"vitamin free" casein (0.14 μg/g of casein; ICN Pharmaceuticals, Inc.). These results confirm the necessity of preliminary testing of sample extract purification effects on the chemical thiamin determination to ensure accuracy during routine analysis.

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In Vitro Rumen Microbial Stability and in Vivo Availability of Polymerized L-Lysine-HCl

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The stability of a urea–L-lysine–HCl–formaldehyde polymer to in vitro rumen microbial degradation and the in vivo lysine availability of the polymer were determined. After incubation with rumen microorganisms for 24 or 48 h, from 51 to 59% of the polymerized L-lysine–HCl was recovered. In contrast, only about 11% of free L-lysine–HCl could be recovered after rumen fermentation. Rat growth studies indicated that essentially all of the L-lysine–HCl in the polymer was available.

Rumen bacteria and protozoa rapidly hydrolyze protein to amino acids, which are immediately deaminated to an organic acid and ammonia (Broderick, 1975). However, research within the past few years has shown that treatment of oilmeal and forage proteins with aldehydes and tannins can effectively reduce rumen microbial degradation of the treated protein and increase the quantity of protein reaching the lower gastrointestinal tract (Amos and Evans, 1976; Nishimuta et al., 1974; Axford and Evans, 1975; Chalupa, 1975). In many instances, increasing the quantity of dietary protein which is not degraded in the rumen has failed to increase animal growth or nitrogen retention. Part of this failure to increase these growth parameters may be due to deficiencies of methionine and lysine in the treated protein. These two amino acids have been shown to be deficient in microbial protein for growing lambs (Nimrick et al., 1970); responses have been shown from these amino acids by lactating cows (Schwab et al., 1976) and in growing steers to lysine (Boila and Devlin, 1972) and to methionine (Steinacker et al., 1970; Fenderson and Bergen, 1975). Since direct supplementation of ruminant diets with a free amino acid is not effective in increasing the quantity of that amino acid available for absorption, due to deamination as cited earlier, alternate methods for supplementing the ruminant diet with amino acids are needed.

The objectives of this work were to determine the in vitro stability to rumen microbial activity and in vivo availability of the lysine present in a urea–formaldehyde–lysine polymer.

EXPERIMENTAL PROCEDURES

The polymerized lysine used in these studies was prepared by dissolving 100 g of urea (1.67 M) in 186 mL of 37% formaldehyde (2.29 M) at room temperature. After complete dissolution of the urea, 100 g of L-lysine–HCl (0.55 M) was added with constant stirring. The L-lysine–HCl-urea–formaldehyde mixture was allowed to react and harden at room temperature (usually 24–36 h). After hardening, the lysine polymer was ground to pass through a 1-mm Wiley mill screen, dried at 39 °C under reduced pressure (10 mmHg) for 24 h, and used in vitro rumen microbial stability and in vivo rat growth studies. Total nitrogen in the lysine polymer was determined by the Kjeldahl procedure (AOAC, 1970). Portions of the polymer were hydrolyzed in 6 N HCl (Wilkinson et al., 1968) and total lysine was determined according to Moore et al. (1958).

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Table I. Recovery of Lysine from in Vitro Fermentations of Control, L-Lysine-HCl, or Polymerized L-Lysine-HCl (CBG,[a] Experiment 1)

Treatment	Observations per treatment	Fermentation time, h: 0	Fermentation time, h: 24	Amount from added lysine[b]
		mg/30 mL		
Inoculum	1	2.8	3.0	
Inoculum + CBG	1	5.0	5.7	
Inoculum + CBG + lysine (1)[c]	1	9.8	5.8	0.1
Inoculum + CBG + lysine (2)[c]	1	14.6	6.8	1.1
Inoculum + CBG + lysine (3)[c]	1	19.4	7.3	1.6
Inoculum + CBG + polymer (1)[d]	1	9.8	8.3	2.6
Inoculum + CBG + polymer (2)[d]	1	14.6	10.9	5.2
Inoculum + CBG + polymer (3)[d]	1	19.4	13.1	7.4

[a] Coastal bermudagrass. [b] Calculated by subtracting inoculum + CBG from total quantity of lysine recovered from each respective treatment. [c] L-Lysine-HCl added at 0 time to provide 4.8, 9.6, and 14.4 mg of L-lysine for levels 1, 2, and 3, respectively. [d] Polymerized L-lysine-HCl added at 0 time to provide 4.8, 9.6, and 14.4 mg of L-lysine for levels 1, 2, and 3, respectively.

Two in vitro rumen experiments were conducted to determine the stability of the L-lysine in the polymer to rumen microbial degradation. Inoculum for these experiments was obtained from a mature rumen cannulated steer fed a Coastal bermudagrass (CBG) diet. In experiment 1, CBG and purified cellulose (Solka-floc BW-200) were used as substrates. The inoculum consisted of 10 mL of strained rumen fluid plus 20 mL of McDougall's buffer (1948) and 400 mg of either substrate. Treatments were: (1) control (substrate + inoculum), (2) control + L-lysine–HCl, or (3) control + polymerized L-lysine–HCl. Incubations were conducted with three levels of L-lysine–HCl and polymerized L-lysine–HCl. The L-lysine–HCl treatment levels were 6.0, 12.0, or 18.0 mg per 30 mL and the polymerized L-lysine–HCl levels were 16.0, 32.0, or 48.0 mg per 30 mL. These levels of L-lysine–HCl and polymer provided 4.8, 9.6, and 14.4 mg of actual L-lysine for each treatment level, respectively. In addition, the L-lysine–HCl treatments also contained 6, 12, or 18 mg of urea to provide the same amount of nitrogen from urea as provided by the three polymer treatments. Four replicate 24-h fermentations were used with each substrate at each treatment level. All fermentations were done at 39 °C in 50 mL centrifuge tubes. The fermentation tubes were closed with rubber stoppers fitted with one-way bunsen values to permit loss of CO_2 and CH_4 produced during substrate fermentation. After 24 h, two replicates were used for the determination of in vitro dry matter disappearance (IVDMD, Tilley and Terry, 1963); and the remaining two replicates were combined, frozen in dry ice, and lyophilized. The IVDMD data were tested by analysis of variance for treatment differences and treatment means were separated by using orthogonal coefficients (Steel and Torrie, 1960). Portions of the lyophilized samples were hydrolyzed and analyzed for lysine as described previously.

In experiment 2, a 4-L Erlenmeyer flask was used as the incubation chamber. Only the CBG substrate and the highest level of L-lysine–HCl (18.0 mg) and polymer (48.0 mg) were used in this experiment. Four 30-mL samples were taken at 0, 3, 6, 12, 24, and 48 h of fermentation and analyzed for IVDMD and lysine as in experiment 1. All other experimental conditions were the same as given previously, except that the incubation flask was bubbled with CO_2 throughout the experiment.

Two rat-growth experiments were conducted to determine the biological availability of the L-lysine in the polymer. Both experiments were conducted in a randomized block design with rats weighing between 85 and 90 g initially. There were five replicates per treatment in experiment 1, a 28-day growth study; and three replicates per treatment in experiment 2, a 14-day growth study. Each replicate contained one rat per treatment. Feed and water were offered ad libitum in each experiment. Body weight changes and feed consumption were determined every 7 days. All data were treated by analysis of variance for treatment effects, and differences between means were determined by orthogonal comparisons (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Urea and formaldehyde when reacted alone form an amorphous polymer by a Mannich type reaction (Barton et al., 1977; House, 1965; Tomita and Hirose, 1976). When lysine is included in the reaction mixture, crosslinking by means of a methylene carbon occurs between the ϵ-amino group of lysine and the urea–formaldehyde polymer. The ^{13}C NMR spectra indicate that after 96 h the reaction has reached equilibrium, no further changes occur in the spectra after this time and that approximately 40% of the lysine is crosslinked to the urea–formaldehyde polymer (Barton et al., 1977). Of the 60% unreacted lysine (not crosslinked), about 20 percentage units were readily solubilized in H_2O within 12 h and the remaining 40 percentage units of lysine are entrapped with the urea–formaldehyde polymer.

The calculated content of L-lysine in the polymer was 30.0 to 30.4%; actual lysine analyses of three preparations of the polymer ranged from 29.9 to 31.2% L-lysine (mean for the three analyses was 30.5% L-lysine). This was a decrease in lysine from about 37.7% in the original mixture and resulted from a 32.0 to 32.2% increase in the weight of product due to the binding of formaldehyde during the polymerization reaction and entrapment of water.

Table I gives the recovery of lysine from the Coastal bermudagrass fermentations containing added lysine. At 0 time, the inoculum contained 2.8 mg of lysine which increased to 3.0 mg after 24-h fermentation. The addition of 400 mg of CBG increased the initial lysine level to 5.0 mg. After 24 h, the CBG + inoculum treatment contained 5.7 mg of lysine. The initial lysine levels for the incubations containing added free L-lysine–HCl were 9.8, 14.6, and 19.4 mg for levels 1, 2, and 3, respectively; but after 24-h fermentation only 5.8, 6.8, and 7.3 mg of lysine were recovered. This indicated that 0.1, 1.1, and 1.6 mg of the added lysine had escaped rumen microbial degradation in vitro; and these amounts represented only 2.0, 11.4, and 11.1% recovery of the added lysine in treatments 1, 2, and 3, respectively. The initial levels of lysine for the polymer treatments were also 9.8, 14.6, and 19.4 mg for levels 1, 2, and 3, respectively. At the end of 24-h incubation, 8.3, 10.9, and 13.1 mg of lysine were recovered and accounted for 2.6, 5.2, and 7.4 mg of the lysine added in the polymer for

Table II. Recovery of Lysine from in Vitro Fermentations of Control, L-Lysine-HCl, or Polymerized L-Lysine-HCl (SF,[a] Experiment 1)

Treatment	Observations per treatment	Fermentation time, h		Amount from added lysine[b]
		0	24	
		mg/30 mL		
Inoculum	1	2.8	2.7	
Inoculum + SF	1	2.8	4.5	
Inoculum + SF + lysine (1)[c]	1	7.6	5.1	0.6
Inoculum + SF + lysine (2)[c]	1	12.4	5.6	1.1
Inoculum + SF + lysine (3)[c]	1	17.2	6.4	1.9
Inoculum + SF + polymer (1)[d]	1	7.6	6.0	1.5
Inoculum + SF + polymer (2)[d]	1	12.4	9.2	4.5
Inoculum + SF + polymer (3)[d]	1	17.2	12.2	7.7

[a] Solka-floc, BW-200. [b] Calculated by subtracting inoculum + SF from total lysine recovered from each respective treatment. [c] L-Lysine-HCl added at 0 time to provide 4.8, 9.6, and 14.4 mg of L-Lysine for levels 1, 2, and 3, respectively. [d] Polymerized L-Lysine-HCl added at 0 time to provide 4.8, 9.6, and 14.4 mg of L-Lysine for levels 1, 2, and 3, respectively.

Table III. Percentage in Vitro Dry Matter Disappearance from CBG[a] and SF[b] Fermentations (Experiment 1)

Treatment	Observations per treatment	Substrate	
		CBG ± SD[c]	SF ± SD[c]
Control	2	55.6 ± 0.2	41.5 ± 0.3
Control + lysine (1)[d]	2	53.1 ± 0.1	45.2 ± 0.1
Control + lysine (2)[d]	2	53.4 ± 0.4	48.4 ± 0.3
Control + lysine (3)[d]	2	58.6 ± 0.6	42.3 ± 0.6
Control + polymer (1)[e,f]	2	51.1 ± 0.1	42.8 ± 0.5
Control + polymer (2)[e,f]	2	46.6 ± 0.3	50.5 ± 0.1
Control + polymer (3)[e,f]	2	44.6 ± 0.3	44.4 ± 0.2

[a] Coastal bermudagrass. [b] Solka-floc, BW-200. [c] Standard deviation. [d] L-Lysine-HCl added to provide 4.8, 9.6, and 14.4 mg of L-lysine at 0 time for 1, 2, and 3, respectively. [e] Polymerized L-Lysine-HCl added to provide 4.8, 9.6, and 14.4 mg of L-lysine for 1, 2, and 3, respectively. [f] Significant linear decrease in IVDMD due to polymer ($P < 0.05$) in CBG treatment.

levels 1, 2, and 3, respectively. These amounts represented 54.2, 54.2, and 51.4% recovery of the polymerized lysine.

Table II gives the lysine recovered from in vitro fermentations of solka-floc plus either free L-lysine–HCl or polymerized L-lysine–HCl. The initial lysine level in the control (inoculum + solka-floc) was 2.8 mg and increased to 4.5 mg after 24-h fermentation. The addition of three levels of lysine gave 0 time lysine levels of 7.6, 12.4, and 17.2 mg, respectively. After 24-h in vitro fermentation these lysine levels had decreased to 5.1, 5.6, and 6.4 mg and represented only 12.5, 11.4, and 13.2% recovery of the free L-lysine added at 0 time. In the fermentations initially containing 4.8, 9.6, and 14.4 mg of L-lysine in polymerized form plus 2.8 mg of lysine from the inoculum, 6.0, 9.2, and 12.2 mg of lysine were recovered after 24-h fermentation. This represented 31.2, 46.9, and 53.5% recovery of the added lysine. Perhaps this somewhat greater loss of lysine from the solka-floc incubations compared to the CBG incubations indicates that nitrogen was limiting in these fermentations. Nitrogen was in excess of microbial needs in the incubations containing CBG (Amos et al., 1976), but the rumen microorganisms may have had to degrade more of the polymer to meet their nitrogen requirements when solka-floc was used as the substrate.

The percentage IVDMD from the incubations containing either CBG or solka-floc are shown in Table III. The percentage IVDMD for CBG was 55.6 and averaged 51.8 for all treatments. However, there was a decrease in percentage IVDMD for the CBG treatments with polymer ($P < 0.05$) as compared to without. The percentage IVDMD (cellulose digestion) when solka-floc was used as the substrate was 41.5 (control) and averaged 45.0 for all treatments. The addition of nitrogen from L-lysine–HCl plus urea or the polymer tended to increase cellulose digestion. The increase again indicates that a deficiency of nitrogen may have limited cellulose digestion in the control treatment. Even though cellulose digestion was greater than control in all incubations containing the polymer, the highest level of polymer addition appeared to decrease cellulose digestion relative to the intermediate level.

Table IV gives the recoveries of lysine from experiment 2. Total lysine for the CBG incubation was 4.5 mg at 0 time, increased to a high of 5.9 mg after 12 h, and decreased thereafter to a low of 4.4 mg after 48-h incubation. Total lysine for CBG + L-lysine–HCl was 16.7 mg at 0 h and decreased gradually to 12.8 mg after 12 h for a loss of 23.4% of the 0-h lysine level. During the second 12-h fermentation total lysine decreased to 6.1 mg (63.5% loss) and further decreased to 4.6 mg after 48 h, a total loss of 12.1 mg of lysine (72.4% loss). In actuality, the addition of 14.4 mg of L-lysine–HCl at 0 h only increased the total

Table IV. Recovery of Lysine from In Vitro Fermentations of Control, L-Lysine-HCl or Polymerized L-Lysine-HCl (CBG,[a] Experiment 2)

Fermentation time, h	Treatment					
	Control (inoculum + CBG)	Loss or gain[b]	L-Lysine-HCl	Loss mg/30 mL	Polymerized L-lysine	Loss
0	4.5		16.7		14.4	
3	5.6	+1.1	15.0	−1.7	13.8	−0.6
6	5.1	+0.6	13.7	−3.0	12.2	−1.2
12	5.9	+1.4	12.8	−3.9	12.2	−1.2
24	5.4	+0.9	6.1	−10.6	11.2	−3.2
48	4.4	−0.1	4.6	−12.1	13.0	−1.4

[a] Coastal bermudagrass. [b] Calculated by subtracting control (0 time value) from each respective time and treatment value.

Table V. Percentage Composition of Rations Used in Rat Growth Studies

Ingredient	Ration 1	2	3	4	5	6	7	8	9	10
Corn starch	57.9	57.6	57.4	57.1	57.2	56.5	55.7	57.0	57.4	56.8
Sunflower meal[a]	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9
Vitamin premix	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Mineral premix	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Lysine-HCl[b]		0.275	0.550	0.825						0.55
Polymer[c]					0.725	1.450	2.175			
L-Glutamic acid								0.90		
Urea									0.55	0.55
Kjeldahl crude protein	16.2	16.5	16.6	16.6	16.6	17.6	19.0	17.1	17.5	18.8

[a] Provided 0.47% L-lysine in total diet. [b] Provided 0.22, 0.44, and 0.66% actual L-lysine. [c] Actual L-lysine analyses of this polymer was 30.4%; therefore treatments 5, 6, and 7 provided 0.22, 0.44, and 0.66% L-lysine.

Table VI. Performance of Rats Fed Control Diet, L-Lysine-HCl, or Polymerized L-Lysine-HCl Supplemented Diets (Experiment 1)[a]

Treatment	Gain,[b] 28 days, g	Feed consumed,[c] 28 days, g	Feed efficiency,[d] g of feed/g of gain
Control	128.4	520.0	4.0
Control + 0.275% L-Lys-HCl	180.0	510.8	2.8
Control + 0.725% polymer	161.8	558.8	3.4
Control + 0.55% L-Lys-HCl	196.2	535.2	2.7
Control + 1.45% polymer	200.8	577.8	2.9
Control + 0.825% L-Lys-HCl	190.2	538.8	2.8
Control + 2.175% polymer	199.0	564.4	2.8

[a] Each value is the mean of five observations per treatment. [b] Significant increase in growth due to lysine supplementation ($P < 0.005$). [c] Significant difference due to source of lysine supplementation ($P < 0.10$). [d] Significant decrease in feed required per unit gain with lysine supplementation ($P < 0.005$).

Table VII. Performance of Rats Fed Control Diet, L-Lysine-HCl, or Polymerized L-Lysine-HCl Supplemented Diets (Experiment 2)[a]

Treatment	Gain,[b] total 14 days, g	Feed consumed,[c] total 14 days, g	Feed efficiency,[d] g of feed/g of gain
Control	52.0	189.6	3.6
Control + 0.275% L-Lys-HCl	76.7	214.3	2.8
Control + 0.725% polymer	76.0	230.0	3.0
Control + 0.550% L-Lys-HCl	86.0	222.2	2.6
Control + 1.450% polymer	85.3	192.7	2.3
Control + 0.825% L-Lys-HCl	87.0	230.0	2.6
Control + 2.175% polymer	86.3	233.7	2.7
Control + 0.90% glutamic	40.3	192.7	4.8
Control + 0.55% urea	35.0	190.7	5.4
Control + 0.55% urea + 0.55% L-Lys-HCl	84.0	208.3	2.5

[a] Each value is the mean of three observations per treatment. [b] Significant linear increase due to lysine supplementation ($P < 0.005$). [c] Significant increase due to lysine supplementation ($P < 0.01$). [d] Significant decrease in feed required per unit gain with lysine supplementation ($P < 0.005$).

lysine recovery by 0.2 mg after 48-h incubation. On the other hand, the total 48-h incubation loss in the presence of the polymer was only 1.4 mg and gave a 90.3% recovery of the 0-h lysine content.

The 0-h lysine recoveries for both the CBG + L-lysine–HCl and CBG + polymerized L-lysine–HCl were lower than expected (18.9) and may indicate some error in sampling. However, the data show the polymerized form of L-lysine was much more stable to rumen microbial attack than free L-lysine–HCl. If the 0-h lysine content was in fact 18.9 mg and 13.0 mg remained after 48 h (a loss of 5.9 mg of lysine), then 59.7% of the lysine in polymer was recovered as compared to only 1.4% recovery of the added L-lysine–HCl.

Rations used in the rat growth experiments for evaluation of the in vivo availability of the polymerized L-lysine–HCl are presented in Table V. Rations 1 to 7 were used in experiment 1, and all 10 rations were used in experiment 2. Sunflower meal, used as the protein supplement, is deficient in lysine for the growing rat (Amos et al., 1975) and provided 0.47% lysine in ration 1 (control). L-Lysine–HCl was added to rations 2, 3, and 4 to provide an additional 0.22, 0.44, and 0.66% L-lysine, respectively. Ration 3 provided lysine at a level of 0.91% of the total diet (dry matter basis) which is essentially the NRC (1972) requirement for lysine in the growing rat. The polymerized lysine addition of 0.725, 1.45, and 2.175% to rations 5, 6, and 7, respectively, also provided 0.22, 0.44, and 0.66% additional lysine. L-Glutamic acid was added to ration 8 to provide nitrogen at the same level as provided by L-lysine–HCl in ration 3. Urea was added to ration 9 to provide the same amount of nitrogen as furnished by the polymer addition to ration 6. Finally, urea and lysine were added to ration 10 to provide lysine and urea at the same level furnished by the polymer in ration 6.

Table VI gives the 28-day growth, feed consumption, and feed efficiency data of rats in experiment 1. Rats receiving the control diet gained 128.4 g, consumed 520.0 g feed, and had a feed efficiency (g of feed/g of gain) of 4.0. There was a highly significant ($P < 0.005$) increase in growth due to the addition of L-lysine–HCl to the control diet. However, there were no differences between means in growth due to source of supplemental L-lysine. Feed consumption was increased ($P < 0.10$) in rats fed diets

containing the polymerized L-lysine-HCl. Perhaps this increase in feed consumed was due to the dilution of energy (starch) by the addition of up to 2.175% of the polymer to the diet; and once the lysine requirement was met the rats ate to satisfy the energy requirement, thus increasing their feed consumption. The amount of feed required to produce a unit of gain was reduced ($P < 0.005$) also by the addition of L-lysine to the diet. However, there was no difference due to source of L-lysine ($P > 0.10$).

The performance of rats in experiment 2 is presented in Table VII. Rats fed the control diet gained 52.0 g, consumed 189.6 g of feed, and had a feed efficiency of 3.6. There was a highly significant ($P < 0.005$) linear increase in growth with increasing levels of supplemental lysine regardless of source of lysine. Feed consumptioni also increased ($P < 0.01$) with lysine supplementation; but in contrast to experiment 1 there was no difference due to the source of lysine. The failure for feed consumption to differ in this study may be due to its shorter duration. As in experiment 1, the amount of feed required to produce a unit of gain was reduced by the addition of lysine ($P < 0.005$), but no difference was found due to source of lysine.

The last three treatment comparisons (Table VII) are quite revealing. Growth of rats fed the diets containing L-glutamic acid or urea was not equal to those fed the control diet for reasons not completely clear at this time. However, the data show that the increase in growth of rats fed the various levels of supplemental lysine was due to lysine per se and not to the simple addition of nitrogen. Also the poorer performance was not due to lowered feed consumption, since feed intake of rats fed rations 8 or 9 was equal to that of rats fed the control diet. Finally, the addition of lysine and urea (ration 10) to the control diet gave a response equal to that obtained from the addition of an equal level of lysine alone (ration 4).

The overall results of this study clearly show that free L-lysine is rapidly degraded by the rumen microbial population in vitro. In contrast the lysine present in a urea-lysine-formaldehyde complex was quite stable to rumen microbial attack for up to 48 h. A minimum of 51–59%, and possibly as high as 90% in some cases, of the polymerized L-lysine-HCl was recovered after 48-h incubation with rumen microorganisms in vitro. Addition of the polymers to in vitro rumen fermentations appeared to depress IVDMD slightly; however, this would be expected to be of minor importance in in vivo studies. The results of the rat growth experiments also vividly show that the lysine in the polymer was essentially completely available to the growing rat. The polymerized L-lysine shows considerable promise as a dietary supplementary amino acid for ruminants where a deficiency of this amino acid may be limiting animal performance.

This mechanism for protecting lysine may also have some utility for protecting other amino acids (entrapment in the polymer). Methionine when reacted with urea, lysine, and formaldehyde was about 52.6% protected from microbial degradation in vivo but only 10.5% protected when reacted with urea and formaldehyde alone (Amos and Evans, 1978). In addition, varying the molar ratios of urea to lysine from 3:1, 2:1 to 1:1 has resulted in a 56.5, 60.4, and 42.2% recovery, respectively, of lysine from the abomasum in in vivo studies (Amos and Evans, unpublished results).

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