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Proteolysis in the Sheep Rumen by Whole and Fractionated Rumen Contents

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SUMMARY: The proteolytic activity of the whole rumen fluid of sheep does not depend on the diet of the animals. The optimum pH value for proteolysis lies between 6 and 7, with an irregular maximum at about pH 6·5. All fractions of rumen contents (protozoa, large bacteria and small bacteria) show activity. Proteolytic activity can be demonstrated in the supernatant liquid after disruption of the microorganisms. The proteolytic activity of the whole rumen fluid was enhanced by cysteine at 0·06 and 0·006 % (w/v) unless the rumen fluid had been collected and processed under highly anaerobic conditions. Potassium cyanide and ascorbic acid were also slightly stimulatory, but sodium thiosulphate, thioglycollic acid, sodium sulphide and Fe^{++}, Ca^{++} and Mg^{++} ions had no effect.

The breakdown of protein in the rumen has been shown by *in vivo* experiments; McDonald (1954, 1957) and Weller, Gray & Pilgrim (1958) showed that a large proportion of the foodstuff protein was broken down and converted to microbial protein. Warner (1956) and Annison (1956) studied the proteolytic activity of sheep rumen contents or some fractions thereof *in vitro*; Hunt (1957) made some observations on bovine rumen contents. Some rumen bacteria, isolated because of their fermentative activities on various carbohydrates, have been found to produce a gelatinase, or in some cases to break down protein during bacteriological tests; but the only systematic search for proteolytic bacteria was made by Appleby (1955) who found *Bacillus licheniformis* to be the main proteolytic bacteria isolated from three sheep, and Hunt & Moore (1958) who isolated a *Flavobacterium* sp. from a cow. Since protein breakdown is one of the main functions of the rumen organisms and, of the large number of rumen bacteria so far characterized Bacillus types have been in the minority, it appeared worth while to investigate this problem further and to try to relate the results of attempts to isolate proteolytic bacteria with the proteolytic activity of the rumen contents as a whole. Since there was little data available at the beginning of the experiments, studies were also made on changes in proteolytic activity of rumen contents and breakdown of protein and redistribution of nitrogenous material during a feeding cycle. The results of these experiments are described in this paper. More extended observations from the *in vivo* experiments (Blackburn & Hobson, 1960) will be reported elsewhere. A preliminary account of some of these results has been given previously (Blackburn & Hobson, 1958; Hobson, 1959).

METHODS

Quantitative determination of protein hydrolysis. In all methods undegraded protein was precipitated by trichloroacetic acid (TCA). This protein was then redissolved and determined either by the biuret reaction or by micro-Kjeldahl nitrogen determinations. Tests showed that the results obtained by these two methods were similar and so the biuret reaction was usually used. In the second method the tyrosine and tryptophan in the filtered supernatant fluid after precipitation of the protein was determined by Folin reagent (Folin & Ciocalteu, 1927). The increase in substances reacting with Folin reagent, estimated as tyrosine, paralleled the decrease in protein determined by the biuret reaction in toluene-treated digests, although the theoretical amount of tyrosine (assuming a completely random hydrolysis) was not formed. All digests were incubated under toluene except where noted, and at 37° in test tubes held vertically.

Biuret determinations. Standard graphs were prepared by using different concentrations of casein dissolved in 0·1 M-phosphate buffer (pH 7·0) and casein precipitated by addition of 4 vol. 0·72 M-TCA and redissolved in N-sodium hydroxide. Although these plots differed slightly in slope, both were linear up to a concentration of *c.* 0·18 mg. casein when 2 ml. casein solution and 8 ml. biuret reagent (Layne, 1957) were used. Suitable portions of the digests were treated in a similar manner. A Unicam colorimeter was used for all measurements.

Determination by Folin reagents. The method used was essentially that of Anson (1938). Although the slope of the standard curve compared to that of a solution of tyrosine in water was altered when TCA in the concentrations used to precipitate protein was present, the curve was linear up to a weight of 250 μg. tyrosine, or to an optical density of 0·9, as the slopes of the curves varied in different experiments. With a high proportion of rumen fluid or with reducing substances added better results were obtained when the filtrates were treated before the Folin estimations by the oxidation procedure which Tracey (1948) used to remove reducing substances in plant juice. The irregularities appeared to be caused by reducing substances which were oxidized to different extents in different digests; this oxidation seemed to be affected both by the making up and by the pH value of the digests.

Reagents. Casein was B.D.H. Light White Casein (British Drug Houses Ltd., Poole, Dorset) or Eastman Casein (Kodak Ltd., Box 14, Hemel Hempstead, Hertfordshire) dissolved in 0·1 M-phosphate buffer (pH 7·0) except where noted. All other reagents were standard A.R. or Laboratory Reagent grades.

Sheep. Unless otherwise stated, rumen contents were obtained from sheep fitted with a permanent rumen cannula and fed on a daily diet of hay (900 g.) and concentrates (1 part crushed oats, 1 part wheat bran, 4 parts ground maize, $\frac{1}{2}$ part linseed cake meal, $\frac{1}{2}$ part white fish meal; 450 g.). Samples were usually taken about 2 hr. after feeding. 'Whole rumen fluid' refers to rumen contents which had been strained through muslin to remove the larger plant particles

and debris. 'Heated rumen fluid' had been placed in a boiling water bath for 5 min.

Description of organisms. An illustrated survey of the main morphological types of rumen bacteria was given by Moir & Masson (1952) and the 'large' and 'small' bacteria mentioned here will be found described in that paper. The rumen protozoa were described by Oxford (1955).

RESULTS

The proteolytic activity of rumen fluid of different sheep

Whole rumen fluid was incorporated into digests containing 0·1 M-phosphate buffer (pH 7·0) 5 ml.; 5% casein, 2 ml.; water or rumen fluid, 2 ml.; 0·6% L-cysteine hydrochloride, 1 ml. The concentration of cysteine hydrochloride chosen was about that used to reduce bacterial culture media. The digests were incubated with controls containing all the constituents except rumen fluid, or with heated rumen fluid. All were incubated at 37° for 48 hr. in air, preliminary results having shown that in the presence of cysteine results were similar whether the digests were incubated aerobically or anaerobically. The undigested casein was determined by biuret reagent. No appreciable proteolysis occurred in digests containing less than 0·1 ml. rumen fluid, so % hydrolysis in digests containing 1 ml. of rumen fluid were compared. The main morphological types of bacteria present in the different samples were also noted. The proteolytic activities are shown in Table 1. There appeared to be no correlation between proteolytic activity and diet or the predominating bacteria in the rumen fluid, so further experiments were done on sheep fed the Institute standard diet of hay and concentrates.

Table 1. *The proteolytic activity of whole rumen fluid from different sheep*

Sheep diet	Activity as hydrolysis of casein (%)	Sheep diet	Activity as hydrolysis of casein (%)
HG	99	HCS	75
HG	97	HG	70
HC	89	HG	63
HC	82	HG	60
HG	85	HG	55
HG	77	HG	48
HC	76		

Diets. H, hay; G, dried grass; C, different concentrates; S, potato starch. The digest constituents are described in the text.

Proteolysis by whole rumen fluid measured by Folin reagent

Digests contained whole rumen fluid, 4 ml.; 0·2 M-phosphate buffer pH 7, 5 ml.; 5% casein, 4 ml.; water, 7 ml. Controls were set up containing heated rumen fluid, without rumen fluid and also without casein. These digests were incubated anaerobically under CO_2 with and without a layer of toluene. The results are shown in Table 2. Incubation for 18 hr. was selected as giving a

measurable degree of proteolysis for further experiments and this time was used except where noted. The increase in non-precipitable material reacting with Folin reagent in the digests without casein was due to hydrolysis of protein already present in the rumen fluid.

Table 2. *Proteolysis by whole rumen fluid measured by Folin reagent*

Incubation temperature = 37°

Digest		Time of incubation (hr.) 1	2	18
		Colorimeter reading (OD)		
Rumen fluid	+casein	0·316	0·265	0·880
	−casein	0·195	0·173	0·215
Rumen fluid*	+casein	0·230	0·312	0·920
	−casein	0·208	0·239	0·301
Heated rumen fluid	+casein	0·155	0·176	0·275
	−casein	0·176	0·195	0·192
No rumen fluid	+casein	0·005	0·009	0·005
	−casein	0·004	0·004	0·008

* Under toluene. The digest constituents are described in the text.

The optimum pH *value for proteolytic activity of whole rumen fluid*

The digests contained: whole rumen fluid, 1 ml.; phosphate + citrate buffers of different pH values, 5 ml.; 0·6% (w/v) cysteine hydrochloride, 1 ml.; water, 1 ml.; 5% casein dissolved in dilute NaCl, 2ml.; control digests were (i) without casein, and (ii) with casein but with heated rumen fluid. The actual pH values of the digests before and after incubation were measured with a Pye pH meter, as they differed slightly from the buffer pH values. The extent of proteolysis was measured with Folin reagent. The results are shown in Fig. 1. Repeat experiments always showed an optimum about pH 6·5, but the sharpness of the optimum varied, and in some cases the curve was comparatively flat between pH 6 and 7. The digests were generally made to pH 7, as this value had been used in the initial experiments.

The effect of various additives and oxidation on proteolysis by whole rumen fluid

To obviate as far as possible effects due to buffer salts, and to obtain a measurable amount of proteolysis in a short time, digests of the following composition were used, the digests being at *c.* pH 6·5: additive solution, 1 ml.; whole rumen fluid, 5 ml.; 3·3% (w/v) casein in dilute NaCl, 3 ml. After incubation for 2 hr. a portion, 2 ml., was removed and the protein precipitated by the addition of 0·72N-TCA, 2 ml.; 0·5 ml. of the filtrate was then used for determination of 'tyrosine' by Folin reagents after the oxidation procedure noted under Methods had been followed. Cysteine hydrochloride stimulated proteolysis at a final concentration in the digests of 0·06 and 0·006% (w/v) (being highest at the latter value), but was inhibitiory at 0·6%; no effect was found below 0·006%. Potassium cyanide was stimulatory at

0·02 M, but had little effect at other concentrations. Ascorbic acid was slightly stimulatory at 0·01 M. Sodium thiosulphate and thioglycollic acid had little effect; and sodium sulphide was inhibitory at concentrations above 0·001 M. Fe^{++}, Ca^{++} and Mg^{++} ions gave a precipitate in concentrations over 0·0001 M and had no effect at lower concentrations.

In the presence of cysteine and a layer of toluene there was not a great deal of difference between digests incubated aerobically and anaerobically (see above). To test the effect of maintaining, as far as possible, completely anaerobic conditions during sampling and preparation of the rumen contents, and the effect of excess aeration, the following experiments were done. Rumen contents were taken from the animal directly into a flask containing liquid paraffin, rapidly strained into a second flask, again under paraffin, and incorporated into digests, as above, which were incubated under toluene in an atmosphere of CO_2. A portion of the strained rumen fluid was also taken and air was bubbled through it for 30 min.; after incorporation of

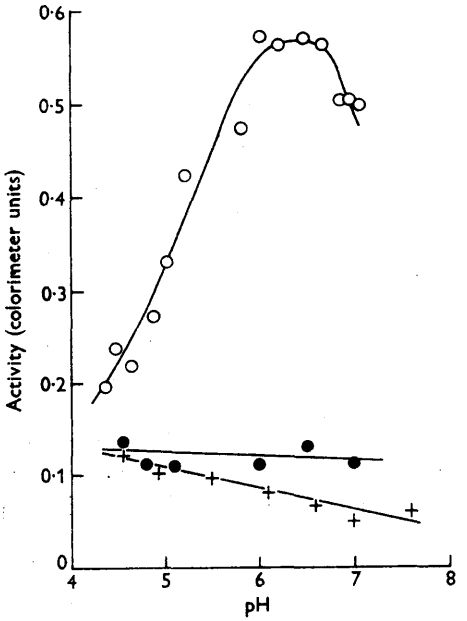

Fig. 1. The effect of pH on proteolysis by whole rumen fluid. ○, rumen fluid plus casein, ●, rumen fluid alone. +, heated rumen fluid plus casein. For digest conditions see text.

this material into digests these were shaken at intervals in air during the incubation period at 37°. The proteolytic activity of the strictly anaerobic digests was somewhat higher than that found in the first experiments in the presence of 0·06 and 0·006 % (w/v) cysteine, and 0·06 % cysteine had no effect under these conditions; 0·01 M-cyanide was slightly inhibitory. The oxidation procedure decreased the proteolytic activity to a value about that found in the first experiments when no activator was present.

Some inactivation of proteinases appeared to take place during the collection and processing of rumen fluid samples in air; this inactivation was not increased by bubbling air through the fluid. The only additives which were under the conditions of the experiments were reducing agents, and the activation seems to be due to their reducing properties, since the effect was lost when the rumen fluid was processed and incubated under anaerobic conditions.

The proteolytic activity of fractionated rumen fluid

Whole rumen fluid was fractionated, as shown in the diagram (Fig. 2), to separate as far as possible protozoa, large bacteria and small bacteria. Fractionation was done at room temperature except for the high-speed centrifugation which was carried out at 0°.

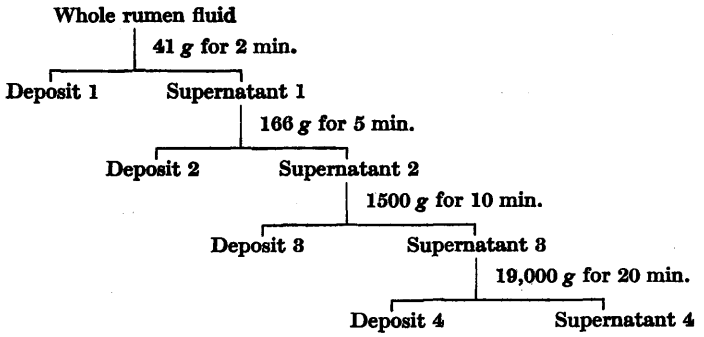

Fig. 2. The fractionation of sheep rumen contents by centrifugation. Fractionation was carried out at room temperature except for the centrifuging at 19,000 *g* which was carried out at 0°.

The first centrifugation (41 g.) was designed to separate the protozoa (deposit 1) with as little damage as possible and with few bacteria. A few protozoa were left in the supernatant (1) and these and some larger debris were removed by the second centrifugation (deposit 2). A small amount of the bacteria was lost in this deposit. Each deposit was washed once in a buffer (pH 7·2) designed originally to give optimum conditions for protozoal activity (Eadie & Oxford, 1955) and suspended in buffer to the volume of the preceding supernatant fluid. The suspensions were examined in wet and stained dried preparations, and a portion was removed for determination of dry weight after correcting for the weight of buffer salts. Finally a portion was incorporated in a digest as follows: fraction, 4 ml.; 0·2 M-phosphate buffer (pH 7·0) 5 ml.; 5% casein solution, 4 ml.; water, 7 ml. A second series of digests incorporated 0·06% (w/v) cysteine hydrochloride and corresponding controls without casein were also incubated. The extent of proteolysis was determined by Folin reagent and the activity determined on an arbitrary scale of (OD of Folin determination/mg. dry wt.) $\times 100$. The results of two experiments are shown in Tables 3 and 4. In all the fractionations carried out the combined activities of the fractions was somewhat higher than that of the whole fluid.

The protozoal fraction (1) always contained the highest percentage of the total activity, followed by the large bacteria (3) and small (4) bacteria fractions, but the large-bacteria fraction had the highest activity/unit weight. The activity of the supernatant (4) was usually very small without cysteine and was increased with it, but not usually to as high a figure as that shown in Table 3 (e.g. in one experiment the increase was from 0 to 0·084). The dry matter in the supernatant (4) was always high and seemed to be made of cell debris, soluble foodstuff material and possibly some very small bacteria. When attempts were made to produce a protozoal fraction entirely free from bacteria, by sedimentation under gravity or by more rigorous washing, the organisms rapidly died and most of the activity was lost. The activity of the large-bacteria fraction was increased when incubated under anaerobic conditions and cysteine had a pronounced effect in the anaerobic digest, the increase

Table 3. *The proteolytic activity of whole and fractionated rumen fluid*

	Cysteine		Expt. 1		
			Cysteine		
	None	Added	None	Added	Expt. 2 no cysteine
	Proteolytic activity (increase in OD with Folin reagent due to hydrolysis of casein)		Activity per unit dry wt.		
Fraction					
Whole fluid	0·468	0·595	1·5	2·0	1·2
Deposit 1	0·208	0·260	4·5	5·8	3·4
Supernatant 2	0·318	0·538	1·6	3·1	1·0
Deposit 3	0·151	0·200	*	*	103·5
Supernatant 3	0·802†	0·202	—	1·1	0·5
Deposit 4	0·103	0·098	20·6	19·6	13·0
Supernatant 4	0·059	0·384	0·5	3·2	0·0

* Weight of deposit too small for accurate measurement.

† Anomalous result.

The constituents of the digests and methods of fractionation are given in the text. A description of the fractions is given in Table 4.

Table 4. *The main constituents of the fractionated rumen fluid*

Fraction	Constituents
Whole fluid	Protozoa plus all bacteria of fractions
Deposit 1	Mainly protozoa, but with long chains of Gram-negative cocci, *Oscillospira*, Quin's ovals and large (2 μ) Gram-negative cocci in pairs. Some small (1 μ) Gram-negative and Gram-positive cocci.
Deposit 3	Mainly large Gram-negative cocci, Quin's ovals and large Gram-negative curved rods, with small Gram-negative curved rods and a few spirilla and 'window pane' groups. Small Gram-positive, Gram-negative cocci, some in chains
Deposit 4	Small Gram-positive, Gram-negative coccoid bacteria. Debris, probably containing small Gram-negative bacteria (0·5 μ)
Supernatant 4	Gram-negative undifferentiated debris

in OD value with Folin reagent, due to proteolysis, being 0·668 and 0·740 in aerobic and anaerobic digests with cysteine and 0·410 in anaerobic digest without cysteine. (A more concentrated suspension of bacteria was used than in the previous experiments.) Addition of Mg^{++} had no effect.

Proteolytic activity of the disintegrated fractions

A fractionation was carried out as in Fig. 2 and samples of deposits 1, 3 and 4 in 0·1 M-phosphate buffer (pH 7·0) 15 ml., shaken with no. 12 ballotini beads (Chance Bros., Smethwick, 5 ml.) in a Mickle tissue disintegrator (Mickle, 1948) at maximum amplitude for 1½ hr. at room temperature. Some apparently whole bacteria, as well as debris, were seen in each fraction after disintegration, but all the protozoa were disintegrated. The treated fractions were cleared of beads and debris by centrifugation and the proteolytic activity measured with and without cysteine as above. The results are shown in Table 5. A better disintegration of the bacteria was obtained by shaking for 2 hr., but the activities of the deposits 3 and 4 were not much changed, while the protozoal extract needed more centrifuging to clear it and lost activity during the treatment.

Table 5. *Proteolytic activity of the cell-free liquid from disintegrated fractions of rumen fluid*

Cell-free extracts from	Activity*/mg. N	
	−cysteine	+cysteine
Deposit 1	0·49	1·24
Deposit 3	1·48	1·87
Deposit 4	0·67	1·50

* From increase in OD with Folin reagent due to hydrolysis of casein. Disintegration by shaking 1½ hr. with ballotini beads, as described in the text. The digest constituents are described in the text.

DISCUSSION

The results described here agree with those of other workers in showing that active proteolysis is caused by whole rumen fluid and is not necessarily connected with active growth of the bacteria. The proteolytic activity of whole rumen fluid varies but does not appear to be dependent on the diet of the sheep; these results agree with those of Annison (1956) and Warner (1956) who tested washed suspensions of bacteria from a smaller number of sheep. By comparison with the results of Hunt (1957) the proteolytic activity of bovine rumen fluid appears to be higher than that of ovine fluid. For proteolysis pH 6–7 was optimum. The proteolytic activity of fractions containing the large bacteria and the small bacteria was relatively constant at pH 6 to 7; the protozoal activity appeared to be optimum at *c.* pH. 6·5. Variations in the protozoal activity may explain variations in the sharpness of the optimum found in different experiments. Annison (1956), who used a washed suspension of rumen bacteria, found little variation in activity at pH 6 to 7·5. Hunt (1957), whilst obtaining

a maximum activity at *c.* pH 6·9, also showed other pH optima at 5·7, 6·3, 7·5 and 8·2. In some experiments with whole rumen fluid and the supernatant fluid after disintegration of the organisms we have found apparent subsidiary pH optima, but the variation was so great in different experiments that it seemed unlikely that these were due to different proteinases unless these differed very much in activity. There is little free proteinase in the rumen fluid; the main activity seems to be connected with the microorganisms, but active solutions can be obtained after their disintegration and the activity of the different fractions appears to be relatively similar, on a nitrogen basis. The protozoa and large bacteria appear to play an active part in rumen proteolysis. Although it is impossible by centrifugation to obtain these fractions entirely free from smaller bacteria it seems unlikely that the activity of these fractions is due to contaminating small bacteria. In other experiments oligotrich protozoa were seen to ingest stained particles of casein which gradually disappeared inside the organisms, again suggesting that the protozoa are actively proteolytic.

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