The use of pivalic acid as a reference substance in measurements of production of volatile fatty acids by rumen micro-organisms *in vitro*

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1. A procedure is described for using pivalic acid as an inert reference substance in determination of changes in concentrations of volatile fatty acids (VFA).

2. Pivalic acid in concentrations of up to 80 mmol/l had no effect on production of methane or VFA by rumen contents.

3. Pivalic acid was inert during incubation with rumen contents from sheep given different diets and with samples taken at different times with respect to feeding.

The production of volatile fatty acids (VFA) by rumen contents can be measured in vitro by determining the net increase in their concentration during incubation under suitable conditions (e.g. see Whitelaw, Hyldgaard-Jensen, Reid & Kay, 1970). The total VFA concentration can be determined by steam-distillation, or by distillation in Conway units, and titration with dilute alkali solution. The determination of concentrations of individual VFA usually involves some type of chromatography. A widely used technique is that of Cottyn & Boucque (1968). In this method, the acidified mixture of VFA contains crotonic acid which acts as an internal standard, but other substances have been used. Some internal standards are non-acid and therefore would be expected to behave differently from the VFA, and others, e.g. hexanoic acid, have inconveniently long retention times.

A good internal standard substance should emerge faster on the chromatograph than the slowest significant peak of the VFA mixture (usually valeric acid) and it should be an acid. It is difficult to obtain a reproducible sample of rumen contents, even during incubation in vitro, and an inert internal standard, that could be added to the incubation mixture at the start, would offer considerable advantages. It would remove much variability due to sampling and would make it unnecessary to correct for volume changes caused by additions and withdrawals of liquids. A useful internal standard should be neither produced nor utilized by rumen micro-organisms and it should have no effect on the usual rumen fermentation.

It would appear that the commercially available tertiary C_5 carboxylic acid, namely pivalic acid, satisfies many of the criteria enumerated above and thus may be useful in the determination of VFA production in vitro.

METHODS

Analytical methods. The concentrations of VFA were determined as described by Cottyn & Boucque (1968), using columns of 50 g carbowax 20M-TPA/kg chromosorb G (Perkin-Elmer, Beaconsfield, Bucks.) in a gas–liquid chromatograph (Model 104; Pye Unicam, Cambridge), at 130° using crotonic acid as a secondary standard. Under these conditions, pivalic acid emerged on the gas–liquid chromatograph with the same retention time as isobutyric acid (Czerkawski & Breckenridge, 1975*a*), and although its peak coincided with that of isobutyric acid, it proved the best reference substance. For instance, *t*-butyl acetic acid had a retention time of 0·82 relative to that of valeric acid, but it was not completely separated from isovaleric acid (relative retention time 0·75). Normally, the proportions of isovaleric acid in the rumen are small, but they are not as small as those of isobutyric and can increase considerably under certain conditions (e.g. inhibition of methane production).

In some of the experiments, the metaphosphoric acid precipitating agent of Cottyn & Boucque (1968) was replaced by phosphoric acid to give a final concentration of 0·2 M.

Incubations. The rumen contents were obtained from sheep through rumen fistulas. The contents were strained through four layers of gauze and incubated at 39° in large test-tubes or the small-scale artificial rumen as described by Czerkawski & Breckenridge (1975*a*). In most incubations vigorous fermentation was maintained by intermittent addition of glucose solutions. The pivalic acid, as the sodium salt, was added at the start of the incubation.

RESULTS

As was mentioned previously, pivalic acid has the same retention time as isobutyric acid, a normal component of rumen VFA. However, the concentration of isobutyric acid in rumen contents is very low; in our experience, the concentration of this acid is rarely greater than 0·3 mmol/l, compared with 30–70 mmol acetic acid/l. Thus, if one adds sufficient pivalic acid to give about 40 mmol/l, the isobutyric acid would contribute less than 1 % of the combined peak area, well within the error of the method. During incubations the concentrations of VFA change by less than 50 %, and therefore the change in the contribution of isobutyric acid to the pivalic acid peak will be very small and can be ignored. Thus, provided that one is not interested in isobutyric acid as such, and provided that pivalic acid is neither produced nor utilized, pivalate could be used as an internal standard during incubations.

In the exploratory experiments, samples of rumen contents (40 ml), from sheep on a diet of sugar-beet nuts and hay, were incubated with 10 ml pivalate solutions at 39° for up to 6 h. Glucose solution (2 ml, 50 mg/ml) was added at the start and after 3 h of incubation. In a typical experiment with 0, 40 and 80 mmol pivalic acid/l the mean methane production was 2·80, 2·74 and 2·78 ml/5 h respectively. This confirmed previous findings (Czerkawski & Breckenridge, 1975*a*) that pivalic acid had no effect on methane production. In the same experiment, the final concentrations of acetic, propionic and butyric acids were the same irrespective of the initial pivalic acid

Table 1. *Effect of pivalic acid on volatile fatty acid production during a typical incubation in vitro of rumen contents with glucose*

(The donor sheep were given sugar-beet nuts and hay, and samples were taken before the morning feed. Glucose (100 mg in 2 ml) was added at the start and after 3 h of incubation)

Pivalate added (mmol/l)	Period of incubation (h)	Concentrations (mmol/l)			
		Acetate	Propionate	Butyrate	Pivalate
0	0	39·0	10·1	6·9	0
	3	45·4	17·8	8·8	0
	6	54·2	26·1	10·8	0
20	0	36·0	9·6	6·1	18·6
	3	46·9	18·5	9·0	19·5
	6	53·0	26·6	11·1	18·5
40	0	37·3	9·8	6·2	39·8
	3	45·5	18·3	8·6	41·0
	6	51·1	25·6	19·8	39·6
Mean	0	37·1	9·8	6·4	—
	3	45·9	18·2	8·8	—
	6	53·1	26·1	10·9	—

Table 2. *Incubation of pivalic acid in vitro with rumen contents of sheep given various diets*

(The initial concentrations (C_0) and the mean increase for 6 h incubation (Δ) are given, calculated from the initial concentration and incubations for 2, 4 and 6 h. The values for pivalic acid are means of four values with their standard errors and all values are expressed in mmol/l)

Diet	Acetate		Propionate		Butyrate		Pivalate	
	C_0	Δ	C_0	Δ	C_0	Δ	Mean	SE
Hay only	65·2	12·6	11·7	4·8	4·3	3·0	1·80	0·01
Hay + goat mix* (4:6, w/w)	54·9	9·6	12·8	6·0	5·8	3·1	1·81	0·01
Silage	36·0	10·2	6·6	7·2	1·4	1·8	1·84	0·01
Hay + sugar-beet nuts (6:3, w/w)	66·3	13·2	21·8	4·7	11·9	11·4	4·20	0·05

* Rolled oats – decorticated cake – bean meal – cooked, flaked maize (22:7:7:4, by wt) (Paterson Connell, Kilmarnock, Scotland).

content (mean of six results ± SE) 57·4 ± 0·3, 20·0 ± 0·2 and 13·4 ± 0·1 mmol/l respectively. Thus the rumen fermentation characteristics did not seem to be affected by the presence of up to 80 mmol pivalic acid/l.

The results of another experiment of this type are summarized in Table 1. The recovery of added pivalate was good and the added pivalate had no significant effect on production of acetic, propionic and butyric acids (mean increases 7·2, 27·7 and 11·7 %/h respectively). Experiments with prefeed samples of rumen contents from sheep given hay and goat mix gave similar results.

A direct comparison of the effect of diet on inert behaviour of pivalic acid was made and the results are given in Table 2. In this experiment, prefeed samples of rumen contents were obtained from sheep on diets of hay only, hay + goat mix, silage and

Table 3. *Concentrations (C) and changes in concentrations (ΔC) of volatile fatty acids in samples of rumen contents taken from a sheep at various intervals with respect to feeding and incubated for 3 h in vitro*

(Pivalate was added to all samples at the start of incubation. Values (mmol/l) are means of duplicate incubations)

Period after feeding h	Acetate		Propionate		Butyrate		Pivalate	
	C	ΔC	C	ΔC	C	ΔC	C	ΔC
Before	47·2	+1·8	8·2	+0·4	3·9	+0·2	14·7	−0·1
0·5	51·8	+12·0	11·7	+3·6	5·5	+1·8	14·9	+0·3
1·0	59·2	+5·4	13·1	+1·2	6·3	+1·0	14·8	−0·2
2·0	61·0	+0·7	13·6	−0·1	6·7	+0·9	14·6	−0·4
3·0	66·9	−1·3	13·8	−0·3	7·6	+0·5	14·9	−0·3
4·0	61·2	−3·3	12·4	−0·6	6·1	+0·7	15·2	−0·3
5·0	64·2	+6·0	12·7	+1·0	7·3	+0·6	14·2	+0·2

hay + sugar-beet nuts. It can be seen that with the concentrations of pivalate used, the diet of the donor animal had no significant effect. Nevertheless, this might not be general, and before making use of pivalate as an inert reference substance, it is advisable to do a simple experiment of the type referred to in Table 2.

So far all samples of rumen contents used in the incubations were taken before feeding. The following experiment was done to ascertain that pivalic acid would remain inert in the presence of postfeed samples of rumen contents. Samples of rumen contents were taken from sheep before feeding (09.00 hours), and at 09.30, 10.00, 11.00, 12.00, 13.00 and 14.00 hours. Samples were strained and 10 ml of each was incubated with 2 ml pivalic acid solution for 3 h at 39°.

Table 3 indicates that although the concentration of acetic, propionic and butyric acids increased soon after feeding, the concentration of pivalic acid did not increase. No additional substrate was added during these incubations, consequently there was a net production of acetate only for 2 h after feeding, i.e. when soluble carbohydrate was available. Later the concentrations of acetate and propionate were found to have a net decrease, indicating an uptake. The concentrations of pivalic acid did not change markedly during incubations, but there was a tendency for the concentration of pivalate to decrease slightly when there was no net production of acetate and propionate. It is possible that under certain conditions there might be a slight utilization of pivalate, but this is unlikely in experiments in vitro when substrate is not in short supply. The mean decrease in concentration of pivalic acid was about 2%, which is less than the usual experimental error.

CONCLUSIONS

The limited number of experiments described above indicate that pivalic acid might be useful as a reference substance in the rumen studies. The possible applicability of the method to the estimation of the production of VFA in the rumen in vivo is presently being studied. The present results established the usefulness of pivalic acid in experiments in vitro and we are using it routinely in our experiments.

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