

Zinc homeostasis in man: studies using a new stable isotope-dilution technique

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1. A new method has been developed for the study of zinc metabolism in man using the stable isotope ^{67}Zn . The technique involves intravenous infusion of the isotope followed by measurements of the plasma and faecal enrichments over a period of days.

2. A procedure for the analysis of Zn isotopes in plasma and faeces is described which requires the separation of Zn from other elements using the chelator dithizone before analysis by thermal-ionization mass spectrometry.

3. The stable isotope technique has been used in conjunction with a metabolic balance study to obtain measurements of Zn absorption and gastrointestinal secretion in a normal subject. Preliminary measurements of the size of the exchangeable pool of Zn have been made as have estimates of the rates of plasma and whole-body Zn turnover.

4. Following an increase in dietary Zn the body appeared to respond in two ways. The gastrointestinal secretion of Zn increased immediately, but only by a relatively small amount. The absorption of Zn initially increased in proportion to the increase in dietary levels but then decreased within 4 d by an amount sufficient to restore Zn balance.

Abnormalities of zinc absorption are now thought to occur in several human pathological conditions such as acrodermatitis enteropathica (Moynahan, 1974), coeliac disease (Elmes *et al.* 1976) and other malabsorptive states (Weisman *et al.* 1978). Despite this, actual measurements of the metabolism of Zn such as rates of absorption, equilibration between tissue pools and secretion have not been widely made, mainly because suitable techniques have not been available for use in man. Some workers have studied Zn absorption using radioactive ^{65}Zn (Lombeck *et al.* 1975; Sandstrom *et al.* 1980), but the long radioactive half-life of this isotope (245 d) has limited its use. Recently other groups (Aamodt *et al.* 1979; Molokhia *et al.* 1980) have used $^{69\text{m}}\text{Zn}$, which has a very short half-life (13.6 h) but the application of this is limited by the practical difficulties of using such a labile isotope. It has been suggested that stable isotopes of Zn might overcome these problems (King *et al.* 1978; Janghorbani & Young, 1980; Janghorbani *et al.* 1981; Turnland *et al.* 1982), although stable isotopes are more expensive and their analysis more complicated than that of their radioactive counterparts.

In this study a new method for the measurement of stable isotopes of Zn in biological materials has been developed using thermal-ionization mass spectrometry. This has enabled experiments to be performed using the relatively cheap and entirely safe stable isotope ^{67}Zn to study the factors determining the rates of Zn absorption and secretion in man. The technique involved the enrichment of the exchangeable body pool by infusion of isotopic Zn and comparison of the enrichment in the faeces and plasma over a period of time when the dietary levels of Zn were varied.

The results obtained demonstrate the suitability of the technique for the determination of rates of absorption and gastrointestinal (GI) secretion of Zn in man and indicate that

the homeostatic control of body Zn involves changes both in absorption and secretion with changes in secretion adapting more rapidly than absorption.

We also present preliminary results showing how the technique can be used to estimate the size of the exchangeable body Zn pools.

METHODS

Subject. The subject was a normal 80 kg male aged 29 years.

Metabolic balance studies. Conventional metabolic balance studies were modified by the use of an internal faecal marker (cuprous thiocyanate) together with visible carmine markers which were taken at the beginning of each 4 d balance period. The carmine markers were used to determine which stool specimens were derived from each dietary period and the cuprous thiocyanate recovery was used to correct for any losses of faecal material during each period.

The diet was chosen by the subject in consultation with the dietitian and was kept constant throughout the study. The main meat source was white chicken, the total daily energy intake 10.46 MJ (2500 kcal) and the daily fibre intake 23 g. The basal total Zn intake was between 110 and 130 $\mu\text{mol/d}$ and this was increased (every two periods) by addition of Zn supplements ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) in capsule form taken after meals evenly throughout the day.

Enrichment of body pools with ^{67}Zn . ^{67}Zn was obtained as 93.11% enriched zinc oxide (Oakridge National Laboratory, Oakridge, Tennessee). This was dissolved in hydrochloric acid and neutralized with sodium hydroxide. The solution was sterilized and diluted with saline (9 g sodium chloride/l) before use. The infused Zn is referred to in the text as the 'spike'.

During the 6 d run-up to the balance whilst taking the standard diet, but not collecting samples, the subject was infused intravenously with 5 mg ^{67}Zn on four consecutive days. Each infusion was in a volume of 10 ml and lasted 1 h. On day 2 after the last infusion the subject took the first carmine marker and started to collect urine and faeces.

Analyses of plasma and faecal ^{67}Zn content. At the mid-point of each 4 d balance period, 40 ml venous blood was taken for analysis. The faecal ^{67}Zn enrichment was obtained from measurements of the pooled 4 d faecal samples from each balance period.

Measurements of the isotopic abundance were performed using thermal-ionization mass spectrometry. To be suitable for this the Zn in the biological samples had first to be separated into a relatively pure aqueous solution in two stages. First the samples were dry ashed to remove organic material and then the Zn was extracted using a chelating agent.

Removal of organic material. Faecal samples (4 d) were pooled, diluted with 4 l deionized distilled water, weighed and homogenized. Portions (50 g) of this homogenate were placed in silica beakers, dried and ashed at 450° for 48 h. The ash was then dissolved in 10 ml hydrochloric acid (20 ml/l, AristaR grade; BDH Chemicals Ltd, Poole, Dorset). Samples of blood plasma (10 ml) were also placed in silica beakers, dried, ashed and dissolved in HCl in the same way.

Extraction of Zn from acid solution. All glassware used in the extraction was cleaned by soaking for 24 h in EDTA solution (5 g/l) followed by rinsing in a large volume of deionized distilled water. All reagents were of AnalaR grade or better and all solutions were further purified by extraction with excess dithizone before use. The Zn was extracted from the HCl-extracts of faeces and plasma using the chelating agent dithizone by a modification of the method of Verdier *et al.* (1957) except that, at the end of the extractions, the aqueous extract was freeze-dried, the residue was then transferred to a small glass tube where it was treated with concentrated nitric acid followed by hydrogen peroxide to destroy any organic matter left from the dithizone extraction.

Mass spectrometry. For isotopic analysis the samples were measured on a solid-source (thermal-ionization) mass spectrometer. The instrument was a Thomson-Houston THN 206 solid-source mass spectrometer fitted with a multi-filament turret source and V. G. Isotopes power supply, control unit and data acquisition system. A silica-phosphoric acid emitter was applied to the sample to ensure stable and enhanced ion-beam production.

The quantity of Zn yielded by the extraction procedure from each sample was sufficient for two separate mass-spectrometric analyses. In tests of the method, reagent Zn samples of 4–6 μg were used and the samples were of a similar magnitude (although very much smaller samples could be analysed with some loss of precision).

Zn has five naturally-occurring isotopes, masses 64, 66, 67, 68 and 70. Although the spike consists mainly of mass 67 (93.11%) it has small amounts of the other isotopes. The samples measured consisted of mixtures of naturally-occurring Zn with various proportions of the spike. Thus the mass spectra were predominantly normal but with a 67:64 value larger than normal.

The main cause of variation in measuring isotope ratios is isotopic fractionation which alters the values by a small amount. This can be corrected by normalizing all the ratios to an accepted value for one of them (Dodson, 1969). Preliminary mass spectrometer runs using reagent Zn produced a set of ratios similar to those of Bainbridge & Nier (1950) but fractionated to the extent of 0.7–1%/mass unit. We have used our own determinations of normal ratios in order to correct for fractionation, but even without this the results would be quite satisfactory for the purposes of this study.

Repeated analyses of standards demonstrated that the mass spectrometric analyses had an excellent precision with an average standard error of 0.09%.

Calculations of Zn absorption and GI secretion. The movement of Zn in and out of the GI tract may be described by a series of pathways (Fig. 1). Of the variables, the dietary intake (D), the urinary output (U) and the faecal output (F) are measured directly in the conventional metabolic-balance study and the over-all balance, B , can be calculated from:

$$B = D - (F + U).$$

After an infusion of ^{67}Zn , measurement of the plasma and faecal enrichment enables the GI secretion to be calculated. If the plasma enrichment is p and the faecal enrichment f

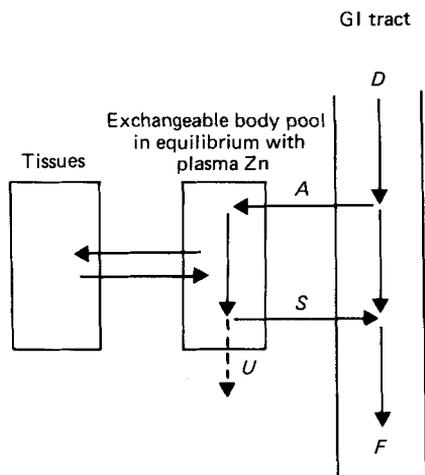


Fig. 1. Diagrammatic representation of the over-all processes of zinc absorption and gastrointestinal (GI) secretion. D , dietary intake of Zn; A , absorbed Zn; S , GI secretion of Zn; U , urinary output of Zn; F , faecal output of Zn.

then the proportion of the total faecal Zn derived from GI secretion is f/p and thus the quantity of Zn secreted in this way (S) is given by:

$$S = F \cdot f/p \quad (1)$$

From Fig. 1 it can be seen that:

$$F = D - A + S,$$

or

$$A = D - F + S,$$

but since

$$S = F \cdot f/p,$$

$$A = D - F(1 - f/p). \quad (2)$$

With a knowledge of the dietary Zn content (D), the faecal content (F), the faecal enrichment (f) and the plasma enrichment (p), the GI Zn secretion and absorption can be calculated from eqns (1) and (2) respectively.

This method of assessing Zn secretion and absorption has not previously been applied to man although it has been validated in animals by Weigand & Kirchgessner (1978) and Evans *et al.* (1979).

RESULTS

Plasma enrichment. The enrichment of the plasma with ^{67}Zn above the naturally-occurring levels as the result of four consecutive intravenous infusions of ^{67}Zn is shown in Table 1 and Fig. 2(a). Blood was taken 4 d after the final infusion and then every 4 d for the next 24 d (six balance periods). The enrichments are expressed as a ratio, amount of spike (i.e. mainly ^{67}Zn): total Zn in the sample, and have been corrected for fractionation during mass spectrometry using the ratio, 66:64 and the ratio, 68:64.

As a consequence of the natural turnover of body Zn the plasma Zn enrichment decreased throughout the 24 d of the study (Fig. 2(a)). A straight line was drawn through the points when plotted on a semi-log basis (Fig. 2(b)) and from this the plasma Zn half-time was calculated as 12.5 d. Extrapolating the line back to zero time, taken as the day of the fourth infusion, gives an estimate of the size of the exchangeable pool in equilibrium with the plasma Zn, in this case 678 mg Zn or approximately 10 mmol.

GI secretion and absorption. During the first 4 d of the run-up the subject was infused with 5 mg ^{67}Zn each day. On day 2 after the last infusion the subject took the first carmine

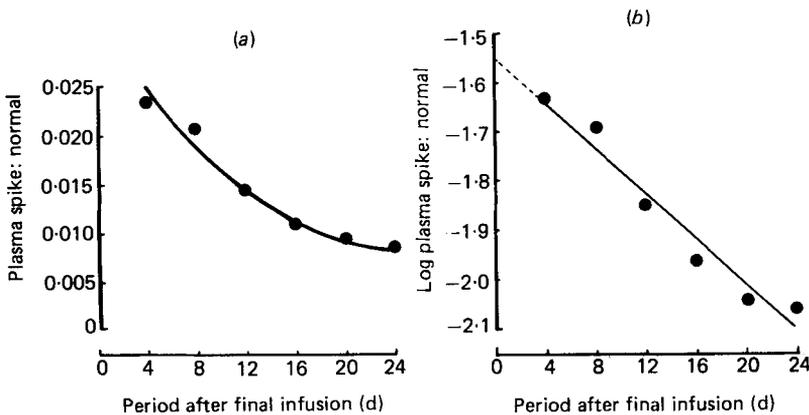


Fig. 2. Plasma enrichment of ^{67}Zn spike (expressed as ratio, spike:normal) plotted *v.* time after the last infusion of ^{67}Zn . The plasma enrichment is plotted as (a) linear and (b) logarithmic values.

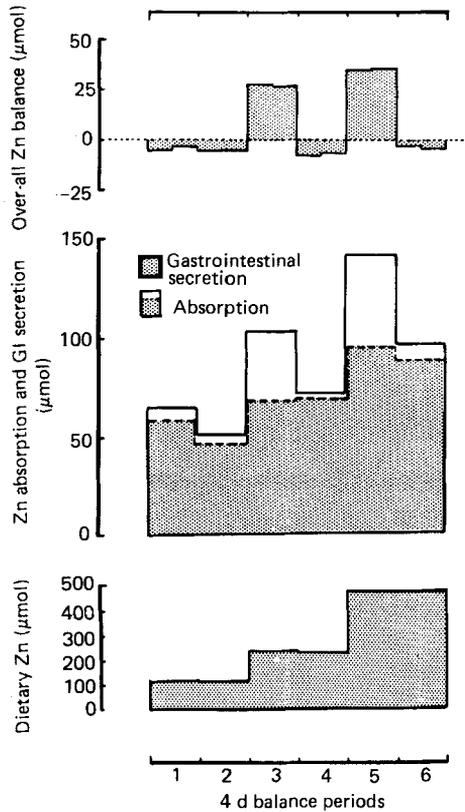


Fig. 3. Comparison of changes in gastrointestinal (GI) absorption and secretion measured by stable isotope techniques with the over-all balance for zinc with different dietary intakes.

marker and began collecting samples. The results for the over-all balance are shown in Fig. 3.

During the first two periods on the basal diet (Zn 110 $\mu\text{mol}/\text{d}$) the subject was in approximately zero Zn balance; when the diet was supplemented with 120 $\mu\text{mol}/\text{d}$ the balance became positive during the third period, with the subject retaining about 25 $\mu\text{mol}/\text{d}$. During period 4, while on the same Zn intake, the balance returned to zero. When the diet was further supplemented, approximately doubling the Zn content, the balance again became positive during period 5, with the subject retaining 35 $\mu\text{mol}/\text{d}$. Again, while on the same Zn intake during period 6, the balance returned to zero.

Blood was taken half-way through each balance period and the plasma analysed for ^{67}Zn abundance. From this and the measured abundance of the faecal Zn the GI secretion and absorption were calculated. Table 1 gives the abundance values together with the faecal Zn content and Table 2 the derived rates of secretion and absorption.

In the first two periods the secretion and absorption were very similar with a mean of about 50 $\mu\text{mol}/\text{d}$. On doubling the Zn intake during period 3, absorption also approximately doubled while the secretion increased by about 50%. In the following period 4, secretion remained at the same level (about 70 $\mu\text{mol}/\text{d}$) while the absorption fell to the same value. Doubling the Zn intake in period 5 produced an almost identical result, the absorption doubled (to about 140 $\mu\text{mol}/\text{d}$) while the secretion increased by a further 50%. In the sixth

Table 1. Total zinc ($\mu\text{mol/d}$) and isotopic composition (spike: normal) of plasma, faecal and urine samples

(The subject was infused with 20 mg ^{67}Zn and the abundance measured in plasma and faecal samples during the course of a balance study with 4 d balance periods. The dietary Zn intake was doubled after every second period. The faecal and plasma abundance and measurements are expressed as ratios, amount of spike in the sample (i.e. the amount of the administered ^{67}Zn tracer): total amount of Zn in the sample)

Period	Dietary Zn	Faecal Zn	Urine Zn	Plasma abundance (spike: normal)	Faecal abundance (spike: normal)
1	111	105	10.6	0.0237	0.0131
2	109	105	9.7	0.0210	0.0094
3	231	196	8.1	0.0143	0.0051
4	224	222	9.2	0.0110	0.0035
5	473	427	10.5	0.0092	0.0021
6	472	464	10.7	0.0088	0.0017

Table 2. Rates of absorption and gastrointestinal (GI) secretion ($\mu\text{mol/d}$) calculated from ^{67}Zn abundance values

(Rates of GI absorption and secretion of Zn calculated from the measurements of ^{67}Zn abundance given in Table 1)

Period	Dietary Zn	GI secretion	Absorption	Percentage of intake absorbed
1	111	58.2	64.6	57.8
2	109	46.5	50.7	46.6
3	231	68.6	103.7	44.8
4	224	70.7	72.6	32.4
5	473	96.4	142.1	30.1
6	472	89.6	97.6	20.7

period, still on the high-Zn intake, the secretion remained at the same level, about $90 \mu\text{mol/d}$, while the absorption decreased to a similar value.

The percentage of the dietary intake absorbed (Table 2) tended to remain constant during the first period after the Zn intake was increased (periods 3 and 5) before falling in the second period (periods 4 and 6) as the measured absorption decreased. These results are summarized in Fig. 3.

There were no significant changes in the plasma Zn levels during the course of the experiment, with all values being within the normal range ($11.4\text{--}17.6 \mu\text{mol/l}$). Likewise there were no significant changes in the urinary Zn excretion (Table 1).

DISCUSSION

The results presented here show that the isotope-dilution method using ^{67}Zn is suitable for the measurement of GI secretion and absorption in man. The results from our one subject indicate the existence of a homeostatic mechanism for Zn which involves changes in absorption and GI secretion of Zn. With suitable modification the methods could also be used to follow the equilibration of Zn between different body pools.

The use of stable isotopes has the major advantage over radioactive methods in that it is entirely safe, but also has the disadvantages that stable isotope analysis tends to be more

complicated and the initial cost of the isotope much higher. Most previous workers with stable isotopes in man have utilized neutron activation analysis (King *et al.* 1978; Janghorbani & Young, 1980; Janghorbani *et al.* 1981). This technique is not widely available and is less precise than thermal-ionization mass spectrometry used in the present study and also in that of Turnland *et al.* (1982). In addition only certain isotopes can be measured by neutron activation whereas all isotopes can be measured by mass spectrometry. This has meant that ^{67}Zn could be used in the present study, an isotope which is relatively cheap compared with ^{70}Zn used in previous studies with stable isotopes.

It is difficult to assess the accuracy of the absorption and GI secretion measurements. The precision of the metabolic-balance technique may be as good as 5% when an internal faecal marker is used in conjunction with visible faecal markers. The analysis of Zn isotopes in aqueous solution by thermal-ionization mass spectrometry is a very precise method and it seems likely that if adequate precautions are taken to avoid contamination during the preparatory stages then the precision of the whole technique will be better than 10%.

Samples for analysis by thermal-ionization mass spectrometry must be relatively pure. In the present study the purification was achieved by selective chelation of the Zn with dithizone, whereas Turnland *et al.* (1982) performed the necessary purification by ion-exchange chromatography. The mass spectrometry method used in the present study required approximately $5\ \mu\text{g Zn/analysis}$, thus duplicate determinations could be obtained from an extract of 10 ml blood plasma.

Infusion of the Zn isotope in conjunction with a concurrent metabolic-balance study for Zn enables quantitative measurements of Zn turnover together with rates of both secretion and absorption in the GI tract to be made. The measurements can also be repeated at convenient intervals so long as the plasma enrichment of ^{67}Zn remains high enough to be measured accurately.

In the present study the Zn was given as divided doses for two reasons. First, it is important not to perturb the Zn metabolism suddenly with a single large dose as there is the suggestion that a bolus of Zn might be handled in a different way from physiological quantities (Aamodt *et al.* 1979). Secondly, in order to calculate rates of secretion, etc., the assumption was made that the plasma values taken at the mid-point in each period represented the mean plasma enrichment during the whole period, i.e. that it is changing in a fairly linear fashion. Immediately after the infusion the plasma Zn will be equilibrating with the very rapidly exchanging pools and the enrichment will be falling in an exponential manner. Spacing the infusions over 4 d and taking the first blood sample 4 d after the last infusion will have minimized the effects of the rapid changes. The dose of ^{67}Zn administered in the present study was relatively large because at the outset there was uncertainty about the size of the exchangeable body pool and the rate of disappearance of isotopic Zn from the plasma. In the event it appears that more than enough ^{67}Zn was used and that a single infusion of 5 mg might have been sufficient.

One of the problems of interpreting absorption values is knowing the bioavailability of the total Zn in the diet. It is generally thought that Zn associated with phytate in high-fibre diets is not available but without precise measurements of intrinsic Zn absorption there must always be some doubt. For measurements of absorption the infusion technique is preferable to the method of oral administration and following the appearance of isotope in the plasma. The infusion method gives the true absorption of intrinsic Zn from the diet instead of extrinsic label admixed with the diet or even given separately as a test meal. The values in Table 2 indicate that on the basal diet the subject absorbed between 46 and 58% of the total dietary Zn which is comparable with the range of 41–84% reported by other workers using oral administration of radioactive Zn (Lombeck *et al.* 1975; Aamodt *et al.* 1979; Molokhia *et al.* 1980).

As the ^{67}Zn was infused over 4 d and the dietary Zn was varied during the present study the experimental design was not suitable for accurate determination of the biological half-life of the isotope in plasma or for estimations of the size of the rapidly exchanging body-Zn pool. However, approximate values can be calculated from values given in Fig. 2(b). The half-life of the plasma Zn was found to be approximately 12.5 d and the size of the exchangeable pool with which it is in equilibrium, approximately 10 mmol.

This exchangeable pool of 10 mmol is well below the likely total body Zn of the 80 kg subject, which would be expected to be about 30 mmol. The total extracellular-fluid Zn contributes to the exchangeable pool and the size of this can be estimated from the approximate extracellular fluid volume (15 l) and the plasma Zn concentration ($14 \mu\text{mol/l}$) giving a total pool size of about 0.2 mmol. The liver is the major organ for Zn metabolism in the body (Cousins, 1979) but would only contribute approximately 1 mmol leaving the bulk of the exchangeable pool in some other tissue of the body.

The half-life of the plasma Zn is the sum of the rates of exchange of the plasma Zn with tissue pools and the loss through GI and urinary excretion. An estimate of the half-time of the secretion of Zn into the GI tract can be made assuming the rate of GI secretion to be $50 \mu\text{mol/d}$ (Table 2) and the rapidly exchangeable pool to be 10 mmol. Using the general exponential decay equation $A = A_0 e^{-kt}$, k is the rate of secretion divided by the pool size (i.e. $50/10000 \mu\text{mol}$) giving a half-time of 138 d. This measurement should give an indication of whole-body turnover and is of the same order as the estimate of whole-body turnover, 89 d (Aamodt *et al.* 1979) from studies with radioactive Zn. The precise value would be expected to vary according to the dietary Zn intake. Our estimates of secretion were based on the first two balance periods when the dietary intake was relatively low ($110 \mu\text{mol/d}$). The calculated whole-body turnover is approximately ten times slower than the observed half-life of the ^{67}Zn in the plasma indicating that the plasma half-life is dominated by the exchange with tissue Zn pools rather than secretion from the body.

The values derived above are only approximations but serve to demonstrate the potential of this stable isotope-dilution method for studies such as this and for planning future experiments. It is apparent that because of the relatively small rapidly exchangeable pool of Zn (i.e. 10 mmol), less ^{67}Zn could be used and only one infusion required. If 5 mg ^{67}Zn were used the enrichment would be sufficient for accurate measurements and the relatively slow plasma half-life means that the plasma enrichment would remain sufficient to allow extended experiments over about 20 d.

In a normal subject the plasma pool of Zn is approximately 0.2 mmol while the rapidly exchangeable pool is about 10 mmol. Infusion of 5 mg Zn (0.075 mmol) might therefore cause a slight perturbation in the plasma Zn but this is likely to be transitory since the quantity of infused Zn is small (1%) compared with the rapidly exchangeable pool.

The results of the metabolic-balance study (Fig. 3) confirm earlier observations (Jackson *et al.* 1980) that when the dietary Zn level is changed there is a delay before balance is re-established. Following both increases in the dietary Zn content an increased retention of Zn occurred during the first 4 d period. In the next period, balance was restored indicating that whatever change had occurred it must have come about during the first 4 d, since during this second period the subject was in zero balance.

Similar changes in Zn balance have been seen in two patients in whom the diet was supplemented. One was a case of acrodermatitis enteropathica (Jackson, 1977) in whom the retention of Zn may well have been due to repletion of tissue Zn, but in the second case, a patient recovering from polymyositis (Edwards *et al.* 1979), Zn supplementation resulted in a transitory positive phase.

A change in body balance may be the result of changes in either absorption or secretion or both. In man there is no direct evidence as to which process is responsible for the control,

although McCance & Widdowson (1942) demonstrated that the majority of an injected dose of Zn was excreted through the GI tract, indicating that secretion may be important. Work with animals has suggested that both secretion and absorption may play a controlling role (Cotzias *et al.* 1962; Jackson *et al.* 1981).

Fig. 3 shows the changes in absorption and secretion as the dietary intake was progressively increased together with the over-all balance which is the sum of the two. On each occasion when the dietary Zn was increased the most striking change was an increase in the Zn absorption, which adapted within 4 d, accompanied by a smaller increase in GI secretion which remained approximately constant at each dietary level. These results indicate that the absorptive process is capable of fairly large adaptive changes, but takes a number of days (< 4) to do so, while there can be smaller but rapid changes in GI secretion.

We have recently described the characteristics of Zn absorption in rats (Jackson *et al.* 1981) and the present results suggest that similar mechanisms may exist in man. In rats on normal diets absorption appears to be carrier-mediated, probably obeying Michaelis-Menten kinetics in that for dietary levels in the normal range the amount absorbed is roughly proportional to the dietary intake.

In the present experiments the proportion of the ingested Zn absorbed was roughly constant during periods 1, 2 and 3 (Table 1) despite a doubling of the intake during period 3 and, likewise, it was almost constant during periods 4 and 5, despite a doubling of Zn intake during period 5. This suggests a carrier responding to an increased substrate concentration. It is, however, not clear what mechanism could account for the decrease in the proportion absorbed between periods 3 and 4 and periods 5 and 6.

In the present study the GI secretion of Zn seems to adapt rapidly (Fig. 3, Table 2) although it has a limited capacity for dealing with changes.

From the results presented here it appears that control of body Zn in man is achieved in two ways. Small daily variations in intake are dealt with by changes in the GI secretion of Zn as this is a rapidly responding mechanism. Larger changes in intake can only be adequately dealt with by changes in absorption which are slower to respond but have the capacity to cope with larger fluctuations in dietary Zn.

Clearly, further work is required in normal subjects to test these findings but the new technique of using infused stable isotopes should facilitate this and it also offers the opportunity of characterizing the defect in conditions where disorders of Zn absorption are suspected, such as acrodermatitis enteropathica.

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