

## **Effect of proteins on availability of zinc**

### **I. Gastrointestinal transit time of casein and whey protein and zinc absorption in weaned rats**

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#### *Summary*

The availability of zinc from isolated casein (CasD) was compared with that from whey protein (WpD) in 23–25 day old rats. The study was designed to show the course of the gastrointestinal transit time of either chyme (radiolabeled by  $^{141}\text{Ce}$  as a non-absorbable marker) or zinc (as  $^{65}\text{Zn}$ ) in groups of 9 to 12 animals each. Animals were killed either 1/4, 1/2, 1, 2, 6, 12 or 24 hours after intragastric intubation of the protein suspensions. Immediately afterwards, intragastric pH was measured and the determination of zinc retention in intestinal tissues and liver as well as in the carcass was performed.

30 and 60 minutes after intubation the intragastric pH of the CasD group was significantly lower than that of the WpD group. The precipitation behaviour of the two protein fractions – compact curd formation by the CasD versus flocculent structure of the WpD – was determining for the pattern of gastric emptying. With the WpD the chyme was emptied according to an exponential function; while the CasD precipitate left the stomach in three distinct phases. With either protein suspension zinc left the stomach earlier than the bulk of the chyme, indicating a partial disintegration of the zinc-protein-complexes. With the WpD, zinc was emptied exponentially, whereas with the CasD a biphasic emptying pattern was found. Ileum was found to be the main zinc absorbing segment, mainly due to the long time of contact with zinc. After 2 and 12 hours zinc retention from the CasD was significantly higher than that from the WpD, however, after 24 hours retention was significantly better from the WpD.

From the present study it can be concluded that, for comparison of zinc availability from diets containing different proteins, short-term experiments are not appropriate. Furthermore, it can be supposed that preabsorptive processes in the stomach are crucial for the availability of zinc.

#### *Zusammenfassung*

Ziel der an 23 bis 25 Tage alten Ratten durchgeführten Untersuchungen war es, die Verfügbarkeit von Zink aus intragastral verabreichten Casein-(CasD-) bzw. Molkenprotein-(WpD-)Suspensionen zu vergleichen. Um den zeitabhängigen Verlauf des gastrointestinalen Durchgangs des Chymus (mit nichtresorbierbarem  $^{141}\text{Ce}$  markiert) und des Zinks (als  $^{65}\text{Zn}$ ) zu ermitteln, wurden je 9 bis 12 Tiere 1/4, 1/2, 1, 2, 6, 12 und 24 Stunden nach dem Sonden getötet. Unmittelbar danach wurde der intragastrale pH-Wert sowie die Retention von Zink im Darmgewebe, Leber und Restkörper gemessen.

30 Minuten nach Intubation der Proteine war der pH-Wert im Magen der CasD-Gruppe signifikant niedriger als der der WpD-Gruppe. Die unterschiedliche intragastrale Präzipitation der beiden Proteine – kompaktes Präzipitat bei Casein, feinflockiges bei Molkenprotein – dürfte ausschlaggebend sein für das Entleerungsmuster aus dem Magen: der Chymus aus WpD wurde einer Exponentialfunktion entsprechend entleert, während der Chymus aus CasD den Magen in drei Phasen mit unterschiedlicher Geschwindigkeit verließ. Das Zink aus beiden Proteinsuspensionen wurde schneller aus dem Magen entleert als die Masse des Chymus, woraus auf eine zumindest partielle Loslösung des Zinks aus der Proteinbindung geschlossen werden kann. Das Zink aus der WpD wurde exponentiell, das Zink aus der CasD nach einem biphasischen Muster entleert. Als Folge der besonders langen Verweildauer des Zinks im Ileum erwies sich dieser Darmabschnitt als Hauptresorptionsort. Die Zink-Retention war 2 und 12 Stunden nach Verabreichung der Nahrungen signifikant höher aus der CasD als aus der WpD, nach 24 Stunden jedoch war aus der WpD signifikant mehr Zink retiniert worden als aus der CasD. Aus dieser Untersuchung ist zu folgern, daß zur Beurteilung der Zink-Verfügbarkeit aus unterschiedlichen Proteinen Kurzzeitversuche ungeeignet sind. Weiterhin muß angenommen werden, daß die präresorptiven Prozesse im Magen für die Zinkverfügbarkeit von wesentlicher Bedeutung sind.

*Key words:* zinc, availability, casein, whey protein, gastrointestinal transit time

## Introduction

The bioavailability of zinc was found to be significantly higher from human than from bovine milk or bovine milk based formulas (20, 8, 13, 14, 4, 22). Studies performed to clarify the reason for these differences focused mainly on the effects of low molecular weight zinc binding ligands such as citrate (17), picolinic acid (9) and prostaglandins (28) all of them found in different quantities in the milk of the two species. They could, however, only partially explain the divergent bioavailabilities.

According to Lönnerdal et al. (16) some 84 % of zinc is bound to casein in bovine milk, where the casein is the major protein. Contrary to this, the human milk whey protein fraction, predominant in this milk, binds more than 50 % of zinc. These fractions – the casein(s) on the one hand and the heterogenous group of the whey proteins on the other hand – are fundamentally different in their chemical and physico-chemical properties. As a consequence, their physiological behaviour in the gastrointestinal tract is likely to be dissimilar too.

Above all, differences in quality and quantity of proteolytic break-down products generated from either protein fraction during the gastrointestinal passage have to be considered. Some of these fragments, especially phosphopeptides and the amino acids histidine, cysteine and glutamic acid are potential zinc binding ligands (12, 25) and may thus influence zinc bioavailability. The distribution of zinc among the binding ligands is determined by the stability constants, by the concentration ratio between the metal and the different ligands and by the pH value of the medium (24).

Our investigations, using rats in the postweaning period, were aimed to compare the bioavailability of zinc from isolated casein and whey protein. Because we assumed that the duration of the contact of zinc with the intestinal tissues is crucial for zinc absorption (11), the study was designed

to show the time course of the gastrointestinal transit of chyme and zinc. As the two proteins were thought to have different effects on the intragastric pH, which in turn could influence the binding of zinc to potential ligands (2, 12), intragastric pH was measured in addition.

## Materials and Methods

The experiments were carried out in 144 male Wistar rats (body weight  $42 \pm 6.7$  g). The weaned 23–25 day old animals were fed a stock diet (Altromin 3014 pellets) with 80 mg zinc per kg which was withdrawn 2 hours before the beginning of the experiment. In order to avoid any influence from the circadian rhythm, each experiment was started at the same hour of day.

The experimental liquid diets contained 20 mg protein per ml suspension and were prepared from isolated lyophilized bovine milk casein (CasD) or whey protein (WpD), respectively. Both diets contained 27  $\mu$ g zinc per ml. Zinc was radiolabeled adding 20  $\mu$ Ci  $^{65}\text{Zn}$ /ml diet ( $^{65}\text{ZnCl}_2$  in 0.1 M HCl; Amersham Buchler, Braunschweig). For radiolabeling of the chyme 20  $\mu$ g  $^{141}\text{cerium} \triangleq 18 \mu\text{Ci } ^{141}\text{CeCl}_3$  in 0.1 M HCl; Amersham Buchler, Braunschweig) was added per ml protein suspension, as a non-absorbable marker (19). The two liquid diets were identical in all components, except the nature of the protein. Their composition was as follows: lactose 4.9; citrate 0.07; Ca 0.4; Na 0.3 and K 0.02 mg/ml.

Each animal was given by flexible gastric tube 0.5 ml of CasD or WpD (38 °C), respectively. The activity of  $^{65}\text{Zn}$  was determined immediately afterwards by whole body gamma-counter (reference: point-shaped  $^{65}\text{Zn}$  standard). Then, 15 and 30 minutes, 1, 2, 6, 12 and 24 hours later, groups of 12 animals each were killed by a blow on the neck. After intubation, rats were kept separately in plastic metabolic cages. They had free access to the drinking water but were not fed. Immediately after killing, the stomach was removed and the pH was measured in the closed organ by inserting a pH microelectrode ( $d = 3$  mm; type N 60, Schott, Mainz) through the pylorus according to Niemegger (21).

The intestinal tract was removed as quickly as possible and cut into the following segments: duodenum (10 cm), jejunum (15 cm), ileum (rest of the small intestine), caecum, colon. With the exception of the caecum, the intestinal content was rinsed out using aqua bidest. radiolabeled with 5.7  $\mu$ g  $^{51}\text{Cr} \triangleq 1$  mCi per l (Amersham Buchler, Braunschweig). By adding the non-absorbable (6) Cr to the rinsing water the real net weight of the intestinal tissues could be estimated by subtracting the amount of adherent rinsing fluid calculated from the  $^{51}\text{Cr}$  activity.

The following data were registered:

- chyme in stomach, intestinal segments and faeces, on the basis of percentage of intubated  $^{141}\text{Ce}$ ;
- “non-absorbed zinc” in the gastrointestinal contents and in faeces, as percentage of intubated  $^{65}\text{Zn}$ ;
- retained zinc in the intestinal tissue (duodenum, jejunum, ileum, colon), in the liver and in the carcass, as percentage of intubated  $^{65}\text{Zn}$ .

For determination of dry weights, tissues were lyophilized. All radionuclides –  $^{65}\text{Zn}$ ,  $^{141}\text{Ce}$  and  $^{51}\text{Cr}$  – were measured in a gamma-counter (LB MAG 510, Berthold, Wildbad). The energy-spectra of the three nuclides were different enough to allow simultaneous determination.

## Statistical analysis

Normal distribution of the variables was tested by program BMDP 5D-histogram and univariate plots; outliers were eliminated by the Nalimov test. Statistical comparisons were performed by paired t-test (BMDP 3D). A p value of less than 0.05

was considered to indicate a statistically "significant" difference between the two mean values. In the present experimental arrangement neither of the groups could be considered as a "control group". Thus, each group was compared with the other.

## Results

### *Intragastric pH and gastric emptying of chyme and zinc*

As shown in Table 1, both 30 and 60 minutes after intubation the intragastric pH value of the CasD group was significantly lower than that of the WpD group. No differences were detected after 15 minutes, or after two hours.

Figure 1 shows the emptying pattern from the stomach for chyme and for zinc. The percentage of intubated protein – radiolabeled with  $^{141}\text{Ce}$  – and of zinc – as  $^{65}\text{Zn}$  – still present in the stomach is plotted against the time after intubation of the suspensions. In the first 15 minutes, the chyme from the WpD left the stomach faster than that from CasD; after 15 minutes the stomach of the CasD group contained significantly more chyme than that of the WpD group. The emptying of the WpD group followed an exponential function, whereas a triphasic emptying pattern was found with the casein diet. After two hours about 90 % of the CasD chyme but only 75 % of the WpD chyme had left the stomach.

As shown in the lower part of Figure 1, the gastric emptying of zinc was not identical with that of the chyme. Generally, zinc left the stomach earlier than the chyme, indicating a disintegration of the bonds between the metal and the chyme. For instance, 30 minutes after intubation 30 % of the zinc from CasD and about 38 % of that from the WpD was found in the

Table 1. Intragastric pH measured after intubation of casein suspension (CasD) and of whey protein suspension (WpD) at different time intervals.

Group	Time after intubation	n*)	pH**)
CasD/0.25	15 min	10	2.25 ± 0.3
CasD/0.5	30 min	11	1.82 ± 0.2
CasD/1	1 h	10	1.80 ± 0.2
CasD/2	2 h	11	2.09 ± 0.3
CasD/6	6 h	11	2.26 ± 0.6
CasD/12	12 h	10	2.06 ± 0.3
CasD/24	24 h	10	2.57 ± 0.2
WpD/0.25	15 min	10	2.36 ± 0.2
WpD/0.5	30 min	10	2.21 ± 0.4
WpD/1	1 h	11	2.30 ± 0.5
WpD/2	2 h	12	2.24 ± 0.4
WpD/6	6 h	9	2.06 ± 0.3
WpD/12	12 h	9	2.25 ± 0.2
WpD/24	24 h	6	2.13 ± 0.3

\*) = number of animals, \*\*)  $\bar{x} \pm \text{s. d.}$

Differences significant ( $p \leq 0.05$ ) between: CasD/0.5 and WpD/0.5, CasD/1 and WpD/1.

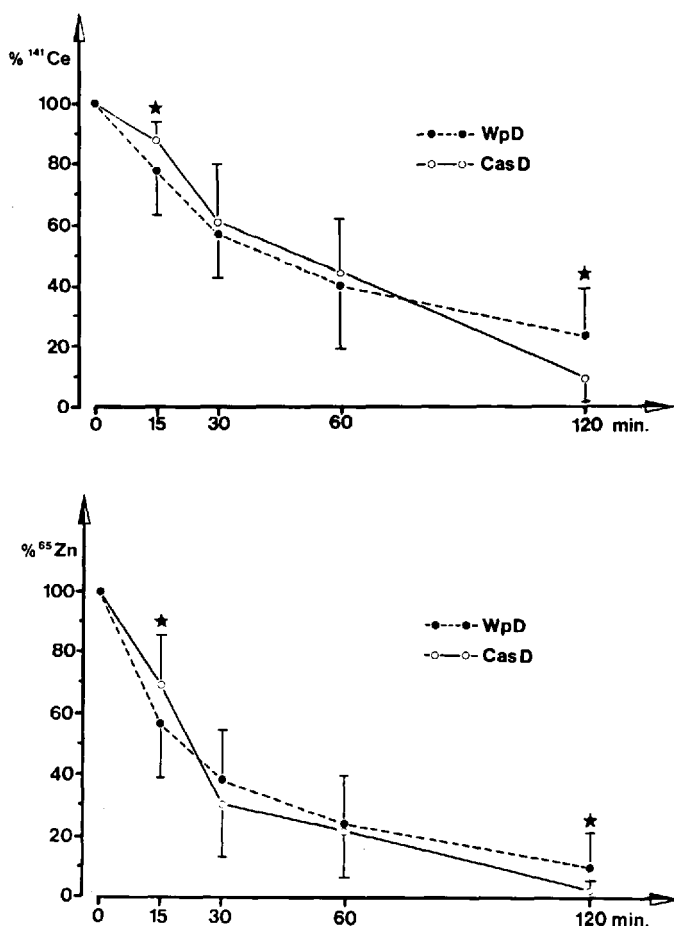


Fig. 1. Percentage of chyme (above) and zinc (below) in the stomach after intragastric intubation of casein (CasD) or whey protein (WpD).  $\bar{x} \pm \text{s.d.}$ ; \*differences between Cas D and WpD significant at  $p \leq 0.05$ .

stomach, whereas still some 60% of the chyme was present. At the beginning of the experimental period zinc from both diets was emptied more rapidly than later. Zinc from the WpD left the stomach obeying an exponential function, whereas the emptying pattern of zinc from CasD was biphasic: a rapid phase lasting up to 30 minutes was followed by a slow one. Almost all the zinc from the CasD but only about 90% of that from the WpD had left the stomach after two hours.

#### *Transit of chyme and of zinc through the intestine*

The transit time of both chyme and zinc through the duodenum and jejunum was short. Some 17–20% of the protein and the zinc was detected in the jejunum 30 minutes after intubation. Two hours after the onset of the experiment only traces of either the protein or the zinc were found in

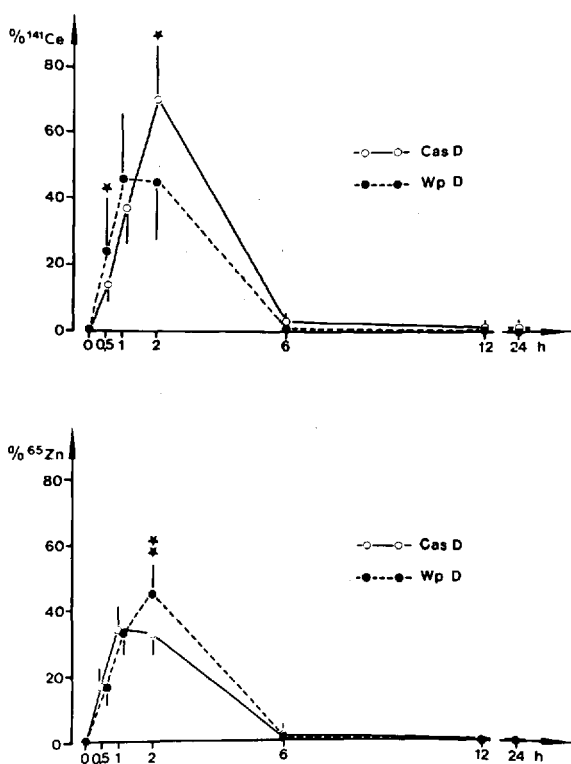


Fig. 2. Percentage of chyme (above) and zinc (below) in the ileal lumen after intragastric intubation of casein (CasD) or whey protein (WpD).  $\bar{x} \pm \text{s.d.}$ ; \*differences between CasD and WpD significant at  $p \leq 0.05$ .

the jejunal lumen. The low concentration of zinc in the lumen of both proximal segments was partially due to the fast transmission of chyme and zinc to the ileum and partially due to the absorption of some of the zinc already in the proximal intestine.

Considerable portions of chyme and of zinc were retained in the ileum for many hours (Figure 2). About 45 % of the WpD chyme was found in this segment after 60–120 minutes. After 6 hours chyme from both diets was almost entirely emptied from the ileum.

Due to the successive absorption of zinc along the intestinal passage, a smaller portion of  $^{65}\text{Zn}$  than of  $^{141}\text{Ce}$  was found in the ileum up to about 60 minutes. The highest portion, about 35 % of the  $^{65}\text{Zn}$  given with the CasD, was present at 60 minutes. The  $^{65}\text{Zn}$  peak (45 %) from the WpD was reached after 2 hours.

The passage through the caecum of chyme and zinc from both diets was slow. Even 24 hours after intubation we detected 8 % of the  $^{65}\text{Zn}$  from the WpD and 16 % from the CasD. At that time, 55 % of the zinc from the CasD and 40 % from that of the WpD was recovered in the faeces.

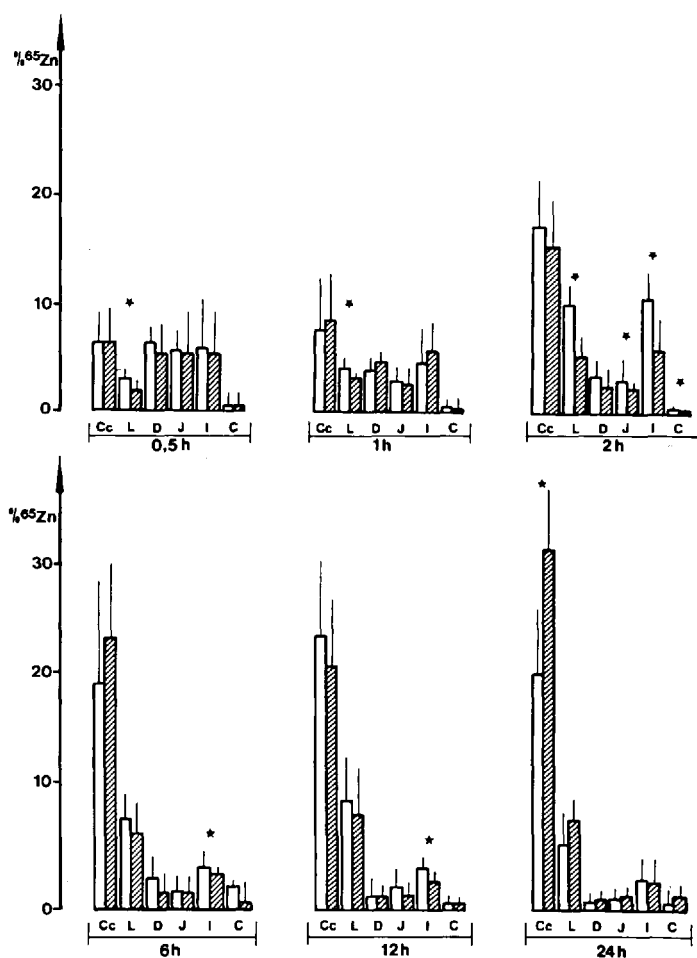


Fig. 3. Percentage of zinc retained in different tissues and in the carcass after intragastric intubation of casein (CasD = white columns) or whey protein (WpD = hatched columns).  $\bar{x} \pm \text{s.d.}$ ; \*differences between CasD and WpD significant at  $p \leq 0.05$ . Cc = carcass, L = liver, D = duodenum, J = jejunum, I = ileum, C = colon.

### Zinc retention as a function of time

From the  $^{65}\text{Zn}$  determination in the duodenal, jejunal, ileal and colon tissue as well as in the liver and in the carcass the percentage of zinc retained was calculated. Distribution of absorbed zinc in the different tissues at different times is presented in Figure 3. At 30, 60 and 120 minutes the liver tissue of the CasD group contained significantly more zinc than that of the WpD group. After two hours, however, differences could no longer be detected anymore. After 24 hours significantly more  $^{65}\text{Zn}$  was retained in the carcass from the WpD than from the CasD, a fact which could not be predicted from the data found in the shorter periods.

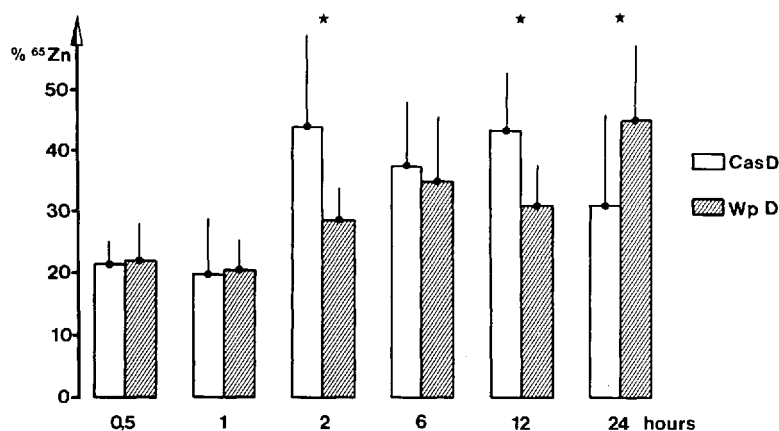


Fig. 4. Total percentage of zinc retained after intragastric intubation of casein (CasD) or whey protein (WpD).  $\bar{x}$  = s.d.; \*differences between CasD and WpD significant at  $p \leq 0.05$ .

The total of  $^{65}\text{Zn}$  retained in all tissues together is shown in Figure 4. After one hour no difference could be detected between the two diet groups. Then, 2, 6 and 12 hours after intubation retention from the CasD was significantly higher than that from the WpD. After 24 hours the situation had changed, in that retention of  $^{65}\text{Zn}$  was now significantly higher in the WpD group.

## Discussion

Compared with the WpD group, the intragastric pH of the CasD group was found to be significantly lower 30 and 60 minutes after intubation of the diet. This phenomenon might be due to different buffering capacities of the two protein fractions. According to the titration curves of these proteins (own experiments, unpublished) the buffering capacity of casein is higher than that of whey protein in a pH-range between 6.1 and 4.2, while at lower pH values it is less than that of the whey protein. Due to this fact, the whey protein is obviously able to buffer more of the secreted HCl and thus to keep the initial pH measured after 15 minutes, whereas casein is not. Furthermore, it has to be assumed that HCl secretion is higher after intubation of casein than after intubation of whey protein. Our experiments carried out in suckling rats (1) also corresponded with this fact. The mechanism by which the quantitatively different acid output after intubation of casein and of whey protein was controlled is not clear. Similar results were reported by Berger et al. (3) who found an increased gastrin secretion in babies fed a casein dominant milk formula compared to a whey dominant one. The significance of this finding in connection with our results remains to be elucidated.

According to George and Lebenthal (10) casein precipitates at low pH values as compact curd. Whey protein, on the other hand, precipitates with



a flocculent structure. Therefore in our experiments, the hard casein curd was retained in the proximal stomach, as usually found with solid food, and the whey protein left the stomach like a liquid (15), indicating an effect due to the kind of protein precipitation. This was also shown by Cavell (5) who compared the gastric emptying of human milk with that of bovine milk formula in preterm infants.

From either protein suspension zinc was emptied from the stomach distinctly before the chyme, indicating a disintegration of the metal protein complexes. The zinc emptying pattern found with the WpD allows the conclusions that zinc was homogeneously distributed in an intragastric liquid or semi-liquid phase. In contrast, due to the low pH (12), zinc seemed to be separated from the casein immediately after the intubation of the CasD. A considerable portion of the metal might, therefore, leave the stomach quickly with the liquid, followed by a second one with the solids. This emptying pattern supports the view of a very inhomogeneous intragastric distribution of zinc from the casein diet.

It can be assumed that the intragastric events of this initial time period concerning the first steps of protein digestion, generation of proteogenic ligands, releasing and rebinding of the metal, intragastric distribution of zinc and time-dependent course of emptying of both ligands and metal, could be significant for the absorptive processes in the intestine and finally for the availability of zinc from different protein. For this reason further characterization of the initial intragastric events appears to be relevant for the understanding of the divergent availability of zinc from casein and whey protein. This point might be of interest especially for the young infant with its immature gastric function.

In agreement with Curtis et al. (6), the passage of chyme from both the proteins through the proximal segments, duodenum and jejunum, was a rapid process. Nevertheless, zinc was possibly absorbed in these upper parts of the intestine already during the first 30 minutes after intubation of the protein suspensions. This corresponds to the results obtained by Methfessel and Spencer (18) in intact rats as well as with *in vivo* ligated intestinal sacs. Although there is no doubt that duodenum and jejunum have a high zinc absorbing capacity (26), in our experiment the main site of zinc absorption was the ileum, mainly due to the long period of time during which chyme and zinc were retained.

The accumulation pattern of chyme and zinc in the ileum reflected the emptying pattern from the stomach. With the uniform clearance of the WpD chyme, a plateau was found in the ileal lumen 60–120 minutes after intubation. Almost identical behaviour was found for zinc from this diet. In contrast, the fast gastric emptying of the CasD chyme led to a sharp peak in the ileum 2 hours after intubation. The long lasting contact of zinc with the ileal epithelia might be the most important among the factors making the ileum the main absorbing site for the metal (11). The luminal and surface pH in this segment, as well as the presence of eventually occurring proteogenic zinc-binding ligands, could promote zinc absorption even further (2).

According to Shaw (7) and Davies (27) zinc in variable amounts is secreted into the intestinal lumen as early as one hour after administration. Intraluminal zinc concentrations cannot therefore be considered as

an exact criterion for the estimation of zinc absorption and thus for zinc availability.

Retention seems to be more appropriate. In this respect, Sandström et al. have chosen the liver as a reference tissue (23). Using different milks, they found that zinc retention in the liver correlates highly with the total of zinc retained, measured by balance studies, and depends on the type of milk being fed. In our experiments, we also found differences in liver zinc retention depending on the dietary protein. However, we additionally saw an effect of time. Two hours after the feeding significantly more zinc was retained from the CasD than from the WpD. At 6 and 12 hours differences were not significant any more. Then after 24 hours zinc retention was significantly higher from the WpD than the CasD. It can therefore be concluded that short-term experiments are not appropriate to test different proteins – or other foods – for their effects on zinc availability. No definite conclusion can be drawn from luminal zinc disappearance either. Therefore, only long-term experiments may be valid – and have been initiated by us – to judge the quality of dietary proteins with respect to their effect on trace element availability. As our results may suggest that preabsorptive processes substantially influence zinc availability from proteins, we will report in a further paper on the early intragastric events following intubation of zinc with either casein or whey protein.

#### Acknowledgements

The financial support of Milupa AG, Friedrichsdorf, ist gratefully acknowledged. The authors are obliged to Prof. Dr. E. L. Sattler for giving them the opportunity to work in the laboratories of the Strahlencentrum (Central Department) of the Justus-Liebig-University, Gießen.

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Received July 16, 1985

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