Nutritional rehabilitation of malnourished rats by di- and tripeptides: nitrogen metabolism and intestinal response

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An enzymic hydrolysate (DTP) rich in di- and tripeptides (75%) was prepared from bovine plasma proteins (BP). Protein-calorie malnourished rats were subjected to refeeding tests with diets having the same nitrogen and caloric contents and differing only in the molecular form of the nitrogen supply: BP, DTP, or an equivalent amino acid mixture (AAM). Growth, food intake, and food efficiency were measured after an eight-day period of rehabilitation; nitrogen balance and digestibility were also determined. Animals refed the DTP diet exhibited the best nutritional parameters, AAM failed to support any rehabilitation. In the intestine measurements of mucosal enzyme activities and morphometric studies strongly suggested the beneficial effect of DTP as indicated by increased both aminopeptidase N level and villus height. The results showed an adaptive response of rats to the molecular form of the nitrogen supply and demonstrated the superior efficacy of DTP for a rapid recovery from protein-calorie malnutrition.

Keywords: refeeding; di and tripeptides; intestinal enzymes and morphology; nutritional adaptation

Introduction

Partial protein hydrolysates or equivalent amino acid mixtures are widely used for medical and therapeutic uses, either for enteral or parenteral nutrition[1,2] in the rehabilitation of undernourished patients with or without intestinal diseases. Thus, it seems preferable to test the efficacy of such products using similar animal models, ie, protein-calorie malnourished rats (low protein level and reduced food intake), rather than starved animals (absence of luminal nutrients).

Undernutrition has been shown to cause lowered small intestinal function[3-6] associated with decreased mucosal enzyme activities[7-9] and severely impaired morphology.[10-16] Resuming food intake results in a rapid restoration of intestinal physiology.[17-20] However, many of these numerous studies have been performed using either acute starvation, protein-free diets, or a litter expansion technique. Moreover, no attempt has been made to investigate the effects of diets differing only in the molecular form of the nitrogen source supplied on the nutritional rehabilitation of undernourished rats. The aim of this work is to determine if rehabilitation is more effective using diets with hydrolyzed protein or amino acids, compared with using diets containing whole protein; thus, we have chosen to conduct this study to examine the effects of bovine plasma proteins, a partial enzymic hydrolysate of these proteins, and a free amino acid mixture equivalent to the hydrolysate, on the intestinal response (ie, enzyme activity and morphology) of malnourished animals. In addition, our attention was turned on their overall nutritional adaptation.

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Materials and methods

Partial hydrolysate (DTP) of plasma proteins (BP)

Blood was collected in a slaughter house. Spray-dried bovine plasma proteins (BP) were hydrolysed by an alkaline bacterial serine proteinase according to Bressollier et al.[21] Resulting peptides were continuously extracted by a membrane ultrafiltration process (molecular weight cut off: 1,000 daltons). Then, each peptide class, characterized by its size, was quantified on the basis of its amino acid content after separation by ligand exchange chromatography using Cu(II)-modifield silica gel. The relative distribution, as moles percentage, was: amino acids, <5; dipeptides, 40 ± 4; tripeptides, 35 ± 4; larger peptides (chain length <6 residues), 20 ± 2 (Verneuil et al., unpublished data). This hydrolysate, rich in di- and tripeptides, was named DTP. The amino acid composition of BP and DTP is further described and reported in *Table 1*.

Animal model and diets

Twenty male rats (Sprague-Dawley strain) weighing 106 ± 3 g were housed in stainless steel metabolic cages with urine-feces separators in an animal room kept at 25° C with a 12-hr light: dark cycle. They were fed a diet containing casein (2.0 g of protein per 100 g of diet) and their food intake was limited to an average of 5.5 g/day in order to achieve body weight loss. Water was given ad libitum. On day 16 five animals were killed (controls). The remaining rats were then divided in three equal groups (five rats per group) based on average body weight; they were switched to isoenergetic and isonitrogenous diets (2.4% N) containing either BP, DTP, or an amino acid mixture (AAM) as the sole nitrogen source (2.4% N × 6.25, ie, 15% protein level). Refeeding lasted for 8 days. Rats were killed on the day 9. Although the composition of the diets was expressed as percentage on a dry basis (*Table 2*), they were given to rats on a gruel form containing 60% of moisture, to avoid food spillage. Proteins, peptides, and amino acids (Merck-Clevenot, Nogent-sur-Marne, France) were in powder form and mixed with the rest of the diet. In diets containing AAM, all the nitrogen was supplied as a mixture of cristalline L-amino acids corresponding to the composition of the DTP mixture. Every day food consumption and body weight were individually recorded. During the last 3 days of the malnutrition and refeeding periods the urine and feces were collected for nitrogen balance and digestibility studies.

Table 1 Amino acid composition of the nitrogen sources

Aminoacid	Composition (amino acid %) BP	DTP
Aspartic acid	7.21	9.70
Threonine	6.73	6.98
Serine	7.03	7.53
Glutamic acid	14.13	14.66
Proline	4.09	4.04
Glycine	3.45	3.38
Alanine	4.85	5.12
Cysteine	1.37	1.44
Valine	6.73	6.80
Methionine	1.01	1.04
Isoleucine	3.10	3.01
Leucine	9.05	8.85
Tyrosine	4.80	4.79
Phenylalanine	4.88	4.67
Tryptophane	1.79	1.35
Lysine	9.05	8.73
Histidine	2.44	2.46
Arginine	5.30	5.36

Table 2 Composition of the experimental diets expressed as g/100g of diet (dry basis)

Ingredients	Dietary groups: Malnutrition Control	Refeeding BP	Refeeding DTP	Refeeding AAM
Casein (13.8% N)	2.32	—	—	—
DL-methionine[a]	0.04	—	—	—
BP (14.1% N)	—	17.05	—	—
DTP (15.5% N)	—	—	15.52	—
AAM (16.0% N)	—	—	—	15.00
Starch	68.37	58.06	59.13	59.50
Glucose	4.88	4.15	4.22	4.25
Cellulose	4.88	4.15	4.22	4.25
Fat	7.81	6.64	6.76	6.80
Salt mixture[b]	10.75	9.12	9.30	9.35
Vitamin mixture[c]	0.98	0.83	0.84	0.85
Protein[d] and energy level in each diet				
N × 6.25	2.0	15.0	15.0	15.0
Kcal (calcd)[e]	392	393	393	393
KJ (calcd)	1638	1643	1643	1643

[a] Casein was supplemented with 2% DL-methionine.
[b] Salt mixture consisted of (g/kg): calcium dihydrogen phosphate: 430.0; potassium chloride: 100.0; sodium chloride: 100.0; magnesium chloride: 50.0; magnesium sulfate: 50.0; ferric oxide: 30.0; manganese sulfate: 2.45; zinc sulfate: 2.00; cupric sulfate: 0.50; cobalt sulfate: 4.10^{-3}; potassium iodide: 8.10^{-3}.
[c] Composition expressed in international units or g per kg of vitamin mix: retinyl acetate: 1980,000 IU; cholecalciferol: 600,000 IU; DL-tocopheryl acetate: 17.0; menadione: 4.0; thiamin-HCl: 2.0; riboflavin: 1.5; calcium pantothenate: 7.0; pyridoxin-HCl: 1.0; inositol: 15.0; cyanocobalamine: 5.10^{-3}; ascorbic acid: 80.0; nicotinic acid: 10.0; choline-HCl: 136.0; folic acid: 0.5; p-aminobenzoic acid: 5.0; D-biotin: 3.10^{-2}.
[d] Calculated as: grams of nitrogenous ingredient × % nitrogen ×6.25.
[e] Calculated as: total carbohydrates × 4 kcal/g; fat × 9 kcal/g; protein × 4 kcal/g.

Experimental procedure

At the end of each experimental period rats were killed by cervical dislocation and the abdomens rapidly opened. The subsequent steps were carried out at 4° C. The intestine was removed intact from the pylorus to the ileocecal valve and three standard segments were excised: duodenum (from pyloric valve to Treitz's ligament), middle jejunum (consisting of the portion 30–45 cm distal to the Treitz's ligament) and ileum (consisting of the portion 15–30 cm proximal to the ileocecal valve). After flushing with ice-cold saline, the intestinal mucosae were scraped and homogenized in the same medium, then stored at −80° C until assays. Brush border alkaline phosphatase (AP, duodenum) and aminopeptidase N (AN, ileum) activities were assayed according to Bessey et al.[22] and Maroux et al.,[23] respectively. Protein was determined by the Lowry procedure.[24]

Morphometric study

Immediately after the sacrifice, 1 cm specimens were taken from identical parts of the digestive tract (beginning of the middle jejunum), fixed in Bouin's solution, embedded in paraffin, cut perpendicular to the longitudinal axis and stained with

Table 3 Effects of malnutrition on nutritional parameters[a]

Parameters	Controls
Initial body weight (g)	102.60 ± 1.90
Final body weight (g)	73.40 ± 3.20
Protein intake (g/day)	0.11 ± 0.01
Caloric intake (kcal/day)	21.95 ± 0.51
N intake (mg/day)	14.50 ± 0.30
Fecal N (mg/day)	13.40 ± 1.60
Urinary N (mg/day)	8.60 ± 0.70
N balance (mg/day)	−8.60 ± 2.00

[a] Values are expressed as mean ± S.E.M.

haemalun-eosin. The lengths of the villi and adjacent crypts were measured by means of a light microscope (Nikon 104, Charanton le Pont, France) equipped with a projective apparatus.

Amino acid analysis

The amino acid determination of nitrogen sources was performed after an acid hydrolysis using 6N HCl at 110° C for 24 hr in an air-oven and under nitrogen at atmospheric pressure; then HCl was evaporated under reduced pressure at 40° C; tryptophan content was measured after alkaline hydrolysis using 4N NaOH at 110° C for 16 hr and under nitrogren atmosphere according to Hugli and Moore.[25] Amino acid analyses were then performed using a high-performance liquid chromatography (HPLC) system (Kontron SFM 25, Zurich, Switzerland) with a Shimadzu integration system. Derivatization procedure used 9-fluorenylmethylchloroformate (FMOC-Cl). All the subsequent steps were carried out according to Einarsson.[26]

Statistical analysis

Results are given as mean ± SEM. Data were compared by analysis of variance (ANOVA); significance was determined by a multiple range Student's *t* test.

Results

The effects of malnutrition are summarized in *Table 3;* the animals lost 29% of their body weight during 16 days of malnutrition. The mean total protein intake represents an amount of protein too low to meet the protein requirement of the adult rat (approximately 1.8–2.0 g/day). On the basis of nitrogen excretion, the animals were in a severe negative balance. It must be pointed out that urinary N was lower than fecal N, giving evidence of the malnutrition status of the rats.

Table 4 shows the nutritional parameters of rats after rehabilitation. The initial body weight differs slightly from the final body weight in the control group (*Table 3*) because five animals were killed at random before the restoration period; therefore, means were modified. Refeeding DTP diet produced a pronounced growth. This diet had the best food efficiency although no significant difference was observed between the three groups in terms of food intake; refeeding BP or AAM failed to reach such an efficiency. Nitrogen retention followed a similar pattern. Nitrogen digestibility was lower in AAM refed rats; nutritional performances of these animals were weaker than those of the other groups and particularly in comparison with the DTP fed group. None of the three diets caused diarrhea.

Intestinal alkaline phosphatase activities (AP) are given in *Figure 1*. After refeeding, activities increased independent of the diet utilized. Activities were identical between BP and DTP refeds, these last values being significantly weaker than in AAM ones. In the ileal segment, a decrease in mucosal protein content dependent on the malnutrition status was observed (*Table 5*); refeeding short peptides resulted in maximal mucosal protein contents. Ileal AN activities expressed per total segment or per milligram of protein (*Table 5*) were significantly lower after malnutrition. Refeeding permitted a repair of enzymic activities, either segmental or specific. Examination of segmental activities revealed that the maximal AN activity was confined to DTP refeds. No discernible differences were noted between BP and DTP refeds concerning the specific activities, however, a weaker difference was noticeable in AAM refeds.

Table 4 Nutritional parameters after refeeding*

	Refeeding		
	BP	DTP	AAM
Initial B.W. (g)	80.6 ± 2.5[a]	80.6 ± 2.5[a]	80.6 ± 2.0[a]
Final B.W. (g)	130.0 ± 2.8	140.0 ± 5.7	107.4 ± 1.9
Growth (g/day)	6.1 ± 0.2	7.4 ± 0.4	3.3 ± 0.4
Food intake (g)	104.4 ± 4.1[a]	103.8 ± 8.0[a]	94.5 ± 9.2[a]
Food efficiency†	3.1 ± 0.1	3.9 ± 0.3	2.1 ± 0.1
N intake (mg/day)	322.2 ± 11.3[a]	315.2± 16.0[a]	290.3 ± 8.8[a]
Fecal N (mg/day)	43.1 ± 3.3[ab]	40.1 ± 3.3[a]	46.0 ± 2.5[b]
Urinary N (mg/day)	125.6 ± 4.8	112.7 ± 6.1	143.0 ± 5.1
N retention (%)‡	54.9 ± 1.0	57.3 ± 2.7	39.2 ± 1.0
CDU (%)§	86.5 ± 1.2[a]	87.3 ± 0.9[a]	82.3 ± 1.7

* Values are expressed as mean ± SEM; values in a same row with identical superscript letters are not significantly different ($P < 0.05$)
† Calculated as body weight gain/protein intake measured over the 8-day period.
‡ Calculated as (N intake − Fecal N − Urinary N)/(N intake − Fecal N) measured during the last 3 days.
§ Nitrogen apparent digestibility = (N intake − Fecal N)/N intake.

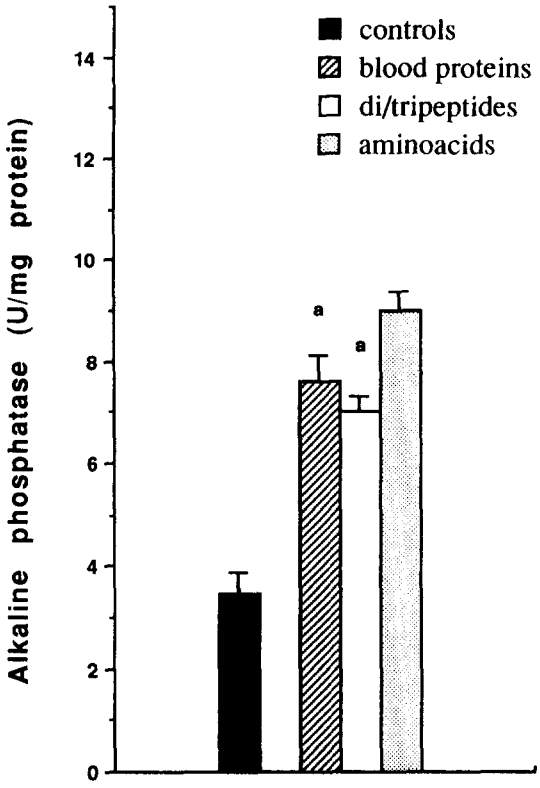

Figure 1 Specific activities (units/mg protein) of alkaline phosphatase after malnutrition (controls, black bars) and after refeeding BP (hatched bars), DTP (clear bars), or AAM (stippled bars). Values represent means of five determinations per group ± SEM; bars with the same superscript letter are not significantly different ($P < 0.05$).

Morphometric data are summarized in *Figure 2;* villus length and crypt depth were significantly lower after protein-calorie malnutrition, accounting for a severe nutrient deprivation. Refeeding DTP was associated with the greatest illus height; crypt depth being different among the three groups.

Discussion

It seems likely that peptide-rich enzymic protein hydrolysates behave differently from equivalent amino acid mixtures when administered per os, in relation to either absorptive or postabsorptive events.[4-6,27] Few studies attempted to detect differences between them in terms of overall nutritional parameters, including food efficiency, nitrogen retention, and digestibility[28] or in terms of intestinal behavior. So, the present experiments were designed to provide a basis for optimal utilization of either a dietary protein, its partial hydrolysate, or the corresponding constituent amino acids.

Data from *Table 4* shows that when protein-calorie malnourished animals were refed at a standard nitrogen level in the diet (2.4% N), only DTP emerged from the three diets in terms of growth, food efficiency, and nitrogen retention. Moreover, rats receiving AAM diet had significantly greater nonfecal nitrogen losses, via increased urinary excretion of unchanged amino acids;

Table 5 Effects of malnutrition and refeeding on ileal aminopeptidase activity and mucosal protein content[a]

	Mucosal protein (mg/segment)	U/segment	Specific activity (mU/mg protein)
Controls	18.2 ± 2.2	1.06 ± 0.1	55.0 ± 4.9
Refeds			
BP	31.3 ± 4.5[a]	3.9 ± 0.4	124.5 ± 6.9[a]
DTP	33.9 ± 4.0	4.4 ± 0.3	126.8 ± 4.5[a]
AAM	31.3 ± 4.4[a]	3.3 ± 0.3	106.2 ± 3.4

[a] Values with a same superscript letter in a column are not significantly different ($P < 0.05$); comparisons were made separately for each malnutrition schedule.

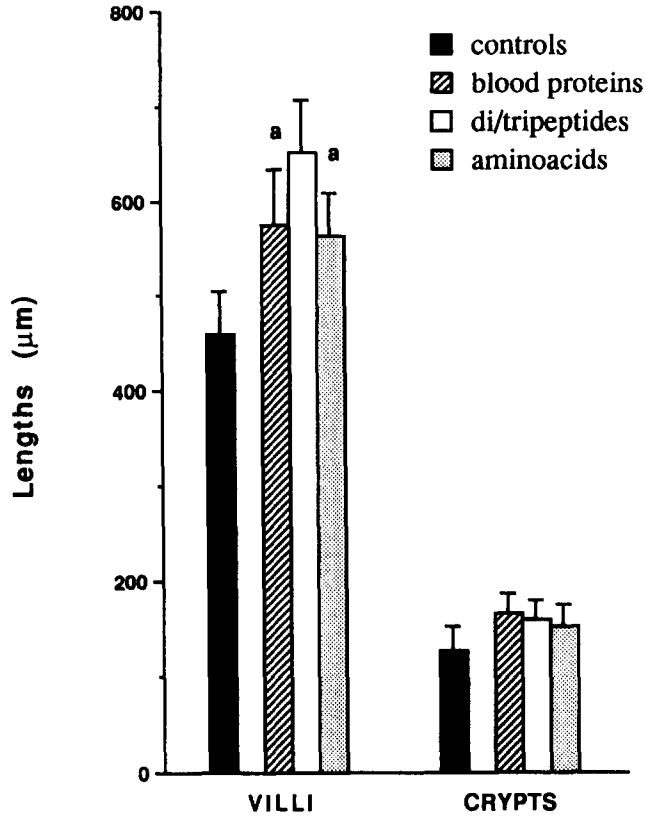

Figure 2 Intestinal histomorphometric study after malnutrition and refeeding. Diets are indicated in Fig. 1. Three sections were examined for each specimen (ie, per rat); fifteen measurements were made per section. Bars with the same superscript letter are not significantly different ($P < 0.05$).

it is likely that the body of such animals cannot utilize all the dietary nitrogen for protein synthesis, suggesting an adaptive response to the moleuclar form of nitrogen supplied. In another respect, proteins are known to be the best substrate of intestinal proteolytic enzymes; replacing BP or DTP by AAM in the diet would be expected to modify the metabolism of endogenous nitrogen as well as the absorption of amino acids from the gut. The poor nitrogen digestibility of amino acid diet is not surprising; we have recently observed (unpublished data) that healthy rats fed 15% AAM diets ad libitum had a four-fold increase in intes-

tinal ^{3}H-thymidine incorporation compared to DTP or BP diets, providing a large enterocyte turnover and a subsequently increased rate for endogenous fecal nitrogen. If true in malnourished rats refed amino acid mixture, (high fecal nitrogen excretion), this might explain the slight coefficient of digestive utilization (CDU) observed. These nutritional data suggested that di- and tripeptides were utilized more efficiently than their equivalent amino acids mixture in rehabilitation of protein-calorie malnourished subjects.

We showed that nutritional rehabilitation of malnourished rats resulted in AP recoveries. The mechanisms of restoration seemed due to the nature of the nitrogen supply. The marked malnutrition status of rats before refeeding emphasized the burst in AP recovery observed in blood proteins and di/tripeptides refeds. Moreover, some authors[29] have shown that force-feeding rats with amino acids stimulated intestinal AP level; in relevance to this, restoration by feeding the amino acid mixture induced high levels of AP activity. Nevertheless, the role of AP in amino acid absorption remains unclear and the mechanism of the stimulation needs further elucidation.

The recovery in AN activities was also achieved after nutritional rehabilitation; nevertheless, these activities, both segmental and specific, varied markedly, depending on the diet administered. However, variations in segmental activities, which take into account mucosal changes and are closely related to the digestive capacity of the gut, were more remarkable than those of the specific activities. Our data suggested that there was a specific action of di- and tripeptides differing from that of proteins (BP) and total hydrolysis products (AAM) on AN activity. These adaptational enzymic differences not only appeared dependent on a specific substrate[30,31] and different pancreatic secretions, but also on the nature and/or the molecular form of the dietary nitrogen. These findings were corroborated by the morphometric study. Similar results were recently reported[32] although experimental conditions such as protein source and malnutrition schedule differed from those used in this report. It remains for future investigation to elucidate the mechanisms involved in these changes. This work also indicates that the alterations of intestinal function and morphology as well as of nutritional parameters caused by malnutrition, can be rapidly reversed during a brief period of refeeding by di- and tripeptides. Increased demands for development of oligopeptides products for a therapeutic use justify such a research.

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