INFLUENCE OF FORMALDEHYDE-TREATED CASEIN SUPPLEMENTS ON THE CONCENTRATION OF ε-*N*-METHYLLYSINE IN SHEEP PLASMA

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Abstract

The amino acid ε-*N*-methyllysine (NML) was identified in sheep plasma by a comparison with authentic NML by means of ion exchange and paper chromatography. NML was present in acid hydrolysates of formaldehyde-treated casein but not of untreated casein.

NML is a normal component of plasma. The mean concentration (±S.E.) in plasma from 18 sheep on a moderate amount of a roughage diet was 5·2±0·25 μmoles/100 ml. The concentration of NML in plasma was not increased by casein added to the diet, but was increased four- to sixfold by supplements of 100 g/day of formaldehyde-treated casein.

Dietary supplements of [^{14}C]formaldehyde-treated casein resulted in the appearance of labelled NML in plasma. No other amino acid was labelled.

Plasma NML concentration was not altered by abomasal infusions of methionine, cystine, or of four proteins (casein, whole egg protein, Promine-D, and wheat gluten). Abomasal infusions of four other proteins (zein, maize gluten, gelatin, and egg albumen) caused small decreases in plasma NML levels.

It was concluded that the concentration of NML in plasma was not markedly influenced by the availability of lysine as a substrate or of methionine as a source of methyl groups, and that the large increase in plasma NML concentration with formaldehyde-treated casein was due to the absorption of NML formed by the reaction of formaldehyde with lysine residues in the protein.

I. Introduction

Formaldehyde treatment was developed as a means of protecting proteins in the diet of ruminants from degradation by rumen microorganisms (Ferguson *et al.* 1967). In a comparison of the nutritive value of casein and formaldehyde-treated casein (Reis and Tunks 1970) it was noted that there was a large increase in the plasma concentration of an unidentified compound, normally present in plasma, in response to the feeding of formaldehyde-treated casein. In contrast, dietary supplements of untreated casein or the infusion of casein into the abomasum did not increase the concentration of the compound in plasma. It was suggested that this compound may be the amino acid ε-*N*-methyllysine (NML), on the basis of a comparison of its elution pattern with that of authentic NML, and the isolation of NML from the

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plasma of Romney sheep receiving dietary supplements of formaldehyde-treated casein (Dr. I. E. B. Fraser, personal communication; subsequently reported by Weatherall and Haden 1969).

NML has been isolated from mammalian histone proteins (Murray 1964; Turberville and Craddock 1971) and from muscle myosin (Hardy *et al.* 1970), and has been identified as a component of blood plasma in humans (Perry *et al.* 1969) and in sheep (Weatherall and Haden 1969; Bergen 1970). Carrico *et al.* (1970) and Fraser and Haden (1970) observed substantial increases in the concentration of NML in plasma when sheep were given supplements of formaldehyde-treated casein. However, they also reported small increases in plasma NML with dietary supplements of untreated casein. Insufficient information is given by the above authors to allow the effects of untreated casein on plasma NML to be assessed, but the standard errors quoted do not support the claims of significant effects. Fraser and Haden (1970) proposed that there was a relationship between plasma NML concentration and wool growth rate, and that plasma NML may be an indicator of cyst(e)ine availability. Bergen and Potter (1971) suggested that methionine availability plays a role in the regulation of NML synthesis in sheep.

Reis and Tunks (1970) suggested that NML may be produced by the reaction of formaldehyde with lysine residues in casein. The present studies were undertaken to obtain evidence on the origin of the increased amounts of NML in plasma with supplements of formaldehyde-treated casein, and to observe the effects of varying the supply of amino acids, especially methionine and cystine, to the tissues on the concentration of NML in plasma. Evidence is presented that the large increase in plasma NML during ingestion of formaldehyde-treated casein is, in fact, due to the reaction of formaldehyde with lysine residues in the protein.

II. Experimental

(*a*) *Formaldehyde Treatment of Casein*

Industrial grade, HCl-precipitated casein was treated with aqueous solutions, prepared from A. R. formaldehyde, by procedures based on those of Hemsley *et al.* (1973).

Dry casein was treated for 3 days in sealed vessels at ambient temperature with two volumes of 1·5% w/v solution of formaldehyde (2 ml/g casein) for use in experiments 1 and 3 and for hydrolysis. A further casein sample was treated for 20 hr at 60°C with two volumes of 5% w/v solution of formaldehyde prior to hydrolysis. After treatment the casein preparations were washed four times with two volumes of water and then dried (50°C). [^{14}C]Formaldehyde was used for some casein preparations. The specific radioactivities of the formaldehyde in the solutions were 2 mCi/mole (experiment 3), 40 μCi/mole (casein treated with 1·5% formaldehyde for hydrolysis), and 0·6 mCi/mole (casein treated with 5% formaldehyde for hydrolysis).

The treated casein for experiment 2 was casein–safflower oil particles treated with formaldehyde as described by Mills *et al.* (1972).

(*b*) *Sheep and Diet*

The experimental sheep were mature Merino wethers, except for some of the sheep in experiment 5 which were Corriedales or English Leicester × Merinos. They were fitted with abomasal or rumen cannulae and were kept in metabolism cages in a room maintained at 23±3°C. The basal diet was a mixture of equal parts by weight of chopped wheaten and lucerne hays; amounts ranging from 600 to 800 g/day were fed either as a loose mix or ground and pelleted. The daily ration was offered once daily or in eight portions at intervals of 3 hr. Water was available *ad libitum*. Blood samples were collected from the jugular vein as indicated in each experiment.

(*c*) *Outline of Experiments*

Experiment 1

Two sheep received a daily ration of 900 g, offered in equal portions every 3 hr, of a pelleted mixture (eight parts basal diet and one part casein). After 2 weeks the casein was replaced with formaldehyde-treated casein for a further 2 weeks. Blood samples were taken on 11 occasions during the experiment (see Fig. 2). The last three blood samples were not taken from one sheep because of the accidental emptying of the rumen following the loss of a rumen cannula.

Experiment 2

One sheep consumed formaldehyde-treated casein–safflower oil particles for at least 3 weeks in the experiments of Mills *et al.* (1972), followed by [^{14}C]formaldehyde-treated particles for 2 days. Blood was then collected from the sheep.

Experiment 3

One sheep received 800 g/day of the basal diet, offered once daily. On one occasion (approximately 1 hr after the daily ration was offered), 105 g of [^{14}C]formaldehyde-treated casein (113 μCi) was poured into the rumen, through a cannula, as a slurry over a period of 45 min. Blood samples were taken during the next 54 hr. Samples used for subsequent detection of radioactivity were: (1) a bulk of seven samples collected between 22 and 48 hr after dosing, and (2) a sample collected 52 hr after dosing.

Experiment 4

Sheep were given abomasal infusions of varying amounts of L- and DL-methionine and L-cystine (see Reis *et al.* 1973*a*), and blood samples were taken as described by Reis *et al.* (1973*b*).

Experiment 5

Sheep were given abomasal infusions of approximately 100 g/day of eight different proteins (see Colebrook and Reis 1969 and Reis and Colebrook 1972); blood samples were taken after at least 5 weeks of protein supplementation.

(*d*) *Analytical*

(i) *Hydrolysis of Casein*

Duplicate samples of casein and formaldehyde-treated casein were hydrolysed in sealed glass tubes *in vacuo* with constant boiling point HCl (*c.* 6N) at 110°C for 20, 68, and 117 hr, and amino acid analyses were performed as described below. The mean value for the three hydrolysis times was used for most amino acids; the values for serine, threonine, and half-cystine were estimated by extrapolating the concentration to zero time, and the maximum values were taken for valine, leucine, and isoleucine.

(ii) *Amino Acid Analysis*

The collection of blood and the preparation of plasma for amino acid analysis were carried out as described by Hogan *et al.* (1968). A sample corresponding to about 0·5 mg casein or 1·0 ml plasma was analysed, except for experiments 2 and 3 when samples corresponding to 7–10 ml plasma were analysed. Analyses were carried out with a Technicon amino acid analyser (Technicon Co., New York) using a 145 by 0·6 cm column with a Technicon type A resin and a gradient elution system based on that of Efron (1966), but with flow rates modified to give adequate resolution in 16 hr. Amino acid concentrations were calculated by reference to norleucine, used as an internal standard.

(iii) *Identication of NML*

The elution pattern of the compound in plasma was compared with that of authentic ε-*N*-methyl-L-lysine (supplied by Calbiochem., Los Angeles, California, U.S.A.) added to plasma and to standard mixtures of amino acids run through the amino acid analyser system. Fractions collected from the resin column were desalted by the method of Drèze *et al.* (1954) and were compared with authentic NML and with lysine by descending paper chromatography in propan-2-ol : water : 0·88 ammonia (8 : 1 : 1).

(iv) *Radioactivity Measurements*

[^{14}C]Formaldehyde solutions were prepared by appropriate addition of [^{14}C]formaldehyde (Radiochemical Centre, Amersham, England) to unlabelled formaldehyde (British Drug Houses; A.R.). The radiochemical purity of the [^{14}C]formaldehyde was at least 98% (Hemsley *et al.* 1973). The bound formaldehyde in casein preparations was estimated by measuring the ^{14}C content (Hemsley *et al.* 1973).

With plasma samples from experiments 2 and 3, *c.* 93% of the effluent from the resin column was diverted from the analytical system, and fractions (*c.* 3·3 ml) were collected at intervals of 5 min into glass vials for the measurement of radioactivity. These fractions were identified by reference to the analytical results. Radioactivity was measured by liquid scintillation counting as described by Downes *et al.* (1970).

III. Results

(*a*) *Identification of NML*

When a standard mixture of amino acids containing NML was run through the analytical system, NML was eluted in the same position as the unknown plasma component. When plasma samples were run with and without added authentic NML, the added NML was eluted with the plasma component as a single peak. The elution pattern of amino acids in the region of lysine is shown in Figure 1.

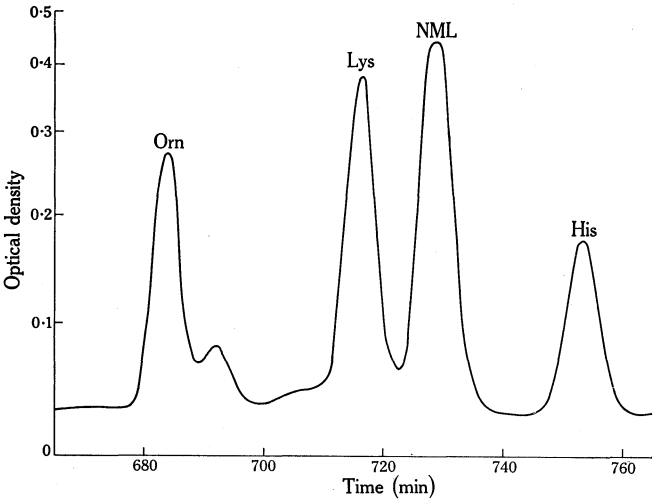

Fig. 1.—Elution pattern of plasma amino acids in the region of lysine. The plasma sample was taken from a sheep in experiment 1 (Fig. 2) 11 days after the start of supplementation with formaldehyde-treated casein. See Section II for elution conditions.

For identification by paper chromatography, fractions were isolated by column chromatography from plasma of a sheep receiving the basal diet and from plasma of a sheep receiving formaldehyde-treated casein (experiment 3). The fractions identified as lysine and NML moved the same distance as authentic lysine and NML respectively, which were well separated in the system.

(*b*) *Isolation of NML from Acid Hydrolysates of Formaldehyde-treated Casein*

Acid hydrolysis of casein and of two treated casein preparations, containing 1·4 and 3·9% bound formaldehyde, yielded the same proportions of most amino acids. Those amino acids whose proportions in casein were altered by formaldehyde

treatment are shown in Table 1. No NML was isolated from untreated casein. Formaldehyde treatment resulted in the appearance of NML; the amounts of NML in the treated preparations were in proportion to the amounts of bound formaldehyde. Other effects of formaldehyde treatment on the amino acids isolated from a casein hydrolysate were the disappearance of cystine and tyrosine and reductions in the amounts of lysine and histidine.

TABLE 1

AMINO ACIDS IN HYDROLYSATES OF CASEIN AND FORMALDEHYDE-TREATED CASEIN

Casein was treated with 1·5% (preparation 1) or 5% w/v formaldehyde (preparation 2) as described in Section II

Amino acid	Concentration (μmoles/g dry casein) in:		
	Untreated casein	Treated casein (1)*	Treated casein (2)*
½Cystine	7·4	0	0
Tyrosine	313	40	0
Lysine	540	478	392
Histidine	172	163	120
ε-*N*-Methyllysine	0	24	66

* Preparations 1 and 2 contained 1·4 and 3·9% bound formaldehyde respectively.

(*c*) *Increase in Plasma NML in Response to Supplements of Formaldehyde-treated Casein*

Previously reported values for the unidentified component in plasma of sheep receiving casein supplements for 7 weeks (Reis and Tunks 1970) have been recalculated as μmoles NML/100 ml plasma. The mean control value (±S.E.) of 4·6±1·1 μmoles/100 ml was not altered by dietary supplements of 100 g/day casein (4·4±1·1 μmoles/100 ml) but was substantially increased by dietary supplements of 100 g/day treated casein (28·3±3·3 μmoles/100 ml).

In experiment 1, two sheep that were receiving 100 g/day casein added to their diet had plasma NML levels of about 4 and 6 μmoles/100 ml (Fig. 2). When the supplement was changed to the same amount of treated casein, increases in plasma NML were detected after 24 hr. One sheep could be sampled for only 4 days, during which time plasma NML increased from 4 to 14 μmoles/100 ml. Plasma NML in the second sheep showed a similar increase and the concentration was about 24 μmoles/100 ml after 10 days.

(*d*) *Labelling of Plasma NML by* [^{14}*C*]*Formaldehyde-treated Casein Supplements*

For experiment 2, blood was obtained from an experiment reported by Mills *et al.* (1972). Lysine and NML did not separate completely on the amino acid analyser. Approximately 8% of the radioactivity emerged from the resin column with the buffer front and the remaining 92% was associated solely with the lysine–NML peak.

Two plasma samples from experiment 3 were chromatographed and, on both occasions, lysine and NML were separated completely. Only two regions of radio-

activity were detected: (1) an area of activity coming off the resin column with the buffer front, and (2) the NML peak. No radioactivity was detected in any other amino acid, including lysine. The proportion of radioactivity detected in the two

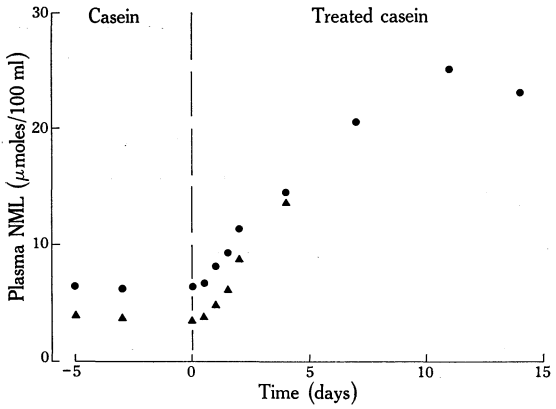

Fig. 2.—Effect of formaldehyde-treated casein on the concentration of NML in plasma. The two sheep in experiment 1 (indicated by different symbols) received dietary supplements of casein (100 g/day) for 2 weeks, followed by the same amount of treated casein for 2 weeks.

regions changed with the time after administration of the [^{14}C]formaldehyde-treated casein as indicated below:

Plasma sample	Proportion of radioactivity	
	Buffer front	NML
1. Bulk representing period 22–48 hr after dosing	49	51
2. Sample 52 hr after dosing	25	75

(*e*) *Concentration of NML in Plasma of Sheep receiving Abomasal Infusions of Sulphur-containing Amino Acids and of Proteins*

Duplicate analyses of plasma from 18 sheep receiving 600–800 g/day of the basal diet prior to experiments 4 and 5 indicated that the mean concentration of NML (±S.E.) was 5·2±0·25 μmoles/100 ml plasma. The range of values, due to differences between individuals, was 2·4–8·2 μmoles/100 ml plasma.

In Figure 3 plasma NML values for sheep receiving abomasal infusions of L-cystine and L- and DL-methionine (experiment 4) are plotted against the amount of amino acid infused. It is apparent that the amount of cystine or methionine available to the tissues had no appreciable effect on plasma NML levels in any of the sheep.

The concentration of NML in plasma was not altered significantly by the abomasal infusion of casein, Promine-D, wheat gluten, or whole egg protein; there were small, but significant, decreases in plasma NML with abomasal infusions of zein, maize gluten, and gelatin ($P < 0{\cdot}01$), and with egg albumen ($P < 0{\cdot}05$) (Table 2; experiment 5).

IV. Discussion

In view of the rigorous identification of NML in the plasma of sheep fed normal diets (Weatherall and Haden 1969; Bergen 1970) and formaldehyde-treated casein supplements (Weatherall and Haden 1969), the comparison with authentic NML by ion-exchange and paper chromatography is regarded as sufficient proof that the compound eluted after lysine in the present study is, in fact, NML.

It is apparent that NML is a normal component of sheep plasma and that the concentration is not influenced appreciably by altering the amounts and proportions of various amino acids available to the tissues. The considerable increases in plasma NML concentration during the consumption of treated casein preparations are almost

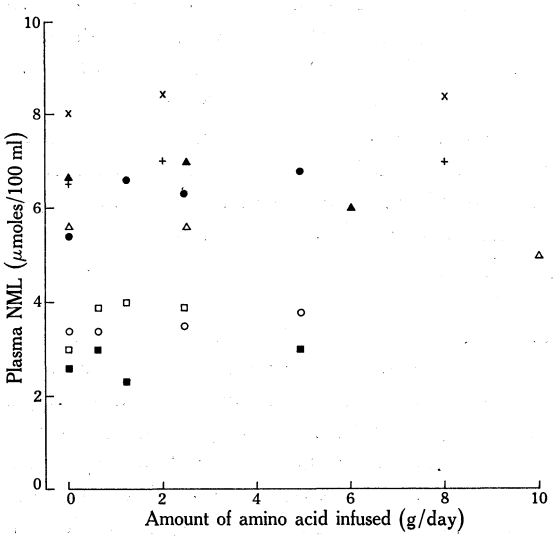

Fig. 3.—Concentration of NML in plasma of sheep receiving abomasal infusions of various amounts of cystine and methionine. Each symbol represents a different sheep receiving L-cystine (×, +), DL-methionine (▲, △), or L-methionine (●, ○, ■, □) in experiment 4.

certainly due to the reaction of formaldehyde with lysine residues in the protein, with consequent absorption of NML. Thus, whilst these elevated levels of NML in plasma may be indicators of effective treatment of casein with formaldehyde, they are unlikely to have any special biological significance.

TABLE 2

CONCENTRATION OF NML IN PLASMA OF SHEEP RECEIVING ABOMASAL INFUSIONS OF PROTEINS

Approximately 100 g/day of the proteins indicated were infused into the abomasum (experiment 5). Values are means (±S.E.) for sheep receiving the basal diet only or for the same sheep receiving the protein infusions. A mean of 20 values (duplicate samples from 10 sheep) is given for casein; a mean of four values (duplicate samples from two sheep) is given for the other proteins

Protein	Plasma NML (μmoles/100 ml)		Protein	Plasma NML (μmoles/100 ml)	
	Basal value	Protein infusion value		Basal value	Protein infusion value
Casein	5·4±0·24	4·6±0·38	Promine-D	5·9±0·54	7·6±0·60
Egg albumen	5·1±0·39	3·9±0·05*	Wheat gluten	4·4±1·04	3·7±0·74
Gelatin	4·7±0·24	3·5±0·06**	Whole egg protein	5·4±0·34	5·1±0·15
Maize gluten	5·8±0·18	4·2±0·27**	Zein	5·4±0·26	3·3±0·34**

* $P < 0·05$. ** $P < 0·01$.

Several pieces of evidence from the present study support the conclusion that the absorption of NML formed by the reaction of formaldehyde with lysine residues causes the elevated plasma NML levels in sheep fed formaldehyde-treated casein:

(1) Increases in plasma NML levels were obtained only with dietary supplements of formaldehyde-treated casein; dietary supplements or abomasal infusions of untreated casein were ineffective. The increases in plasma NML cannot, therefore, be due to alterations in the supply of amino acids to the tissues, in particular lysine as substrate or methionine as a methyl donor. Also, limited data from two sheep indicated that plasma NML levels were not influenced by the supply of formaldehyde to the tissues. Abomasal infusion of formaldehyde (1 g/day) for 10 days failed to increase plasma NML.

(2) The evidence with [^{14}C]formaldehyde-treated casein supplements (experiments 2 and 3) indicated that the formaldehyde carbon was transferred to NML. As no other amino acid in plasma was labelled it is probable that [^{14}C]NML was absorbed as such from the intestines. Other amino acids should have been labelled if the [^{14}C]NML was derived by way of metabolic conversions in the sheep's tissues.

(3) The amino acid composition of hydrolysates of the formaldehyde-treated casein (1·4% bound formaldehyde) indicates that treated casein could be the source of the increased amounts of NML found in plasma. Assuming a plasma volume of 2 litres for a sheep of 40 kg body weight, the amount of NML circulating in the plasma of a sheep with a plasma level of 20–25 μmoles/100 ml would be 400–500 μmoles. Taking the NML content of an optimally treated casein (Hemsley *et al.* 1973) as 24 μmoles/g (Table 1), a supplement of 100 g/day would provide 2400 μmoles.

The presence of NML in hydrolysates of treated casein is not conclusive evidence for its presence in the casein preparation. It is possible that free formaldehyde and lysine reacted during the hydrolysis to form NML. Gruber and Mellon (1968) observed that the presence of formaldehyde during acid hydrolysis influenced the recovery of several amino acids; in particular, tyrosine was completely lost, recoveries of cystine and histidine were substantially reduced, and that of lysine was slightly reduced. Similar changes in amino acid composition were observed in the present study when casein was treated with formaldehyde (Table 1). Together with small losses which could have occurred during hydrolysis, the formation of NML from lysine probably accounts for the appreciable reduction of lysine in hydrolysates of treated casein.

While the present results confirm the findings of Fraser and Haden (1970) and of Carrico *et al.* (1970) that supplements of formaldehyde-treated casein produce substantial increases in plasma NML, they do not confirm the suggestions of Fraser and Haden (1970) regarding the metabolic significance of the changes. In particular, the claim that dietary supplements of untreated casein cause increases in plasma NML (Carrico *et al.* 1970; Fraser and Haden 1970) do not agree with our results. However, as mentioned previously, the significance of these reported increases is not clear. Individual variation may be involved in the responses reported by Fraser and Haden (1970), although this cannot be assessed. All the data in the present paper, which showed no effect of untreated casein, were derived from direct comparisons in the same sheep receiving untreated or treated casein and would not be biased by the high degree of individual variation which occurs (Fig. 3). It is obvious from experiments 4

and 5 that various protein and amino acid infusions, which increase wool growth rate, do not increase plasma NML levels. Therefore the relationship between plasma NML and wool growth in sheep receiving formaldehyde-treated casein (Fraser and Haden 1970) must be fortuitous, and can probably be explained by the effects of absorbed amino acids on wool growth and the effect of absorbed NML on plasma NML. Also, the results of experiment 4 with cystine and methionine infusions would seem to invalidate the suggestion that plasma NML is an indicator of cyst(e)ine availability (Fraser and Haden 1970).

The results of experiments 4 and 5, with methionine, cystine, or a variety of proteins given as abomasal infusions, indicated that large changes in the amount or composition of amino acids supplied to the tissues have little influence on NML levels in plasma. The NML normally found in sheep plasma is probably derived from turnover of histone proteins. The NML in histones is most probably formed by methylation of lysine already incorporated into the peptide chain (Allfrey *et al.* 1964); it is known that methionine acts as a methyl donor for this reaction via *S*-adenosylmethionine (Murray 1964; Kim and Paik 1965). However, it would appear from the results with methionine infusions (experiment 4) that the supply of methionine would not normally restrict NML synthesis. The results with various proteins given as abomasal infusions indicate that there may be a slight lowering of plasma NML levels with rather unbalanced mixtures of amino acids, especially those lacking in lysine (zein and maize gluten) or in methionine (gelatin). Bergen and Potter (1971) suggested that methionine availability plays a role in the regulation of NML synthesis in sheep. Our results indicate that this may only be so when methionine availability is markedly restricted.

V. Acknowledgments

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