Rumen protein degradation and biosynthesis

1. A new method for determination of protein degradation in rumen fluid in vitro

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1. A method is described for the determination of protein degradation based on measurements of ammonia concentration and gas production (Menke *et al.* 1979) when a feedingstuff was incubated with rumen fluid in vitro.

2. NH_3 liberated during incubation is in part used for microbial protein synthesis. Production of carbon dioxide and methane can be regarded as a measure of energy available for protein synthesis. The ratio, gas production: incorporation of NH_3-nitrogen was estimated by addition of starch to the substrate. The response in gas production was linear in the range 0–200 mg starch, when starch was added to 0–200 mg feedingstuff dry matter and 30 ml rumen fluid-medium mixture.

3. Linear regression between NH_3-N concentration (y, mg) and gas production (x, ml) yielded an intercept (b_0) representing that amount of NH_3-N which would be released when no fermentable carbohydrates were available and consequently no bacterial protein synthesis took place.

4. The difference between this intercept b_0 and NH_3-N content in the blank (rumen fluid without substrate added) indicated the amount of NH_3 liberated from protein and other N-containing compounds of the feedingstuff incubated. In vitro-degradable N (IVDN) was calculated as a proportion of total N by the equation:

$$\text{IVDN} = \frac{\text{NH}_3\text{-N at zero gas production } (b_0) - \text{NH}_3\text{-N of blank}}{\text{Total N of feedingstuff incubated}}$$

Degradability of protein in the rumen is an important value to be used for the prediction of protein passing undegraded to the small intestine and for the calculation of protein utilization and protein requirements of ruminants. Attempts have been made to estimate protein degradation from solubility tests (Crooker *et al.* 1978; Jarrige *et al.* 1978; Chamberlain & Thomas, 1979) or by the use of polyester bags to be incubated in the rumen of a fistulated animal (Ørskov & Mehrez, 1977). Difficulties in measuring protein degradation from the release of ammonia (Chamberlain & Thomas, 1979) arise from the fact that protein degradation and bacterial protein synthesis are processes which occur simultaneously.

For separate determination of bacterial protein synthesis either certain characteristic constituents of micro-organisms such as diaminopimelic acid, RNA or DNA (Ling & Buttery, 1978; Siddons *et al.* 1979), or isotopes such as ^{35}S, ^{32}P or ^{15}N can be used (Pilgrim *et al.* 1970; Hume, 1975; Walker & Nader, 1975; McMeniman *et al.* 1976; Van Nevel & Demeyer, 1977).

The method described here uses the known relationship between fermentation of carbohydrates and microbial protein synthesis for the determination of NH_3-N incorporated into microbial proteins. The total amount of NH_3-N liberated is found by extrapolation of the linear regression to zero protein synthesis.

EXPERIMENTAL

Material and methods

Incubations were carried out in 100-ml calibrated syringes, according to the method described by Menke *et al.* (1979). Two rotating holders each capable of supporting fifty-eight syringes were used and positioned in ventilated drying ovens at 39° (accuracy 0·5°).

Protein sources to be incubated were analysed for crude protein (N × 6·25) and dry matter

* For reprints.

Table 1. *Expts 1 and 2. Crude protein (nitrogen × 6·25) content of feedingstuff, sample weight and amount of starch added*

Expt no.	Feedingstuff	Crude protein (g/kg DM)	Sample wt* (mg DM)	Amount of rice starch (mg) added (895 g DM/kg)				
1	Casein	921	28·2	50	100	150	200	250
	Soya-bean meal	487	55·4	50	100	150	200	250
	Rape-seed meal	389	68·4	50	100	150	200	250
	Hay	134	194·0	50	100	150	200	—
	Feather meal	980	26·5	50	100	150	200	—
2	Casein	921	28·2	50	100	150	200	250
	Linseed-cake meal	330	80·0	50	100	150	200	250
	Kapok residue	275	94·5	50	100	150	200	250
	Hay	134	194·0	50	100	150	200	—
	Maize-gluten feed	227	114·5	50	100	150	200	—

DM, dry matter.
* Sample weights containing 26 mg crude protein.

(DM). In all experiments sample weight was chosen such that it corresponded to 26 mg crude protein (e.g. 130 g crude protein/kg in 200 mg DM). This material and appropriate amounts of starch or other carbohydrates were first introduced into a syringe.

Rumen fluid was drawn from a rumen-cannulated dairy cow, receiving 2·6 kg hay, 3·3 kg barley, 0·6 kg soya-bean meal and 70 g of a mixture of minerals and vitamins. The ration was adjusted to the requirement of the cow without changing these proportions. Rumen fluid was withdrawn in the morning before feeding and filtered through two layers of cheese cloth into a warm flask of approximately 2 l volume filled with carbon dioxide. Rumen fluid was mixed with two parts of a medium (for details of composition, see Menke *et al.* 1979) and the mixture dispensed in 30 ml portions to the syringes, using a CO_2 stream to maintain anaerobic conditions.

At the end of incubation the amount of gas produced was determined by reading the position of the plug (accuracy 0·5 ml). Then the syringe was put into an ice-bath at 0° in order to prevent further microbial activity. The contents of the syringes were transferred quantitatively to Kjeldahl flasks for determination of NH_3 by distillation with magnesium oxide, according to Bremner (1965). Liberated NH_3 was trapped in 2–3 ml 0·2 M-sulphuric acid and titrated with 0·1 M-sodium hydroxide.

All incubations were run in duplicate (Expts 1, 2 and 4) or triplicate (Expts 3 and 5). In addition, three syringes with rumen fluid-medium mixture were incubated as blanks. Incubations with rumen fluid from different days were repeated with some of the protein sources.

Expt 1

The following feedingstuffs were used to study the effects of increasing amounts of rice starch on gas production and NH_3-N concentration during fermentation: casein, extracted soya-bean meal, extracted rape-seed meal, hay and non-hydrolysed feather meal.

Expt 2

Casein and hay were studied again in this experiment; in addition linseed-cake meal, kapok residues and maize-gluten feed were also used (Table 1).

Table 2. *Expt 4. Composition (g/kg on a dry matter (DM) basis), crude protein (nitrogen × 6·25) content and sample weights of rations tested*

(Amounts of starch added were always 30, 60 and 90 mg air-DM)

Ration	1	2*	3	4	5
Maize silage†	325	325	430	430	430
Hay	165	165	210	210	210
Concentrate	500	500	360	360	360
Mineral feed	10	10	—	—	—
Crude protein	149	153	138	138	102
NPN:N value	0	0	0	0·26	0
Sample weight (mg DM)‡	174	170	188	188	255

NPN, non-protein-N.
* Containing formaldehyde-treated soya-bean protein.
† Dried material (60°) was used for in vitro incubation.
‡ Whole diets were incubated.

Expt 3

Pectin, inulin, xylan, sorbitol, glucose, cellobiose, cellulose (chemically pure) and unpurified cellulose (toilet paper) were used, alone or in combinations, in place of maize starch to compare the effects on in vitro-degradable N (IVDN) determination of casein, extracted soya-bean meal and artificially-dried grass (Table 6, see p. 575).

Expt 4

Five rations, previously tested in studies with cannulated dairy cows at the Agricultural Research Station in Braunschweig (FAL), were taken for comparison with the in vitro method described. In vivo degradability of protein was measured using ^{15}N (Brandt & Rohr, 1981). The compositions of the rations are given in Table 2. In ration 2 half the protein was formaldehyde-treated soya-bean protein. Ration 1 was similar in composition but with untreated soya-bean protein. Ration 4 differed from ration 5 in having a supplement of urea, bringing the crude protein content to 138 g/kg, compared with 102 g/kg in ration 5. All rations were incubated for 8 and 24 h. Ration 2 was additionally tested at 6, 12, 15 and 17 h of incubation.

Expt 5

The effect of period of incubation on starch fermentation, pH and volatile fatty acid (VFA) production as well as the composition of the gas phase after 8 h incubation was investigated here.

The relationship between starch fermentation and gas production has been studied by incubation of constant amounts of rice starch (180 mg DM) for different periods of incubation. For determination of residual starch, the rumen fluid was adjusted to pH 4·8 with acetate buffer and incubated with 50 mg amyloglucosidase (*EC* 3.2.1.3) for 22 h (Salewski *et al.* 1974). The glucose formed was determined by the method of Luff-Schoorl (Naumann *et al.* 1976).

Immediately after addition of starch and subsequent cooling of the rumen fluid, 99% of the starch added was recovered. Incubations were stopped after 1·5, 3·0, 4·5, 6·0, 7·5 and 8 h to measure gas production and determine residual starch.

Changes in pH and composition of the VFA fraction were studied in a separate experiment, in which 150 mg starch and 8·9 mg urea were incubated as indicated in Table 8 (see p. 577). The composition of gas in the syringes was analysed after an 8 h incubation

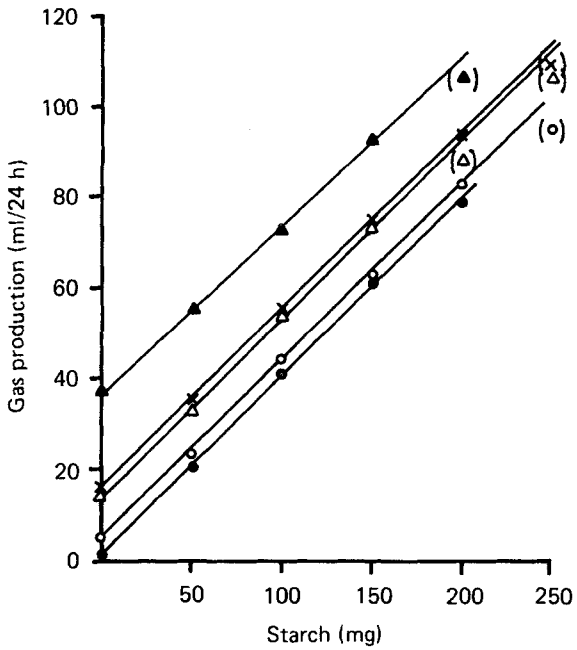

Fig. 1. Expt 1. Gas production (ml/24 h) in vitro with different amounts of starch (mg) added. Regression equations:

			R
Casein	(○)	$y = 5{\cdot}10 + 0{\cdot}3935\ x$	0·9994
Soya-bean meal	(△)	$y = 13{\cdot}83 + 0{\cdot}4050\ x$	0·9991
Rape-seed meal	(×)	$y = 16{\cdot}15 + 0{\cdot}3945\ x$	0·9997
Hay	(▲)	$y = 36{\cdot}53 + 0{\cdot}3800\ x$	0·9946
Feather meal	(●)	$y = 1{\cdot}70 + 0{\cdot}3965\ x$	0·9994

period. VFA, CO_2, methane and hydrogen were determined using a Hewlett-Packard gas-liquid chromatograph (for details of procedures, see Cafantaris, 1981).

Estimates of protein degradability

Estimates were calculated from appropriate linear regressions of NH_3-N concentration (y, mg) v. gas production (x, ml). The intercept b_0 represented that amount of NH_3-N which would be released when no fermentable carbohydrates were available and consequently no bacterial protein synthesis took place. The difference between this intercept b_0 and NH_3-N content in the blank (rumen fluid without substrate added) indicated the amount of NH_3 liberated from protein and other N-containing compounds of the feedingstuff incubated. IVDN was calculated as a proportion of total N by the equation:

$$\text{IVDN} = \frac{NH_3\text{-N at zero gas production } (b_0) - NH_3\text{-N of blank}}{\text{total N of feedingstuff incubated}}$$

The period of incubation adopted was generally 24 h. When different incubation periods (h) were used this is indicated by a subscript (e.g. $IVDN_8$).

RESULTS

Expts 1 and 2

With all feedingstuffs tested a linear increase in gas production was observed with increasing amounts of added starch (Figs. 1 and 2). The regression equations differed in their intercepts but had almost the same regression coefficients (approximately 0·39 ml gas/mg starch

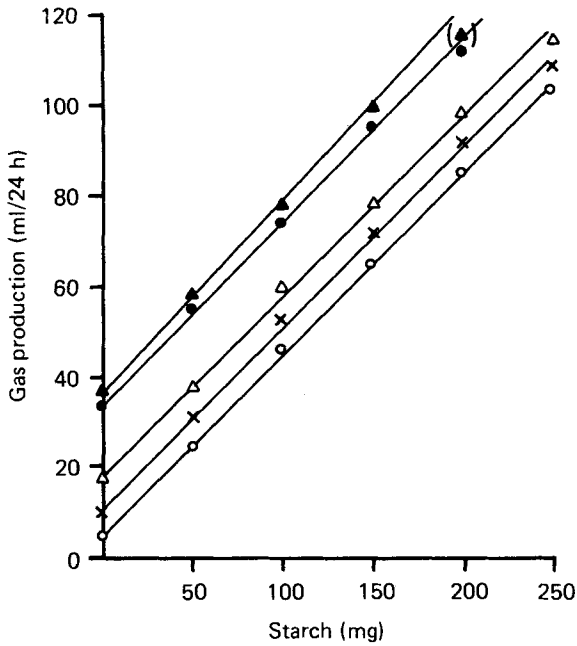

Fig. 2. Expt 2. Gas production (ml/24 h) in vitro with different amounts of starch (mg) added. Regression equations:

			R
Casein	(○)	$y = 5{\cdot}92 + 0{\cdot}3940\ x$	0·9996
Linseed-cake meal	(△)	$y = 20{\cdot}19 + 0{\cdot}3869\ x$	0·9983
Kapok residue	(×)	$y = 11{\cdot}99 + 0{\cdot}3940\ x$	0·9994
Hay	(▲)	$y = 37{\cdot}41 + 0{\cdot}4214\ x$	0·9978
Maize-gluten feed	(●)	$y = 34{\cdot}60 + 0{\cdot}4000\ x$	0·9954

added). Only when gas production rates were greater than 90 ml/24 h did lower increments occur. These values were omitted when calculating the regression equations.

NH_3-N contents measured after 24 h incubation decreased with increasing amounts of added starch. Again the intercepts were different between the feedingstuffs incubated, indicating differences in the degradability of the protein (Table 3). Regression coefficients also differed between feedingstuffs. For all feedingstuffs tested (except hay, which could be tested with three levels of starch only) linear regression equations were highly significant. The same was true for the relationship between gas production and NH_3-N content after 24 h (Table 4).

Mean (with SEM) values for blanks (*n* 3) were 4·68 (0·05) and 5·93 (0·09) mg NH_3-N in Expts 1 and 2 respectively. Subtraction of these blanks from the intercepts of regression equations in Table 4, and division of the difference by the total amount of N in the feedingstuff (4·16 mg) gave the IVDN values shown in Table 5. Expts 1 and 2 have in part been repeated with incubation periods of 12 h. The results are included in Table 5.

Expt 3

When starch is used as an energy source for bacterial protein synthesis, the assumption is made that gas production is a measure of energy available for protein synthesis, independent of the kind of carbohydrate fermented. Differences in fermentation pattern (VFA production) may, however, cause different ATP:CO_2 production values, and these may be reflected in the regression lines between gas production and NH_3-N utilization.

In Expt 3 maize starch was replaced by some other carbohydrates in IVDN determinations.

Table 3. *Regression equations (with standard errors of intercepts and regression coefficients in parentheses) showing the effect of the amount of starch added* (x, *mg*) *on ammonia-nitrogen content of rumen fluid after 24 h incubation* (y, *mg*)

Expt no.	Feedingstuff	Regression equation	*n*	*R*
1	Casein	$y = 8{\cdot}542\ (0{\cdot}0405) - 0{\cdot}0230\ (0{\cdot}0006)\ x$	10	−0·997
	Soya-bean meal	$y = 7{\cdot}955\ (0{\cdot}0520) - 0{\cdot}0220\ (0{\cdot}0007)\ x$	8	−0·995
	Rape-seed meal	$y = 7{\cdot}322\ (0{\cdot}0159) - 0{\cdot}0205\ (0{\cdot}0002)\ x$	10	−0·999
	Hay	$y = 4{\cdot}499\ (0{\cdot}0903) - 0{\cdot}0189\ (0{\cdot}0023)\ x$	6	−0·972
	Feather meal	$y = 5{\cdot}299\ (0{\cdot}0409) - 0{\cdot}0186\ (0{\cdot}0007)\ x$	8	−0·996
2	Casein	$y = 9{\cdot}948\ (0{\cdot}0486) - 0{\cdot}0181\ (0{\cdot}0006)\ x$	11	−0·995
	Linseed-cake meal	$y = 8{\cdot}712\ (0{\cdot}0564) - 0{\cdot}0146\ (0{\cdot}0009)\ x$	8	−0·986
	Kapok residue	$y = 8{\cdot}318\ (0{\cdot}0465) - 0{\cdot}0156\ (0{\cdot}0006)\ x$	11	−0·994
	Hay	$y = 6{\cdot}084\ (0{\cdot}0412) - 0{\cdot}0152\ (0{\cdot}0009)\ x$	7	−0·990
	Maize-gluten feed	$y = 7{\cdot}049\ (0{\cdot}0477) - 0{\cdot}0162\ (0{\cdot}0010)\ x$	5	−0·991

Table 4. *Regression equations (with standard errors of intercepts and regression coefficients in parentheses) showing the relationship between gas production* (x, *ml/24 h*) *and ammonia-nitrogen content of rumen fluid after 24 h incubation* (y, *mg*)

Expt no.	Feedingstuff	Regression equation	*n*	*R*
1	Casein	$y = 8{\cdot}80\ (0{\cdot}062) - 0{\cdot}0573\ (0{\cdot}0024)\ x$	10	−0·993
	Soya-bean meal	$y = 8{\cdot}61\ (0{\cdot}048) - 0{\cdot}0510\ (0{\cdot}0023)\ x$	8	−0·994
	Rape-seed meal	$y = 8{\cdot}19\ (0{\cdot}030) - 0{\cdot}0526\ (0{\cdot}0012)\ x$	10	−0·998
	Hay	$y = 6{\cdot}26\ (0{\cdot}108) - 0{\cdot}0491\ (0{\cdot}0068)\ x$	6	−0·963
	Feather meal	$y = 5{\cdot}38\ (0{\cdot}040) - 0{\cdot}0468\ (0{\cdot}0016)\ x$	8	−0·996
2	Casein	$y = 10{\cdot}22\ (0{\cdot}057) - 0{\cdot}0458\ (0{\cdot}0018)\ x$	11	−0·993
	Linseed-cake meal	$y = 9{\cdot}48\ (0{\cdot}053) - 0{\cdot}0387\ (0{\cdot}0018)\ x$	8	−0·994
	Kapok residue	$y = 8{\cdot}79\ (0{\cdot}052) - 0{\cdot}0395\ (0{\cdot}0016)\ x$	11	−0·993
	Hay	$y = 7{\cdot}41\ (0{\cdot}067) - 0{\cdot}0356\ (0{\cdot}0031)\ x$	7	−0·982
	Maize-gluten feed	$y = 8{\cdot}58\ (0{\cdot}014) - 0{\cdot}0432\ (0{\cdot}0008)\ x$	5	−0·999

Table 5. *Expts 1 and 2. In vitro-degradable nitrogen (proportion of total N)* after 12 h ($IVDN_{12}$) and 24 h ($IVDN_{24}$) of incubation*

Expt no.	Feedingstuff	$IVDN_{12}$	$IVDN_{24}$	Expt no.	Feedingstuff	$IVDN_{12}$	$IVDN_{24}$
1	Casein	0·86	0·99	2	Casein	—	1·03
	Soya-been meal	0·78	0·94		Linseed meal	0·72	0·85
	Rape-seed meal	0·71	0·84		Kapok residue	—	0·69
	Hay	—	0·38		Hay	—	0·36
	Feather meal	—	0·17		Maize-gluten feed	43	0·64

* $\dfrac{NH_3\text{-N at zero gas production } (b_0) - NH_3\text{-N of blank}}{\text{Total N of feedingstuff incubated}}$

Table 6. *Expt 3. In vitro-degradable nitrogen (IVDN)* of casein, soya-bean meal and artificially-dried grass, determined with different carbohydrates (24 h incubation)*

Carbohydrate added (% of 150 mg air-DM)										IVDN of:						
										Casein	Soya-bean meal				Artificially-dried grass	
Maize starch	Pectin	Inulin	Xylan	Sorbitol	Glucose	Cellobiose	Cellulose, purified	Cellulose not purified	Run...	1	2	3	4	5	6	7
									Sample wt (mg air-DM)...	30	56	56	56	56	115	115
									mg N...	4·47	4·16	4·16	4·16	4·16	4·16	4·16
100	—	—	—	—	—	—	—	—		0·97	0·79	0·79	0·77	0·78	0·60	0·65
70	5	—	5	—	—	10	—	10		—	—	—	0·80	0·82	0·63	0·63
40	15	—	10	—	—	15	—	20		—	—	—	0·81	0·79	0·63	0·68
10	25	—	15	—	—	20	—	30		—	—	—	0·79	0·81	0·63	0·62
—	100	—	—	—	—	—	—	—		0·94	0·75	0·74	—	—	—	—
—	—	100	—	—	—	—	—	—		0·98	0·81	0·80	—	—	—	—
—	—	—	100	—	—	—	—	—		0·98	0·83	0·83	—	—	—	—
—	—	—	—	100	—	—	—	—		0·95	0·78	0·79	—	—	—	—
—	—	—	—	—	100	—	—	—		0·96	0·78	0·79	—	—	—	—
—	—	—	—	—	—	100	—	—		0·96	0·81	0·79	—	—	—	—
—	—	—	—	—	—	—	100	—		1·04	0·89	0·88	—	—	—	—
—	—	—	—	—	—	—	—	100		1·01	0·84	0·85	—	—	—	—
SEM†										0·0057	0·0056	0·0061	0·0065	0·0058	0·0138	0·0203

DM, dry matter.
* Calculated from extreme measurements using the equation given in Table 9.
† Average standard error of the means of three observations.

Table 7. *Expt 4. Comparison of in vitro-degradable nitrogen (IVDN), determined after different incubation times (h) shown as subscripts, with rations previously tested in ^{15}N experiments with cannulated dairy cows, from which rumen degradability (RDN) has been calculated (Brandt, 1979; Brandt* et al. *1981)**

Ration no.	$IVDN_6$	$IVDN_8$	$IVDN_{12}$	$IVDN_{15}$	$IVDN_{17}$	$IVDN_{24}$		RDN*
1	—	0·67	—	—	—	0·76	0·78†	0·78
2	0·41	0·49	0·51	0·54	0·66	0·83	—	0·64
3	—	0·67	—	—	—	0·86	0·87†	0·82
4	—	0·55	—	—	—	0·72	0·65†	0·74
5	—	0·74	—	—	—	0·74	0·75†	0·76

* ^{15}N-labelled urea was infused continuously into the rumen during the first 4 d of a 5-d digesta sampling period. ^{15}N-excess was measured in bacterial and protozoal N isolated from duodenal digesta and rumen fluid, in non-ammonia-N of duodenal digesta and in milk (as an estimate of ^{15}N-excess in endogenous protein from postruminal secretion). Undegraded dietary N was calculated from microbial N and non-NH_3-N at the duodenum, corrected for endogenous N.

† Repeated with different rumen fluid.

The results in Table 6 show lower IVDN estimates with pectin and higher values with xylan and cellulose when these were added in amounts of 150 mg to soya-bean meal (56 mg). No other carbohydrates (inulin, sorbitol, glucose, cellobiose) were different from starch in their effects on IVDN. With casein, only the effects of cellulose and inulin were significantly different from starch. Mixtures of 700, 400 and 100 g starch/kg with 300, 600 and 900 g of other carbohydrates (pectin, xylan, cellobiose and unpurified cellulose)/kg resulted in higher IVDN estimates of casein, soya-bean meal and artificially-dried grass, compared with those produced with starch. The different proportions of starch in these carbohydrate mixtures seemed to have no effect on IVDN. There appeared to be a marked difference in the effects of chemically-purified and unpurified cellulose, the latter being more-readily fermented and causing lower IVDN values.

Expt 4

The results of in vitro incubations with rations previously tested in ^{15}N experiments with cannulated dairy cows are shown in Table 7 and compared with protein degradabilities obtained in vivo. In the first trial, these rations were tested in a 24 h incubation period. The results show fairly good agreement with the in vivo values for rations 1, 3, 4 and 5. In order to find an explanation for the difference found between these two methods when applied to ration 2, which contained protected protein, all rations were tested in an 8 h incubation period. In addition, samples of ration 2 were incubated for 6, 12, 15 and 17 h. The results are included in Table 7 and demonstrate that protection of protein is efficient up to 15 h of incubation but seems to be destroyed thereafter. Therefore, protein degradation after 24 h is as high in ration 2 as in ration 1, which contained untreated soya-bean protein. More than 80% of non-protected protein degraded within 24 h was degraded in the first 8 h.

Expt 5

The results of Expt 4 indicated that it may become necessary to study degradability of protein for different periods of incubation. Therefore, an experiment was set up in which disappearance of starch was measured for different periods of incubation. As can be seen from Fig. 3, a decrease in starch content in the rumen fluid was closely related to gas production, showing a small deviation from linearity in the first hours of incubation only.

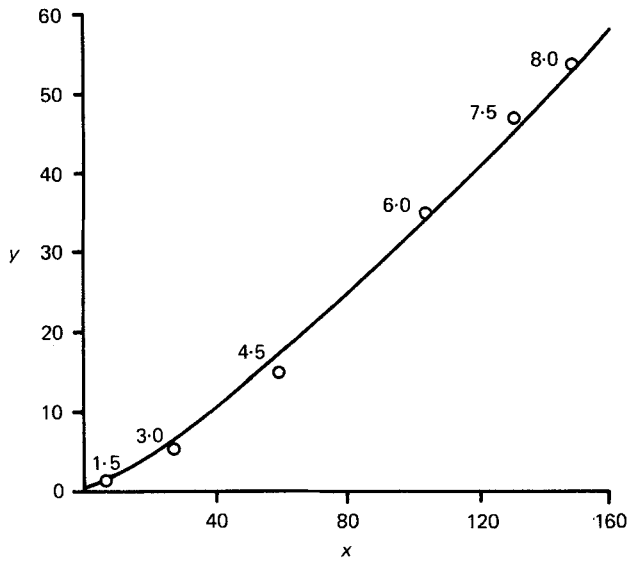

Fig. 3. Relationship between gas production (y, ml/24 h) and disappearance of starch (x, mg) during 1·5, 3·0, 4·5, 6·0 and 8·0 h of incubation (n 6).

$y = 0{\cdot}114\ x^{1{\cdot}233}$; R 0·999; residual SD 0·08.

Table 8. *Effect of period of incubation on pH and volatile fatty acids (VFA) in rumen fluid, and composition of gas during studies on protein degradation* (150 *mg starch and* 8·9 *mg urea*)

Period of incubation (h)...	4	6	8	10	12	SEM*
pH	—	—	6·69	6·61	6·56	0·02
Gas production (ml)	22·0	44·5	53·1	57·3	—	1·11
VFA production (μmol)	428	743	817	917	—	51
Acetate (mmol/mol)	710	635	649	660	—	12
Propionate (mmol/mol)	209	220	206	210	—	5
Butyrate (mmol/mol)	81	146	145	130	—	11
Gas production (μmol)	—	—	1775	—	—	20
Carbon dioxide (% total gas production)	—	—	83·9	—	—	0·6
Methane (% total gas production)	—	—	16·1	—	—	0·6
Hydrogen (% total gas production)	—	—	0·02	—	—	—

* Average standard error of the means of four to five observations.

Mean values for measurements of pH and VFA after 4–12 h incubation are given in Table 8. pH did not fall below 6·5. VFA production was approximately 17 μmol/ml gas produced. Acetate, propionate and butyrate were present in normal proportions (0·6–0·7, 0·2–0·22 and 0·08–0·15 respectively). In the first hours butyrate increased at the expense of acetate. Propionate proportions were fairly constant.

The gas phase of the syringes contained CO_2 and methane in the proportions 0·84:0·16 after 8 h of incubation. Hydrogen occurred in very small amounts.

DISCUSSION

Relationships between gas production and disappearance of NH_3-N

The results of Expts 1 and 2 demonstrated linearity between the amount of starch added and gas production on the one hand (Figs. 1 and 2) and a decrease in NH_3-N in rumen fluid on the other (Table 3). Consequently, the relationship between gas production and decrease in NH_3-N was linear also (Table 4). Deviations from linearity appeared with gas production rates of more than 90 ml/24 h, which corresponded to 200 mg starch added to 30–100 mg feedingstuff (DM basis) when the feedingstuff was high in protein (e.g. soya-bean meal, rape-seed meal) or the addition of 150 mg starch to 100–200 mg feedingstuff with less than 250 g crude protein/kg (e.g. hay, maize-gluten). The amount of protein incubated should be more than 20 mg in order to obtain reliable results. This may lead to rather high sample weights with feedingstuffs low in protein and to smaller ranges for the calculation of regression lines. It is to be expected, therefore, that IVDN values of those feedingstuffs will have higher standard deviations.

Effect of rumen fluid

Regression coefficients in Figs. 1 and 2 showed closely similar relationships between starch fermentation and gas production, irrespective of feedingstuff incubated. There were differences in regression coefficients in Tables 3 and 4, however, indicating effects of both rumen fluid and feedingstuff incubated on the disappearance of NH_3-N per unit carbohydrate fermented. The latter value was related to the efficiency of energy utilization for microbial protein synthesis, expressed as mg microbial N synthesized per g truly-digestible organic matter (MN/TDOM) or mg microbial DM synthesized per mmol ATP (Y_{ATP}). The regression coefficient for casein in Expt 1, for example, was 0·023 (SEM 0·0006) mg NH_3-N/mg added starch, corresponding to 23 mg microbial N/g TDOM (assuming starch to be 100% truly-digested) and 15·7 Y_{ATP} (calculated according to Hespell & Bryant, 1979 and Harrison & McAllan, 1980). In Expt 2 the regression coefficient for casein was significantly lower: 0·0181 (SEM 0·0006) mg NH_3-N/mg added starch, corresponding to 18·1 mg N/g TDOM and 12·3 Y_{ATP}. In spite of this difference, there seemed to be no effect of rumen fluid on IVDN values of casein (99, 103) and hay (38, 36) calculated from these regression coefficients (Table 5). In other words, different slopes met at the same point on the *y*-axis. This was confirmed in Expt 3 where IVDN measurements of soya-bean meal were made with four different batches of rumen fluid in time intervals of 1–10 weeks (Table 6, runs 2–5). Taking account also of other values (K. H. Menke, L. Raab, B. Cafantaris, K. Matthes and T. Jilg, unpublished observations) the following mean (with SEM) values of $IVDN_{24}$ estimates with rumen fluid from different days (*n*) were: casein 97·9 (1·1) (*n* 8), extracted soya-bean meal 90·9 (0·7) (*n* 6), extracted rape-seed meal 78·9 (1·3) (*n* 4), hay 37·8 (1·2) (*n* 3). (Average SEM of five observations on gas production and NH_3 concentration were 0·59 ml gas and 0·026 mg NH_3-N; Cafantaris, 1981.)

Casein seemed to be the most sensitive indicator of rumen fluid quality with regard to IVDN determination but, when compared with gas production from standard feedingstuffs routinely used for in vitro digestibility studies (hay and starch; Menke *et al.* 1979), there was no significant change of IVDN when gas production of these standard feedingstuffs varied by up to 5%. Larger deviations in gas production, however, should not be used for correction of IVDN estimates, since different protein sources are affected to a different extent. They simply may indicate when a series has to be discarded.

Effects of carbohydrates other than starch

The effects of different carbohydrates as energy sources in IVDN determinations have been studied in Expt 3. The results showed no significant difference in IVDN vaues when inulin, sorbitol, glucose or cellobiose was used in place of starch as an energy source (Table 6). Pectin caused somewhat lower, and cellulose higher, IVDN values than starch. Differences in microbial efficiency and in IVDN seem to be related to the rate of fermentation (ml gas/24 h). Gas production (ml/24 h) was particularly low with purified cellulose (40–42) compared with starch (67–70) or pectin (73–75). The following equations have been found with 56 mg soya-bean meal and 150 mg of the different carbohydrates (Table 6, runs 2, 3, 4 and 5):

$$y_1 = 150{\cdot}4 - 1{\cdot}39x; \quad r - 0{\cdot}88 \quad n\ 26$$
$$y_2 = 101{\cdot}4 - 0{\cdot}33x; \quad r - 0{\cdot}88 \quad n\ 26$$

where y_1 is NH_3-N disappearance (μg/ml gas produced) in 24 h, y_s is $IVDN_{24}$ (24 h incubation), x is gas production (ml) in 24 h.

With casein (run 1) and artificially-dried grass (runs 6 and 7) similar correlations were found. This relationship cannot be explained by differences in gas production per mg carbohydrate. Steingass (1983) found identical gas production from starch and from purified cellulose, when corrected for residual carbohydrate not fermented during incubation. There was a difference, however, in the time-course of fermentation. Gas production from starch was high in the first 12 h of incubation but rather low thereafter, indicating a period of starvation and microbial lysis. Fermentation of purified cellulose was low in the first 8 h and increased at a constant rate without inclination to a saturation point within a 24 h period. The extent of microbial lysis, therefore, must be higher with easily-fermentable carbohydrates, when the time of incubation was longer than needed for a given amount of substrate (150 mg in Expt 3). NH_3-N from microbial lysis was added to that NH_3-N not utilized and would thus reduce microbial efficiency (y_1). Again, the effect on IVDN (y_2) was less pronounced, as can be seen from comparison of the regression coefficients (1·39 *v.* 0·33). For a discussion of the relationship between rate of fermentation, incubation time and the efficiency of energy utilization for microbial protein synthesis, see Raab (1980).

There were no significant differences in IVDN determined with mixtures (g/kg) in the range of 100–700 starch with 50–250 pectin, 50–150 xylan, 100–200 cellobiose and 100–300 unpurified cellulose (Table 6, runs 4–7). Incubations with pure starch resulted in approximately 2% lower IVDN values. The back projection of a line determined with starch would differ in this order of magnitude from that ratio caused by the carbohydrate of the feedingstuff, provided its composition was in the range of mixtures used in Expt 3. Further experiments would show whether this difference is constant for all feedingstuffs of interest, thus allowing an additive correction of values determined with starch.

Estimation of protein synthesis from NH_3-N disappearance and gas production may include the utilization of amino acids and peptides for microbial protein synthesis, provided that the following assumptions are valid: (1) all preformed monomers are deaminated, when no gas production occurs in 24 h incubation; (2) there is a linear relationship between non-deaminated amino acids and gas production (see Fig. 4).

The first assumption is difficult to prove. Amino acids present after 24 h of starvation can be the result of microbial lysis and may not be due to incomplete deamination of amino acids deliberated from the protein of the feedingstuff added. The ratio of amino acids derived from microbial lysis to those deliberated from feed protein but not deaminated, may change with time of fermentation and amount of starch added.

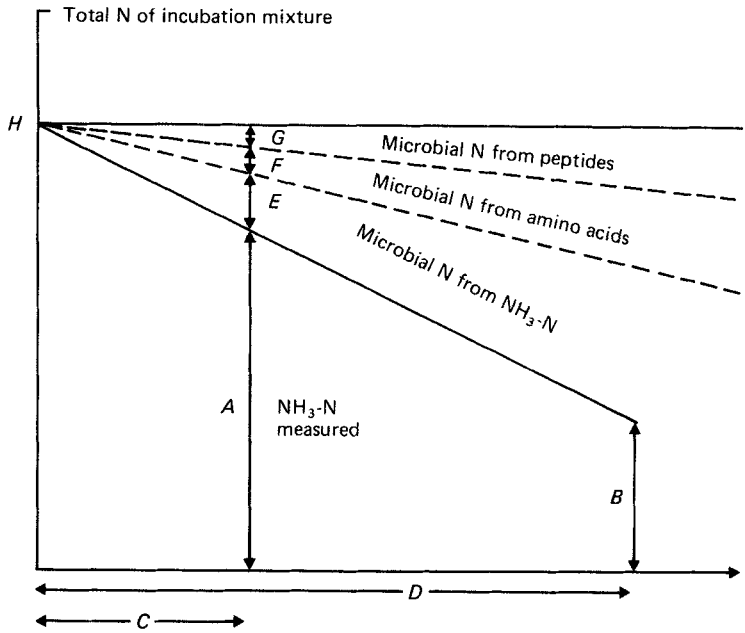

Fig. 4. Schematic representation of the relationship between protein degradation and protein synthesis in rumen fluid in vitro.
A, ammonia-nitrogen content after incubation of feedingstuff alone;
B, ammonia-nitrogen content after incubation of feedingstuff + starch;
C, gas production from feedingstuff alone;
D, gas productions from feedingstuff + starch;
E, *F*, *G*, microbial N expected to be derived from NH_3–N, amino acids and peptides, when the feedingstuff is incubated alone;
H, NH_3-N expected to be present at zero gas production.

Protein degradation and residence time in the rumen

In vitro methods not only have the advantage of being less expensive and less time-consuming, but also give the chance to maintain experimental conditions more precisely than in vivo trials permit. On the other hand, application of in vitro results to the prediction of in vivo events is dependent on how well the conditions are known of the in vivo event in question. Rumen degradability of protein, for example, is not only related to the kind of feedingstuff and nature of protein, but it is also a function of the residence time of the feedingstuff in the rumen. Ørskov & McDonald (1979) determined the rate of passage of soya-bean meal (treated with sodium dichromate) in the rumen of sheep and corrected protein disappearance from polyester bags using an exponential equation. In this way they calculated different final degradabilities for restricted (0·71) and *ad lib*-fed animals (0·66), which correspond to rumen incubation times of 12·5 and 10·5 h respectively. Mathers & Miller (1981) derived incubation times of 7·6–11·2 h from their experiments with sheep given chopped lucerne (*Medicago sativa*) and rolled barley, using the same exponential equation as Ørskov & McDonald (1979). Dilution rate of rumen fluid varied widely between sheep within diets, and individual animals showed no consistent trend across diets in these experiments. It seems even more difficult to derive a function from the many other experiments on mean retentions times in the rumen, reported in the literature (Evans, 1981; Jilg, 1982).

In Expt 4 (Table 7) IVDN values were close to in vivo degradabilities when incubations

Table 9. *In vitro-degradable nitrogen (IVDN) for 24 h incubations derived from four extreme measurements (two with 200 mg starch and two without starch), compared with IVDN values derived from regressions in Table 4, including all measurements* (n *5–11*)

	IVDN values calculated from:			
	Extreme measurements		All measurements	
Feedingstuff	*n*	$IVDN^{*}_{24}$	*n*	$IVDN_{24}$
Casein	4	1·01	10	0·99
Soya-bean meal	4	0·95	8	0·94
Rape-seed meal	4	0·83	10	0·84
Hay	4	0·42	6	0·38
Feather meal	4	0·18	8	0·17
Casein	4	1·00	11	1·03
Linseed-cake meal	4	0·83	8	0·85
Kapok residue	4	0·68	11	0·69
Hay	4	0·34	7	0·36
Maize-gluten feed	4	0·64	5	0·64

$$* \text{ IVDN} = \frac{A - \left(\frac{A-B}{C-D}\right) \times C - (\text{NH}_3\text{-N of blank})}{\text{total N of feedingstuffincubated}},$$

A is mg NH_3-N after 24 h incubation, when no carbohydrate is added; *B* is mg NH_3-N after 24 h incubation, with carbohydrate added; *C* is ml gas production in 24 h, when no carbohydrate is added; *D* is ml gas production in 24 h, with carbohydrate added.

were stopped after 17 h. The results indicate differences in the time-course of degradation of normal (rations 1, 3, 4 and 5) and protected proteins (ration 2). The latter reached only 60% of the value measured after 24 h within 8 h, whereas normal proteins reach 80% and more of the final value within 8 h. It may not be justified, therefore, to express protein degradability in vitro as a rate-constant to be adjusted to different retention times by a general function. We need to know the mean retention time of the feedingstuff in question, as a function of ration composition, level of feeding and animal characteristics, to be able to choose the appropriate incubation time for in vitro degradability studies. Further experiments must show whether factors other than incubation time cause marked differences between in vivo and in vitro determinations of protein degradation in the system used.

The ratio, substrate:inoculum was rather low (maximum 350 mg DM in 10 ml rumen fluid + 20 ml medium), thus pH did not fall below 6·2 in 24 h and accumulation of end-products of fermentation did not reach a critical level. At the end of incubation a dense population of protozoa could still be observed under the microscope, indicating a normal anaerobic fermentation. Lactic acid and hydrogen were present in traces only. The system tolerates up to 2 ml oxygen, introduced into the syringe at the beginning of incubation (Steingass, 1983), provided contamination with O_2 has been avoided when the rumen fluid is taken and the medium is CO_2-saturated before mixing with the rumen fluid.

For IVDN calculation from regressions, triplicates of samples with four levels of carbohydrate and a blank have to be incubated; in total, fifteen syringes. A reduction in the number of syringes to be used for one determination seems possible, when taking into consideration that the factors of the regression equations are much more affected by extremes (with and without a maximum of starch) than by measurements in between. When these extremes are used for the calculation of protein degradation, the results are close to these IVDN values derived from regression equations in Table 4 with up to eleven single incubations (Table 9).

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