# Nutritional availability of amino acids from protein cross-linked to protect against degradation in the rumen

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1. Casein was labelled with pairs of radioactive amino acids, lysine, tyrosine and leucine, one with $^{14}C$ and the other with $^{3}H$, by jugular infusion into lactating goats followed by isolation of the double-labelled casein from the milk. Total milk protein was similarly labelled by jugular infusion of [$^{35}S$]cystine. U-$^{14}C$-labelled fraction-1 leaf protein was isolated from lucerne (*Medicago sativa*) grown in an atmosphere of $^{14}CO_2$.

2. The proteins were treated with different levels (333 and 667 mmol/kg protein) of formaldehyde, glutaraldehyde and glyoxal.

3. Absorption from the small intestine was measured in sheep with fistulas in the abomasum and terminal ileum, using Cr-EDTA as the digesta flow marker, by introducing radioactive casein into the abomasum.

4. Lysine, tyrosine and cystine became increasingly unavailable for absorption from the small intestine of sheep with increasing levels of aldehyde. At the lower level (333 mmol/kg) the proportions of the amino acids that were unavailable were 0·192, 0·051 and 0·123 respectively. At the higher level of formaldehyde (667 mmol/kg) the corresponding values were 0·335, 0·201 and 0·432 respectively. Leucine was not made unavailable with formaldehyde.

5. The proportions of lysine, tyrosine and leucine that were unavailable were higher, on a molar basis, after treatment of the proteins with the dialdehydes glutaraldehyde and glyoxal than after treatment with formaldehyde. However, the extent of protein protection provided by the dialdehydes in the rumen, measured using an in vitro procedure, was lower.

Dietary protein has been protected against microbial degradation in the rumen by introducing cross-links by various physical and chemical treatments (Chalupa, 1975; Ferguson, 1975). When ruminants are given protected protein, increased amounts of non-ammonia-nitrogen, mostly in the form of amino acids, are made available post-ruminally for absorption in the small intestine (Faichney & Weston 1971; MacRae *et al.* 1972; Faichney, 1974; Sharma *et al*: 1974; Williams & Smith, 1976; Hagemeister, 1977; Faichney & White, 1979). Increased wool production has been reported in sheep given protected proteins, especially casein (Ferguson, 1975), the responses probably being related to the content of sulphur amino acids in the treated protein (Barry, 1976). However, responses obtained in milk production or in growth rate (measured as N retention or live-weight gain) on feeding protected protein have been smaller and more variable than those reported for wool growth (Hudson *et al.* 1969; Peter *et al.* 1971; Schmidt *et al.* 1973; Waldo *et al.* 1973; Clark *et al.* 1974; Clark, 1975*a*; Johnson & Hatfield, 1975; Hartnell & Satter, 1978). Body growth and milk production may require a more finely balanced supply of essential amino acids post-ruminally, as demonstrated by responses obtained in milk production on abomasal infusion of casein in high-yielding cows (Clark, 1975*b*; Clark *et al.* 1977).

The fact that protected protein passes through the rumen undegraded and reaches the lower gut for digestion does not necessarily mean that it is digested efficiently nor, once

digested, that the amino acid profile is such that it provides a better balance of amino acids for milk production and growth (Ørskov *et al.* 1980). The work of Verite & Journet (1977) and Thomas *et al.* (1979) who obtained positive resposes in milk production and growth respectively at low levels of formaldehyde treatment (200 – 233·5 mmol/kg protein) also lends support to the hypothesis that amino acid availability and balance in protected proteins could be important. The present paper describes methods developed to determine amino acid absorption–availability in vivo and the results obtained with protein cross-linked using different aldehydes. Implications of these results in protecting proteins for ruminants are discussed.

## EXPERIMENTAL

### *Materials and methods*

*Chemicals.* Labelled amino acids, L-[U-$^{14}$C]lysine monohydrochloride 10 mCi/mmol, L-[4,5-$^{3}$H]lysine monohydrochloride 75–100 Ci/mmol, L-[U-$^{14}$C]leucine 300 mCi/mmol, L-[$^{35}$S]cystine hydrochloride 1–250 mCi/mmol, L-[2,6-$^{3}$H]tyrosine 30–60 Ci/mmol, [$^{14}$C]formaldehyde 10–20 mCi/mmol, were obtained from Amersham (Australia) Pty Ltd. [U-$^{14}$C]-labelled fraction-1 leaf protein was prepared at the AFRC Institute of Animal Physiology, Cambridge, UK. Other chemicals used were of analytical reagent grade.

*Preparation of labelled casein and total milk protein.* For each preparation amino acids, one containing a $^{14}$C label (100–200 $\mu$Ci) and the other containing a $^{3}$H label (250–1000 $\mu$Ci) were dissolved in normal saline (9 g sodium chloride/l) and infused over 24 h into the jugular vein of lactating Saanen goats. The animals were milked twice daily and the milk for each day was pooled separately. Milk was collected for 3 d post infusion. Casein was prepared from this milk as described by Dunn (1949).

Casein obtained after infusion of [$^{35}$S]cystine had very low levels of radioactivity reflecting its low cystine content, although whey protein was highly radioactive. Consequently, total milk protein was prepared from milk obtained after [$^{35}$S]cystine infusion by centrifugation to remove the cream. The skim milk obtained was dialysed at 4° against two changes of deionized water over a 24 h period and the total milk protein recovered after freeze-drying.

*Aldehyde treatment of protein.* Protein (10 g) was dissolved in 53 ml water, containing 62·5 mmol sodium hydroxide, using an Ultra-Turrax mixer (Janke & Kunkel, Staufen, Germany). The final pH of the solution was adjusted to 9·0 with 5·0 M-NaOH. The requisite volume of aldehyde solution was added slowly under vigorous mixing, until a thick and rubbery gel was obtained. The aldehyde solutions, as commercially available, contained 360, 250 and 400 g formaldehyde, glutaraldehyde and glyoxal respectively/l.

The beakers containing the treated protein were sealed and allowed to stand overnight at room temperature for the reaction to proceed. The gel was broken up into small fragments and dried in a vacuum oven at 40°. The treated proteins were stored over silica gel for a minimum of 1 month to ensure adequate cross-linking before introducing them into the abomasum.

*Animals and experimental procedures.* Two sheep were used for the absorption studies and were surgically prepared with permanent fistulas in the abomasum near the pylorus and small intestine 100–150 mm anterior to the ileo-caecal junction, as described by Hecker (1974). During the experimental period the animals were maintained in metabolic cages in an air-conditioned room and given dried lucerne (*Medicago sativa*) pellets (800 g/d per sheep) using interval feeders designed to offer an equal feed portion of the ration every 3 h over a 24 h cycle.

Treated proteins (5·0 g, containing approximately 0·4 $\mu$Ci $^{14}$C- and 1·0 $\mu$Ci $^{3}$H-isotopes or 0·4 $\mu$Ci $^{35}$S-isotope) were hydrated in 40 ml distilled water at pH 7·0 overnight, while the unprotected proteins were dissolved just before infusion and the pH adjusted to 7·0 with

5 M-NaOH. Before use, 10 ml Cr-EDTA solution prepared according to Binnerts *et al.* (1968), was added to the protein suspension–solution and the mixture homogenized to give fine particles using an Ultra-Turrax mixer. The mixture was transferred into the abomasum using a modified 50 ml syringe. The syringe was rinsed with abomasal contents (50 ml), 100 ml of which were collected just before the transfer of the Cr-EDTA–protein suspension. This was done to minimize any change in the volume of digesta and consequent change in flow. Digesta samples (approximately 20 g) were collected every 0·5 h at the terminal ileum, the exact time of the flow from the cannulas being recorded. The samples were cooled immediately and stored frozen until required for analysis.

### *Analytical methods*

*Determination of Cr.* Digesta samples were diluted tenfold with distilled water, centrifuged at 2000 ***g*** for 10 min and the supernatant fraction analysed for Cr using an atomic absorption spectrophotometer (Binnerts *et al.* 1968).

*Radioactivity determination in digesta and protein samples.* Samples of digesta (approximately 1·0 g) were absorbed into cotton wool (approximately 125 mg) placed in aluminium foil and dried at 80° for 16 h. These samples, together with samples of labelled proteins or [$^{14}$C]formaldehyde-treated proteins (25 mg), were combusted in Schoniger flasks (Romer Glass and Physics Laboratory, Sydney, Australia) and the products of combustion absorbed in 20 ml of a methyl cellosolve–ethanolamine (9:1, v/v) mixture (Kalberer & Rutschmann, 1961); 10 ml of this solution were taken and counted for $^{14}$C, $^{3}$H or $^{35}$S by liquid-scintillation counting techniques. The radioactivity (disintegrations/min) was then calculated by taking into account counting efficiency; this was determined by spiking samples with isotopes of known radioactivity.

*Hydrolysis of protein and amino acid analysis.* Protein (125 mg) was hydrolysed in an atmosphere of $N_2$ at 110° for 24 h in 10 ml 6 M-hydrochloric acid containing 2·0 mg stannous chloride using 19 mm × 100 mm Pierce vacuum hydrolysis tubes (Pierce, Rockford, Illinois). Amino acid analysis was done by ion-exchange chromatography using a Technicon TSM amino acid analyser.

*Protein protection.* The net ammonia production from treated and untreated caseins was determined by in vitro anaerobic incubation with strained rumen fluid at 37° for 20 h, as described by Ashes *et al.* (1979). The protein protection was calculated as follows:

$$\text{protection } (\%) = 100 - \left(\frac{\text{net } NH_3 \text{ production with treated casein}}{\text{net } NH_3 \text{ production with untreated casein}}\right) \times 100.$$

*Tightly-bound formaldehyde.* This was determined as the difference between the total bound formaldehyde and that recovered from distillation of [$^{14}$C]formaldehyde-treated proteins in the presence of phosphoric acid by the method of Van Dooren (1976). Total bound formaldehyde was determined by combustion of [$^{14}$C]formaldehyde-treated casein. Liquid-scintillation techniques were used to assess the quantities of formaldehyde present.

*Calculation of amino acids remaining unabsorbed.* The concentrations of Cr ($\mu$g/g digesta) and radioactivity (disintegrations/min per g digesta) in ileal digesta were plotted against the time-scale used for sampling. The area under each curve was determined by planimeter. The area for Cr was related to the amount of Cr originally introduced into the abomasum. It was then used to calculate the amount of isotope appearing at the terminal ileum, i.e.

$$\text{total amount of isotope (disintegrations/min)} = \frac{\text{Cr added } (\mu g) \times \text{area of isotope} \times SF}{\text{area of Cr}},$$

where SF is the scale factor determined in such a way that 1 $\mu$g = 1 disintegration/min, in terms of unit area.

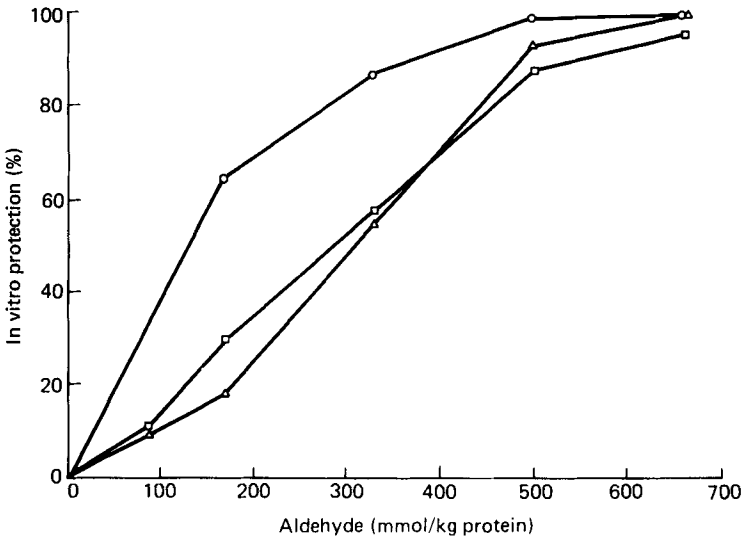


Fig. 1. The relationships between in vitro protection to microbial degradation of casein treated with various levels of formaldehyde (○), glyoxal (□) and glutaraldehyde (△). Each point is the mean of two determinations. The standard error of the difference between any two means for each of the aldehyde concentrations 166, 333, 501 and 667 mmol/kg protein was 11·1, 6·9, 11·0 and 3·9 respectively.

*Statistical analysis.* Significant differences between the means of experimental treatments were determined using the procedure of one-way analysis of variance and applying the criterion of least significant difference. The test was applied to the unabsorbed amino acid values obtained for lysine and tyrosine at aldehyde concentrations of 333 and 667 mmol/kg protein (Table 2, see p. 245) and to values relating aldehyde concentration and in vitro protein protection (Fig. 1).

## RESULTS

Protection against microbial degradation in the rumen determined using an in vitro technique of casein treated with different levels of aldehyde is shown in Fig. 1. At lower levels (166 and 333 mmol/kg) of treatment the protection provided by formaldehyde was significantly higher ($P < 0{\cdot}05$) than that provided by the other aldehydes.

Table 1 shows the total amount of formaldehyde added and that which was bound to casein after treatment. When 333 mmol [$^{14}$C]formaldehyde was added to casein and the product dried 238 mmol (71·4%) of the formaldehyde was actually bound to casein compared with 550 mmol (55%) when 1000 mmol was added. After 2 weeks of storage at the lower level of treatment there were 62 mmol formaldehyde tightly bound. This amount remained relatively constant after 2 years' storage. At the higher levels of treatment (667 and 1000 mmol/kg), a similar percentage but approximately twice this quantity was tightly bound after 2 weeks' storage which further increased after 2 years.

The typical pattern of emergence at the terminal ileum of radioactivity measured as $^{3}$H and $^{14}$C after the abomasal transfer of casein labelled with [$^{3}$H]tyrosine and [$^{14}$C]lysine is shown in Fig. 2(*a*) and that of the same batch of casein treated with 667 mmol formaldehyde/kg protein is shown in Fig. 2(*b*). Fig. 2(*a*,*b*) also shows the emergence of Cr-EDTA used as a marker. It is apparent that the peak concentrations with respect to

Table 1. *Proportion of unabsorbed amino acid residues at the terminal ileum*

(Mean values with their standard errors)

| | Formaldehyde (mmol/kg protein) | | | | | | | | | Glutaraldehyde (mmol/kg protein) | | | | | | Glyoxal (mmol/kg protein) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 333* | | | 667† | | | 333* | | | 667† | | | 333* | | | 667† | | |
| Amino Acid | Mean | SE | *n* | Mean | SE | *n* | Mean | SE | *n* | Mean | SE | *n* | Mean | SE | *n* | Mean | SE | *n* | Mean | SE | *n* |
| Lysine | 0·029 | 0·005 | 3 | 0·192 | 0·012 | 3 | 0·335 | 0·045 | 3 | 0·498 | 0·122 | 4 | 0·775 | 0·082 | 4 | 0·447 | 0·041 | 4 | 0·503 | 0·092 | 3 |
| Tyrosine | 0·024 | 0·001 | 2 | 0·051 | 0·007 | 2 | 0·201 | 0·050 | 2 | 0·116 | 0·006 | 2 | 0·273 | 0·031 | 2 | 0·152 | 0·025 | 2 | 0·121 | | 1 |
| Leucine | 0·014 | | 1 | 0·027 | | 1 | 0·065 | | 1 | 0·115 | 0·011 | 2 | 0·167 | 0·019 | 2 | 0·031 | 0·009 | 2 | 0·082 | 0·004 | 2 |
| Cystine | 0·058 | 0·002 | 2 | 0·123 | 0·021 | 2 | 0·432 | 0·111 | 2 | — | | | — | | | — | | | — | | |
| [U-$^{14}$C]-labelled fraction-1 leaf protein | — | | | — | | | 0·118 | | 1 | — | | | — | | | — | | | — | | |

* Error mean square for aldehyde level 333 mmol was 0·008 and 0·001 for lysine and tyrosine respectively.
† Error mean square for aldehyde level 667 mmol was 0·008 and 0·004 for lysine and tyrosine respectively.

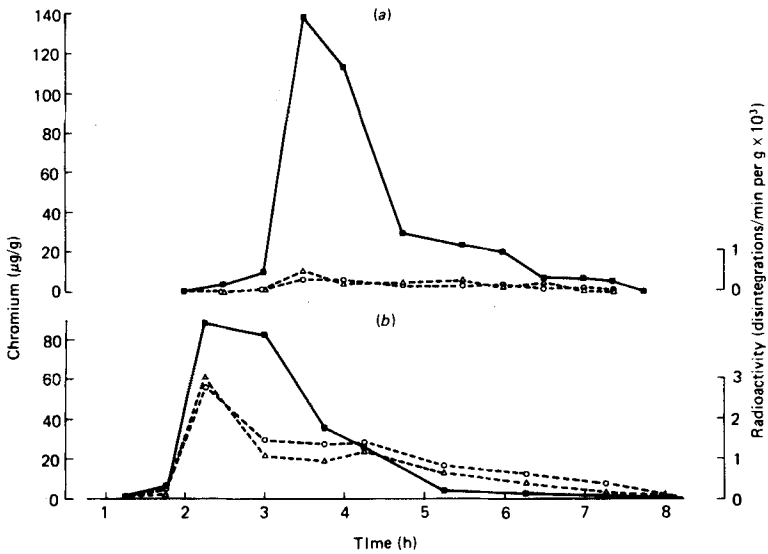


Fig. 2. The appearance with time at the terminal ileum of sheep of chromium marker (■) and radioactivity associated with L-[2,6-$^{3}$H]tyrosine (△) and L-[U-$^{14}$C]lysine (○) residues representative of a single experiment with (*a*) untreated casein, (*b*) casein treated with formaldehyde (667 mmol/kg protein). Both marker and casein were simultaneously transferred into the abomasum at zero time.

radioactivity and Cr marker were reached simultaneously in digesta samples collected at the terminal ileum. However, in some other experiments, irrespective of the treatment, the radioactivity peaks lagged behind the Cr marker by about 0·5 h; this did not affect the results as the areas were integrated over the entire collection period.

The proportions of amino acids remaining unabsorbed from different batches of casein and other proteins either untreated or after aldehyde treatment are summarized in Table 2. The values for lysine pertain to casein batches some of which were labelled with [$^{14}$C]lysine and[$^{3}$H]tyrosine and others with [$^{3}$H]lysine and [$^{14}$C]leucine.

Proportions of lysine, tyrosine and leucine from the untreated casein remaining unabsorbed from the small intestine were below 0·03 and that of cystine from untreated total milk protein was 0·06. From formaldehyde (333 mmol/kg)-treated casein, proportions of unabsorbed lysine and cystine rose to 0·192 and 0·123 respectively, while those of tyrosine and leucine showed a very small effect. At the higher level (667 mmol/kg) of formaldehyde treatment the proportions of unabsorbed cystine rose more sharply (to 0·432) than those of lysine (0·335) and tyrosine (0·201). Leucine absorption was least affected.

Proportions of unabsorbed lysine from casein treated with glutaraldehyde were 0·498 and 0·775 at the lower and higher levels of treatment respectively. These were significantly higher ($P < 0·05$) than the corresponding values obtained with formaldehyde treatment. The proportion of unabsorbed tyrosine (0·116) at the lower level of glutaraldehyde treatment was also significantly higher ($P < 0·05$) than that obtained with the corresponding level of formaldehyde treatment. Glyoxal treatments produced effects with respect to lysine and leucine absorption intermediate to those produced by the other two aldehydes. The results obtained with tyrosine were variable.

When U-$^{14}$C-labelled fraction-1 leaf protein was treated with 667 mmol formaldehyde/kg protein and subjected to absorption studies, the proportion of radioactivity recovered at the terminal ileum was 0·12 of that originally infused.

Table 2. *The extent of* [$^{14}$C]*formaldehyde binding with casein*
(Mean values with their standard errors for three observations)

| Amount of [$^{14}$C]formaldehyde added to casein (mmol/kg protein)... | Extent of binding | | | | | |
|---|---|---|---|---|---|---|
| | 333 | | 667 | | 1000 | |
| | Mean | SE | Mean | SE | Mean | SE |
| Total bound to casein (determined after drying) (mmol/kg) | 238 | 7 | 463 | 18 | 550 | 29 |
| Amount tightly bound (not recovered by distillation) (mmol/kg): | | | | | | |
| 2 weeks after treatment | 62 | 1 | 141 | 2 | 107 | 9 |
| 2 years after treatment | 65 | 1 | 200 | 30 | 164 | 4 |

## DISCUSSION

Results reported here on the amino acid digestibility are based on a number of assumptions including: (1) that the labelled amino acids were incorporated as such into casein or total milk protein and that the degradation products, if any, did not contribute to the radioactivity in these proteins; (2) that during the absorption process recycling of absorbed labelled amino acids did not occur; (3) that the residual activity at the terminal ileum represented unabsorbed amino acids either in the free form or as peptide fragments modified through cross-linking. When casein hydrolysate was subjected to ion-exchange chromatography and the emergence of each amino acid monitored by liquid-scintillation procedures (Mangan & Bounden, 1965), only a single peak pertaining to the $^{14}$C-labelled amino acid originally perfused could be detected, supporting the first assumption. During the passage of a single bolus of radioactive protein through the abomasum and small intestine, due to the short time-span, the likelihood of amino acid recycling is minimal. The main advantage of the technique we employed was that the true absorption of amino acids could be measured more reliably without the errors introduced by indirect estimations of contributions made by endogenous secretions in conventional methods.

The results for amino acid absorption from untreated casein show that the values obtained for the four amino acids are well within the range of those reported on true digestibility for simple-stomached animals (0·93–1·00) by various workers (Varnish & Carpenter, 1975; Hurrell & Carpenter, 1978; Bodwell *et al.* 1980; Wilson & Leibholz, 1981). Precise determinations of digestibility and absorption of cyst(e)ine from the ovine small intestine have been complicated by a relative lack of reliable analytical procedures. Consequently, either there is a lack of information, or there are wide variations in the values quoted for apparent digestibility, i.e. from 0·25 to 0·73 (Coelho da Silva *et al.* 1972; Faichney & White, 1979). True absorption of cyst(e)ine from $^{35}$S-labelled rumen microbial protein has been reported to be 0·72 (Elliot & Little, 1977). Our results show that from high-quality milk protein, true digestibility could reach 0·94, which is similar to that reported for simple-stomached animals.

The results obtained for the proportions of individual amino acids remaining unabsorbed from formaldehyde-treated proteins (Table 2) are consistent with suggested mechanisms of the formaldehyde reaction with lysine, cyst(e)ine and tyrosine side-chains on the protein molecule (P. H. Van Dooren, personal communication; Feeney *et al.* 1975). Since the leucine side-chain does not react with formaldehyde it must be presumed that absorption is affected only indirectly through inaccessibility brought about by the proximity of other amino acid

side-chains cross-linked with aldehydes. Although the proportions of lysine, cystine and tyrosine remaining unabsorbed increased greatly from proteins treated with high levels of formaldehyde (667 mmol/kg protein), the overall absorption of the protein, as measured from the absorption of uniformly-labelled protein treated at the same level, was only slightly reduced. These findings are in agreement with the conclusions of Hagemeister (1977).

The reactions of protein with formaldehyde lead to the formation of complexes resistant to acid-distillation, the amount of which increased at higher levels of treatment (Table 1). It is probable that the formation of these complexes is associated with the nutritional unavailability of certain amino acids similar to those reported in Table 2.

The absorption of lysine and tyrosine from casein was more seriously affected at both the levels of glutaraldehyde and at the lower level of glyoxal treatments than from casein treated with corresponding levels of formaldehyde. These differences could arise from the nature of the reaction between the amino acid side-chain and the aldehyde. Dialdehydes such as glutaraldehyde and glyoxal react with the side chains of amino acids such as lysine to form Schiff's bases; formaldehyde, on the other hand, forms hydroxyl methyl derivatives which can react further with formaldehyde or other amino acid side chains to form cross-links in the form of methylene bridges (Walker, 1964; Feeney *et al.* 1975; Hurrell & Carpenter, 1978). The extent to which these linkages can be reversed is not known, but the results here suggest that the formaldehyde cross-link is more readily broken. However, it could be related to the fact that more than one molecule of formaldehyde can be associated with a single cross-link, compared with dialdehydes where only a single molecule can be involved with an effective cross-link. It would appear that when the level of protein protection provided, and the amino acid absorption are considered together, formaldehyde is the preferred aldehyde when treating proteins of the type described in the present work.

When judged from the extent of absorption in the small intestine the availability of certain amino acids may be overestimated. Reis & Tunks (1973) showed that from formaldehyde-treated casein, cross-linked lysine is absorbed and appears as $\epsilon$-N-methyl lysine in the blood plasma, the latter increasing in concentration four- to sixfold over the normal values (Faichney, 1974; Corbet & Edey, 1977). The extent to which $\epsilon$-N-methyl lysine can substitute for lysine in ruminants is unknown; however, it is a poor substitute for lysine in mice (Friedman & Gumbmann, 1981).

From work reported earlier (Hurrell & Carpenter, 1978; Achinewhu & Hewitt, 1979) it is reasonable to assume that the loss of amino acid availability, as judged by the growth depression in chickens and rats, is much greater than can be predicted from the loss in digestibility of lysine, and presumably of other amino acids, from formaldehyde- or heat-treated proteins. In the ultimate analysis a positive response to dietary protected protein depends on the extent to which the pattern of essential amino acid supply is modified by the method of protection in relation to the amino acid pattern required by the animal for various body functions. The level of treatment which minimally affects essential amino acid availability and provides adequate protection against rumen degradation, requires further critical examination for each food protein.

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