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Effect of proteins on availability of zinc
II. Bioavailability of zinc from casein and whey protein – retention study in young rats

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Summary: The availability of zinc from two semi-synthetic diets with isolated whey protein (Wp D) or with isolated casein (Cas D) as protein component (20 % W/W) was compared in a 21-day study with growing male rats (initial weight 40 g; 14 animals/group). Zinc concentration in both diets (18 ppm) was adequate to meet the requirements of the animals fed ad libitum. For radiolabeling ~ 13μg ^{65}Zn (= 4 μ Ci) was given daily by intragastric intubation to each animal. The investigation was designed primarily as a retention study, but also general parameters like weight development, food and water intake, organ weights etc. were registered and the activity of alkaline phosphatase was determined in serum and femur tissue. A significantly higher percentage of ^{65}Zn was retained in the whole body from the Wp D (36.5 %) than from the Cas D (31.6 %) during the experimental period. The same is valid for the percentage retention of ^{65}Zn in the femur and for the ^{65}Zn concentration in femur and hair as well as for the total zinc concentration (^{65}Zn and non-labeled zinc) of the femur. The other parameters determined were not unequivocally influenced by the protein component of the diet. The study clearly demonstrated that the availability of zinc by the growing rat was better from a diet with whey protein than from one with casein as the protein component. The reason on this phenomenon has to be elucidated by further investigations.

Zusammenfassung: Ziel der Untersuchung war es, die Verfügbarkeit von Zink aus zwei semisynthethischen Kostformen, die isoliertes Molkenprotein (Wp D) bzw. isoliertes Casein (Cas D) als Proteinkomponente (20 Gew. %) enthielten, zu vergleichen. Je 14 wachsende männliche Ratten pro Gruppe (Anfangsgew. 40 g) wurden mit diesen Diäten 21 Tage lang ad libitum gefüttert. Die Kostformen enthielten mit 18 ppm eine bedarfsdeckende Zinkdosis. Zur radioaktiven Markierung des Zinks wurden jedem Tier täglich ~ 13 μg ^{65}Zn (= 4 μ Ci) intragastral gesondet. Die Untersuchung war hauptsächlich als Retentionsstudie angesetzt, es wurden jedoch auch allgemeine Parameter wie Gewichtsentwicklung, Futter- und Trinkwasseraufnahme, Organgewichte u. a. erfaßt und die Aktivität der alk. Phosphatase in Serum und Femurgewebe bestimmt. Während der Versuchsperiode wurde aus der Wp D signifikant mehr ^{65}Zn (36,5 %) im Ganzkörper retiniert als aus der Cas D (31,6 %). Analoges gilt für die ^{65}Zn-Retention im Femur und für die ^{65}Zn-Konzentration im Femur und Haar sowie für die Gesamt-Zink-Konzentration (^{65}Zn und nicht markiertes Zink) im Femur. Bei den sonstigen Parametern war kein eindeutiger Einfluß der Proteinkomponente festzustellen. Die Untersuchung hat eindeutig bewiesen, daß das Zink aus einer Kostform mit Molkenprotein besser verfügbar ist als

aus einer Diät mit Casein als Proteinkomponente. Den Grund hierfür sollen weitere Experimente klären.

Key words: zinc availability, casein, whey protein, retention study

Introduction

In a previous study the availability of zinc from casein or whey protein suspensions was investigated in weaned rats over a 24-hour period (11). From the time course of these experiments we established that short-term investigations are not appropriate for comparing different diets for their effects on bioavailability of zinc – and of other trace elements: depending on when the experiment was terminated opposite results could be obtained. Therefore, we concluded that only long-term investigations may be suitable to judge the quality of dietary proteins with regard to their favourable or unfavourable effect on zinc availability. This would hold true especially if only slight variations are expected. For the same reason, the present investigation was designed primarily as a zinc retention study. As zinc supply by the two diets was adequate for the experimental animals it could not be anticipated that growth would be affected. A good correlation between zinc status and alkaline phosphatase activity was frequently discussed. Thus, this parameter was included in the experiments.

Materials and Methods

The experiments were carried out in 28 *weaned male rats* (initial body weight 40.0 ± 1.3 g) divided into two groups. The animals were kept separately in plastic metabolic cages for 21 days. The experimental device made it possible to quantify the food and drinking water intake as well as to collect urine and feces separately. In order to prevent contamination with zinc, drinking water tubes, food boxes and cages were cleaned regularly with EDTA-solution. The experimental animals were kept at 25 °C in a room with 60 % relative humidity and light-dark periods lasting 12 hours.

The animals were fed an *experimental semi-synthetic diet* with the following composition (in percent by weight): casein from bovine milk[1]) or whey protein from bovine milk 20 %, rice starch 63 %, saccharose 5 % lard 5 %, minerals 5 %[2]) and vitamins 2 %[2]). The zinc content of the diets – determined by atomic absorption spectrometry – was 18.1 ppm in the casein (Cas D) and 18.4 ppm in the whey protein diet (Wp D). These powdered diets as well as aqua bidest. were accessible ad libitum. Additionally, each animal was intubated 0.5 ml of a *protein suspension* containing 10 mg of casein or whey protein, respectively and 14 µg radiolabeled zinc (^{65}Zn = 0.98 µCi; Amersham Buchler, Braunschweig) every day. To control the intragastric administration of ^{65}Zn , each rat was measured by a whole body gamma-counter for laboratory animals against a point-shaped ^{65}Zn-standard immediately after intubation of the nuclide. In order to be able to consider the amount of ^{65}Zn already incorporated, a ^{65}Zn determination was carried out in the whole body counter against a spread ^{65}Zn standard before the estimation mentioned above, and the difference between both values was calculated.

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[1]) supplemented with DL-methionine (3.5 g/kg diet)

[2]) essential minerals and vitamins adequate to meet the requirement of growing rats (9)

The following *parameters* were *registered:*

a. Daily:
- body weight, which is considered as a good indicator for the utilization of experimental diets as a whole in growing organisms;
- food and thus, Zn intake;
- drinking water intake;
- urine volume and ^{65}Zn in urine;
- feces weight and ^{65}Zn in feces;

b. After termination of the experiment:
- whole body retention of ^{65}Zn
- retention of ^{65}Zn in liver, kidney, testes, pancreas, spleen, femur, whole blood, serum and hair; in this context bone and liver were considered as organs in which a high proportion of the total body zinc is retained, whereas pancreas and serum are marked by pronounced zinc dynamics;
- weight of organs mentioned above, especially as a reference basis for the retention calculation;
- activity of alkaline phosphatase (EC 3.1.3.1) in serum and femur, as indicator enzyme of zinc supply status;
- protein concentration in serum and femur, as a reference basis.

Sample preparation

On the 22nd day, i.e. 24 hours after the last intubation of ^{65}Zn, the animals were killed by decapitation. From the blood collected in heparinised tubes non-hemolytic serum was prepared by centrifugation. A hair sample was cut off from an 1 cm^2 area of the abdomen. Liver, kidneys, pancreas, spleen and testes were removed from the abdominal cavity and both femur bones were dissected. The left femur was stored in ice for estimation of the alkaline phosphatase activity. After determination of ^{65}Zn the right femur was ashed for determination of total zinc content.

Analytical methods

Immediately after killing total ^{65}Zn retained was measured by a whole body gamma-counter for laboratory animals. ^{65}Zn in the organs and biological fluids was determined in a gamma-counter (LB MAG 510, Berthold, Wildbad).

Total zinc (non-labeled and labeled) in femur ash was determined by atomic absorption spectrometry according to Parker (10) (AAS Perkin Elmer 360).

Determination of the alkaline phosphatase activity in serum and femur tissue was carried out corresponding to the "optimized standard method" of the Deutsche Gesellschaft für Klinische Chemie (1), using diethanolamine buffer. For the preparation of the bone sample a method described by Roth and Kirchgessner (13) was used.

Protein concentration in serum and femur was measured according to Lowry et al. (5).

Statistical analysis

Data are stated as mean values of the groups ± standard deviation of the individual values. Normal distribution of the variables was tested by programm BMDP 5D-histogram and univariate plots. Outliers were eliminated by the Nalimov-Test. Differences between mean values were evaluated with Student's t-test (BMDP 3D). A p-value less than 0.05 was considered to indicate a statistically "significant" difference between two mean values.

Results

The *food intake* over the whole experimental period in the Cas D group (141.2 ± 20.6 g) and in the Wp D group (138.6 ± 7.4 g) was not significantly different. Thus, the total zinc intake (labeled and non-labeled) during this time was also almost identical: 2.62 ± 0.4 mg in the Cas D group and 2.61 ± 0.1 mg in the Wp D group.

The *food efficiency ratio* (g weight gain/100 g food) was calculated to be 31.7 ± 3.8 g for the Cas D and 30.1 ± 6.0 g for the Wp D. The difference between the groups was not significant. As a consequence of the higher food intake on the one hand and of the higher food efficiency ratio on the other hand, a slightly higher *body weight* was registered in the Cas D group (85.8 ± 4.3 g) than in the Wp D group (81.6 ± 8.2 g) at the termination of the experiment. The daily *drinking water intake* in the Cas D group (9.9 ± 1.8 ml) was significantly lower than in the Wp D group (12.6 ± 3.4 ml). For the urine volume, on the other hand, only unsignificant differences were registered (Wp D 5.32 ± 2.1 ml/d; CasD 4.88 ± 1.9 ml/d).

The absolute *weights* of *kidney* (790 ± 30 mg) and *pancreas* (350 ± 30 mg) in the Cas D group were significantly higher than in the Wp D group (760 ± 30 mg and 290 ± 50 mg). But these differences became statistically non significant when calculated per 100 g body weight. The weight of the other organs was independent of the kind of diet.

^{65}Zn retained from the Cas D and from the Wp D in the whole body and in different organs at termination of the experiment is summarized in Table 1. The percentage of ^{65}Zn (Table 1a) retained from the Wp D in the whole body (36.5 %) was significantly higher than that retained from the Cas D (31.6 %). From the organs considered, the femur was the only one where a significantly higher percentage of ^{65}Zn was retained from the Wp D than from the Cas D.

The concentration of ^{65}Zn per g wet weight (Table 1b) was significantly higher in femur and hair of the Wp D group. Serum ^{65}Zn concentration on the other hand was significantly lower in the Wp D group. However, the exact determination of the very low serum ^{65}Zn-concentration was affected by a certain inaccuracy.

In Figure 1 the *time course* ^{65}Zn retention in the whole body is registered as a percentage of ^{65}Zn intubated. The only time at which ^{65}Zn retention

Table 1a. ^{65}Zn retained from the casein and the whey protein diet after 21 days (% of ^{65}Zn intubated)*.

	Cas D	n**	Wp D	n**	Significance
Liver	2.22 ± 0.4	13	2.03 ± 0.2	11	n.s.
Kidney	0.36 ± 0.1	14	0.38 ± 0.1	14	n.s.
Testes	0.59 ± 0.3	14	0.59 ± 0.3	14	n.s.
Pancreas	0.25 ± 0.1	13	0.23 ± 0.1	12	n.s.
Spleen	0.11 ± 0.0	14	0.12 ± 0.0	14	n.s.
Femur	0.52 ± 0.2	14	0.66 ± 0.1	13	p = 0.016
Whole body	31.60 ± 4.5	12	36.51 ± 6.0	14	p = 0.026

Table 1b. ^{65}Zn retained from the casein and the whey protein diet after 21 days (µg per g wet weight)*.

	Cas D	n**	Wp D	n**	Significance
Liver	0.68 ± 0.15	14	0.63 ± 0.07	11	n.s.
Kidney	0.45 ± 0.09	14	0.49 ± 0.09	14	n.s.
Testes	0.49 ± 0.13	14	0.48 ± 0.09	14	n.s.
Pancreas	0.76 ± 0.17	13	0.84 ± 0.15	14	n.s.
Spleen	0.51 ± 0.11	12	0.50 ± 0.10	13	n.s.
Femur	1.82 ± 0.34	14	2.07 ± 0.08	14	p = 0.016
Hair	0.45 ± 0.11	13	0.55 ± 0.08	13	p = 0.05
Whole blood	0.13 ± 0.03	14	0.13 ± 0.03	14	n.s.
Serum	0.08 ± 0.02	14	0.07 ± 0.01	13	p = 0.02

* $\bar{X}$ ± s.d.; ** number of animals

from the Cas D was higher than that from Wp D was the second experimental day. As from the third day the ^{65}Zn retention curve of the Wp D group runs above the curve of the Cas D group, resulting in a significantly higher ^{65}Zn retention from the Wp D as an integral of the whole experimental period.

The total *zinc concentration* (^{65}Zn and non-labeled Zn) of the femur – determined by AAS – was 115.5 ± 2.8 µg/g ww in the Wp D group and

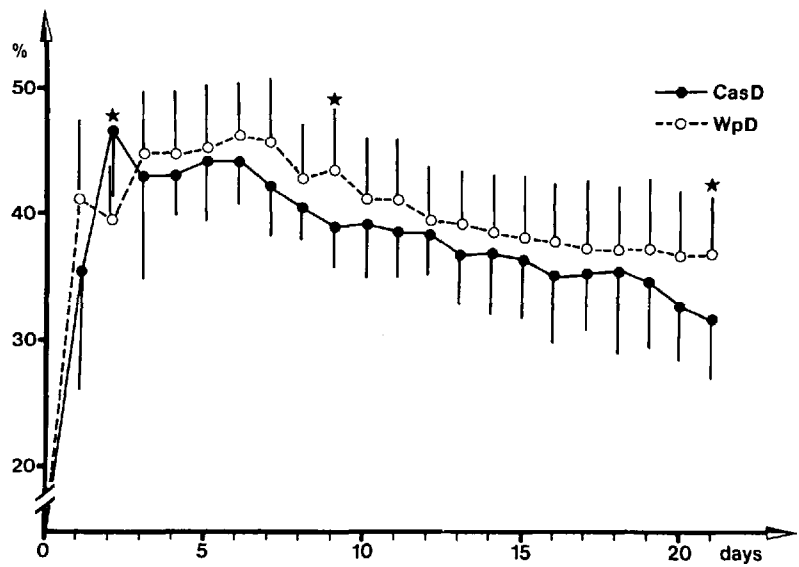

Fig. 1. Time course of ^{65}Zn retention in the whole body as percentage of ^{65}Zn intubated. Cas D = Casein diet; Wp D = Whey protein diet; $\bar{X}$ ± s.d.; *differences between Cas D and Wp D significant at $p \leq 0.05$.

Table 2. Protein concentration and activity of alkaline phosphatase (a Pase) in serum*.

	Cas D	n**	Wp D	n**	Significance
Protein (g/100 ml)	5.44± 0.4	12	6.57± 0.1	14	p = 0.009
a Pase (U/l)	861.3 ±35.5	9	832.4 ±131.3	11	n.s.
a Pase (U/g prot.)	14.4 ± 2.2	13	12.3 ± 1.3	12	p = 0.01

* $\bar{X}$ ± s.d.; ** number of animals

Table 3. Protein concentration and activity of alkaline phosphatase (a Pase) in femur*.

	Cas D	n**	Wp D	n**	Significance
Protein (mg/g ww)	0.12± 0.02	11	0.09± 0.03	14	p = 0.02
a Pase (U/g ww)	40.8 ± 3.1	14	36.7 ± 3.5	13	p = 0.04
a Pase (U/g prot.)	1134.2 ±236.4	14	1190.0 ±261.2	14	n.s.

* $\bar{X}$ ± s.d.; ** number of animals

106.1 ± 4.7 µg/g ww in the Cas D group. The difference was found to be significant at p = 0.001.

The *activity* of the *serum alkaline phosphatase* (Table 2) – related to the volume – was not significantly different in the two groups. However, related to the serum protein, the concentration of which was lower in the Cas D group, alkaline phosphatase activity of the latter group was significantly higher.

The protein concentration of the *femur* tissue was higher in the Cas D group (Table 3). As a consequence, *alkaline phosphatase* activity related to the protein did not markedly differ in the two groups, although the activity of this enzyme calculated per g wet weight was significantly higher in the Cas D group.

Discussion

The present study was scheduled as a long-term experiment in order to estimate the availability of zinc from two semi-synthetic diets with identical zinc concentrations, but different protein components: casein on the one hand and whey protein on the other. The zinc supply of the animals before and during the experimental period was adequate according to the recommendations for growing rats (9). Retention of zinc was chosen as the main criterion, but also some general parameters e.g. body and organ weights, as well as biochemical data were included in the investigations.

Neither the food intake nor the food efficiency ratio were dissimilar in the two groups. An almost identical body weight and also equal organ weights resulted from this, especially when the latter was calculated per 100 g body weight. This fact can be jugded as a favourable basis for a retention study. The similar course of body weight development in both groups could not be anticipated at all, because the protein efficiency ratio value (weight gain per g protein) for casein is 2.5 compared with 3.5 to 4.0 for whey protein (12). The supplementation of the Cas D with DL-methionine obviously led to an equalization of the efficiency of the two diets.

The percentage of ^{65}Zn retained from the Wp D in the whole body (36.5 %) was significantly highher than that from the Cas D (31.6 %). An analogous statement is valid for the percentage retention of ^{65}Zn in the femur as well as for the ^{65}Zn concentration in femur and hair. The ^{65}Zn retention and concentration in the other organs considered were not influenced by the protein component of the diet, as the single variable of the experiment. The only exception was the serum ^{65}Zn concentration which was lower in the Wp D group. But it has to be stressed, that the inaccuracy of the ^{65}Zn determination in the very low concentrations found in this biological fluid is considerable. Thus, it is difficult to make any conclusions from ^{65}Zn serum level concerning zinc availability.

Total zinc concentration (^{65}Zn and non-labeled zinc) of the femur was also determined to be significantly higher in the Wp D group. Accordingly, bone tissue clearly reflected zinc status of the organism. This is in agreement with the view of other authors (2, 7, 8). Considering that in the present study the zinc supply with both diets was adequate, and that nevertheless distinct differences could be detected between the groups, it can be concluded that zinc retention in the bone is a very sensitive criterion for zinc availability. But this organ system can obviously only be used as an indicator in animal experiments.

Hair was the only other tissue in which ^{65}Zn concentration was significantly higher in the Wp D group. Zinc content of the hair was frequently recommended as a criterion for estimation of trace element status, especially of young children (3, 6). Despite the controversial discussion of the suitability of this parameter in the different studies, our results speak for its applicability even for detection of low differences in zinc status.

The determination of alkaline phosphatase activity yielded some confusing results. The specific activity of serum alkaline phosphatase (U/g protein) was found to be higher in the Cas D group, which would be in agreement with the higher serum ^{65}Zn concentration of this group. However, on the basis of the significantly different drinking water intake it can be assumed that the water balance of the two groups was also very dissimilar. Thus, the differencies in the serum protein concentrations could result from the disparity of the intravascular water. This point of view was not considered when planning the experiments because shifting of this parameter could not be relied upon.

The activity of femur alkaline phosphatase did not reflect the significantly higher zinc concentration of this tissue in the Wp D group. As a

result of our experiments it can be asserted that the activity of alkaline phosphatase in serum – and also in femur tissue – is inappropriate for estimation of marginal differences of zinc availability. Due to the great number of various factors influencing the activity of this enzyme, no correlation with zinc supply can be established, especially if the differences between the zinc dosages or the availability of this trace element are slight. Studies from which the suitability of alkaline phosphatase activity as a criterion of zinc status was concluded were scheduled as depletion-repletion experiments (4) and thus differed significantly from the arrangement of our investigation, with an adequate zinc supply over the whole period.

Unlike our short-term experiments (11) the present study plainly demonstrated that the availability of zinc by the growing organism was better from a diet with whey protein than from one with casein as the protein component. Further investigations have been initiated to elucidate the reason on this phenomenon. The superiority of a long-time study compared with short-term investigations could also be anticipated from the fact that several days passed until exchange equilibrium between the radiolabeled zinc and the stable zinc of the body pool was obtained.

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