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Factors Affecting the Evaluation of the Nutritional Value of Severely Alkali-Treated Casein

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ABSTRACT

When alkali-treated casein (NaOH, 0·2N, 80°C, 1h) was given as sole protein source at a suboptimal level (10% dry matter) to rats, reduced food intake (46%) and an arrest of growth were observed. Both these parameters were increased, to a level similar to control rats receiving the 10% protein diet, by either increase of protein level from 10% to 20% or by supplementation with CYS, MET, VAL, THR, TYR. In adult rats, food intake was not impaired but growth rate was greatly reduced.

In young rats given a synthetic inhibitor of trypsin, food intake and growth rate were also reduced. Supplementation by the amino acid mixture restored growth.

The food intake restriction and poor growth rate of rats receiving the alkali-treated casein could probably be related to an alteration in content or availability of amino acids and the formation of trypsin inhibitors, but not to a toxic factor.

INTRODUCTION

Alkaline treatment of proteins induces chemical changes related to treatment duration, pH, and temperature. The changes involve partial destruction of some amino acids such as cysteine, threonine, and the synthesis of new compounds such as lysinoalanine (De Groot & Slump, 1969; Provansal *et al.*, 1975; Friedman *et al.*, 1984). Lysinoalanine was reported to induce kidney lesions (Woodard *et al.*, 1975, Struthers *et al.*,

1978). However, all the newly synthesized compounds are perhaps not known and their eventual toxicity, therefore, remains unknown (Friedman, 1977). Alkaline treatment also induces isomerization of amino acids (Provansal *et al.*, 1975, Liardon & Hurrel, 1983). Trypsin and chymotrypsin inhibitor formation has been described in severely alkali-treated casein (Vimont-Rispoli *et al.*, 1980).

These changes produce a decrease of protein digestibility and nutritional value (De Groot & Slump, 1969) but, except in some cases, diarrhoea and kidney lesions (Karayiannis & MacGregor, 1976), no sign of toxicity was related. The effects of these modifications could depend on the digestive capacity and amino acid needs of the animals. These parameters vary with their age and their physiological condition. So according to the protein concentration in the diet, the nutritional value of protein may differ.

The aim of this study was to investigate the influences of protein concentration in the diet and of the age of rats on the nutritional value and eventual adverse effects of a severely alkali-treated casein.

In order to determine to what extent amino acid requirements are modified by a factor affecting only protein digestibility, rats were given a synthetic inhibitor of trypsin, benzamidine (Mares-Guia & Shaw, 1965) and the problem was dealt with by amino acid supplementation. Diets containing alkali-treated casein were also studied.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (IFFA-CREDO, 1′ Arbreles, France) were divided randomly into groups of six and housed individually in steel wire bottomed cages, in air conditioned rooms at 26 ± 1°C with a 12 h light–dark cycle. Water and food were provided *ad libitum*. The experimental period was 21 days long. To assess dry matter and nitrogen digestibility, feces and urine were collected during the last seven days.

Diets

Diets were composed of 10% or 20% protein (w/dry w) provided either by untreated casein or by alkali-treated casein. The casein treatment and the complete diet composition have been described elsewhere (Berger & Possompes, 1987).

In two experiments, 10% of protein supply was replaced by an amino acid mixture: CYS 1%, MET 2%, THR 3%, VAL 1%, TYR 3% (w/w of total

protein). In another experiment, diets containing 10% untreated casein were treated with 0·3% benzamidine (w/w).

Experimental design

Experiment 1: Effect of protein concentration
Four groups of rats, weighing approximately 100 g, received diets containing 10% or 20% protein provided by untreated casein (C10 or C20) or by alkali-treated casein (ATC10 or ATC20).

Experiment 2: Effect of age and nutritional status
Rats weighing 320 g were divided randomly into three groups. One group (FATC10) received a protein-free diet for 6 days to induce malnutrition and then the diet containing the treated casein for 21 days. One of the remaining groups received the control diet during 6 days and then the diet with alkali-treated casein (ATC10). The third group was given the control diet (C10) during all the experimental period.

Experiments 3 and 4: Effect of the amino acid supplementation
Rats weighing 100 g were given the amino acid supplementation in diets containing the untreated casein (C10 + AA), the alkali-treated casein (ATC10 + AA) and the untreated casein to which was added benzamidine (C10 + B) and (C10 + B + AA). All diets contained 10% protein.

Analytical methods

Nitrogen assay
Nitrogen content of diets and feces was determined by the Kjeldahl method, using a Bouat–Crouzet apparatus for distillation.

The nutritional value of the protein was expressed by the Protein Efficiency (PE: weight gain/protein intake, calculated on the 21-day period).

The dry matter and nitrogen digestibility was determined according to:

$$D = \frac{I - F}{I} \times 100$$

I and F represent, respectively, the dry matter or nitrogen intake and the fecal dry matter or nitrogen.

Amino acid assay
The free amino acids were assayed on pooled plasma from all rats in experiment 3. After deproteinization by 5% sulfosalicylic acid, analyses

were then completed using a Technicon NC1 autoanalyzer with a 60°C thermostated column of chromobeads C2 resin (75 cm × 0·6 cm). Elution was performed with sodium citrate buffers. After coloration by ninhydrin, the absorbance of the eluate was read at 440 and 570 nm.

The amino acids of caseins were analyzed in the same way after hydrolysis with 6N hydrochloric acid at 110°C for a 24 h period under nitrogen atmosphere.

The fluorodinitrobenzene reactive lysine was performed according to Carpenter (1960).

Statistical analysis

Results are expressed as mean ± SEM. Means were compared by Student's *t*-test for paired data. In one experiment (Table 6 see below) one-way analysis of variance was performed.

RESULTS

Chemical score

The alkaline treatment induced a decrease of the Chemical Score (calculated according to FAO, 1973) from 91 to 72, due to a partial destruction of cysteine and threonine. Lysine assay using FDNB showed a Chemical Score of 101 for the treated casein versus 120 for untreated casein. The lysinoalanine accounted for 1·9% of the treated casein (Table 1).

Nutritional value

Effect of the protein concentration

Rats receiving the 10% treated protein diets (ATC10) restrained their food intake to 54% of the control rats (C10). This restriction, added to a lowered protein digestibility (Table 2), could explain growth arrest and the poor PE of the treated casein (Table 2).

With the 20% protein diets, food intake was similar in both groups (ATC20 and C20) but the growth of (ATC20) rats remained 30% lower than of (C20) rats. The PE of treated casein increased with the protein concentration but remained, however, lower than the PE of untreated casein. Although the digestibility of the nitrogen did not appear statistically different in control and experimental groups, the fecal nitrogen concentration was higher in the (ATC20) group. A slight pancreatic hypertrophy

TABLE 1
Effect of Alkali Treatment on Casein Composition and Chemical Score

	Casein		*Alkali-treated casein*	
	%[a]	*CS*[b]	*%*	*CS*
Aspartic acid	6·6		5·9	
Threonine	3·9	97	2·9	72
Serine	5·2		3·2	
Glutamic acid	20·8		20·1	
Proline	10·7		9·7	
Glycine	1·8		1·7	
Alanine	2·7		2·7	
1/2 cystine	0·4	94	0·2	76
Methionine	2·9		2·5	
Isoleucine	5·1	127	4·9	122
Leucine	8·7	124	9·5	87
Tyrosine	5·2	168	4·9	158
Phenylalanine	4·9		4·6	
Lysinoalanine	0		1·9	
Lysine	7·5	136	6·3	114
Histidine	2·5		2·6	
Arginine	5·5		3·5	
Lysine (assayed with FDNB)	6·6	120	5·6	101

[a] Expressed as g/100g protein.
[b] CS: chemical score calculated with the reference protein (FAO, 1973).

was observed in this latter group when compared to control rats (C20) (Table 2).

Effect of age

In adult rats, the supply of protein in the diet (ATC10) by alkali-treated casein did not impair food intake (Table 3) but did not allow a similar growth rate as untreated protein (C10). Rats of (FATC10) group lost an average of 44 g during the protein deprivation period and did not recover their weight deficit. The alkali-treated protein PE seemed higher in adult than in young rats when the protein concentration in the diet was suboptimal ($0{\cdot}02 \pm 0{\cdot}16$ versus $0{\cdot}44 \pm 0{\cdot}41$, $p < 0{\cdot}05$). An opposite phenomenon was observed with the control casein ($3{\cdot}09 \pm 0{\cdot}08$ versus $0{\cdot}44 \pm 0{\cdot}07$, $p < 0{\cdot}05$).

The nitrogen digestibility was consistently lower in adult rats compared to young rats ($66{\cdot}2 \pm 2{\cdot}4$ versus $80{\cdot}5 \pm 1{\cdot}6$, $p < 0{\cdot}05$, for control groups). This parameter was little affected by consumption of the alkali-treated casein in adult rats, but a pancreatic hypertrophy was found in this latter group.

TABLE 2

Effect of Treated or Untreated Protein Concentration on Food Intake, Nutritiona! Value, Growth and Pancreas Relative Weight in Young Rats

	Casein		*Alkali-treated casein*	
	C10	*C20*	*ATC10*	*ATC20*
Protein level in the diet	10%	20%	10%	20%
Weight gain (g/day)	4·67 ± 0·27*a*	7·71 ± 0·35*b*	0·05 ± 0·11*a*	5·43 ± 0·16*b*
Food intake (g/day) (dry matter)	15·1 ± 0·5*a*	16·1 ± 0·4	8·2 ± 0·6*a*	16·1 ± 0·4
Dry matter digestibility (%)	88·1 ± 0·8	92·2 ± 0·4	88·4 ± 1·1	92·4 ± 1·4
Apparent nitrogen digestibility (%)	80·5 ± 1·6*a*	88·8 ± 2·5	72·2 ± 1·7*a*	86·8 ± 1·4
PE	3·09 ± 0·08*a*	2·40 ± 0·06*b*	0·02 ± 0·16*a*	1·68 ± 0·05*b*
Fecal nitrogen (% dry matter)	2·45 ± 0·30*a*	2·96 ± 0·37*b*	4·02 ± 0·42*a*	5·55 ± 0·11*b*
Body weight (g)	234 ± 8	277 ± 7	121 ± 4	207 ± 4
Pancreas weight (g)	ND	0·33 ± 0·02*b*	0·41 ± 0·02	0·43 ± 0·01*b*

Results are expressed as mean ± SEM. Results from diets C10 and C20 are, respectively, matched to results from diets ATC10 and ATC20. Values with the same letter are significantly different ($p < 0·01$).

TABLE 3

Food Intake, Nutritional Value, Growth and Pancreas Relative Weight in Adult Rats receiving Control or Alkali-Treated Casein diets

	Casein (C10)	*Treated casein (ATC10)*	*Treated casein (after protein-free diet) (FATC10)*
Dry matter intake (g/day)	18·6 ± 0·36	18·4 ± 0·52	16·5 ± 0·43
Weight gain (g/day)	2·28 ± 0·16*a*	0·72 ± 0·25*ab*	1·83 ± 0·14*b*
Dry matter digestibility (%)	87·1 ± 1·3	88·9 ± 1·7	87·5 ± 0·4
Apparent nitrogen digestibility (%)	66·2 ± 2·4	54·3 ± 3·9	64·6 ± 3·3
Fecal nitrogen (% dry matter)	5·2 ± 0·2	5·4 ± 0·3	4·3 ± 0·2
PE	1·44 ± 0·07	0·44 ± 0·41	1·27 ± 0·16
Body weight (g)	387 ± 4	341 ± 4	311 ± 4
Pancreas weight (g)	0·33 ± 0·01	0·41 ± 0·01	0·37 ± 0·01

Values with the same letter are significantly different ($p < 0·01$).

TABLE 4

Effect of the Supplementation with an Amino Acid Mixture of a Diet containing Alkali-Treated Casein as Protein Source on Food Intake, Growth and Pancreas Relative Weight

	Casein (C10)	*Treated casein (ATC10)*	*Supplemented treated casein (ATC10 + AA)*
Weight gain (g/day)	$3{\cdot}30 \pm 0{\cdot}24a$	$0{\cdot}10 \pm 0{\cdot}07ab$	$3{\cdot}08 \pm 0{\cdot}14b$
Dry matter intake (g/day)	$10{\cdot}4 \pm 0{\cdot}6a$	$5{\cdot}5 \pm 0{\cdot}6b$	$9{\cdot}5 \pm 0{\cdot}2b$
Apparent nitrogen digestibility (%)	$88 \pm 1ab$	$75 \pm 4a$	$72 \pm 2b$
PE	$3{\cdot}15 \pm 0{\cdot}12a$	$0{\cdot}20 \pm 0{\cdot}14ab$	$3{\cdot}22 \pm 0{\cdot}2b$
Body weight (g)	$140 \pm 5a$	$73 \pm 2b$	$138 \pm 3a$

Values with the same letter are significantly different ($p < 0{\cdot}01$).

TABLE 5

Relative Concentration (g/100g amino acids) of Plasmatic Amino Acids in Rats receiving 10% Protein Diets

	Casein (C10)	*Treated casein (ATC10)*	*Supplemented treated casein (ATC10 + AA)*
Aspartic acid	0·9	0·8	0·7
Threonine[a]	24·7	16·9	34·3
Serine[b]	8·4	11·6	8·3
Glutamic acid	8·3	6·6	9·7
Proline[c]	5·3	5·0	5·7
Glycine	3·3	3·9	2·7
Alanine	10·4	12·8	12·2
Valine	3·4	3·4	3·6
Cystine	—	—	—
Methionine	0·9	1·0	0·9
Isoleucine	2·1	2·1	1·6
Leucine	5·5	5·8	4·5
Tyrosine	2·3	1·9	2·0
Phenylalanine	1·8	2·0	1·6
Ornithine	1·6	2·8	3·1
Lysine	13·1	15·8	4·5
Histidine	1·8	2·7	1·8
Arginine	3·4	4·7	3·0
Total (μM/100ml)	495	416	424

Possible interference with
[a] asparagine
[b] glutamine
[c] citrulline

TABLE 6

Influence of the Supplementation with an Amino Acid Mixture (LYS, MET, THR, TYR, VAL) on Food Intake, Growth, and Pancreas Relative Weight of Rats receiving Casein or Casein with a Trypsin Inhibitor

	C10	*C10 + AA*	*C10 + B*	*C10 + B + AA*
Dry matter intake (g/day)	14·5 ± 0·6*a*	15·1 ± 0·4*b*	10·8 ± 0·7*a*	10·9 ± 0·7*b*
Weight gain (g/day)	4·50 ± 0·13*a*	6·08 ± 0·13*b*	1·68 ± 0·12*ac*	3·69 ± 0·23*bc*
Dry matter digestibility (%)	91 ± 1	91 ± 1	93 ± 1	91 ± 1
Apparent nitrogen digestibility (%)	85 ± 1	89 ± 1	83 ± 2	86 ± 1
Fecal nitrogen (% dry matter)	1·84 ± 0·09	1·98 ± 0·09	2·32 ± 0·04	2·32 ± 0·05
PE	3·24 ± 0·15*ac*	4·02 ± 0·08*a*	1·52 ± 0·73*cb*	3·38 ± 0·11*b*
Body weight (g)	181 ± 12*a*	212 ± 5*b*	120 ± 7*ac*	161 ± 6*bc*
Pancreas weight (g)	0·30 ± 0·02*a*	0·33 ± 0·02*b*	0·42 ± 0·03*a*	0·42 ± 0·02*b*

All diets contained 10% protein (dry matter).
C10, casein; C10 + AA, casein + amino acid mixture; C10 + B, casein + benzamidine; C10 + B + AA, casein + benzamidine + amino acid mixture.
Values with the same letter are significantly different ($p < 0·01$).

Effect of the amino acid supplementation

Supplementation of the alkali-treated casein with amino acids given to rats in 10% protein diets (ATC10 + AA) led to an increase of food intake and growth gate (Table 4) that became similar to that of control rats (C10). PE was also restored to a value similar to that of control rats but remained lower than with control casein supplemented with the same amino acid mixture (C10 + AA). Plasma amino acid analysis showed that lysine concentration in (ATC10 + AA) rats was very low (Table 5). It could be concluded that lysine became limitative in supplemented alkali-treated casein.

Effect of the trypsin inhibitor

Benzamidine, added to the control diet (C10 + B) induced decreased food intake and growth (Table 6). Supplementation with the amino acid mixture (C10 + B + AA) did not modify food intake but growth rate was increased and the nutritional value of the protein (expressed as PE) was as the control casein (C10). The consumption of benzamidine induced a pancreatic hypertrophy when (C10 + B + AA) was compared to (C10) group.

DISCUSSION

Consumption of alkali-treated casein, in a 10% protein diet, induced a decreased food intake and growth rate in young Sprague–Dawley rats, but

these were restored by increasing protein concentration to 20% of dry matter in the diet. However, in Wistar rats receiving the same diet, under similar experimental conditions, a severe diarrhoea occurred (Berger, 1983). So, the restriction of food intake in our experiments did not seem to result from the toxicity of a newly synthesized compound, but from a deficiency or a partial unavailability of essential amino acids induced by the alkaline treatment. The positive effect of supplementation with amino acids corroborates this hypothesis.

Essential amino acid deficiency is a consequence of cysteine and threonine destruction (both amino acids are limitative in casein) and also of the lowered digestibility of the treated protein. This decrease is probably due to the racemization of amino acid residues (Friedman *et al.*, 1981), and to the formation of intra- and intermolecular links which could explain the production of pancreatic protease inhibitors (Vimont-Rispoli *et al.*, 1980). All these transformations could induce an accumulation of indigestible material in intestine and the diarrhoea occurring in Wistar rats.

Supplementation with CYS, MET, VAL, THR, TYR, increased the nutritional value of the control casein by about 20%. The same supplementation improved the PE of alkali-treated casein only to a level similar to non-supplemented control casein. This could be ascribed either to the effect of protease inhibitors or to a deficiency in lysine supply. The lower concentration of lysine in the plasma of rats fed the supplemented treated casein when compared to both the other groups of rats fed control or treated caseins indicates a deficiency in this amino acid. Casein contains high amounts of lysine and the lysine remaining after treatment when assayed by FDNB method, seemed sufficient even in spite of a presumed 13% racemization rate (Liardon & Hurrel, 1983). The unsupplemented treated casein met only the maintenance needs while the casein supplemented by the amino acid mixture allowed growth. In this last case the lysine supply could be inadequate.

Protease inhibitors could also interfere. To assess this hypothesis, rats were given benzamidine. The protein digestibility was not affected and the slight increase of fecal nitrogen could be due in part to benzamidine. So the decreased growth rate would not be related to an amino acid deficiency although the presence of an inhibitor could increase the needs in specific amino acids (Liener & Kakade, 1969) but rather to the anorectic effect of the inhibitor. Thus, during preliminary studies it was noticed that in spite of the large amount of aromatic amino acids in casein, addition of tyrosine in CYS–MET–THR–VAL mixture stimulated growth. Substitution of L-tyrosine by L-phenylalanine, only in meals containing benzamidine or alkali-treated casein, induced a temporary decrease of food intake (Possompes, unpublished data). The anorectic effect of both protease inhibitors and L-phenylalanine is well known (Baile *et al.*, 1986). However, in our

experiments, this effect could be seen only in young rats. As with alkali-treated casein, benzamidine did not induce a restriction in food intake in adult rats (Possompes, unpublished data).

In conclusion, factors affecting pancreatic secretion, intestinal digestive and absorptive mechanisms could influence the feeding behaviour of rats and then, indirectly, modify the nutritional value of protein when assessed by PER. Adaptation capacities seems to depend on age and strain of rats. Technological treatments could impair the nutritional value of protein not only by destroying and decreasing availability of amino acids, but by disturbing endocrine and metabolic balances connected to digestive physiology.

REFERENCES

Baile, C. A., McLaughlin, C. L. & Della-Fera, M. A. (1986). Role of cholecystokinine and opioïd peptides in control of food intake. *Physiol. Rev.*, **66**(1), 172–234.

Berger, J. (1983). Effets physiopathologiques dus à l'ingestion de protéines modifiées en milieu alcalin. Etude sur le pancréas endocrine et l'intestin chez le rat. Thèse de 3eme cycle, USTL, Montpellier.

Berger, J. & Possompes, B. (1987). Effects of severely alkali-treated casein on *in vitro* and *in vivo* digestibility and selected intestinal enzyme activities. *Nutr. Rept. Int.*, **35**(1), 69–78.

Carpenter, K. J. (1960). The estimation of available lysine in animal protein foods. *Biochem. J.*, **77**, 604–10.

De Groot, A. P. & Slump, P. (1969). Effects of severe alkali treatment of proteins on amino acid composition and nutritive value. *J. Nutr.*, **98**, 45–56.

FAO/WHO (1973). Energy and protein requirements. Report of a Joint FAO/WHO Ad Hoc Expert Committee. FAO Nutr. Meeting Report Series No 51, Rome, and WHO Tech. Rep. Series No 522, Geneva.

Friedman, M. (1977). Crosslinking amino-acids—Stereochemistry and nomenclature. *Adv. Exptl. Med. Biol.*, **86B**, 1–27.

Friedman, M., Zahnley, J. C. & Masters, P. M. (1981). Relationship between *in vitro* digestibility of casein and its content of lysinoalanine and D-amino acids. *J. Food Sci.*, **46**, 127–31.

Friedman, M., Levin, C. E. & Noma, A. T. (1984). Factors governing lysinoalanine formation in soy proteins. *J. Food Sci.*, **49**(5), 1282–8.

Karayiannis, N. & MacGregor, J. T. (1976). Lysinoalanine content and biological effects of alkali-treated proteins. 172nd Congress of American Chemical Society, San Francisco.

Liardon, R. & Hurrel, R. F. (1983). Amino acid racemization in heated and alkali-treated casein. *J. Agric. Food Chem.*, **31**, 432–7.

Liener, I. E. & Kakade, M. L. (1969). Proteases inhibitors. In *Toxic Constitutents of Plant Foodstuffs*. Academic Press, NY, pp. 8–69.

Mares-Guia, M. & Shaw, E. (1965). Studies on the active center of trypsin. The binding of amidines and guanidines as models of the substrates side chain. *J. Biol. Chem.*, **240**, 1579–85.

Possompes, B., Berger, J. & Cuq, J. L. (1985). Traitement alcalin et inhibiteur de la trypsine: effet des supplémentations en acides aminés sur la croissance des rats. *Journées Sciences des Aliments.* Association Française de Nutrition, Paris.

Provansal, M., Cuq, J. L. & Cheftel, J. C. (1975). Chemical and nutritional modification of sunflower proteins due to alkaline processing. Formation of amino cross-links and isomerization of lysine residue. *Agric. Food Chem.*, **23**(5), 938–43.

Struthers, B. J., Hopkins, D. T., Prescher, E. E. & Dahlgreen, R. R. (1978). Effects of protein bound lysinoalanine *N*-DL-(2-amino-2-carboxyethyl)-L-lysine on fetal and neonatal rats. *J. Nutr.* **108**, 954–8.

Vimont-Rispoli, S., Possompes, B. & Besançon, P. (1980). Mise en evidence de la formation d'inactivateurs de la trypsine au cours de traitements alcalins des protéines. *C.R. Acad. Sci. Paris*, **291**, 945–8.

Woodard, J. C., Short, D. D., Alvarez, M. R. & Reyniers, J. (1975). Biological effects of *N* (DL-2 amino-2 carboxyethyl)-L-lysine, lysinoalanine. In *Protein Nutritional Quality of Foods and Feeds. Part* 2. ed. M. Friedman, M. Dekker Inc., New York, pp. 595–616.