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Colorimetric determination of *in vitro* feed protein degradation

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A colorimetric method for determining protein degradation using a diazonium chromophore was compared with the dacron bag technique. Diazotized fish meal, maize gluten meal and soya-bean were incubated with strained rumen fluid and a suitable growth medium. Only 49% and 18% of the colour bound to soya-bean was bound to fish meal and corn gluten meal, respectively. Degradation values expressed as a percentage of the total amount actually bound to the dye were in reasonable agreement with dacron bag estimates. However, initial hydrolysis of the diazo feeds was very rapid with little increment in breakdown after two hours incubation. It was concluded that this problem was probably largely a result of limited penetration of the chromophore to potentially reactive sites within the feed particles owing to the large molecular size of the diazonium chromophore.

'n Kolorimetriese metode vir die bepaling van proteïenafbraak met 'n diazonium kromofoor is met die dakronsak tegniek vergelyk. Diazo vismeel, mielie glutenmeel en sojaboon is met rumenvloeistof en groeimedium geinkubeer. Slegs 49% en 18% van die kleur gebind aan sojaboon is aan vismeel en mielie glutenmeel onderskeidelik gebind. Proteïenafbraak waardes, uitgedruk as 'n persentasie van die totale hoeveelheid gebind deur die kleurstof was in redelike ooreenstemming met dakronsak waardes. Aanvanklike hidrolise van die diazo-voere was baie vinnig, met slegs klein veranderings in afbraak na twee ure. Daar is tot die gevolgtrekking gekom dat hierdie probleem waarskynlik toe te skryf is aan die beperkte indringing van die kromofoor na potensieel reaktiewe punte binne die voer partikels weens die groot molekulêre grootte van die diazonium kromofoor.

Keywords: Nitrogen, protein, degradability, diazotization, *in vitro*, dacron bag

Introduction

Various methods are available for determining ruminal degradation of feed proteins. However, the need for a quick and easy laboratory method which is capable of handling large numbers of samples still remains. Solubility of feed

protein in various buffers or autoclaved rumen fluid remains an empirical method and affords no estimate of the rate of degradation, while the dacron bag method is subject to considerable variation and is influenced by the basal diet fed to the sheep. Thus a colorimetric method to estimate the rate and extent of protein degradation was developed from that reported by Mahadevan, Erfle & Sauer (1979) and compared with the dacron bag technique. The colorimetric technique involves treating feed proteins to form a highly coloured diazotized derivative, the colour of which is retained for as long as the protein remains undegraded. After exposure to microbial attack, the rate and extent of degradation is determined colorimetrically from the amount of undegraded acid-precipitable diazo-protein remaining. This method offers considerable advantages over existing methods in that large numbers of samples can be accommodated, and since only one component in the diet is marked, the degradation of one specific feed protein source in the presence of NPN or other feed proteins may be determined. The technique is thus eminently suitable for studying the effect of basal diet on protein degradation.

Materials and Methods

Diazotization Technique

Fish meal, maize gluten meal and uncooked soya-bean were diazotized as described by Mahadevan *et al.* (1979). Whereas the latter authors incubated the diazo-protein with a microbial suspension in potassium phosphate buffer, it was decided that the use of strained rumen fluid and a suitable growth medium would, for the purposes of this experiment, provide a closer simulation of *in vivo* degradation.

An amount of diazo-feed equivalent to 6,25 mg CP was incubated in 10 ml test tubes to which was added 2 ml rumen fluid (strained through two layers of cheesecloth) and 2 ml medium (Short, 1978). The medium contained 0,1% each of starch and glucose, 0,15% cellulose and 0,3% xylan. The tubes were gassed with a mixture containing 65% N_2, 30% CO_2 and 5% H_2, stoppered with bunsen valves and incubated in a waterbath at 39°C.

Triplicate tubes were withdrawn after 1, 2, 4, 6, 8, 12 & 22 hours, and 50 µl 60% perchloric acid was added to terminate bacterial action and to precipitate undigested protein. Samples were then centrifuged at 4 000 *x* g for 30 min, and the precipitate was stored at − 20°C until further analysis. The precipitated protein was solubilized by addition of 5 mg purified bacterial protease (Pronase, Boehringer Mannheim) in 2 ml potassium phosphate buffer (pH 7,6) at 37°C for 20 − 21 h. After incubation, samples were centrifuged and a 200 µl aliquot of the supernatant was mixed with 2,8 ml of 1,5 N NaOH. Absorbance was read at 456 nm using a 2 nm slit width on a varian DMS 90 double beam spectrophotometer.

Dacron Bag Technique

Polyester bags (pore size 53 µm) measuring 16 × 9 cm were used to suspend a 5 g feed sample in the rumen of sheep fed a diet of Lucerne hay *ad lib.* Three sheep were used simultaneously and the experiment was repeated once. Six bags were placed in the rumen of each sheep and removed at intervals, washed in tap water and dried. Residual N was determined by the macro-Kjeldahl method. Curves were fitted to the data by an iterative least squares procedure (Orskov & McDonald, 1979).

Results and Discussion

The total colour yield of 6,25 mg CP of the relevant feed was determined as above, except that no strained rumen fluid was added, and clarified rumen fluid was omitted from the medium. The amount of colour bound to the feeds differed considerably as reflected by maximum absorbance values of 0,659; 0,322; 0,115 for soya-bean, fish meal and maize gluten meal, respectively. These differences are probably related to differences in amino acid composition and permeability.

Standard curves relating absorbance to CP for each feed were determined and curves fitted to the data by linear regression. The diazo crude protein equivalent of the unknowns expressed as a percentage of that initially added to the tubes, i.e. 6,25 mg was then calculated by regression.

Calculated on this basis (Table 1) values for degradation appear to be unrealistically high. As the diazonium compound only binds to certain amino acids, in particular the tyrosine, histidine and lysine residues, it is probable that only a certain fraction of the protein was marked. Since the amount of colour bound to the various feeds differed, the values shown in Table 1 are representative only of a certain fraction of the feed, and not of the feed as a whole. If this fraction represents that fraction which is susceptible to bacterial degradation, the total extent of degradation should be expressed relative to some highly degradable feed such as soya-bean or casein. The absorbance value resulting from such a feed could then be equated with maximum (100%) diazo-binding.

Table 1 Percentage crude protein degradation for three feeds as estimated by two methods

	% Degradation					
	− diazo method			− dacron bag method		
Incubation time (h)	Soya-bean	Fish meal	Maize gluten	Soya-bean	Fish meal	Maize gluten
1	89,9	69,4	51,1	64,8	25,8	13,5
2	91,3	76,9	54,4	73,7	26,7	14,2
4	90,7	78,4	45,9	85,0	28,4	15,7
6	95,9	78,3	32,5	91,2	30,0	17,0
8	95,9	78,4	45,5	94,5	31,7	18,4
12	95,9	80,6	63,2	97,3	35,0	21,0
22	96,1	79,3	46,2			
24				98,5	44,7	28,4

In the following calculations the colour equivalents of fish meal and corn gluten meal were expressed relative to that of soya-bean, since a degradability of 98,5% was obtained for this feed after 24 h incubation in the rumen using the dacron bag technique. Expressed on this basis, only 48,9% and 17,5% of the colour bound to the soya-bean was bound to fish meal and corn gluten meal, respectively. If the

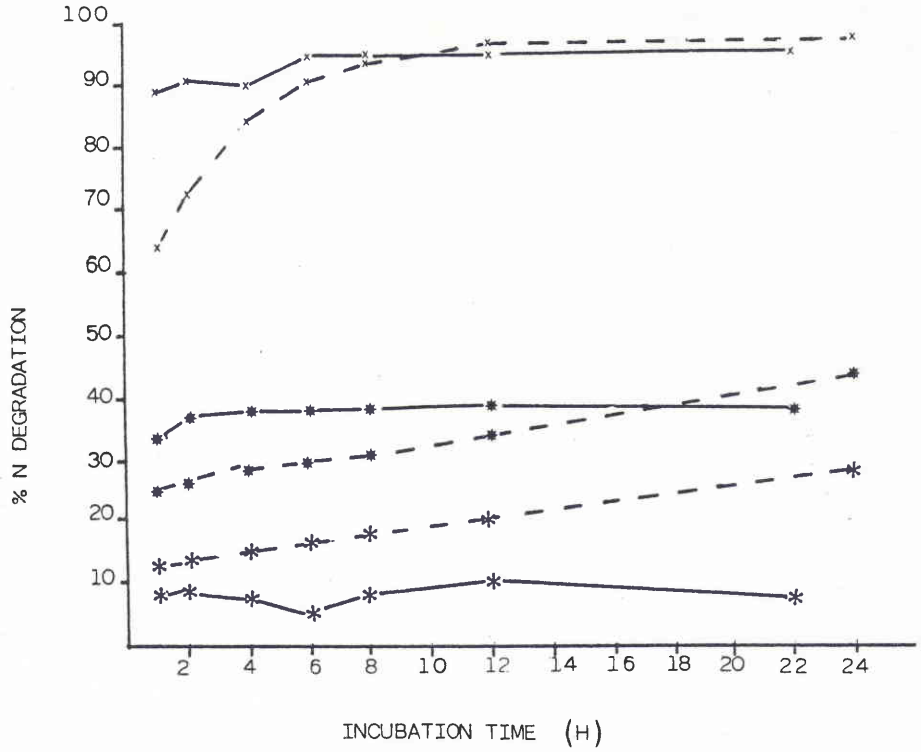

Figure 1 Crude protein degradation for soya-bean (x), fish meal (★), maize gluten (*) as determined colorimetrically (solid line) or by the dacron bag technique (broken line)

degradation values (Table 1) are then expressed as a percentage of the total amount actually bound to the dye, a more realistic picture emerges (Table 2). These degradation curves were then compared with curves obtained by the dacron bag method (Figure 1). Although reasonable agreement between the two methods was obtained, the very rapid initial hydrolysis and small increment in breakdown after 2 h incubation with the diazo method suggests that the diazonium chromophore was removed at a rate faster than the rate at which protein was degraded by the dacron bag method. This discrepancy between the two methods is especially apparent in the case of soya-bean. The rapid rate of hydrolysis could possibly be related to superactivation of proteolytic enzyme specificity owing to the bulky side group modification which was effected. An alternative hypothesis could be that the large molecular size of the diazonium chromophore restricted complete penetration to all potentially reactive sites, especially those buried within the feed particles. This is a problem even at molecular level. Limited penetration would result in modification mainly of superficial amino acids which would be the first to be degraded by proteolytic enzymes. This being the case, the achievement of more extensive penetration through the use of a smaller molecule such as ^{125}I could possibly provide a solution without necessitating finer grinding of the feed which would alter the susceptibility of proteins to bacterial attack.

Table 2 Proportional degradation of crude protein as determined colorimetrically

Incubation time (h)	% Degradation		
	Soya-bean	Fish meal	Maize gluten
1	89,9	33,9	8,9
2	91,3	37,6	9,5
4	90,7	38,3	8,0
6	95,9	38,3	5,7
8	95,9	38,3	8,0
12	95,9	39,4	11,0
22	96,1	38,7	8,1

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