Evaluation of modified [^{35}S]methionine and [^{35}S]casein preparations as supplements for sheep

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1. Doses of L-[^{35}S]methionine (2 g) and [^{35}S]casein (20 g) were given to sheep in the diet or via the abomasum, and the patterns of ^{35}S-labelling in blood, wool and excreta were studied during the 7 d following administration of the dose.

2. Doses given via the abomasum resulted in substantial labelling of the plasma proteins and wool; only small amounts of the dose were recovered in the urine and faeces. In contrast, doses given in the diet resulted in much less labelling of plasma proteins and wool and in greater losses in excreta. These results provide the basis of a rapid system for testing the effectiveness of various methods of protecting methionine and casein from degradation in the rumen.

3. From the patterns obtained with *N*-formyl-DL-[^{35}S]methionine and [^{35}S]polymethionine (mol. wt $7–8 \times 10^4$) it appears unlikely that these compounds would stimulate wool growth if given as dietary supplements.

4. [^{35}S]Casein which had been treated with an 8% aqueous solution of formaldehyde gave results which showed that the casein had been protected from destruction in the rumen without markedly reducing its subsequent digestibility. Treatment of [^{35}S]casein with excess formaldehyde (a 40% aqueous solution) gave a product which was completely indigestible.

5. It is concluded that the radioisotope technique could be applied to other [^{35}S]amino acids or their derivatives and to other [^{35}S]proteins, and should also be applicable to larger ruminants such as cattle.

The administration of casein or of the sulphur-containing amino acids, L-cysteine and DL-methionine, direct into the abomasum of sheep usually causes large increases in the rate of wool growth (Reis & Schinckel, 1963, 1964; Reis, 1967, 1969). When these substances are included in the diet they are largely degraded by the rumen micro-organisms (McDonald, 1969) and have little or no effect on wool growth (Marston, 1932; Du Toit, Malan, Groenewald & Botha, 1935; Colebrook, Ferguson, Hemsley, Hogan, Reis & Weston, 1968; Reis, 1969). However, if casein is allowed to react with a small amount of formaldehyde its degradation in the rumen can be prevented without seriously affecting its intestinal digestion or ability to stimulate wool growth (Ferguson, Hemsley & Reis, 1967; Reis & Tunks, 1969). It should also be possible to devise ways of protecting cystine, cysteine and methionine from degradation in the rumen.

The degree of protection in the rumen can be assessed in vitro by measuring the resistance of the treated amino acid or protein to degradation when incubated with rumen micro-organisms (Ferguson *et al.* 1967). However, it is still necessary to demonstrate that the product can be utilized by the animal. A rapid method for conducting such in vivo tests has been developed from studies of the metabolic fate of ^{35}S-labelled compounds. Results obtained with methionine and casein are described here.

EXPERIMENTAL

Sheep and diet

Two Merino and two Merino × English Leicester wethers were kept in metabolism cages in a room maintained at 20 ± 3°. They were given a constant daily ration of 800 g of equal parts of chopped wheaten and lucerne hays. Three of the sheep had abomasal cannulas. The supplements were either infused into the abomasum at a steady rate over 6 h starting at feeding time (10.00 hours) or mixed with the daily ration. When in the diet, the supplement was consumed within 4 h.

Radiochemicals

L-[^{35}S]Methionine (280 mCi/m-mole) was obtained from the Radiochemical Centre, Amersham, England. [^{35}S]Methionine of lower specific activity (5 μCi/g) was prepared by dilution with methionine in aqueous solution. *N*-Formyl-DL-[^{35}S]methionine was prepared from DL-[^{35}S]methionine (obtained from Commissariat à l'Energie Atomique, France) as described by Greenstein & Winitz (1961). L-[^{35}S]Polymethionine (mol. wt 7–8 × 10^4) was kindly prepared from L-[^{35}S]methionine by Dr I. W. Stapleton of the CSIRO Division of Protein Chemistry using published methods (Bloom, Fasman, de Lozé & Blout, 1962). [^{35}S]Casein was prepared (Dunn, 1949) from the milk of a goat to which L-[^{35}S]methionine (3 mCi; 250 mCi/m-mole) had been administered intravenously. Methionine is incorporated into milk proteins with high efficiency (Askonas, Campbell & Work, 1954) and accounts for most of the sulphur in casein (Block & Bolling, 1951). The [^{35}S]casein used for abomasal infusions was dissolved in phosphate (Sorenson) buffer pH 7.

[^{35}S]Casein was treated with formaldehyde by two methods based on unpublished experiments (J. A. Hemsley, P. J. Reis & A. M. Downes). (*a*) Formaldehyde (5 ml 8%, w/v, aqueous solution) was sprayed on to [^{35}S]casein (20 g; 0·5 μCi/g) with continuous mixing in polyethylene containers, which were then sealed and kept at room temperature (20–25°) for 6 d. The preparation was thoroughly washed with water before addition to the diet. (*b*) The same procedure was followed except for the greater strength of the formaldehyde solution (5 ml 40%, w/v, aqueous solution) used.

Sampling

Blood samples (5 ml) were taken from the jugular vein, transferred to tubes containing heparin (0·05 ml 1%, w/v, in saline) and centrifuged (6500*g*; 15 min; 2°). The amounts of ^{35}S in protein-bound plasma components were determined after separation on a column of Sephadex G 50 (1·7 cm diam. × 6 cm).

Urine and faeces were collected usually for 14 d after administration of each supplement, and samples were taken for assay of radioactivity. The rates of wool growth were estimated by the tattooed patch method (Reis, 1967) and the results converted into units of g/d by multiplying by previously determined fleece:patch ratios for each sheep. From this information and from measured specific radioactivity of plucked wool samples, the total amounts of ^{35}S in the wool on the sheep were estimated.

Two liquid scintillation solutions were used in the preparation of samples for assay

of radioactivity. Solution A consisted of toluene with diphenyloxazole (0·4%, w/v) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (0·01%, w/v). Solution B contained 7 parts of solution A by volume mixed with 6 parts of Triton X-100 (supplied by Robert Bryce and Co. Ltd, Sydney) (Patterson & Greene, 1965).

The plasma or urine samples (1 ml) were added to glass vials containing water (6·5 ml) and solution B (10 ml) was added. The samples were shaken vigorously and allowed to stand at 5° for at least 2 h to form a clear gel before counting. All samples were assayed at the 'balance point' (Packard Instrument Manual) in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3375). The counting efficiency for each sample was determined by using the automatic external standard (supplied with the spectrometer) after calibration against standards containing known amounts of ^{35}S.

Most of the wool samples (20–100 mg) were assayed (Downes & Till, 1963) as suspensions in solution A (10 ml) but, in order to determine the counting efficiency, representative samples from each sheep were assayed after combustion (Kalberer & Rutschmann, 1961). Dried faecal samples (100 mg) were also assayed after combustion.

The counting efficiencies were as follows: combusted samples, 69%; plasma and wool samples, 60–69%; and urine samples, 40–60%.

RESULTS

L-[^{35}S]*Methionine and derivatives*

Fig. 1 shows the results obtained when 2 g L-[^{35}S]methionine were included in the diet of two sheep and, several weeks later, introduced into the abomasum of the same sheep. A daily abomasal supplement of 2 g of methionine is usually effective in stimulating wool growth (Reis, 1967). After the sheep had ingested the labelled methionine, ^{35}S rapidly appeared in non-protein components of the blood plasma (Fig. 1*a*). The amount of ^{35}S in these components reached a maximum about 6 h after dosing and then decreased until, after about 3 d, most of the ^{35}S remaining in the plasma was present in the proteins. The ^{35}S in the non-protein fraction was presumably present mainly as [^{35}S]sulphate, the end-product produced by destruction of the methionine in the rumen followed by absorption of ^{35}S-labelled metabolites and conversion into sulphate in the tissues. A very different distribution of ^{35}S in the plasma was observed after administering L-[^{35}S]methionine via the abomasum (Fig. 1*b*). A rapid rise in plasma ^{35}S was again observed but this ^{35}S was mainly present in the proteins, and the extent of incorporation into plasma proteins was about four times that observed after adding [^{35}S]methionine to the diet.

The rates of excretion of ^{35}S-labelled metabolites in the urine and the proportions of the dose incorporated into the wool also clearly reflected the different modes of administration. About 40% of the dose appeared in the urine during the 1st day after addition of the dose to the diet (Fig. 1*a*) whereas less than 5% appeared during the same time after administration of the methionine abomasally (Fig. 1*b*). The incorporation of ^{35}S into the wool in 7 d was approximately five times higher with the abomasal dose than with the ingested dose and approached values previously observed after administration of L-[^{35}S]cystine intravenously (Downes, 1961).

When *N*-formyl-DL-[^{35}S]methionine (7 g; 1 μCi/g) was included in the diet the results were similar to those in Fig. 1*a*, indicating substantial destruction in the rumen; incorporation of ^{35}S into wool was about the same as that obtained with [^{35}S]methionine in the diet. After [^{35}S]polymethionine (2 g; 5·5 μCi/g) had been added to the diet a negligible amount of ^{35}S was detected in the blood and urine, and more than 95% of the administered ^{35}S was excreted in the faeces within 4 d. This preparation was therefore not degraded in the rumen but was indigestible in the intestines.

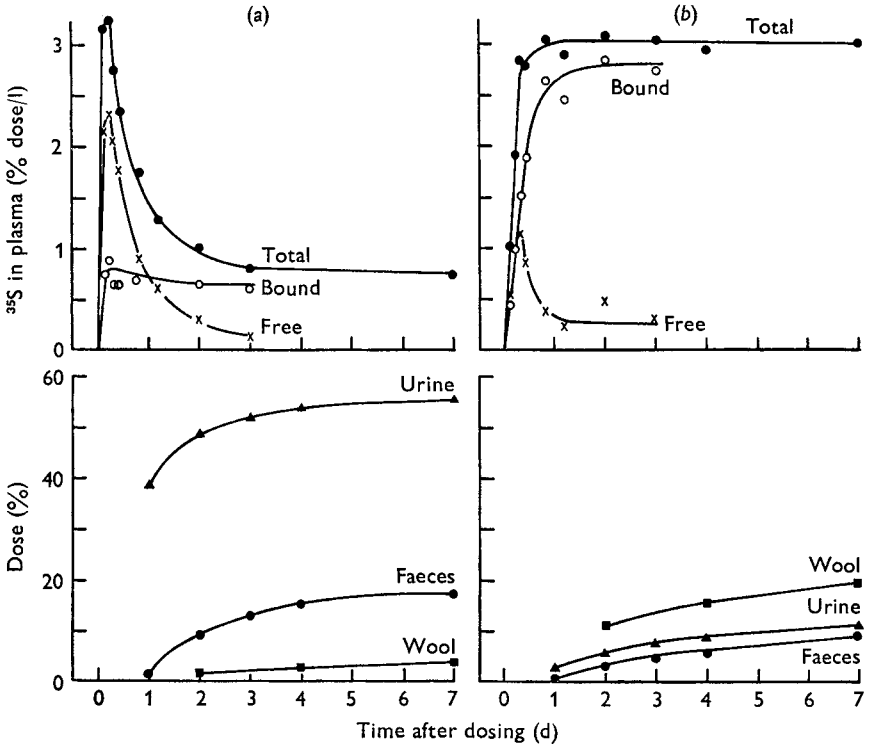

Fig. 1. Distribution of ^{35}S in blood plasma, and the recovery of ^{35}S in urine, faeces and wool following administration of L-[^{35}S]methionine (2 g; 5 μCi/g) to sheep via (*a*) the diet and (*b*) the abomasum. The results are mean values for two sheep.

[^{35}*S*]*Casein*

The results in Fig. 2*a* show the fate of the ^{35}S when untreated [^{35}S]casein (20 g; 0·5 μCi/g) was added to the diet. Similar results were obtained with supplements of 5 or 50 g of [^{35}S]casein. Some of the ^{35}S (less than 10%) in the plasma was in the non-protein fraction, but the proportion was much less than that observed with [^{35}S]methionine. Similarly, much less ^{35}S was excreted in the urine. The results obtained when [^{35}S]casein was infused into the abomasum (Fig. 2*b*) show a different pattern, with a relatively high incorporation into the plasma proteins and into wool, and only a small amount of ^{35}S excreted in the urine and faeces during the first few days.

Treatment of [^{35}S]casein with formaldehyde by method (*a*) gave a product which was protected from destruction in the rumen without markedly reducing its digestibility (J. A. Hemsley, P. J. Reis & A. M. Downes, unpublished). When the treated [^{35}S]casein (20 g; 0·5 μCi/g) was eaten by the sheep as part of its ration, a negligible amount of ^{35}S appeared in the plasma during the first 10 h (Fig. 3*a*), but the subsequent incorporation into plasma proteins was similar to that obtained by infusing 20 g of untreated [^{35}S]casein into the abomasum (Fig. 2*b*). The incorporation of ^{35}S into wool

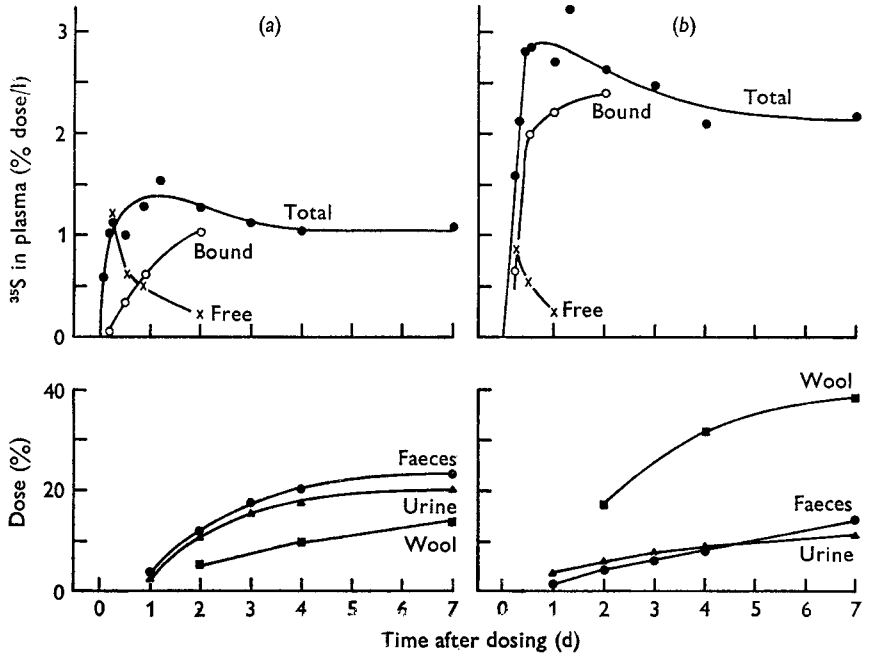

Fig. 2. Distribution of ^{35}S in blood plasma, and the recovery of ^{35}S in urine, faeces and wool following administration of [^{35}S]casein (20 g; 0·5 μCi/g) to sheep via (*a*) the diet and (*b*) the abomasum. The results are mean values for two sheep.

was less than that observed with abomasal infusion, but the difference may not be significant because of the experimental errors involved in this measurement. As shown in Fig. 3*b*, when [^{35}S]casein was allowed to react with a much larger amount of formaldehyde (method (*b*)), the product was almost completely indigestible. Approximately 80% of the ^{35}S in this preparation was excreted in the faeces within 4 d and correspondingly small amounts of ^{35}S were detected in the urine, blood plasma and wool.

DISCUSSION

It is apparent that [^{35}S]methionine given as part of the diet produces very different labelling patterns (Fig. 1*a*) from those obtained by infusing the amino acid direct into the abomasum (Fig. 1*b*) thus avoiding the rumen. These differences therefore provide the basis of a rapid system for testing the effectiveness of various methods of protecting methionine from degradation in the rumen. The feeding of an ideally

protected [^{35}S]methionine preparation, that is methionine completely protected from destruction in the rumen and completely released in the abomasum, would be expected to produce results similar to those shown in Fig. 1*b*, except that the absorption and excretion of the ^{35}S would be delayed until the labelled material passed from the rumen to the abomasum. The results obtained with *N*-formyl-DL-[^{35}S]methionine and with [^{35}S]polymethionine illustrate the use of the method. The *N*-formyl deriva-

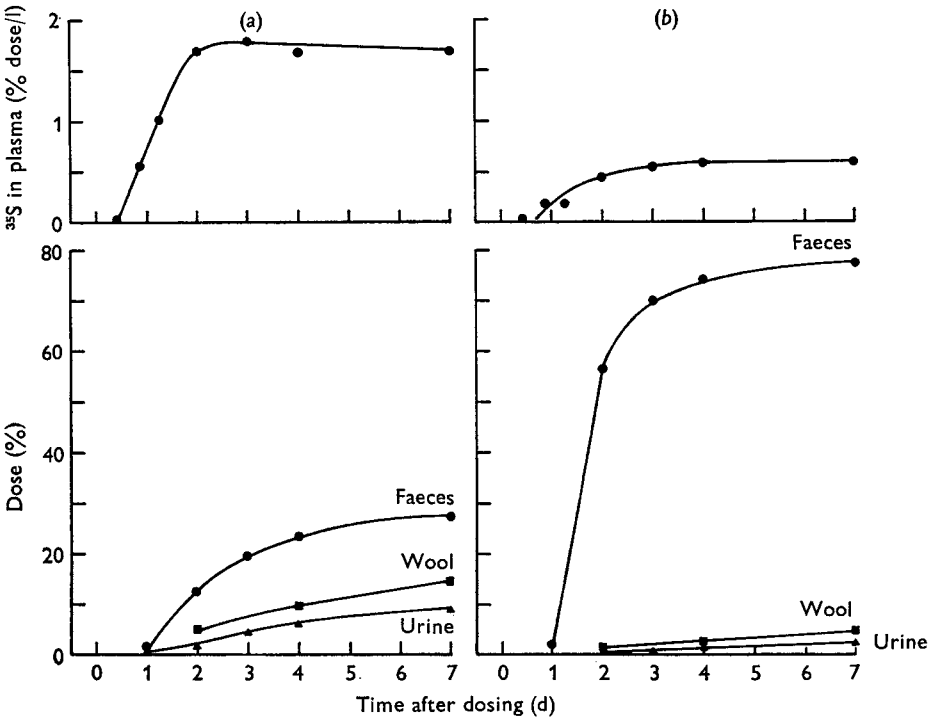

Fig. 3. Amount of ^{35}S in blood plasma, and the recovery of ^{35}S in urine, faeces and wool following administration to sheep of formaldehyde-treated [^{35}S]casein (20 g; 0·5 μCi/g) in the diet. (*a*) casein prepared by method (*a*) (see p. 1084); (*b*) casein prepared by method (*b*) (see p. 1084). The results are mean values for two sheep.

tive gave results essentially the same as those obtained with methionine itself, while the polymer was almost completely indigestible. These comparatively rapid in vivo tests show that the addition of these compounds to the diet would not have been expected to stimulate wool growth.

As mentioned on p. 1083, casein may be protected from degradation in the rumen by treatment with solutions of formaldehyde. There are many possible variations in the method of treatment; for example, time and temperature of reaction, and the amount, concentration, and pH of solution may all be varied. Experiments were therefore conducted to see whether [^{35}S]casein could be used to test the usefulness of various treatments in a way similar to that outlined for [^{35}S]methionine.

The results (for the plasma) after untreated [^{35}S]casein was added to the diet, show

that a comparatively small proportion of the methionine residues from casein was destroyed in the rumen (cf. Figs. 2*a* and 1*a*), possibly because more of them were used for microbial protein synthesis (McDonald & Hall, 1957). Nevertheless, after feeding with the 'optimally' treated [^{35}S]casein, ^{35}S was almost completely absent in the plasma during the first 10 h, in contrast to the rapid appearance of ^{35}S in the plasma after feeding with untreated [^{35}S]casein. The delayed response can be accounted for by the time casein particles take to pass from the rumen to the abomasum. These results indicate that this particular treatment of casein with formaldehyde (method (*a*)) genuinely protected the protein in the rumen with subsequent release of amino acids in the abomasum and small intestines (Ferguson *et al.* 1967; Reis & Tunks, 1969). The results obtained with [^{35}S]casein treated with the larger amount of formaldehyde (Fig. 3*b*) clearly show that this product would not be a useful food supplement.

The techniques described above are easily applied to [^{35}S]methionine, [^{35}S]cystine and [^{35}S]cysteine which are commercially available. [^{35}S]casein is easy to prepare and should have many uses as a model protein in nutritional research, particularly in testing alternative methods of protection. Since small doses (5–10 μCi) of ^{35}S are sufficient for studies with sheep when liquid-scintillation counting methods are used, similar studies with larger ruminants, such as cattle, should be practicable. The same sheep may be used repeatedly to test various ^{35}S-labelled preparations, provided the pre-test levels of ^{35}S in the plasma are low. For initial screening, measurements of ^{35}S in the urine and blood plasma during the first 3 d should clearly indicate whether a preparation is worth testing on a larger scale.

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