Hydrolysis of ^{14}C-labelled proteins by rumen micro-organisms and by proteolytic enzymes prepared from rumen bacteria

BY R. J. WALLACE

Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

(*Received 22 December 1982 – Accepted 8 April 1983*)

1. Proteins were labelled with ^{14}C in a limited reductive methylation using [^{14}C]formaldehyde and sodium borohydride.

2. The rate of hydrolysis of purified proteins was little (< 10%) affected by methylation and the ^{14}C-labelled digestion products were not incorporated into microbial protein during a 5 h incubation with rumen fluid in vitro. It was therefore concluded that proteins labelled with ^{14}C in this way are valid substrates for study with rumen micro-organisms.

3. The patterns of digestion of ^{14}C-labelled fish meal, linseed meal and groundnut-protein meal by rumen micro-organisms in vitro were similar to those found in vivo.

4. The rates of hydrolysis of a number of ^{14}C-labelled proteins, including glycoprotein II and lectin from kidney beans (*Phaseolus vulgaris*), were determined with mixed rumen micro-organisms and with proteases extracted from rumen bacteria. Different soluble proteins were digested at quite different rates, with casein being most readily hydrolysed.

5. Proteins modified by performic acid oxidation, by cross-linking using 1,6-di-iso-cyanatohexane or by diazotization were labelled with ^{14}C. Performic acid treatment generally increased the susceptibility of proteins to digestion, so that the rates of hydrolysis of performic acid-treated proteins were more comparable than those of the unmodified proteins. Cross-linking resulted in a decreased rate of hydrolysis except with the insoluble proteins, hide powder azure and elastin congo red. Diazotization had little effect on the rate of hydrolysis of lactoglobulin and albumin, but inhibited casein hydrolysis and stimulated the breakdown of γ-globulin.

Measurements of the proteolytic activity of rumen micro-organisms and of the rates of digestion of different proteins in the rumen have been approached in several ways. The rate of digestion of feed proteins in vivo can be estimated by suspending nylon bags containing the material under study in the rumen of fistulated cattle or sheep (Ørskov *et al.* 1981). The loss of nitrogen from the dry matter is taken to represent the digestion of protein, although the soluble protein which is lost from the bag need not necessarily be hydrolysed. The digestion of soluble proteins in vivo has been studied with casein, using its high content of phosphorus to distinguish it from other proteins (Mangan, 1972); with zein, from its solubility in ethanol and lack of lysine (McDonald, 1954); and with ovalbumin, using soluble protein-N as the criterion of undigested protein (Mangan, 1972). In vitro, the production of ammonia-N and amino acid- and peptide-N from proteins incubated with rumen fluid can be used to measure proteolytic activity (Annison, 1956; Blackburn & Hobson, 1960*a*) although labelling of proteins by diazotization and following the release of colour into trichloroacetic acid (TCA)-soluble material is a much more convenient method (Mahadevan *et al.* 1979; Dinsdale *et al.* 1980; Brock *et al.* 1982; Wallace & Kopecny, 1983) and avoids difficulties associated with the microbial assimilation of the released N. The sensitivity of the diazotization method is, however, limited by the extent of modification which is possible with different proteins and also by the presence in rumen fluid of pigments which absorb at the same wavelength as the hydrolysis products. There may also be an influence of diazotization on the rate of digestion of the protein as diazotization slows the hydrolysis of casein (Hazlewood & Edwards, 1981; Kopecny & Wallace, 1982; Wallace & Kopecny, 1983) although it had no influence on the digestion of other proteins tested (Mahadevan *et al.* 1979; Wallace & Kopecny, 1983). The most

sensitive method so far described for measuring proteolysis in rumen fluid is one using as substrate lucerne (*Medicago sativa*) fraction I protein prepared from lucerne grown in the presence of $^{14}CO_2$ (Nugent & Mangan, 1978, 1981). Clearly the method is of considerable value in studying proteolysis, but it cannot be applied directly to many other proteins.

The aims of the experiments described in the present paper were to develop a method for the radioactive labelling of proteins, to evaluate these labelled proteins as substrates for proteolysis by rumen micro-organisms, and to examine further the relation between the structure of a protein and its susceptibility to proteolytic digestion in the rumen.

METHODS

Animals, diets and sampling

Six mature sheep fitted with rumen fistulas received twice daily a diet consisting of two parts hay or dried grass and one part of a barley-based concentrate. Samples of rumen fluid used in the measurement of proteolysis were removed 3 h after feeding and immediately strained through four layers of muslin. The rumen fluid was used in incubations without further treatment.

Preparation of proteases from rumen bacteria

A bacterial fraction was prepared from rumen fluid by differential centrifugation (Kopecny & Wallace, 1982). Proteases were extracted from the bacteria in this fraction by mechanical treatment in a Waring blender, which removes coat and capsular material from bacteria without cell breakage (Kopecny & Wallace, 1982). Since much of the proteolytic activity of the bacteria is extracted in this material, these enzymes will henceforth be termed 'coat proteases'.

Preparation of ^{14}C-labelled proteins

Oxidation of proteins by performic acid (Mahadevan *et al.* 1980), cross-linking with 1,6-di-iso-cyanatohexane (Ozawa, 1967) and diazotization (Kopecny & Wallace, 1982) were carried out before the ^{14}C-labelling procedure.

Casein was prepared by the method described by Blackburn & Hobson (1960*b*). White fish meal, linseed meal and groundnut meal were milled (mesh 0·7 mm) before use. The N content of casein, white fish meal, linseed meal and groundnut meal was 13·9, 11·5, 5·2 and 7·7% respectively. The other proteins were used in the form supplied by the manufacturers. Hide power azure and elastin congo red were fine powders and completely insoluble. The other proteins were soluble.

The labelling procedure, adapted from that used by Means & Feeney (1971) for the reductive alkylation of proteins, was carried out with 10 mg/ml suspensions or solutions of the above proteins. These were kept on ice and 0·015 vol. freshly-prepared sodium borohydride solution (0·5 mg/ml) was added. A few seconds later, 0·05 vol. [^{14}C]formaldehyde solution (0·1 mg/ml) was added. The mixture was kept on ice for 30 min, then dialysed against distilled water at 4° and freeze-dried. The specific radioactivity of the [^{14}C]formaldehyde used in these experiments was 0·1 Ci/g and that of the labelled protein product 3·6–19·4 μCi/g.

Measurement of proteolysis using ^{14}C-labelled proteins

Pronase. Pronase, a mixture of proteolytic enzymes prepared from *Streptomyces griseus*, was used to determine if the rate of release of radioactivity from ^{14}C-labelled proteins was equivalent to the rate of release of amino acids, and also if the label altered the susceptibility of proteins to proteolytic attack. The reaction mixtures, incubated at 39°, contained 25 mM-potassium phosphate buffer, pH 7·5, and 2 mg protein/ml. The pronase concentration

used depended on the protein substrate. With casein and its derivatives, 1·5 μg pronase/ml was used and with albumin, haemoglobin and γ-globulin the concentrations were 6, 25 and 250 μg/ml respectively. Samples were removed periodically into TCA to a final concentration of 50 g/l. The undigested protein was removed by centrifugation (31,000 *g*, 10 min, 4°) and the supernatant fluid was analysed for amino acids using the Folin reagent (Lowry *et al.* 1951) and radioactivity by liquid scintillation spectrometry.

Rumen fluid. In an experiment where the progress of digestion of native, ^{14}C-labelled and diazotized casein was followed, the reaction mixture was the same as that used for pronase incubations, except that pronase was replaced by strained rumen fluid at a final concentration of 500 ml/l. Samples were removed periodically and chilled, then centrifuged rapidly in a microcentrifuge (12000 *g*, 2 min) to remove micro-organisms. Undigested protein was then precipitated and the supernatant fluid and resuspended pellet were analysed as described above.

In another experiment in which the digestion of ^{14}C-labelled casein, fish meal, linseed meal and groundnut meal by rumen fluid was compared, the labelled substrate (2 mg/ml) was incubated in a shaking water bath with strained rumen fluid. The radioactivity released into TCA-soluble material was measured as before. Neutrase, a mixture of proteases from *Bacillus subtilis*, was added at a concentration of 20 ml/l to incubation mixtures in parallel with these, in order to determine the total radioactivity capable of being released by proteolysis, and to find out if the released radioactivity could be incorporated into microbial protein during the time-scale of these experiments.

The third type of experiment done with strained rumen fluid was to compare the rates of digestion of various ^{14}C-labelled purified proteins. The reaction mixture again contained strained rumen fluid (500 ml/l) in 25 mM-potassium phosphate buffer, pH 7·5, with 2 mg ^{14}C-labelled protein/ml. Replicate samples were removed at zero time and after 1 h, except with ^{14}C-labelled casein and lactoglobulin which were sampled after 30 min. The extent of digestion of the substrate was determined either by precipitation with 50 g TCA/l and centrifugation as before to measure acid-soluble radioactivity, or by precipitation of undigested protein on a glass-fibre filter (GFC; Whatman Laboratory Products Ltd, Maidstone, Kent) and measurement of the radioactivity remaining in acid-insoluble material. In the latter method, a 25 mm diameter filter-disc was soaked with TCA (250 g/l) and 20 μl of sample was applied to the filter. After 30 s, the filter was washed twice with 1 ml TCA (50 g/l) and the radioactivity remaining on the filter was measured by liquid scintillation spectrometry. The efficacy of this method was compared with the centrifugation method using azocasein and found to be satisfactory.

Coat proteases. The reaction mixture again contained 2 mg ^{14}C-labelled protein/ml of 25 mM-potassium phosphate buffer, pH 7·5, and cysteine hydrochloride (2·5 mM) and EDTA (10 mM) were added to stimulate the activity of the coat proteases (Kopecny & Wallace, 1982). The concentration of coat proteases depended on the rate of digestion of the substrate protein. With casein, for example, coat proteases were used at 0·5 mg/ml, whereas with albumin and γ-globulin they were 5 mg/ml. Digestion was measured by either the centrifugation or glass-fibre filter disc method described previously.

Haemagglutination

The haemagglutinin activity of native and ^{14}C-labelled lectins was assayed by the method described by Pusztai & Watt (1970).

Materials

Pure proteins were obtained from Sigma Chemical Company (Poole, Dorset), except for glycoprotein II and lectin, which were isolated from 0·1 M-borate, pH 8·0, extracts of

Phaseolus vulgaris using SP-Sephadex (Manen & Pusztai, 1982) and fetuin, which was prepared by the method of Spiro (1960). The pure proteins were lactoglobulin from bovine milk, bovine serum albumin, bovine Cohn fraction II γ-globulins, bovine haemoglobin type I and myoglobin from equine muscle. [^{14}C]formaldehyde was obtained from Amersham International, Amersham, Bucks. Pronase was from Calbiochem-Behring, Bishops Stortford, Herts and Neutrase solution was from Novo Enzymes Ltd, Windsor, Berkshire.

RESULTS

The concentration of formaldehyde used in labelling proteins in these experiments (5 μg/ml) was 0·5% of that used in the reductive methylation described by Means & Feeney (1971). At this concentration, maximum labelling of casein occurred at a $NaBH_4$ concentration of 7·5 μg/ml. Approximately 30% of the [^{14}C]formaldehyde became protein-bound during the reaction with casein as substrate, and a similar order of incorporation with other soluble proteins yielded ^{14}C-labelled proteins whose specific radioactivity varied from 8·4 μCi/g with lectin to 19·4 μCi/g with albumin. The feed proteins and insoluble proteins were less extensively modified, from 3·6 μCi/g elastin to 8·9 μCi/g fish meal. Stepwise addition of [^{14}C]formaldehyde, as recommended by Means & Feeney (1971), did not increase the labelling efficiency.

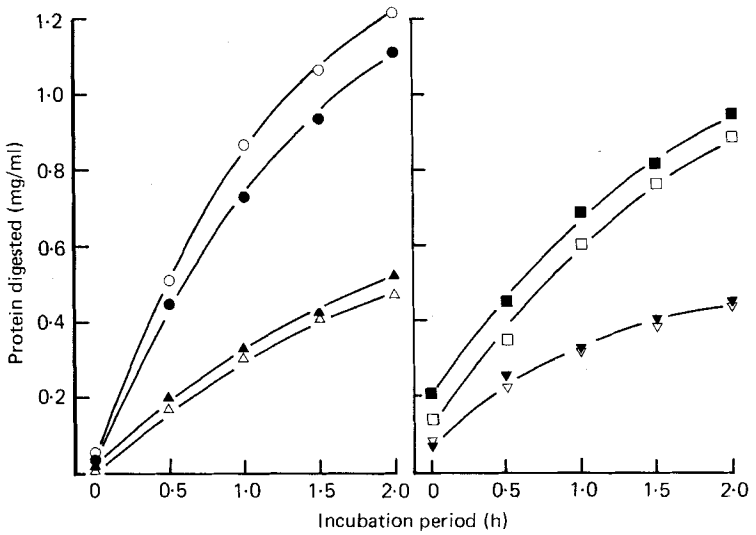

Fig. 1. Digestion of native proteins (○, △, □, ▽) and proteins labelled with ^{14}C by reductive methylation (●, ▲, ■, ▼) by pronase. The reaction mixture contained 2 mg protein/ml 25 mM-potassium phosphate buffer, pH 7·5, and pronase at 1·5 μg/ml with casein (○,●), 6 μg/ml with albumin (△, ▲), 25 μg/ml with haemoglobin (□, ■) and 250 μg/ml with γ-globulin (▽, ▼). Amino acids released into acid-soluble material were measured by the method of Lowry *et al.* (1951).

Proteins oxidized by performic acid had a similar reactivity to the native proteins, although the amount of label taken up by some oxidized proteins was increased while with others it was decreased. As would be expected, since both formaldehyde and the bifunctional cross-linking reagent 1,6-di-iso-cyanatohexane react principally with amino groups in proteins (Means & Feeney, 1971), previous reaction with 1,6-di-iso-cyanatohexane decreased the subsequent reactivity of protein with [^{14}C]formaldehyde. The soluble proteins treated in this way had a final specific radioactivity of approximately half that of the labelled

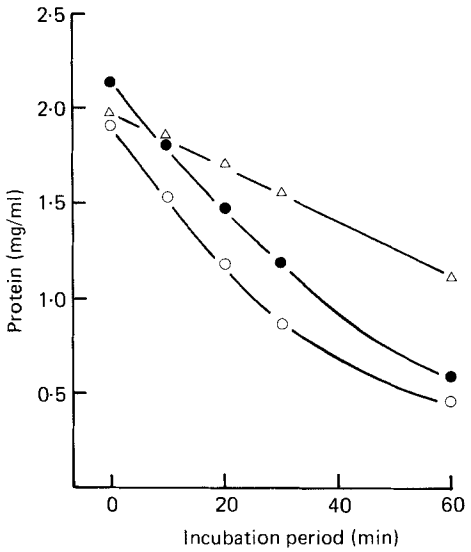

Fig. 2. Digestion of native (○), ^{14}C-labelled (●) and diazotized (△) casein by rumen fluid taken via the rumen cannula of a sheep receiving a grass+concentrate (2:1, w/w) diet. The reaction mixture contained 2 mg protein and 0·5 ml strained rumen fluid/ml 25 mM-potassium phosphate buffer, pH 7·5. Samples were chilled and centrifuged immediately (12000 *g*, 2 min) and the protein content of the supernatant fluid was measured by the method of Lowry *et al.* (1951) by precipitating in 50 g trichloroacetic acid/l and centrifuging.

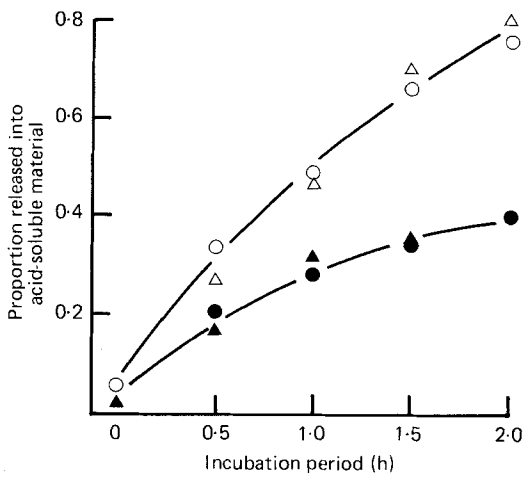

Fig. 3. Release of amino acids (○, ●) and radioactivity (△, ▲) into acid-soluble material during digestion of ^{14}C-labelled casein (○, △) and ^{14}C-labelled albumin (●, ▲) by pronase. Conditions were the same as for Fig. 1.

unmodified proteins. Similarly, diazotization modifies amino groups among others (Means & Feeney, 1971) and the efficiency of labelling of diazotized proteins with ^{14}C was on average 30% lower than that of untreated proteins.

The rates of digestion of albumin, haemoglobin and γ-globulin by pronase were unchanged by ^{14}C-labelling, while the rate of hydrolysis of casein was decreased by only about 8% (Fig. 1). A similar extent of inhibition of casein hydrolysis was observed with

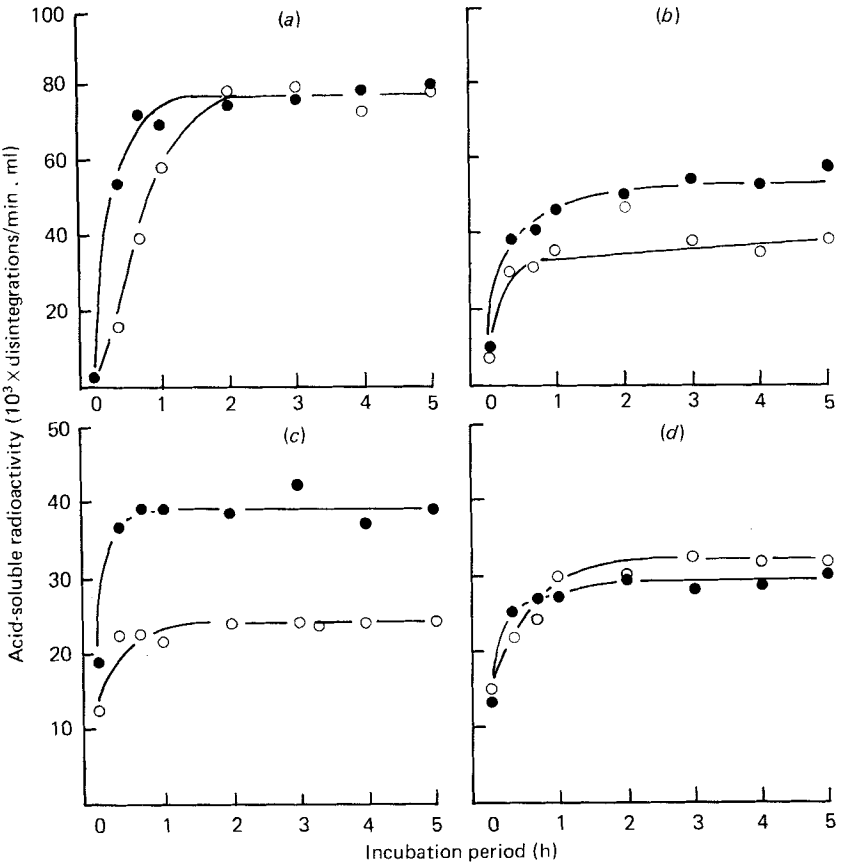

Fig. 4. Digestion of ^{14}C-labelled proteins by rumen fluid taken from a sheep receiving grass + concentrates. Proteins were suspended to a concentration of 2 mg/ml in strained rumen fluid (○) or strained rumen fluid + Neutrase (20 ml/l) (●) and the appearance of ^{14}C in acid-soluble material was followed: (*a*) casein, (*b*) linseed meal, (*c*) fish meal, (*d*) groundnut meal.

rumen fluid (Fig. 2). This compared with a variable inhibition caused by diazotization (Fig. 2, Table 2). A further indication of how little protein structure was changed by ^{14}C-methylation was that the haemagglutinin activity of lectin prepared from *Phaseolus vulgaris* was not at all diminished in the ^{14}C-labelled protein. Although lectin was labelled least of all the pure soluble proteins, its specific radioactivity was still of a similar order to the other proteins, indicating that their structure is likely to be little more modified than that of lectin. It was therefore concluded that the structural properties of proteins were not significantly changed by the reductive methylation described previously.

If ^{14}C-labelled proteins were to be valid substrates for proteolysis, it was necessary that the label should be uniformly distributed within the protein, so that the release of radioactivity would give a true measure of the rate of breakdown of the polypeptide chain. During the digestion of [^{14}C]casein and [^{14}C]albumin by pronase, the appearance of ^{14}C in acid-soluble material closely paralleled that of amino acid (Fig. 3), indicating that this requirement was satisfied.

The ^{14}C-labelled products of casein breakdown by Neutrase, another broad-specificity bacterial protease preparation, were not re-incorporated into acid-insoluble material during a 5 h incubation with rumen fluid (Fig. 4(*a*)) or in a similar incubation with mixed rumen

bacteria (not shown). The same was also true for ^{14}C-labelled linseed meal, fish meal and groundnut meal (Fig. 4(*b*, *c*, *d*)). Thus the appearance of ^{14}C in acid-soluble material can be used as an alternative to measuring its loss from acid-insoluble material in proteolytic measurements.

Time-courses of the digestion of ^{14}C-labelled proteins in rumen fluid in vitro (Fig. 4) showed that, while casein was completely hydrolysed within 2 h and the digestion of groundnut meal was complete after approximately 3 h, neither linseed meal nor fish meal was completely digested by the rumen micro-organisms after 5 h. The initial rate of digestion of fish meal was more rapid than groundnut meal, but the former was digested to an extent of only 45% compared with 100% for groundnut meal.

Individual pure proteins were hydrolysed in rumen fluid at very different rates (Table 1). Casein was digested most quickly, at 6·5 (SE 1·1) mg/ml per h in rumen fluid from sheep fed on dried grass and concentrates and 1·9 (SE 0·3) mg/ml per h in sheep fed on hay and concentrates. Lactoglobulin was digested at 64% of this rate and other proteins were digested at much slower rates. γ-Globulin was particularly resistant, at 6% of the rate of casein, and the rate of hydrolysis of ^{14}C-labelled elastin congo red was so slow as to be impossible to measure accurately (Table 1). A similar pattern was seen when these ^{14}C-labelled proteins were subjected to digestion by bacterial coat proteases, except with hide powder azure which was digested relatively much more quickly by the coat proteases than by rumen fluid (Table 1).

Table 1. *Digestion of ^{14}C-labelled purified proteins by rumen fluid and by coat proteases extracted from rumen bacteria*

(Activity is expressed as a percentage of the rate of hydrolysis of [^{14}C]casein. The activity of strained rumen fluid was (mean with SE) 6·51 (1·08) mg [^{14}C]casein digested/ml per h for sheep receiving a dried-grass+concentrates (G) diet and 1·93 (0·34) mg [^{14}C]casein digested/ml per h for sheep receiving a hay+concentrate (H) diet. Coat proteases were prepared from the rumen fluid of sheep receiving diet G, and their activity was 0·77 (0·40) mg [^{14}C]casein digested/mg coat proteases per h. The results are expressed as means and standard deviations of *n* determinations, each done in duplicate, using different samples of rumen fluid or coat proteases taken from different sheep or on different days)

		Rumen fluid			Coat proteases		
Protein	Diet	Mean	SD	*n*	Mean	SD	*n*
Casein	H/G	100	—	—	100	—	—
Lactoglobulin	G	63·8	16·2	6	49·1	16·9	6
Haemoglobin	G	30·9	13·6	8	18·6	10·9	6
Glycoprotein II	H	14·0	5·2	14	7·6	0·9	4
Lectin	H	14·0	5·2	14	—	—	—
Hide powder azure	G	13·4	9·9	6	70·6	23·7	9
Fetuin	G	10·9	7·6	10	—	—	—
Myoglobin	G	8·6	2·6	6	7·2	2·4	4
Albumin	H	9·7	4·6	14	—	—	—
Albumin	G	4·7	3·6	7	5·7	5·5	6
γ-Globulin	G	6·2	1·0	6	2·5	1·7	6
Elastin congo red	G	0·4	2·8	6	2·0	1·4	6

Chemical modification of some of these proteins prior to labelling with ^{14}C caused changes in their rate of digestion by coat proteases (Table 2). Oxidation by performic acid increased the rate of digestion of all proteins except casein, which was depressed slightly. The effect was most marked with the more resistant soluble proteins, albumin and γ-globulin, whose rates of digestion were increased by factors of 18 and 29 respectively. Indeed, the performic acid-treated proteins were digested at quite similar rates compared with the

untreated proteins, whose rates varied over nearly two orders of magnitude. The only exception was ^{14}C-labelled elastin congo red, whose rate of digestion was increased by performic acid, but which still remained highly resistant.

Cross-linking by 1,6-di-iso-cyanatohexane caused a decreased rate of hydrolysis of the soluble proteins but had no influence on the insoluble proteins, hide powder azure and elastin congo red (Table 2). Unlike performic acid oxidation, the effect was of similar magnitude with each of the soluble proteins, varying from 53 to 74% inhibition.

Diazotization of the soluble proteins had little effect on the rate of digestion of lactoglobulin or albumin by coat proteases (Table 2). Casein digestion was inhibited by diazotization (Table 2) but to a lesser extent than with rumen fluid (Fig. 2). In contrast, diazotized γ-globulin subsequently labelled with ^{14}C was digested much more rapidly than the ^{14}C-labelled native protein (Table 2).

Table 2. *Influence of chemical modification on the rate of digestion of ^{14}C-labelled proteins by coat proteases*

(Coat proteases were prepared from rumen bacteria of sheep receiving a dried-grass + concentrates diet. Oxidation of proteins by performic acid (P), cross-linking by 1,6-di-iso-cyanatohexane (H) and diazotization (D) were carried out as described on p. 346 before labelling with ^{14}C. Activity is expressed as mg protein digested/mg proteases per h and values are means and standard deviations for four determinations)

Treatment...	None		P		H		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Casein	0·995	0·143	0·839	0·091	0·471	0·079	0·839	0·131
Lactoglobulin	0·484	0·073	1·038	0·066	0·126	0·033	0·618	0·060
Albumin	0·072	0·020	1·311	0·063	0·027	0·016	0·075	0·028
γ-Globulin	0·023	0·006	0·675	0·273	0·010	0·003	0·110	0·020
Hide powder azure	0·649	0·128	0·942	0·100	0·665	0·047	—	—
Elastin congo red	0·009	0·004	0·031	0·006	0·019	0·005	—	—

DISCUSSION

The use of radioactively-labelled proteins as substrates for the study of proteolysis of rumen micro-organisms is not a new idea. Mahadevan *et al.* (1979) suggested, in the conclusion to their study of diazotized proteins as substrates, that ^{14}C-labelled sulphanilic acid could be used in the diazotization reaction to increase the sensitivity of their assay, but this does not seem to have been pursued. Nugent & Mangan (1978, 1981) found lucerne fraction I protein isolated from plants grown in a $^{14}CO_2$ atmosphere to be a valuable substrate in their studies of proteolysis, as it enabled them not only to measure protein degradation but also to follow the binding of protein to microbial cell surfaces as a preliminary step in its digestion. The method described in the present paper is much more widely applicable than the latter because any protein, including feed proteins of both animal and plant origin, can be labelled with ^{14}C by reductive methylation. It also has the advantage that, unlike unmodified ^{14}C-labelled amino acids, the hydrolysis products of [^{14}C]formaldehyde-labelled proteins are not reincorporated into microbial protein during the time-scale of most digestion experiments (Fig. 4). Increases in acid-soluble radioactivity can therefore be used to measure digestion instead of decreases in acid-insoluble ^{14}C-protein, which means that low proteolytic activities can be measured more accurately.

Proteins labelled with [^{14}C]formaldehyde by the procedure described in the present paper appear to be valid substrates for proteolysis, as they were seen to be uniformly labelled

(Fig. 3) and were digested at or close to the same rate as native proteins by pronase (Fig. 1) and rumen fluid (Fig. 2). The method of labelling was based on the one described by Means & Feeney (1971) who sought maximum methylation of amino groups of proteins and achieved > 80% modification of amino groups. In this work, in which minimal but high specific activity modification was required, 0·5% of the formaldehyde concentration of Means & Feeney (1971) was used. A further indication of how little protein conformation had been altered was that the agglutinin activity of ^{14}C-labelled lectin was not diminished at all compared with the native protein.

A protein substrate concentration of 2 mg/ml was chosen in these experiments because it is a concentration at which the rate of casein hydrolysis is maximal. It greatly exceeds the K_m for casein, which is approximately 0·1–0·2 mg/ml (R. J. Wallace, unpublished results) but it is, nevertheless, a compromise, since some substrate inhibition occurs in this range (R. J. Wallace, unpublished experiments) as was also found with the proteolytic *Bacteroides ruminicola* strain R8/4 (Hazlewood & Edwards, 1981). Furthermore, the K_m of other proteins is almost certainly higher than that of casein, so the comparisons drawn in Tables 1 and 2 should not be assumed to be at V_{max}. These tables thus only give an indication of the comparative susceptibility of different proteins to attack by microbial proteolytic enzymes at protein concentrations which might occur in vivo.

^{14}C-labelled casein was hydrolysed by strained rumen fluid in vitro at rates which varied from 1·9 to 6·5 mg/ml per h (Table 1, Figs 2 and 4). Mangan (1972) reported that the half-life of casein in the bovine rumen was 5·6–21·5 min for a 100 g administration to a 60 l rumen. Thus, there was a range of rate constants of 1·9–7·4/h in these experiments, from which a range of initial rates of casein digestion can be calculated to be 3·2–12·3 mg casein digested/ml rumen fluid per h, approximately twice the rate observed in the present experiments. However, straining of rumen fluid, used here to obtain a homogeneous preparation, is likely to remove at least half of the micro-organisms present (Forsberg & Lam, 1977). It can therefore be concluded that this in vitro method gives rates of hydrolysis similar to those found in vivo. The finding reported here that proteolytic activity is higher on a grass+concentrate diet than on a hay+concentrate diet (Table 1) is an example of how the method may be applied to in vivo work, as similar results have been found with sheep and cattle (Nugent & Mangan, 1981).

The degradation of ^{14}C-labelled feed proteins (Fig. 4) also gave results which were readily identifiable with those seen in vivo (Ørskov *et al.* 1981). Fish meal followed the expected pattern for animal protein of being rapidly degraded initially but with about half or more of the protein remaining undegraded on longer periods of incubation. Groundnut meal was rapidly and completely degraded, as is typically found in nylon bag experiments in vivo. Linseed meal, more slowly but also completely degraded in vivo, was not completely degraded during these incubations, but may well have been if the incubation time had been prolonged. Thus the hydrolysis of ^{14}C-labelled feed proteins in vitro can be a guide to their degradation characteristics in vivo. At this stage, these results are of qualitative rather than quantitative value, however. A quantitative method will depend on finding the optimum particle size (the protein meals were milled to a uniform particle size of 0·7 mm in the present experiments) and other conditions for incubations in vitro, and determining how these can be extrapolated to the properties of the protein meal in vivo. Furthermore, it has not been established here that labelling of non-protein amino groups, such as those of amino sugars, nucleic acids and lipids, is insignificant. The radioactivity released in these incubations may therefore originate from these sources as well as protein.

Digestions of pure ^{14}C-proteins by rumen micro-organisms and by coat proteases of rumen bacteria (Table 1) showed that different soluble proteins are hydrolysed at different rates. The insoluble hide powder azure was rapidly digested by coat proteases, but less so

by whole rumen micro-organisms, indicating a possible restriction in the association of rumen micro-organisms with insoluble proteins. However, even with rumen fluid, hide powder azure was digested at a rate similar to that of most soluble proteins. It is therefore concluded, as it was also by Nugent & Mangan (1978), Mahadevan *et al.* (1979) and Wallace & Kopecny (1983), that solubility alone is not a good index of the susceptibility of a protein to digestion by rumen micro-organisms and that secondary and tertiary structure are also important. Albumin, fetuin and γ-globulin, each with a high extent of disulphide cross-linking, were most resistant to degradation, while casein and lactoglobulin, with very little ordered structure, were easily degraded. The highly-cross-linked elastin congo red was the most resistant of the proteins examined.

The lectin from the kidney bean was degraded at a rate similar to glycoprotein II from the same source and at a rate fairly average for most proteins (Table 1). Thus there is no abnormal resistance of lectin to microbial digestion. It would be expected that little would escape the rumen sufficiently intact to cause the intestinal lesions seen in single-stomached animals, provided that the protein itself is physically available to rumen micro-organisms.

When proteins were oxidized with performic acid, the rates of digestion by coat proteases were similar, despite the large differences observed before oxidation (Table 2). This is consistent with the loss by destruction of disulphide bonds of constraints on protein conformation which are reflected in different rates of proteolysis (Nugent & Mangan, 1978; Mahadevan *et al.* 1980; Wallace & Kopecny, 1983). Conversely, when C_6 cross-links between amino groups were introduced into proteins and the structure was thereby stabilized, the proteins increased their resistance to digestion (Table 2).

Diazotization had little influence on the hydrolysis of ^{14}C-labelled lactoglobulin and albumin but inhibited [^{14}C]casein digestion, as reported previously (Wallace & Kopecny, 1983). In contrast, ^{14}C-labelled diazotized γ-globulin was digested by coat proteases much more quickly than the ^{14}C-labelled native protein (Table 2). Hence the initial rate of diazotized γ-globulin digestion reported previously (Wallace & Kopecny, 1983) is an over-estimate of the rate of digestion of the native protein both because of the slower digestion of azocasein and the increased digestion of diazotized γ-globulin.

The ^{14}C-labelling method described here is therefore a useful and valid method for studying the digestion of protein by rumen micro-organisms. Its sensitivity will be limited only by the specific radioactivity of the [^{14}C]formaldehyde used to label the protein.

The author thanks Dr R. Begbie for his gifts of fetuin and *P. vulgaris* proteins and for his advice and encouragement; also Margaret L. Brammall for skilled technical assistance, Linda More for haemagglutinin assays and W. Shand for his supervision of the animals.

REFERENCES

Annison, E. F. (1956). *Biochemical Journal* **64**, 705–714.
Blackburn, T. H. & Hobson, P. N. (1960*a*). *Journal of General Microbiology* **22**, 272–281.
Blackburn, T. H. & Hobson, P. N. (1960*b*). *Journal of General Microbiology* **22**, 290–294.
Brock, F. M., Forsberg, C. W. & Buchanan-Smith, J. G. (1982). *Applied and Environmental Microbiology* **44**, 561–569.
Dinsdale, D., Cheng, K.-J., Wallace, R. J. & Goodlad, R. A. (1980). *Applied and Environmental Microbiology* **39**, 1059–1066.
Forsberg, C. W. & Lam, K. (1977). *Applied and Environmental Microbiology* **33**, 528–537.
Hazlewood, G. P. & Edwards, R. (1981). *Journal of General Microbiology* **125**, 11–15.
Kopecny, J. & Wallace, R. J. (1982). *Applied and Environmental Microbiology* **43**, 1026–1033.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *Journal of Biological Chemistry* **193**, 265–275.
McDonald, I. W. (1954). *Biochemical Journal* **56**, 120–125.
Mahadevan, S., Erfle, J. D. & Sauer, F. D. (1979). *Journal of Animal Science* **48**, 947–953.
Mahadevan, S., Erfle, J. D. & Sauer, F. D. (1980). *Journal of Animal Science* **50**, 723–728.

Manen, J. F. & Pusztai, A. (1982). *Planta* **155**, 328–334.
Mangan, J. L. (1972). *British Journal of Nutrition* **27**, 261–283.
Means, G. E. & Feeney, R. E. (1971). *Chemical Modification of Proteins*. San Francisco: Holden-Day.
Nugent, J. H. A. & Mangan, J. L. (1978). *Proceedings of the Nutrition Society* **37**, 48A.
Nugent, J. H. A. & Mangan, J. L. (1981). *British Journal of Nutrition* **46**, 39–58.
Ørskov, E. R., Hughes-Jones, M. & McDonald, I. (1981). In *Recent Advances in Animal Nutrition – 1980*, pp. 85–98 [W. Haresign, editor] London: Butterworths.
Ozawa, H. (1967). *Journal of Biochemistry, Tokyo* **62**, 419–423.
Pusztai, A. & Watt, W. B. (1970). *Biochimica et Biophysica Acta* **207**, 413–431.
Spiro, R. G. (1960). *Journal of Biological Chemistry* **235**, 2860–2869.
Wallace, R. J. & Kopecny, J. (1983). *Applied and Environmental Microbiology* **45**, 212–217.

Printed in Great Britain