane sulphonate	588
TCE isobutyrate	833	Trichloromethane sulphonic acid	9
TCE valerate	1250	TCE trichloromethane sulphonate	93
TCE isovalerate	1000		
TCE hexanoate	1250	Ethyl trichloromethane sulphonate	64
TCE isohexanoate	625	TCE tosylate	294
TCE palmitate	278	Trichloro C_3 compounds	
TCE versatate	217	Trichloroacetone	340
TCE linolenate	51	Trichloro-2-propanol	454
Esters of dibasic acids		Trichloro-2-propanol pivalate	214
TCE succinate	1000	General	
TCE glutarate	1667	Chloral hydrate	526
TCE adipate	1428	TCA	714
TBE succinate	5000	TCE benzoate	500
TBE adipate	2500		
Isobutyl adipate	13		

* For details of experimental procedure, see pp. 430–1.

$RIA = (C/T) \times 100$, where C and T are the concentrations of TCE-P and the test compound respectively, which have 50 % inhibition of methane production, when incubated at the same time. The positive control (TCE-P) has a value of 1000 on this scale. The assessment of the inhibitors might be as follows: < 10 non-inhibitors, 10–100 poor, 100–500 moderate, 500–1500 good, > 1500 excellent.

DISCUSSION

Experiments with halogen-substituted alcohols and their esters have shown that the number and type of halogen atoms were of considerable importance for inhibition of methane production. The monochloro and the trifluoro compounds were virtually non-inhibitory. The dichloro compounds were relatively weak inhibitors; the alcohol

Table 8. *Comparison of inhibitory activity of various trichloro-substituted compounds with respect to methane production during incubation of sheep rumen contents in vitro**

| | Concentration to give 50 % inhibition (μmol/l) | | |
Compound	Individual estimations	Comparative experiment†	Literature values
Chloral hydrate	63	23·9	140‡
Trichloroacetic acid (TCA)	3300	3010·0	800§
Trichloroethanol (TCE)	40	21·3	—
TCE pivalate	35	12·8	—
Chloroform	8·1	—	8·0‡
Trichloroacetamide	17	—	12·0§
Isobutyl TCA	344	—	—
TCE TCA	22	—	—
TCE adipate	27	—	—

* For details of experimental procedure, see pp. 430–1.
† All compounds were tested concurrently, using concentrations that would give about 50 % inhibition.
‡ Prins, Van Nevel & Demeyer (1972).
§ Trei, Parish, Singh & Scott (1971).

was three to four times more active as an inhibitor than the ester. The tribromoethyl alcohol and its pivalate ester were the most potent inhibitors tested and again the alcohol was about four times more effective than the ester. Subsequent work with the trichloro derivatives of propan-2-ol also showed that the ester was less potent than the alcohol or ketone. On the other hand, numerous experiments with TCE and its pivalate ester showed that this alcohol and its ester had about the same activity. This property was shared by the esters of other C_5 acids, and to a certain extent by the esters of C_4 and C_6 acids, but not those of C_{16} and C_{10} branched-chain acids.

The solubility of TCE-P was low (32 mg/l) and the ester was hydrolysed slowly. It is unlikely that the similarity in inhibitory activities of this ester and its constituent alcohol would be due to a very rapid hydrolysis. It is more likely that the ester is taken up intact by bacteria and that its ability to enter the microbial cell is a function of its structure. It is interesting to consider the inductive effects of the two ends of the molecule. The alcohol has three chlorine atoms on one C atom, which is one of the most electron-attracting groups known, and the acid has the most electron-releasing group $((CH_3)_3C)$. Yet this might not be the basic property that governs the activity of these compounds because valeryl and isovaleryl residues in TCE esters did not result in the inhibitors being less efficient than the pivalate.

There is much evidence that the methyl derivative of vitamin B_{12} is an intermediate in the production of methane. The accepted pathways involve the transfer of a methyl group from 5-N-methyltetrahydrofolic acid to the reduced vitamin B_{12} and the liberation of methane from methyl vitamin B_{12} in the presence of an electron donor system in which ferrodoxin has been implicated. It was shown by Hogenkamp (1965) that methyl vitamin B_{12} can be photolysed, but the products of the reaction are different depending on whether oxygen is in excess or in restricted supply. In the presence of excess oxygen the products are aquocobalamin and formaldehyde, but with traces of

oxygen the products are reduced vitamin B_{12}, methane, ethane and formaldehyde. Brot, Taylor & Weissbach (1966) maintained that methyl transfer from 5-N-methyltetrahydrofolic acid involves enzyme-bound cobalamin, while the methyl transfer from methyl vitamin B_{12} (and possibly methane production) does not involve enzyme-bound cobalamin. On the other hand, Wood & Wolfe (1966) showed that the cobalamin enzyme involved in methane formation could be inactivated by propylation and reactivated by photolysis. Wood *et al.* (1968) prepared several halogenated cobalamin derivatives, including methyl chloride, methylene chloride, chloroform and carbon tetrachloride. They showed that the photolytic cleavage of the dichloromethyl ligand was not analogous with the cleavage of the methyl ligand and that one product with the dichloromethyl ligand was a reactive chlorocarbene. The over-all reaction appears to be:

$$\overset{\displaystyle CHCl_2}{\underset{\displaystyle >Co<}{\mid}} + 1\cdot 5H_2 \rightarrow\ > Co <\ +CH_3Cl + HCl, \tag{1}$$
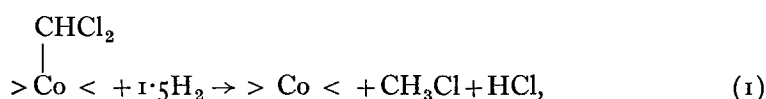
where $> Co <$ is cobalamin. It was also suggested that the formation of the polyhalogen ligands takes place according to the reaction:

$$> Co <\ +CX_nH_m \rightarrow\ \overset{\displaystyle CX_{n-1}H_m}{\underset{\displaystyle >Co<}{\mid}} X^-, \tag{2}$$
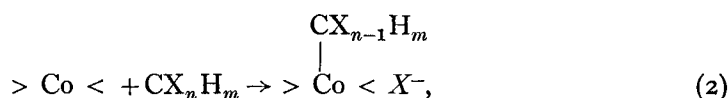
where n is 1, 2, 3 or 4, $n+m = 4$, X is the halogen and the cobalamin ($> Co <$) is in a reduced monovalent state.

Bauchop (1967) found that chloroform and related compounds inhibited methane production in the rumen. Wood *et al.* (1968), using an in vitro system, found that these polyhalogen compounds inhibited methane production competitively. The inhibited system could be reactivated by photolysis, but reactivation was less with chloroform than with dichloride and the authors suggest that the formation of reactive free radicals and their addition to the corrin nucleus or to an amino acid close to the catalytic site could interfere strongly with enzyme action. Prins (1965) incubated rumen contents with chloral hydrate at 2·5 mmol/l and concluded that chloral hydrate was partly converted to TCE and partly decomposed to chloroform and formic acid. No measurements of methane production were made, but the trichloro derivative caused an increase in the propionic acid:acetic acid ratio and a change from Gramnegative to Gram-positive microflora. It is known that when chloral is heated with concentrated alkali, chloroform and formic acid are produced but there is little evidence that these products may be found under physiological conditions.

TCA is a weak inhibitor but it is not known whether this is because it is too stable to be dissimilated into active products or because it cannot enter the cells. If the latter is accepted, then it must be assumed that the cells are permeable to the esters of TCA and that the acid becomes inhibitory once inside the cell, either directly, or after conversion to chloral and chloroform.

Like other TCA esters, neopentyl TCA is a more potent inhibitor than either of its hydrolysis products. This suggests that the structure of the microbial cell surface

is such that the cells are permeable to this compound. Neopentyl TCA and its isomer TCE-P have very similar structures and if we accept that the former can enter the cell without previous hydrolysis, the cells should be permeable to the latter. However, the nature of the actual inhibitory compound remains uncertain. Some individual and comparative results of inhibitory activity of various related compounds are given in Table 8. Clearly, chloroform appears to be the most active compound, whereas TCE and TCE-P, although weaker than chloroform, are better inhibitors than chloral hydrate and much better than TCA. When small amounts of TCA, TCE or chloral hydrate were heated with conc. sodium hydroxide, only chloral hydrate was converted to chloroform. TCA did not change and although TCE reacted to give a brown product there was no chloroform. If similar reactions take place under physiological conditions, and if chloroform is the actual inhibitor, then one would have to assume that both TCE and TCA or their esters would have to be converted to chloral and then to chloroform, before inhibition would ensue. Yet chloral hydrate does not appear to be a better inhibitor than TCE or some of its esters. It is possible that the reactive substance is chloral rather than the hydrate and that chloral hydrate cannot enter the cells as readily as the alcohol or some of its esters. The conversion to the alcohol and oxidation to the aldehyde would then take place inside the cell.

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