energy change that is proportional to the surface area of the proteins. Therefore, it was suggested that the denatured form of the protein, which has a larger surface area, is less stable thermodynamically than the nondenatured form, and thus the protein tends not to denature. It is possible that certain amino compounds such as TMAO protect against protein denaturation in a similar way.

Registry No. TMAO, 1184-78-7; betaine, 107-43-7.

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Nutritional Characteristics of Alkali-Treated Zein

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Batches of commercial zein, a lysine-free protein, were treated with either 0.1 N NaOH or $Ca(OH)_2$ (4 h at 85 °C), neutralized, washed, and freeze-dried. Amino acid analysis showed major losses of cystine and lesser losses of serine and threonine. The average proportions of D-amino acids in the NaOH- and $Ca(OH)_2$-treated samples were 20.3 and 15.3% vs. 1.5% in the untreated zein. The order of racemization was Asp and Ser > Phe, Glu, Tyr, and Thr > Met > Ala > Val, Leu, Ile, and Pro. Young rats receiving 4% N from untreated zein and liberal amino acid supplements grew well. With NaOH-treated zein they failed to grow and showed severe diarrhea; effects with $Ca(OH)_2$-treated zein were less severe. These adverse effects were not fully explained by differences in digestibility, nor were they reproduced by adding D-serine or D-alloisoleucine to diets containing untreated zein. Replacing supplementary L-threonine with D-allothreonine gave depressed growth but did not product diarrhea. Our results show that alkali treatment can cause nutritional damage to a protein without the formation of lysinoalanine.

Adverse nutritional effects from the alkaline treatment of proteins have most commonly been linked to the formation of lysinoalanine (LAL), which can be toxic under certain conditions (De Groot and Slump, 1969; Karayiannis et al., 1979). The special interest of work with zein is that is contains no lysine so that there is no possibility of LAL formation by the Michael addition between the ϵ-amino group of lysine units and dehydroalanine, which may be formed by decomposition of cystine and serine. Our plan was to study the chemical changes in zein treated with either NaOH or $Ca(OH)_2$ and to feed it to young rats. Because of the multiple amino acid deficiencies of zein, feeding experiments require that it be supplemented with a range of amino acids. Our first hypothesis was that if the treatments proved nutritionally deleterious, this could be explained by the formation of D-serine, which has been reported to cause severe kidney damage in rats (Wachstein, 1947a; Kaltenbach et al., 1979). When we did find changes not apparently explained by the formation of D-serine, we

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Table I. Amino Acid Composition[a] of Treated Zeins in Comparison with Untreated Material

	untreated zeins			NaOH zeins		$Ca(OH)_2$ zeins		
amino acid	C-175[b]	C-196[c]	unmodified[d]	C-177[b]	C-197[c]	C-176[b]	C-200[c]	G-200[d]
Ala	12.2	9.7	10.7	93	106	98	101	101
Val	4.4	3.6	3.2	89	106	100	110	123
Leu	25.8	21.1	20.6	97	113	107	109	108
Ile	5.2	4.1	3.1	87	91	92	94	127
Cys	n.d.[e]	1.6	1.1	n.d.	43	n.d.	36	23
Met	n.d.	1.9	2.0	n.d.	118	n.d.	106	n.d.
Phe	9.4	7.9	7.4	87	119	95	99	121
Asp	6.6	5.8	5.6	94	108	100	104	111
Glu	27.7	24.7	25.9	90	110	92	105	101
Ser	6.8	5.6	6.9	71	70	75	63	83
Thr	3.5	2.8	3.2	71	71	74	71	94
Tyr	6.8	5.8	n.d.	84	100	91	92	n.d.
His	1.7	1.4	n.d.	88	117	94	117	n.d.
Pro	12.6	10.7	11.0	86	104	92	93	99
Arg	2.1	1.8	1.6	86	88	67	106	90
Gly	1.5	1.2	1.4	93	111	100	111	95

[a] Values for control zeins are expressed as grams per 16 g of N and those for the treated samples as percentages of the control value in the same series. [b] Samples analyzed in laboratory I. [c] Samples analyzed in laboratory II. [d] The values in these columns were obtained by Boundy et al. (1967) for unmodified zein and a commercial zein (G-200) that had received mild treatment with $Ca(OH)_2$ in its preparation. [e] n.d. = not determined.

studied the changes in digestibility of zein with alkali treatment and also the possible effects of two other racemic products, D-alloisoleucine and D-allothreonine.

MATERIALS AND METHODS

Test Materials. Zein was purchased from ICN Nutritional Biochemicals, Cleveland, OH, and casein from Teklad Test Diets, Madison, WI.

For the first set of alkali treatments, 2 kg of zein (C-175) was suspended, with constant stirring, in 27 L of water; 99.9 g of $Ca(OH)_2$ was added and the temperature raised rapidly to 85 ± 5 °C with an external steam jacket. Water was added as needed to keep the volume constant. After 4 h, the mixture was allowed to cool, then adjusted to pH 3.0 with 6 N HCL, and stored overnight at 4 °C. The curd was then separated, filtered, and rinsed with 50 L of water, freeze-dried, and ground to a powder (C-176) that passed a 40-mesh sieve. The same procedure was than followed with 27 L of 0.1 N NaOH instead of $Ca(OH)_2$, to produce a second produce (C-177).

For the second set of alkali treatments, another batch of zein (C-196) was used. Four kilograms was first suspended in 27 L of water and then 27 L of 0.2 N NaOH was added with stirring. After 4 h at 80–85 °C, the mixture was cooled to 60 °C, neutralized with 6 N HCl, and stored overnight at 4 °C. the mixture was then further adjusted to pH 4.5 and the curd filtered. It was then redispersed in water with a homogenizer, refiltered 4 times, and freeze-dried, forming a fine powder (C-197). A further aliquot of 1.5 kg of zein was dispersed in 12 L of water; 74.1 g of $Ca(OH)_2$ was suspended in 8 L of water and added to the mixture. As the temperature was raised, a powerful homogenizer was used to disperse clumps. After 4 h at 80–85 °C the pH was adjusted to 4.5. After storage overnight at 4 °C, the material was rinsed and refiltered 4 times without difficulty. After freeze-drying, the product was ground to a powder (C-200). The quantities recovered were 1680 g with NaOH and 790 g with $Ca(OH)_2$.

In small-scale runs, the initial pH at 21 °C was 12.7 with NaOH and 12.6 with $Ca(OH)_2$. After 20–25 min, when the temperature reached 80–85 °C, the values were 10.7 and 10.6, and they fell gradually over the 4 h to 9.7 and 9.2, respectively.

Amino Acid Analysis. The procedures used for amino acid analysis are described by Liardon and Hurrell (1983). For the second set of samples, analyzed in laboratory II, sulfur amino acid analysis (using performic acid oxidation) was included but not for the first set of samples analyzed in laboratory I. There was no need for a separate digestion procedure for tryptophan as it is not present in zein. All the values given in Table I are the means of duplicate analyses.

Measurement of D and L Enantiomers. In laboratory I, following the hydrolysis of the test proteins in 6 N HCl, the amino acids were successively reacted with 2-propanol (containing HCl at 3 N) and pentafluoropropionic acid anhydride. The enantiomeric separation of the derivatives was achieved by capillary gas chromatography using an optically active coating (L-valine 3-butylamide cross-linked to a polysiloxane backbone) (Applied Science Laboratories, State College, PA; C.G.C. Analytic, Moessingen, FRG). In laboratory I, the analyses were carried out in a Hewlett-Packard Model 5840 gas chromatograph equipped with a flame ionization detector (Tovar, 1981). In laboratory II, the determinations followed the procedure of Liardon et al. (1981), involving the hydrolysis of the test samples in deuterated HCl and the monitoring of the GC effluent by multiple ion detection mass spectrometry (Hewlett-Packard Model 5992 GC/MS). By this approach, racemization that occurred during the acid hydrolysis stage of the analysis could be distinguished from that already present in the test samples. The values listed in Table II are the means of duplicate analyses.

Other Analyses. Nitrogen was determined in test materials and rat feces by the Kjeldahl procedure (AOAC, 1980). The chromium contents of diets and excreta were measured as previously described (Varnish and Carpenter, 1975), but on a one-fifth scale. Calcium and sodium were determined by ashing 1-g samples followed by standard atomic absorption spectrophotometry (AOAC, 1980).

Rat Experiments. Five experiments were carried out, using commercially reared male, Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA, for experiments 1 and 2 and Bantin and Kingman, Inc., Fremont, CA, for the later tests). They were received at approximately 21 days of age and fed on a control diet for 4–7 days until they weighed approximately 70 g. They were then allocated to the experimental diets by a randomization procedure. The rats in experiment 1 were housed two to a cage; for later experiments they were caged individually. Each cage had a raised wire screen floor; water was provided ad libitum and also the diet, in powder form, in jars. There was

Table II. Racemization[a] of Amino Acids in Untreated and Alkali-Treated Zein

amino acid	untreated		NaOH treated		$Ca(OH)_2$ treated	
	C-175[b]	C-196[c]	C-177[b]	C-197[c]	C-176[b]	C-200[c]
Ala	2	0.7	15	17.6	8	11.5
Val	2	0.4	3	2.9	2	1.6
Leu	<3[d]	0.7	<9[d]	5.0	<7[d]	3.2
Ile	*d*	0.4	*d*	4.8	*d*	3.4
Cys		2.1		40[e]		40[e]
Met		0.9		19.5		15.3
Asp	5	3.4	37	40.2	33	39.0
Glu	3	1.5	26	30.4	17	21.7
Ser	2	1.5	33	35.2	35	34.0
Thr		1.6		26.7		17.2
Pro	1	0.2	5	1.3	3	1.3
Tyr		5.0		28.4		14.0
Phe	4	2.2	27	31.3	15	21.3
Orn				26.2		16.2

[a] D/(D + L) (%). [b] Samples analyzed in laboratory I. Several amino acids could not be analyzed in this system, and the values are not corrected for racemization during acid hydrolysis. [c] Samples analyzed in laboratory II; values corrected for racemization induced during acid hydrolysis. [d] It was known that isomers of Ile, and possibly other compounds, superimposed the peak for D-Leu. [e] Only traces of Cys were detected in these hydrolysates and the values are approximate.

significant spillage on some treatments, which was measured to determine net food consumption. Each rat was weighed at least twice per week.

In each experiment the apparent digestibility of dietary N was measured. This involved adding 0.15% Cr_2O_3 to each diet (in the form of a 15% concentrate) (Kane et al., 1950). Fecal samples were collected over a 3-day period from each cage, freeze-dried, and ground before analysis. Any fecal material contaminated with split food was not included in the collection. Samples of diet and feces were then analyzed for both N and Cr_2O_3. On the assumption that there was no absorption of Cr_2O_3, the apparent digestibility of N could be calculated from the change in N:Cr_2O_3 ratios between diet and feces. It was assumed that the free amino acids in the diets were completely digested. The digestibility of casein was calculated from diets in which it was the sole protein source, and it was assumed to have the same digestibility when mixed with zein. In experiment 1, the chromium concentrate was not included in the diet until the last week of the experiment, and the rats had been on the test diets for 18–21 days during the fecal collection period. In the later experiments, the concentrate was included from the beginning and feces collected on days 3–6 of the experiment.

At the end of experiments 1–3, the rats were killed and a kidney (experiment 1) or pancreas (experiments 2 and 3) were removed and weighed. The kidneys were cooled immediately to −20 °C and stored prior to enzyme assay.

Enzyme Assay. γ-Glutamyl transpeptidase (GGTP) activity was measured in homogenates of the frozen kidneys using γ-L-glutamyl-*p*-nitroanilide as the substrate and glycylglycine as the acceptor molecule for the glutamyl group released (Naftalin et al., 1969).

Experimental Diets. Each diet contained corn oil 5%, α-cellulose 3%, mineral mixture 3.5%, and vitamin mixture 1%. After the variable ingredients had been added, the diet was made to 100% with a mixture (85:15) of corn starch and sucrose. The mineral and vitamin mixtures were slight modifications (Tovar, 1981) of the AIN-76 mixtures (Bieri et al., 1977). Where the $Ca(OH)_2$-treated zeins were used, the calcium content of the mineral mixture was adjusted to keep the total calcium content of the diets similar (Tovar, 1981). The same was done for sodium with the NaOH-treated zein in experiment 1. The second batch (C-197) was of low sodium content and no adjustment was made in the later experiments.

In experiment 1, each of the three zein preparations, C-175, C-176, and C-177, were included at a level contributing 4.0% N to the diet, except for one diet where the level was reduced slightly to compensate for the extra N supplied by glycyl-D-serine. Each diet containing zein also had both a supplement of casein and a mixture of L-amino acids. In experiment 1 this consisted of lysine hydrochloride 1.27, tryptophan 0.21, arginine 0.26, methionine 0.5, threonine 0.13, histidine 0.2, and proline 0.47% of the diet. These supplements were designed to compensate for the amino acid deficiencies of zein for the young rat. In addition, further amino acid supplements were made to individual diets as listed in Table III. The two diets (1 and 2) containing casein, without zein, included just the amino acid supplements shown in Table III. The second casein diet, of lower N content than the others and with D-serine added, was designed to be comparable to that found by Wachstein (1947) to cause acute kidney necrosis. Likewise, the high levels of treated zein in diets 7A and 8 were chosen because they contributed approximately the same level (i.e., 0.5%) of D-serine. Additional comparisons were provided by diets 4 and 5 with the same level of untreated zein and 0.5% D-serine or the equivalent of glycyl-D-serine, as well by diet 3 with 0.5% added L-serine.

In experiments 2–5, the second series of zein products (C-196, C-197, and C-200) were used. For each diet con-

Table III. Response of Rats to Different Diets in Experiment 1

diet no.	treatment of zein	% N from zein	% N from casein	amino acid supplement, % of diet	wt gain, g rat^{-1} day^{-1}	food eaten, g rat^{-1} day^{-1}	food efficiency	apparent N digestibility, %	GGTP activity
1			4.86	L-Met 0.2	5.6[d]	13.2[d]	0.421[d]	97.5[d]	136.3[d]
2			2.34	D-Ser 0.5 + L-Met 0.15	5.2[d]	13.5[d]	0.384[e]	96.9[d]	136.3[d]
3	untreated (C-175)	4.0	0.34	L-Ser 0.5	4.5[e,f]	13.0[d]	0.345[f]	88.2[e]	157.2[d]
4	untreated (C-175)	4.0	0.34	D-Ser 0.5	4.2[e,f,g]	12.9[d]	0.322[f]	86.2[e]	122.8[d]
5	untreated (C-175)	3.9[c]	0.34	Gly-D-Ser 0.78	4.7[e]	12.9[d]	0.362[e,f]	90.1[e]	144.4[d]
6	untreated (C-175)	4.0	0.34	LAL 0.23 + Gly 0.13	3.8[g]	11.1[e]	0.344[f]	90.7[e]	62.9[e]
7A	NaOH (C-177)	4.0	0.96	Gly 0.36	0.3	8.6	0.032	62.3	161.9
8	$Ca(OH)_2$ (C-176)	4.0	0.47	Gly 0.36	4.0[f,g]	11.6[e]	0.34[f]	73.0[f]	154.0[d]
pooled SE of treatment means					0.15	0.32	0.010	1.6	13.8

[a] The experiment lasted 23 days; every diet was given to four cages, each containing two rats. Diet 7A was fed only for the last 7 days, to the six surviving rats; it contained extra casein as shown. [b] Expressed in units per mg of kidney. Units are nanomoles of *p*-nitroaniline released per minute at 25 °C. [c] In diet 5, the level of Gly-D-Ser was chosen to contain the equivalent of 0.5 part of Ser and the level of zein was reduced to keep the total dietary N at 4.88%. [d–g] If two numbers within a column do not share a common superscript letter, they are significantly different ($p < 0.05$). Diet 7A was not included in this analysis.

taining 2.0% N from zein, the following supplement of L-amino acids was also used: arginine 0.36, glutamic acid 1.40, histidine 0.11, isoleucine 0.18, lysine hydrochloride 0.88, methionine 0.35, threonine 0.10, tryptophan 0.15, and valine 0.33% of the diet. The amino acid additions were calculated to bring the dietary level of each to 100% of the National Research Council (1978) requirement, except that isoleucine and valine were brought to 140% to compensate for the imbalancing effect of excess leucine. Where a zein product was fed at the 4.0% N level, the amino acid supplement was also doubled.

The diets containing 2.0% N from casein had as their standard L-amino acid supplement arginine 0.27, glutamine 2.16, methionine 0.35, and threonine 0.03%. These supplements were designed to bring the overall level of each essential amino acid to 125% of the young rat's requirement. Where 4.0% N from casein was fed, the amino acid supplement was also doubled. When 2.0% N from zein and 2.0% N from casein was fed, both sets of amino acid supplements were included.

Statistical Analysis. This was based on analysis of variance to obtain pooled estimates for the standard errors of treatment means, followed by the Newman–Keuls multiple range test for significant differences (Keuls, 1952).

RESULTS

Amino Acid Analysis. The results are summarized in Table I. There is general agreement between the analyses of our two untreated batches of zein, carried out in different laboratories. There are also only small differences from the values published by Boundy et al. (1967), and reproduced in Table I for "unmodified" zein prepared from corn in their laboratory. These workers also found that a commercial zein (HV 9) prepared without an alkali treatment was of similar composition.

The major differences in our alkali-treated samples were the much lower levels of cystine (i.e., 60–70%) and approximately 30% lower levels of both threonine and serine. In addition, a single analysis of the $Ca(OH)_2$-treated material in the first series gave a 33% lower value for arginine. No difference of this magnitude was seen when the second set of samples was analyzed in Laboratory II or in either set for the NaOH treatment.

It is only possible to make a limited comparison with the results of Boundy et al. (1967) because their alkali-treated sample was from a different starting material and the conditions were not completely defined but believed to have been much milder. Again there was a major loss of cystine and a significant loss of serine.

Racemization. The results are set out in Table II. Aspartic acid and serine showed an average racemization of over 30%, with a tendency for the treatment with NaOH to give somewhat higher values. Glutamic acid, threonine, phenylalanine, and tyrosine also showed over 20% average racemization, as did the traces of ornithine seen in the processed samples. Alanine and methionine gave values over 10%.

Response of Rats. The results from experiment 1 are summarized in Table III. The rats except for those receiving the alkali-treated zeins, immediately began to gain weight. Those receiving the $Ca(OH)_2$-treated material showed mild diarrhea, lost a few grams in the first 4 days and then began to gain for the remainder of the 23-day experiment. The rats receiving the zein treated with NaOH lost approximately 10 g each in the first 5 days and showed severe diarrhea; one died on day 5 and its cage mate was in such poor condition that it was killed. The rest were transferred to diet 1 until day 16. With this treatment they immediately began to gain at a similar rate to those that had been fed continually on diet 1. They were then put back on the modified diet (7A) containing the NaOH-treated zein but with a higher level of casein (7.1% instead of 3.5%). Over the last 7 days they showed little change of weight on this treatment.

Looking at weight gains over the whole experimental period, diet 1, with casein as the sole protein, had the highest weight gain, but this was not significantly different from diet 2 with a lower level of casein and added D-serine. The rats receiving untreated zein gained at approximately 80% of the rate of those on diet 1 whether or not they were receiving either D-serine or glycyl-D-serine. In each case their mean daily intake of D-serine, or its equivalent, was 65 mg. With 0.23% supplementary LAL, food intake and weight gain were both significantly lower. The rats receiving the $Ca(OH)_2$-treated zein ate and gained at approximately 90% of the rate of those receiving untreated zein. As already mentioned, the NaOH-treated zein diet (7A) was given for the final week only, and their poor results were not included in the statistical analyses.

The data for apparent N digestibility indicate high values (97%) for the casein diets, intermediate values (86–91%) for the diets containing untreated zein, and lower values for the alkali-treated zeins.

The GGTP enzyme assay showed significantly depressed values in the kidneys only for the rats that had received LAL. Microscopic examination of kidney sections indicated enlargement of nuclei in the proximal tubular cells of rats that had received LAL. The animals receiving D-serine showed the same changes to a lesser extent. Those on diet 2 also showed extensive necrosis of tubular epithelium in the subcortical zone; a similar, but smaller, effect was seen with diet 7A.

The growth results from experiment 2 are summarized in Table IV. The two diets (11 and 12) with untreated zein as their sole protein source supported less gain than the corresponding diets (9 and 10) based on casein. However, the difference was not quite statistically significant for the diet (12) of higher protein content. Rats receiving the mixture of 2% N each from casein and untreated zein grew as well as those receiving 4% N from casein alone. The rats receiving NaOH-treated zein as their sole protein source showed no significant growth, and those receiving the mixture of casein and this zein also both ate and gained at less than one-third the rate of the corresponding diet (13) with casein and untreated zein. All the groups receiving the NaOH-treated zein showed severe diarrhea from the second day on.

The rats used in experiment 3 were from a different breeding colony and grew faster than for corresponding diets used in experiment 2. However, where alkali-treated zeins were the sole source of protein (diets 15, 17 and 18), they still ate less and grew poorly. The mixture of casein and $Ca(OH)_2$-treated zein (diet 19) supported 84% of the gain on 4% N from casein alone (diet 12), but surprisingly, these rats showed severe diarrhea throughout the experiment.

In experiments 2 and 3, pancreas weights, expressed as percent of body weight, did not differ significantly between treatments containing control and alkali-treated zeins.

The results from experiment 4 are summarized in Table V. Regardless of which batch of untreated zein was used, the rats grew at least as well as with 4% N from casein. The addition of 0.07% D-alloisoleucine (diet 23) had no significant effect. Rats on diet 24 with 0.25% L-threonine replaced by D-allothreonine gained weight at 65% the rate of those on diet 22, although they ate 90% as much. However, they did not have diarrhea and appeared healthy.

Table IV. Response of Rats in Experiments 2 and 3[a]

diet no.	type of zein	% N from zein	% N from casein	wt gain, g rat^{-1} day^{-1} [b] exp 2	wt gain, g rat^{-1} day^{-1} [b] exp 3	food eaten, g rat^{-1} day^{-1} exp 2	food eaten, g rat^{-1} day^{-1} exp 3	food efficiency exp 2	food efficiency exp 3
9	none	0	2	5.8[d]	7.5[d]	12.7[d]	17.0[d]	0.453[d]	0.441[d]
10	none	0	4	5.0[d]	6.1[e]	11.1[d]	15.6[d]	0.450[d]	0.393[d,e]
11	untreated (C-196)	2	0	3.1[e]	n.d.	11.9[d]	n.d.	0.253[f]	n.d.
12	untreated (C-196)	4	0	4.4[d]	6.4[e]	12.7[d]	16.2[d]	0.343[e]	0.398[d,e]
13	untreated (C-196)	2	2	5.3[d]	n.d.	12.3[d]	n.d.	0.431[d]	n.d.
14	NaOH (C-197)	2	0	−0.3[g] (DDD)	n.d.	7.5[e]	n.d.	−0.042[i]	n.d.
15	NaOH (C-197)	4	0	0.3[g] (DDD)	0.8[h] (DD)	8.3[e]	10.6[e]	0.033[h]	0.078[g]
16	NaOH (C-197)	2	2	1.6[f] (DDD)	n.d.	10.1[d]	n.d.	0.161[g]	n.d.
17	$Ca(OH)_2$ (C-200)	2	0	n.d.	−0.2[i]	n.d.	8.8[e]	n.d.	−0.017[h]
18	$Ca(OH)_2$ (C-200)	4	0	n.d.	2.1[g] (D)	n.d.	10.9[e]	n.d.	0.194[f]
19	$Ca(OH)_2$ (C-200)	2	2	n.d.	5.1[f] (DD)	n.d.	14.7[d]	n.d.	0.348[e]
pooled SE of treatment means[c]				0.36	0.23	0.55	0.82	0.0235	0.014

[a] Experiment 2 lasted 10 days and used five Simonsen rats per diet; experiment 3 lasted 13 days and used five Bantin-Kingman rats per diet. [b] D, DD, and DDD represent increasing severity of diarrhea in the groups indicated. [c] One rat died on diet 15 in experiment 2 so that the estimated SE of the means on this treatment are higher. [d-i] If two numbers within a column do not share a common superscript letter, they are significantly different ($p < 0.05$).

Table V. Response of Rats in Experiment 4

diet no.	no. of rats	dietary supplements[a]	wt gain, g rat^{-1} day^{-1} 0–6 days	wt gain, g rat^{-1} day^{-1} 0–10 days	food eaten, g rat^{-1} day^{-1} 0–6 days	food eaten, g rat^{-1} day^{-1} 0–10 days	food efficiency 0–6 days	food efficiency 0–10 days
20	5	casein	5.2[b]	5.9[c]	12.0[b]	13.5[c]	0.437[b]	0.442[b]
21	3	untreated zein (C-175)	6.1[b]		13.1[b]		0.466[b]	
22	5	untreated zein (C-196)	6.0[b]	6.9[b]	13.5[b]	15.4[b]	0.466[b]	0.447[b]
23	3	no. 22 + 0.07% D-alloisoleucine instead of 0.07% L-isoleucine	5.8[b]	6.4[b,c]	12.8[b]	15.1[b]	0.453[b]	0.425[b]
24	3	no. 22 + 0.25% D-allothreonine instead of 0.2% L-threonine	3.9[c]		12.2[b]		0.321[c]	
pooled SE of means of 5			0.22	0.19	0.35	0.29	0.014	0.014
pooled SE of means of 3			0.29	0.24	0.45	0.38	0.017	0.017

[a] All the diets contained 4% N from casein or zein, each with the same standard amino acid supplements as diets 10 and 12, respectively. [b-c] If two numbers within a column do not share a common superscript letter, they are significantly different ($p < 0.05$).

Table VI. Nitrogen Digestibility

treatment of zein	diet composition: % N from zein	diet composition: % N from casein	diet composition: % N from amino acids	apparent digestibility of total dietary N, %: exp 2	exp 3	exp 4	exp 5	estimated true N digestibility of zein,[a] %
none	0	2	0.54	95.1	93.0			
none	0	4	1.08		95.7	95.4		
untreated (C-196)	2	0	0.54	58.5				(2) 53.6
untreated (C-196)	4	0	1.08		59.7	61.7	59.7 [65.9]	(3) 55.0; (4) 57.7; (5) 55.0 [62.9][b]
untreated (C-175)	4	0	1.08			62.9		(4) 59.3
NaOH (C-197)	2	0	0.54	52.4				(2) 45.9
NaOH (C-197)	4	0	1.08		46.1			(3) 37.9
$Ca(OH)_2$ (C-200)	2	0	0.54		59.9			(3) 55.5
$Ca(OH)_2$ (C-200)	4	0	1.08		62.8			(3) 59.1
$Ca(OH)_2$ (C-200)	2	2	1.08		77.3			(3) 55.1

[a] The numbers 2–5 in parentheses refer to the experiment from which each result was obtained. [b] The values in brackets were determined from fecal samples collected over days 18–21 of the experiment. All other values were from samples collected over days 3–6.

Only one diet was used in experiment 5. This was identical with diet 22 used in experiment 4, and the growth rate of the rats was similar to that obtained previously (Table V).

Digestibility. The results for the apparent digestibility of total dietary N in experiment 1 are given in Table III. The casein diets gave very high values; the diets containing untreated zein gave, on the average, values 9 percentage units lower and did not differ among themselves. Both alkali-treated zeins gave considerably lower values: 62% for diet 7A and 73% for diet 8.

The results for the later experiments are given in Table VI. The standard deviation of the estimates varied inversely with the digestibility values, and the pooled standard error of each treatment was best estimated as being 4.5% of the apparent indigestibility of the nitrogen for the diet concerned.

The diets having casein and amino acids as their sole sources of N had approximately 95% apparent N digestibility. On the assumption that these are essentially completely digestible and that the 5% "apparently indigestible N" is of metabolic origin, we have estimated that

all the indigestible N in excess of 5%, in the diets containing zein, represents truly indigestible zein material. The resulting estimates of true digestibility for zein are listed in Table VI.

The results from experiments 2–5 are in contrast with those from experiment 1 in that (a) the untreated zein appears less digestible and (b) the $Ca(OH)_2$-treated material is no different from the untreated. Two possible explanations for the first discrepancy were investigated in experiments 4 and 5. In experiment 4 the two batches of untreated zein were compared, but the values obtained (59.3 and 57.7%) were not significantly different. In experiment 5 the digestibility of untreated zein (C-196) was measured from fecal samples collected over both days 3–6 (as in experiments 2–4) and days 18–21 (as in experiment 1). The apparent digestibility of the two diets was significantly different, but the estimated true digestibility of the zein of 63% at the end of the experiment (as compared with 55% at the beginning) was still well below the estimate of 89% obtained from experiment 1.

As regards the NaOH-treated zein samples, there is agreement among all the experiments that it is of lower digestibility as well as causing more diarrhea and growth depression.

DISCUSSION

Amino Acid Composition. Bietz et al. (1979) have studied the structure of zein and found small differences in its composition for different varieties of corn. The analyses of our starting materials have given generally similar values to those previously published (Boundy et al., 1967; Abe et al., 1981), though our methionine and cystine values are higher.

The considerably lower cystine levels in the alkali-processed samples are in line with the results of Boundy et al. (1967) and also with those of Liardon and Hurrell (1983) for alkali-treated casein. Again, serine and threonine were the other amino acids showing significantly lower levels in each case.

When whole corn was treated with alkali, only a small loss of cystine and arginine and negligible changes in other amino acids were reported (Sanderson et al., 1978).

The ranking of the amino acids by their degree of racemization is generally similar to that found for casein heated in 0.2 N NaOH at 80 °C for 1 h (Liardon and Hurrell, 1983). However, the zein showed rather less than expected racemization of methionine and serine and rather more of tyrosine as compared with casein. Bunjapamai et al. (1982) found a generally similar gradation, except that threonine was most racemized. Masters and Friedman (1979), working with four proteins, but a more restricted number of amino acids, also found that phenylalanine, aspartic acid, and glutamic acid were consistently most racemized, alanine was intermediate, and leucine, valine, and proline were least affected; racemization of serine and threonine was not measured.

Rat Results. In several early papers it was reported that zein, even with amino acid supplements calculated to correct its deficiencies, would not support good growth in young rats [e.g., Geiger and Hagerty, (1949) and Harris et al. (1943)]. However, with additional valine the growth depression was largely overcome and it was concluded that zein, though of low digestibility, contained no toxic factor (Kliger and Krehl, 1950, 1952; Geiger et al., 1952). At that time it was thought that the need for the additional supplement must be due to the valine present in zein having a particularly low availability for rats. However, with the discovery of the antagonism between leucine and the other branched chain amino acids (Harper et al., 1954), it was to be expected that rats receiving a protein as rich in leucine as zein would have increased requirements for both valine and isoleucine.

In experiments 2–4, zein, heavily fortified with amino acids including both valine and isoleucine, supported excellent rates of growth when fed at a level contributing 4% N. This confirms that zein does not contain a "toxic" growth inhibitor for young rats. However, the protein itself was then being depended on as the major source of only one essential amino acid, threonine. For these diets the L-threonine supplement was 0.2%, which met 40% of the rat's estimated requirement of 0.5 g/100 g of diet (National Research Council, 1978). The zein supplied 0.8%, so with approximately 60% digestibility, one would expect it to contribute 0.48% digestible threonine. The total effective supply would then be 0.68%, which exceeds the standard of 0.5 g/100 g of diet, though the requirement is probably somewhat greater for a diet of such high protein content.

With untreated zein as the sole protein source at the 2% N level (experiment 2) the rats grew at about 55% of the rate with 2% N from casein. In view of an estimated digestibility of the protein of approximately 60%, the calculated level of digestible threonine was only 0.34%, compared with the requirement standard of 0.5 g/100 g of diet. The slower growth may therefore be explained by partial threonine deficiency.

It is clear that both types of alkali treatment have drastically reduced the value of zein for young rats. The effect was not reproduced by adding D-serine or glycyl-D-serine to a control zein diet, at a level equivalent to those contributed by alkali-treated zein, so that racemization of this amino acid does not appear to provide an explanation. The production of acute lesions in young rats has been induced by giving intraperitoneal injections of D-serine (Kaltenbach et al., 1979), though a high-protein diet appeared to confer protection (Wachstein, 1947a,b). Neither the alkali-treated zeins nor the addition of D-serine reduced the GGTP activity in the rat's kidneys in experiment 1, although the group receiving 0.23% free LAL did show lower activity. This is in contrast to the work of Struthers et al. (1977) in which 0.3% LAL in protein-bound form had no effect.

Although there could not have been LAL present in the treated zeins, we did consider the possibility of ornithinoalanine being formed as a result of possible breakdown of arginine to ornithine. Ornithine was detected in the treated zeins, but only in traces, and there was no consistent loss of a significant proportion of the arginine. Both the leucine and threonine isomers used in experiment 4 have been reported to be nutritionally inactive for rats (Greenstein and Winitz, 1961). Adding D-alloisoleucine at the low level contributed by the treated zeins had no obvious effect. When D-allothreonine replaced L-threonine at levels slightly above those contributed by treated zeins, growth and food efficienty were lower. As discussed above, the threonine supplied by the zein may have been marginal in the high-protein diet so that part, at least, of the poorer performance may have been due to lack of usable threonine rather than to any toxic effect. In any case, there was no diarrhea, so that release of the threonine isomer from the treated material cannot fully explain its changed characteristics.

The digestibility data has shown some apparent inconsistencies. The NaOH-treated zein has always given lower values than the untreated zein, but the digestibility of the $Ca(OH)_2$-treated zein by very young rats has been equal to that of untreated material. Even though rats, as they grow, may improve their ability to digest untreated zein,

the great difference in response to $Ca(OH)_2$-treated zein is already seen in the first days of feeding experiments when there is essentially no difference in N digestibility. We cannot therefore explain the depressed growth or the diarrhea in terms of an overall depression of protein digestibility. In a separate study with these materials, they were digested with Pronase and infused into isolated sections of rat gut; there was significantly less absorption from digests of zein that had been alkali treated (Schwass et al., 1983).

Several workers (Dakin and Dudley, 1913; Hayashi and Kameda, 1980; Friedman et al., 1981; Bunjapamai et al., 1982) have found that alkali treatment of casein reduced the rate at which it is attacked by proteolytic enzymes in vitro. Casein, of course, differs greatly from zein in that it is almost completely digestible by rats when unprocessed. Possompes et al. (1979) have also reported that alkali-treated casein produced diarrhea in rats, though the transit time through the gut was not significantly changed. They also refer in their abstract to pancreatic hypertrophy, but we did not find this in our rats that had received alkali-treated zein. Diarrhea can be caused by an excess of salts in the diet, but with thorough washing we were able to reduce the Na and Cl in the NaOH-treated zein to negligible levels. The level of Ca in the diets containing $Ca(OH)_2$-treated zein was kept constant by adjusting the mineral supplement.

It may be that the indigestible N of untreated zein was in the form of insoluble particles of undigested protein. With the alkali-treated zein, the indigestible N may have been largely in the form of smaller peptide fragments that could not be further digested because they contained some racemized amino acyl residues. If this were so, the same amount of N would have a greater osmotic pressure and be more conducive to diarrhea. We cannot explain the different degrees of diarrhea among the three groups receiving $Ca(OH)_2$-treated zein in experiment 3.

If the effects of alkali treatment were simply to produce a toxic factor, one might expect the animals on the 4% N diets to do worse than those on 2% N diets, but this was not so. On the other hand, if the only change in the treated zeins were a loss of available nutrients, we might expect the diets containing 2% N from casein and 2% N from treated zein to grow well, since the fortified casein alone had supported rapid growth. With the $Ca(OH)_2$-treated zein, growth was quite good despite continuing diarrhea but not with the NaOH-treated zein.

We can only suggest that there is more than one effect responsible for the changed nutritional characteristics of the alkali-treated zein. The findings force a reconsideration of the effects of alkali treatment of proteins; it is clear that all effects cannot be attributed to the formation of lysinoalanine.

The traditional treatment of whole corn in the preparation of tortillas in Central America and Mexico begins by soaking it in lime of the same or higher strength than was used here, and at a similar temperature, but for less than 1 h (Cravioto et al., 1945). This has no adverse effect on the quality of corn protein for the rat (Harper et al., 1958; Tovar and Carpenter, 1982). This may be due partly to the milder conditions within the kernel as opposed to a protein in solution. However, the process has a positive nutritional aspect because of the release of niacin from bound forms resistant to digestion (Carter and Carpenter, 1982).

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Registry No. D-Serine, 312-84-5; cystine, 56-89-3; L-threonine, 72-19-5; D-alloisoleucine, 1509-35-9; sodium hydroxide, 1310-73-2; calcium hydroxide, 1305-62-0.

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