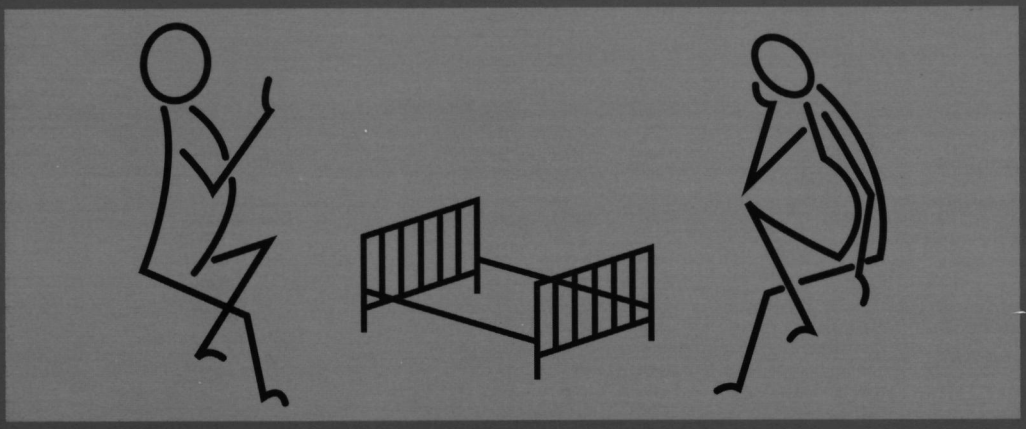
The assessment of malnutrition in non-surgical patients from macronutrient to micronutrient



Ton Naber

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from macronutrient to micronutrient,

with special emphasis on zinc

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"In mijn college dictaat stond een detail dat me bij eerste lezing was ontgaan, namelijk dat zink, zo zacht en week als het is, zo weerloos tegen zuren, die het in een hap opschrokken, zich heel anders gedraagt als het heel zuiver is: dan houdt het als het aangevallen wordt koppig stand. Je kon daar twee elkaar tegenovergestelde filosofische consequenties uit trekken: de lof van de zuiverheid die het pantser is tegen het kwaad; de lof van de onzuiverheid, die de kiem is van de verandering en dus van het leven. Ik schoof de eerste walgelijk moraliserende stelling terzijde en dacht na over de tweede, die mij beter lag."

Primo Levi

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ABSTRACT

The assessment of malnutrition in patients is controversial. The principal questions of this thesis are: what is the prevalence of malnutrition of macronutrients and micronutrients in non-surgical patients, and what is the validity of the methods to assess malnutrition? The last question can be separated into: - Can the currently available methods to determine nutritional deficiency distinguish between nutritional depletion and a normal nutritional status? - Can the currently available methods to determine nutritional deficiency distinguish nutritional status from disease activity?

We demonstrated that malnutrition was present in 40 to 60 % of the non-surgical hospitalized patients at admission, depending on the method that was applied for the assessment of malnutrition. Over 50% of the patients with active inflammatory bowel disease were malnourished. Malnourished patients suffered a higher risk of complications. In part this correlation between malnutrition and complications, could be explained by the disease process. A study in healthy volunteers was performed, using the same methods. Less than 5 % of the healthy volunteers were malnourished, except for elderly persons in which the percentage was more.

The prevalence of malnutrition of zinc was studied in patients with Crohn 's disease. A decrease of serum zinc concentration, one of the most important micronutrients, was observed in 68% of the patients with more or less active Crohn's disease. The question whether the currently available method may distinguish nutritional status from disease activity, was studied in rats. Serum zinc concentration was influenced by the zinc status, but also by the inflammatory process caused by a redistribution of zinc into the liver and a decrease of the zinc transporting serum protein: albumin.

We studied the in vitro uptake of zinc by blood cells of rats fed a zinc deficient diet, rats with a decrease in serum zinc induced by an inflammation, and control rats. We demonstrated that by this method nutritional depletion could be distinguished from a normal zinc status. This method could also distinguish real zinc deficiency from apparent zinc deficiency caused by inflammation.

Alkaline phosphatase is a zinc dependent metallo-enzyme. In case of zinc deficiency serum alkaline phosphatase activity is decreased. In rats total serum alkaline phosphatase activity was decreased in zinc deficiency, but alkaline phosphatase activity was also influenced by growth and acute inflammation, but not by chronic inflammation. Zinc deficiency influenced the activity of the enzyme. Other factors like growth are mainly influencing the concentration of the enzyme. We hypothesized that the difference in serum

alkaline phosphatase activity, using buffers low and high in zinc, was greater in case of zinc deficiency. This hypothesis was confirmed. This method thus can differentiate between zinc deficiency and the normal zinc status. This hypothesis was also confirmed in a situation with an change in the alkaline phosphatase enzyme concentration: growth.

In conclusion, malnutrition of both macronutrients and micronutrients is frequent, but outcomes of methods frequently used to determine malnutrition are also influenced by the disease itself. Especially for zinc, new methods were studied which are less influenced by the disease process.

ABBREVIATIONS

MNC = Mononuclear Cells

PMNC = Polymorphonuclear cells

GPB = Glucose Phosphate Buffer

CDAI = Crohn's Disease Activity Index

CHAPTER 1

AIM OF THE THESIS

One of the essential phenomena in all living organisms is the intake of nutritional substances for the generation of energy and for growth and maintenance of the organism. Insufficient food intake will result in malnutrition, and subsequently in disease and death. In Third World Countries the symptoms of malnutrition are well known. It is less obvious that malnutrition may occur in patients in prosperous countries such as the Netherlands. Weight loss and deficiency of specific nutrients are in most cases attributed to the disease process. But the classical symptoms of malnutrition may also be caused by insufficient food intake or malabsorption of nutrients from the gut lumen. Furthermore the disease may have an influence on the metabolism of the organism.

The central questions of this thesis are:

What is the prevalence of malnutrition of macronutrients and micronutrients in non-surgical patients, and what is the validity of the methods to assess malnutrition?
The second question can be divided into the following questions:

- Can the currently available methods to determine nutritional deficiency distinguish between nutritional depletion and a normal nutritional status?
- Can the currently available methods distinguish nutritional deficiency caused by poor nutritional intake from that caused by disease activity?

CHAPTER 2

INTRODUCTION TO MALNUTRITION

with special emphasis on macronutrients and micronutrients in general, and zinc in particular.

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INTRODUCTION

In this thesis the assessment of malnutrition, both of macronutrients and micronutrients, in patients will be discussed.
In this chapter an introduction is provided to the definition of the terminology, nutritional depletion of macronutrients and micronutrients, the influence of disease on metabolism, the assessment of the nutritional status and the relevance of the assessment of malnutrition. This is essential to comprehend the next chapters of this thesis.

DEFINITION OF TERMINOLOGY

Malnutrition can be defined as a status resulting from a shortage of nutrients, resulting in reduction in biological functions. The expression 'malnutrition' suggests a shortage of food intake. The expression 'malnutrition' does not do enough justice to the effect of the disease process in the patient. A disease process may cause anorexia, maldigestion and malabsorption, all resulting in a reduction of food intake. A disease process may also result in an increase in loss of nutrients or an increase in metabolism of these substances, all causing an increase of need for these nutrients (1,2,3). The stock of the nutrients will decrease if the intake of nutrients is not increased. This is why the term 'nutritional depletion' is more suitable for this situation of 'malnutrition' in patients. But 'malnutrition' is a more suitable word in clinical practice. In the next chapters frequently the expression 'malnutrition' will be used. By 'malnutrition', 'nutritional depletion' is meant.

Besides malnutrition and nutritional depletion, the terms deficiency and nutritional status are used. In most cases depletion and deficiency are exchangeable terms but in some cases deficiency is used with a special adjective, indicating special conditions of deficiency: subclinical deficiency and apparent deficiency. Subclinical deficiency is the status in which laboratory methods do not reveal abnormalities, and no symptoms of deficiency are present, but these symptoms become present in a situation of increased needs. The classical example is the refeeding syndrome, in patients suffering a significant loss of weight. For example, in a malnourished patient with Crohn's disease a stenosis is resected and the patient gains weight. Although the serum concentrations of trace elements and minerals were normal before the surgical procedure, a fast decrease in serum calcium, potassium, phosphate, magnesium and zinc may occur, with clinical symptoms of deficiencies. In this situation the stock of these nutrients was decreased before the surgical intervention.

Apparent deficiency is a situation in which laboratory data suggest a deficiency, but no symptoms of deficiency are present. For example, in case of inflammation serum albumin is decreased. Albumin is the main transporting protein for calcium in the serum, so the total serum calcium concentration will be decreased during inflammation. The concentration of free calcium still remains normal and clinical symptoms (tetany) will not occur. There is a need for methods that can differentiate real from apparent deficiencies.

The total of all of these expressions can be summarized by the term nutritional status which also includes the normal situation and the situation of intoxication.

NUTRITIONAL DEPLETION OF MACRONUTRIENTS AND MICRONUTRIENTS

Macronutrients

Nutritional depletion is subdivided into depletion of macronutrients and depletion of micronutrients or single depletions. Depletion of macronutrients may be divided into caloric malnutrition: i.e. depletion of carbohydrates and fat, and protein malnutrition: depletion of amino acids. This subdivision has been derived from tropical medicine: marasmus and kwashiorkor referring to respectively, too little intake of food in general, and too little intake of protein owing to a cheap one-sided diet.

In patients the cause of nutritional depletion is related to the disease process itself. If the patient is anorectic, if the absorption of nutrients is disturbed or if the need for caloric nutrients is increased, a marasmus-like nutritional depletion will develop. This type of nutritional depletion is often seen in patients with chronic diseases and will develop in a period of months to years. The diagnosis is not difficult because weight loss is evident. Marasmus-like nutritional depletion has a low mortality risk.

In general in Western Countries the nutritional intake is well balanced and in healthy subjects protein malnutrition (kwashiorkor) is very rare; but in patients a kwashiorkor-like type of nutritional depletion is seen frequently. This is caused by an inflammatory process. In case of inflammation the production of albumin will be down regulated immediately and serum albumin is leaking out of the capillary arteries (1, 4-6). Serum albumin will decrease and the colloid osmotic pressure in the capillaries will decrease. Fluids will leave the capillaries for the extracellular space, resulting in oedema. This situation may develop within a day. Sometimes this type of nutritional depletion is difficult to recognize because

the patient is not losing weight (5-7). Kwashiorkor-like nutritional depletion has a high mortality rate. In many patients an intermediate type of nutritional depletion is present. The terms protein-caloric-malnutrition and marasmus-like-kwashiorkor are used. This condition is the consequence of chronic nutritional depletion complicated by an acute disease: both weight loss and oedema are present (3-5,7). These features are connected with a very high mortality rate (4).

Micronutrients

Trace elements are defined as those elements whose individual total body quantity accounts for less than 0.01% of dry bodyweight. Some are non-essential, like lead, and others are essential for normal health, function and development. Both essential and non-essential elements can be toxic in case of high concentrations in tissues and fluids. Zinc is one of the most prominent of the at least ten essential trace elements in man (Fe, Zn, Se, Mo, Mn, Cr, Co, Cu, I, F).

Zinc absorption takes place in the small intestine both by saturable, specific carrier-mediated mechanisms and by non-specific pathways. Most of the zinc is lost by the stools. The urinary zinc loss is about 300 µg and the loss by hair, skin and transpiration is about 500 µg out of a total loss of 1.5 to 4 mg per day.

Expressed as concentration the zinc content is highest in spermatozoa, prostate and ovaries. In absolute quantity most zinc is found in muscle, bone, skin/hair and liver (Table 1 (8)). The total body quantity of zinc is 1.5 to 4 g of which 98% is intracellular. An adult needs 12 to 15 mg of zinc per day, of which about 30 % is absorbed. The availability of zinc in food is highly variable (Table 2 (9)).

Zinc is important for the function of more than 200 enzymes (metallo-enzymes) like alkaline phosphatase, alcohol dehydrogenase, carboanhydrase, glutamate dehydrogenase, etc), and a cofactor in zinc finger proteins, important for the transcription of DNA. Without zinc, the synthesis of RNA and subsequently of proteins is limited. Zinc is also important for the stabilisation of cell membranes, because it is essential for an optimal function of the membrane enzymes superoxide dismutase, hydroperoxides, glutathione peroxidase and protein kinase C. The activation of receptors by hormones like insulin, growth hormone and steroids is zinc dependent. Because zinc is a structural part in many enzymes/proteins, zinc is important for optimal function of the sensorium (taste, sense of hearing, visual faculty), cell growth and differentiation, immune function and sexual function.

Table 1

Zinc in tissues in man

	Total Zn content	% of total body Zn
Muscle	1.53	57
Bone	0.77	28
Skin and hair	0.21	6
Liver	0.13	5
Brain	0.04	1.5
Gastrointestinal tract and pancreas	0.03	1
Kidney	0.02	0.7
Heart	0.01	0.3
Spleen	0.003	0.01
Blood plasma	<0.01	0.01

Zinc deficiency is caused by

1) inadequate intake,
2) maldigestion and malabsorption,
3) increased loss and
4) increased utilisation (10).

Its deficiency causes hair loss, acrodermatitis enteropathica and other skin lesions (11), dysfunction of the immune system (12), behaviour disorders (13), hypogonadism (10), diarrhoea and growth retardation (14). In patients with wounds, treatment with zinc sulphate will accelerate wound healing (15). In the USA, zinc deficiency is estimated to be present in 2% of the population (16). Zinc deficiency has been demonstrated in congenital absorption disorders (acrodermatitis enteropathica), liver diseases, chronic inflammatory bowel disease, coeliac disease, severe diarrhoea especially in children, chronic renal diseases, and parenteral nutrition (17).

Zinc intoxication is rare. In one case history a person ingested 12 g of zinc and became lethargic and light-headed with staggering and difficulty in writing (18). It may cause nausea, vomiting, abdominal pain and bloody diarrhoea (19). Zinc salts can be caustic in high concentrations, if ingested by mouth. Lower dosages of zinc ingested for therapy may induce copper deficiency (20,21).

The function of zinc can be illustrated by animal experiments. In growing rats a zinc-deficient diet will reduce the food intake after four days (22). After six days growth will slow

Table 2

Zinc concentrations in food (mg/100g)

good zinc source		fair zinc source		poor zinc source	
cheese	2-4	milk	0.4	brown bread	4
milk powder	2-4	chicken	0.9	wheat	13
eggs	1.3	pork meat	2	dry peas	4
cows meat	4	liver	5-8	oats	4
shrimps	2.3	fish	0.5-1.5	soya milk	5
oysters	> 7	white bread	0.5	cocoa powder	3.5
		white beans	3		
		vegetables	0.2-1		
		potatoes	0.27		
		fruit	0.1-0.25		
		chocolate	2		

down and after ten days growth is completely ceased. After 40 to 50 days the rat will die. When in zinc-deficient rats zinc is added to the diet, rats will start eating within hours, and within days the skin lesions will disappear. Because in this situation only 1% of the total body zinc has been supplied, this rapid reaction is an argument for activation of enzymes. Growth retardation can be explained by the reduction in food intake due to anorexia caused by zinc deficiency. But in rats which are given forced tube feeding also growth retardation is observed. If this forced tube feeding is continued the rats will die after 12 days (40 to 50 days in case of a free diet), so the reduction in food intake prevents rats from dying in an early phase. Zinc-deficient rats prefer a diet low in proteins. In zinc-deficient rats blood urea and ammonia concentrations are increased. The nitrogen loss in stools is higher in zinc-deficient diet, which in part is explained by less activity of enzymes active in the digestion process. This increase of nitrogen loss by stools can not explain the growth retardation completely. This indicates an influence of zinc on the intermediate metabolism.

NUTRITIONAL DEPLETION EXPLAINED BY THE INFLUENCE OF DISEASE ON METABOLISM

The influence of disease on metabolism can be divided into effects on protein, glucose and fat metabolism, together with the influence on the metabolism of trace elements, vitamins and minerals.

Carbohydrate metabolism

Severe disease is accompanied by hyperglycaemia, an increased production of glucose out of glycogen and proteins in the cell, and glucose intollerance. Glucose production may increase from about 250 g/day to 400-500 g/day. The sensitivity of the peripheral body cell for insulin may be reduced. In case of insulin resistance the uptake of glucose by the cell is decreased and hyperglycaemia is the result. Consequently the administration of glucose in patients with severe disease will have less effect than in healthy volunteers. The excess of glucose in the extracellular space will be taken up by the liver, because the uptake of glucose by the liver cell is insulin independent. In the liver cell, glucose will be converted into fat. In part, this insulin resistance can be compensated by administration of exogenous insulin.

Fat metabolism

Fat is made out of triglycerides: three fatty acids are attached to one glycerol molecule. It is hydrolysed into free fatty acids and glycerol. Both can be used as an energy source. In acute disease lipolysis may increase two fold. This will result in an increase of VLDL and free fatty acids production, which are used as a source of oxidation. Free fatty acids are used as an energy source by lung, muscle, kidney and liver. Oxidation, to generate energy, takes place in the mitochondria of the cell. The uptake of long-chain fatty acids in the mitochondria requires carnitine. In periods of acute disease the concentration of carnitine is decreased, so that the uptake of long chain fatty acids by the mitochondria and subsequent oxidation are also decreased. For oxidation of medium-chain fatty acids, carnitine is not necessary and in theory medium-chain fatty acids are utilized better by the cells in case of severe disease.

Protein metabolism

During illness in general the net result of protein synthesis and breakdown is a negative protein balance manifesting itself with a loss of muscle mass and of functional capacity. The endogenic protein break-down is increased by the influence of a disease process. In most cases a disease process will decrease protein synthesis. In acute disease the consumption of amino acids by the liver is increased. These amino acids are used for the production of acute phase proteins like ceruloplasmin, fibrinogen and α1-macroglobulin. The production of albumin by the liver will be severely depressed.
Large quantities of parenteral nutrition can reduce but rarely abolish this difference. Protein synthesis is stimulated to a maximum by 1.7 g of protein per kilogram bodyweight, per day. Protein synthesis can be stimulated by growth hormone. Insulin administration may reduce the breakdown of proteins.

Metabolism of trace elements and minerals

A disease process may decrease the uptake, increase the loss, and increase the need for trace elements and minerals, but may also cause a shift from one body compartment to another. In acute disease the serum zinc concentration will decrease because of a decrease of the zinc transporting protein albumin, like in calcium metabolism, and because of a shift of serum zinc into the liver (23). In the liver, zinc will be bound up by metallothionin. The increase of zinc uptake by the liver can be explained by the involvement of zinc in the production of acute phase proteins in the liver, but zinc can also be stored in the liver for later use, in case of an anabolic situation later on. In acute disease the excretion of zinc by urine will be increased resulting from leakage of amino acids, like histidine, by the kidneys and zinc will be excreted as a zinc amino acid complex. The nitrogen balance is reduced in zinc deficiency. In part this can be explained by an increase in nitrogen loss in stools, but also by the influence of zinc on metabolism. Metabolism is regulated by hormones. Growth hormone and subsequently the insulin growth factor I are essential for growth. In zinc-deficient rats the IGF I concentration is decreased (24). Also the cell binding of growth hormone is reduced. Insulin has a positive effect on protein synthesis. In zinc-deficient rats the insulin concentration is reduced (24). Zinc is also on influence in protein synthesis by other actions. DNA polymerase and thymidine kinase are zinc dependent enzymes. Zinc is of influence on the gene transcription by zinc finger proteins, transcription factor IIIA

and RNA-polymerase. For the signal transfer of hormones, inositol triphosphate and calmodulin are essential. The calmodulin activity is reduced in zinc-deficient rats (25). Concentrations of other trace-elements and minerals also change. The copper concentration will increase because of an increase of the transporting protein, ceruloplasmin, which is an acute phase protein (26). Iron will pass from the blood into the liver and the reticulo-endothelial system.

In conclusion a disease process will influence all parts of the metabolism and consequently the nutritional status. This will result in a decrease of fat and protein mass. The reduction in muscle mass will result in a reduction in the functional capacities of the organism. In part this catabolic process can be reversed by nutritional intervention: administration of additional nutrients, growth hormone and insulin. Therefore it is essential to determine the nutritional status, and start nutritional intervention in case of nutritional depletion.

THE ASSESSMENT OF THE NUTRITIONAL STATUS

The assessment of nutritional depletion of macronutrients.

For the assessment of the nutritional status no 'Gold Standard' is available (27-30). Subjective parameters are medical and nutritional questionnaires and physical examination. Objective parameters, not influenced by the personal opinion of the patient or the investigator, are also used and listed in table 3. Besides laboratory methods like blood con-

Table 3

Objective parameters

Blood	Anthropometry	Immune system	Urine
- albumin	- weight loss	- total number of	- nitrogen balance
- pre-albumin	- % ideal weight	lymphocytes	- creatinine-height
- transferrin	- triceps skin fold	- delayed type skin	index
- retinol binding	thickness	hypersensitivity	
protein	- mid arm circumference		
- total protein	- squeeze force of		
concentration	the hand		
- cholesterol	- weight-height index		

centrations of nutrients or serum proteins, tests of the immune system and urine analysis, anthropometry and the assessment of the body composition (31) using isotopes, radiological methods and bio-impedance of the body are used. Currently much attention is being paid to the functional capacity: for instance respiratory function and hand grip.
None of these parameters is in itself specific and sensitive enough to determine the nutritional status. Serum albumin for instance is influenced by the nutritional status but also by the disease process, as has been explained previously. The total number of lymphocytes will decrease in case of malnutrition but increase if the malnourished patient is suffering an infection cause by a virus.
By combining individual parameters, multivariate methods (indices) have been developed (32-40). These indices were developed and used in healthy volunteers, or, on the contrary, developed and used in patients with a specific disease. It is not evident if the index, just as the single parameters, is influenced by the disease, and validation of the method by determination of specificity and (if possible) sensitivity has in most cases not been performed. A validation of methods used for assessment of the nutritional status in patients has not been performed in healthy volunteers. Also the aim of the indices varies: assessment of the nutritional status, monitoring the nutritional status or prediction of postoperative complications. Table 4 lists the most frequently used indices, their constituents and aims.

The prevalence of nutritional depletion in hospitals has been studied frequently but mainly in surgical patients. Table 5 (41-59) shows the most important studies with the prevalence of malnutrition and the methods by which the nutritional status is assessed: individual parameters or an index. The number of studies concerning non-surgical patients is limited. Various methods are used so the results are difficult to compare. None of the studies compares these indices by application of different indices to the same population. The present studies indicate that nutritional depletion is not infrequent (median surgical patients 41%, geriatric patients 33 % and general population 44% prevalence of malnutrition), but its clinical relevance must be established.

The assessment of nutritional depletion of micronutrients, in particular of zinc

Several methods are used to determine the zinc status, e.g., serum zinc (60), serum alkaline phosphatase activity levels (61), zinc concentrations in urine (62), hair (63), and blood cells (64). All of these methods have their limitations. The serum zinc concentration is used most frequently, but besides by the zinc status serum zinc is influenced by other

Table 4

Indices for Nutritional Status

Abbreviation	Reference	Full name	Constituents	Aim
PNI	32	Prognostic Nutritional Index	albumin delayed type skin hypersensitivity transferrin triceps skin fold thickness	prediction of complications
HPI	33	Hospital Prognostic Index	albumin delayed type skin hypersensitivity inflammation cancer	prediction of mortality
NIM	34	Nutritional Index Maastricht	albumin pre-albumin total number of lymphocytes % ideal weight	assessment of nutritional status
NRI	35,36	Nutritional Risk Index	albumin % of usual weight	identification of patients with an increased risk of complications
LOM-score	37	Likelihood of Malnutrition Score	folate ascorbic acid albumin % of ideal weight triceps skin fold thickness mid arm circumference total number of lymphocytes	identification of malnutrition
IUN	38	Index of Undernutrition	weight loss mid arm circumference triceps skin fold thickness albumin transferrin	prediction of complications
SGA	28, 39, 40	Subjective Global Assessment	questionnaire physical examination	identification of malnutrition

factors like inflammation. Apparent zinc deficiency, expressed by a low serum zinc level without symptoms of zinc deficiency, may be the result of a low serum albumin concentration, because over 70% of serum zinc is bound to albumin (65), and/or redistribution

Table 5

Published figures for the prevalence of malnutrition in random samples of hospitalized patients

Patients	Reference	Individual parameters	Index	N	Prevalence
Surgical					
	Postma (41)	-	NIM	422	23%
	Hall (38)	-	IOU	367	29%
	Pettigrew (42)	W/H,DSH	-	198	32%
	McWhirter (43)	W/H,TSF,MAC,UW	-	200	33%
	Bistrian (44)	D,A,W/H,ALB,TSF,MAC	-	131	40%
	Detsky (45)	-	CI	202	44%
	Reilly (46)	-	LOM	406	48%
	Buzby (47)	-	PNI	100	62%
	Mean				41%
Geriatric					
	Larsson (48)	W/H,TSF,MAC,ALB,PALB,DSH	-	500	29%
	Füllöp (49)	ALB,PALB	-	552	34%
	Constans (50)	MAC,TSF,ALB,RBP	-	324	37%
	Sullivan (51)	ALB,W/H,CHOL	-	110	38%
	Mean				33%
General Medicine					
	Larsson* (52)	-	CI	382	29%
	Willard* (53)	IDEAL,W/H,TSF,MAC,ALB,TLC	-	200	32%
	Coats (54)	-	LOM	228	38%
	McWhirter (43)	W/H,TSF,MAC,UW	-	300	45%
	Weinsier (55)	-	LOM	134	48%
	Robinson (56)	-	CI	100	56%
	Reilly (46)	-	LOM	365	59%
	Bistrian (57)	W/H, TSF, MAC, ALB, Ht	-	251	44%
	Mean				44%

* = part of these samples were surgical patients.

ALB = albumin, CHOL = cholesterol, D = diagnosis, UW = Usual weight, W/H = weight/height, IDEAL = ideal weight, A = age, MAC = mid arm circumference, PALB = pre-albumin, RBE = retinol binding protein, TSF = triceps skin fold, DSH = delayed type skin hypersensitivity, TLC = total lymphocyte count, CI = clinical impression.

of zinc into the liver as a result of inflammation (23,66,67). The zinc concentration in urine shows no correlation with the degree of zinc deficiency (67). The concentration of zinc in hair is decreased in the early phase of zinc deficiency, but, because zinc deficiency can slow down hair growth, the zinc concentration may return to normal (68). The activity of zinc dependent enzymes, e.g., serum alkaline phosphatase will, in theory, be influenced by many other factors such as liver function and bone turnover. Tissue zinc concentrations may be a more appropriate standard to determine the zinc status but tissue specimens are difficult to obtain in general. In addition, as a reaction to certain pathophysiological circumstances, the changes in concentration of macro- and micronutrients in an organism, may vary from tissue to tissue.

Blood cells, which are more easily obtained, are a kind of tissue, so maybe the concentration of zinc in these cells reflect the zinc status. However, zinc concentrations in mononuclear cell suspensions and polymorphonuclear cell suspensions are low. Also the risk of zinc contamination of the suspension is high. Therefore determination of zinc in blood cells is difficult (69), although this method is widely used in humans to determine the zinc status.

The whole-body retention as a function of time of a parenteral dose of ^{65}Zn seems to reflect the zinc status: retention and zinc deficiency appear to increase simultaneously (70). It may be a suitable tool for research but not for daily clinical practice (71).

There is a need for new methods to determine zinc deficiency that can distinguish between real and apparent zinc deficiency and can be applied in clinical practice.

THE CLINICAL RELEVANCE OF NUTRITIONAL DEPLETION

What is the purpose of determining the nutritional status; what is the underlying goal? Many studies focused on the improvement of nutritional parameters. This includes the hypothesis that by improvement of the nutritional parameters the nutritional status will improve as well. But improvement of the nutritional status as a single phenomenon only is not really important. The aim of nutritional assessment and subsequent nutritional intervention should be an improvement of the nutritional status of a patient which results in a better quality of life: less mortality and morbidity, and improvement of functional capacities of a person in all of respects. This has been studied rarely, and mainly in surgical patients.

Only in a limited number of studies the correlation between nutritional depletion

and complications has been determined in surgical patients (46,58). Several studies were performed in populations of geriatric patients. Sullivan performed studies (51,59,72) in which he demonstrated that a decrease of serum albumin and functional status are prognostic for complications. Serum albumin and pre-albumin are also prognostic for mortality (49,50). Larsson (52) recently studied the prevalence of malnutrition in Swedish hospitals in both surgical and non-surgical patients, and correlated complications with the nutritional status according to the Subjective Global Assessment. The relative risk of complications was 1.92 for malnourished patients versus well-nourished patients. Reilly (46), in a retrospective study, calculated a relative risk of 2.6 in malnourished patients according to the LOM. Concerning the population of "general medical patients" it should be stressed that such a population mentioned in these studies is not always identical to a population in a ward for internal patients in a Dutch hospital.

In a malnourished patient, the question arises, to what extent nutritional deficiency, and to what extent the disease process is responsible for complications during the course of a disease. Can the currently available methods to determine nutritional deficiency distinguish nutritional status from disease activity? The answer to this question is important because if complications are (in part) caused by nutritional deficiency, the number of complications could be reduced by improving of the nutritional status. None of presently available studies answers this important question.

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PREVALENCE OF MALNUTRITION IN NON-SURGICAL HOSPITALIZED PATIENTS AND ITS ASSOCIATION WITH DISEASE COMPLICATIONS

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ABSTRACT

The prevalence of malnutrition and its predictive value for the incidence of complications was determined in 155 patients hospitalized for internal or gastrointestinal diseases. Malnutrition was present at admission in 45% of patients according to Subjective Global Assessment (physical examination plus questionnaire), in 57% according to the Nutrition Risk Index ((1.5 x albumin) + (41.7 x present/usual weight)), and in 62% according to the Maastricht Index ((21 - (0.24 x albumin) - (19 x transthyretin (pre-albumin)) - (1.9 x lymphocytes) - (0.04 x ideal weight)). More than 50% of the patients with inflammatory bowel disease were malnourished. Crude Odds Ratio's for the incidence of any complication in malnourished versus well-nourished patients during hospitalization were 2.7 (95% confidence interval 1.4 to 5.3) for the Subjective Global Assessment, 2.8 (1.5 to 5.5) for the Nutritional Risk Index and 3.1 (1.5 to 6.4) for the Maastricht Index. Odds Ratio's were reduced to 1.7 (0.8 to 3.6), 1.6 (0.7 to 3.3) and 2.4 (1.1 to 5.4), respectively, after a multivariate analysis that included disease category and disease severity. Because the confounding factors adjusted for are not only a measure of the severity of the disease but may also be influenced by malnutrition itself, the actual risk for complications due to malnutrition could be higher than the adjusted Odds Ratio's.

In conclusion, malnutrition was frequent in gastrointestinal and internal medicine patients at the time of admission. The severity of malnutrition predicted the occurrence of complications during hospital stay, and this association was not completely explained by confounding factors.

INTRODUCTION

Many hospitalized patients are malnourished. However, the relation between malnutrition, disease, and complications is unclear, especially for non-surgical patients.

About 30 % of patients on surgical wards have been found to be malnourished at admission (1-10). There is limited information about the nutritional status of non-surgical hospital patients (5, 7, 11, 12, 13). Nutritional depletion is usually caused by the joint action of an underlying disease, e.g. cancer, and dietary deficiency (Figure 1). It is not clear to which extent each of these two factors is responsible. If insufficient food intake is a factor in the development of nutritional depletion and also of the associated complications, then treatment should not only be focused on the disease but also on nutritional inter-

vention. In malnourished surgical patients peri-operative parenteral nutrition may indeed reduce the rate of post-operative complications (14,15). The association between malnutrition and occurrence of complications in non-surgical patients is less clear. We therefore assessed the nutritional status of patients at admission to a ward for internal diseases and a gastro-intestinal ward, and the association of nutritional status with the subsequent development of complications.

METHODS

Design

The University Hospital of Nijmegen University Medical School serves as the tertiary referral hospital for an area of 30 by 60 miles in the South-Eastern part of the Netherlands with a catchment population of 2.3 million. We assessed nutritional status in patients admitted to the gastrointestinal and internal medicine wards over one 4 months period and another 2.5 months period 9 months later. No nutrition support team was active on the wards. The nutritional status at entry was evaluated; during the first study period the nutritional status was also evaluated at discharge. No single nutritional index is considered a reference standard; we therefore applied three previously investigated well-established methods simultaneously. The occurrence of disease complications during hospital stay was studied as a function of the nutrition status at entry.

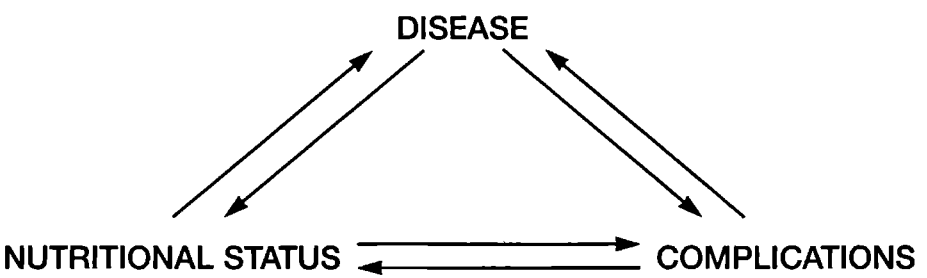

Figure 1 - Mutual relations between nutritional status, underlying disease and complications during the course of the disease.

Patients

Only seriously ill patients are nowadays hospitalized in the Netherlands; others are treated as outpatients. We excluded patients admitted only for observation after endoscopic treatment, patients who were unconscious or clinically unstable, and all those unable or unwilling to give their informed consent. All 155 eligible patients who gave their informed consent entered the study. Nutritional status was assessed within 24 hours after admission. The study was approved by the Committee for Ethics and Research in Humans.

Assessment of nutritional status

The Subjective Global Assessment (2,16,17) is a clinical score. It was performed by a trained independent physician, using a standardized questionnaire concerning food intake and complaints such as vomiting, diarrhoea, and loss of weight. A physical examination focused on nutritional status, and weights were corrected for oedema, ascites and dehydration. Based on these data the physician classified the patient as not, mildly, moderately or severely malnourished (see appendix). The physician had no knowledge of the medical history, diagnosis, results of laboratory tests, or the reason for admission of the patient in question.

The Nutritional Risk Index (18,19) is derived from the serum albumin concentration and the ratio of actual to usual weight using the equation

Nutritional Risk Index = (1.489 x serum albumin (g/L)) +
(41.7 x (present weight/ usual weight)).

A Nutritional Risk Index > 100 indicates that the patient is not malnourished; 100-97.5 is mild malnourishment; 83.5 - <97.5 is moderate malnourishment and <83.5 is severe malnourishment. The usual weight was defined as the stable weight at least 6 months before admission. The actual weight was determined with the patient sitting on a calibrated Seca balance (Hamburg, Germany).

The Maastricht Index (20) involves serum albumin and transthyretin (pre-albumin) concentrations, blood lymphocyte count and percentage of ideal weight using the equation:

Maastricht Index = 20.68 - (0.24 x albumin (g/L)) - (19.21 x
transthyretin (pre-albumin) (g/L)) - (1.86 x lymphocytes (10^6/L))
- (4 x present weight/ideal weight)).

This index was called Nutritional Index by the investigators in Maastricht (20) who developed it; we use the term 'Maastricht Index' to avoid confusion with the Nutritional Risk Index. Patients with a Maastricht Index of > 0 are considered malnourished. We measured height (Seca, Hamburg, Germany) and wrist circumference (Stanley Tools, New Britain, Connecticut, USA) and then derived ideal weight from the tables of the Metropolitan Life Insurance Company were used (21). The nutritional status was graded as malnourished or not malnourished in the Maastricht Index, and as none, mild, moderate or severe in the other two methods.

In the 90 patients studied in the first four-month period nutritional status was assessed twice, once at admission and once at discharge. We also assessed the Nutritional Risk Index and the Maastricht Index in 175 healthy blood donors and in 34 healthy elderly participants in the strenuous Nijmegen Four Days Walking March. In these 209 healthy subjects the apparent percentage of malnourished persons was 1.9% according to the Nutritional Risk Index and 3.8% according to the Maastricht Index. These low values show that in our hands a high percentage of malnutrition in patients is not likely to be caused by false-positive diagnoses (22).

We also merged the results of the Subjective Global Assessment, the Nutritional Risk Index and the Maastricht Index into a single combined index. We considered a patient malnourished according to this combined method (COMBI) if he was malnourished to any degree according to at least two of the three underlying methods.

Albumin was determined by photometry on a BM Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan), transthyretin (pre-albumin) by immunonephelometry (Cobas Fara II, Hoffmann-La Roche, Basel, Switzerland) using a rabbit anti-human transthyretin (pre-albumin) anti-serum (Dako, Copenhagen, Denmark), and total number of blood lymphocytes by an automatic blood cell counter (Sysmex NE 8000, TOA Medical Electronics, Kobe, Japan). A pool of serum from 209 healthy donors was used as working standard for transthyretin (pre-albumin). It was calibrated against the CRM-470 (CRM = Certified Reference Material) international reference preparation of transthyretin (pre-albumin) of the International Federation of Clinical Chemistry. In the present study albumin and transthyretin (pre-albumin) serum concentrations were correlated with each other ($r=0.39$, $p=0.001$).

We recorded the presence or absence of cancer or non-malignant disease as a potential determinant of complications. Non-malignant disease was further divided into 'gastrointestinal' and 'non-gastrointestinal' (Table 1).

In case of multiple diagnoses, the diagnosis which was the reason for admission was chosen. We recorded 'number of drugs used', 'duration of hospital stay' and 'functional capacity' as proxies of the severity of the disease. The functional capacity was graded into three categories:

Category I: the patient can take care of his/her personal hygiene such as washing and shaving, can eat without help and has no limitation in his/her daily activities such as walking and reading. Category II: the patient needs assistance for his/her personal hygiene and in

Table 1

Diagnoses of the 155 patients at admission to a non-surgical ward

Non- malignant				Cancer	
Gastro-intestinal		Non-gastrointestinal			
Crohn's disease	10	Diabetes	10	Oesophagus	4
Abdominal pain e.c.i.*	8	Hypo-y-globulinemia	4	Pancreas	4
Liver cirrhosis	5	Anaemia	4	Hepatocellular	3
Hepatic encephalopathy	3	Cardiac decompensation	4	Colon	3
Pancreatitis	3	Pneumonia	3	Gastric	2
Ulcerative colitis	3	Thrombosis	3	Lung	2
Short bowel syndrome	3	AIDS	3	Osteosarcoma	1
Rectal bleeding	3	COPD**	2	Leukaemia	1
Gastrointestinal bleeding	3	Hypertension	2	M. Kahler	1
Acute pancreatitis	2	Fever e.c.i.*	2	Non-Hodgkin	1
Vomiting	2	Others	12	Thyroid	1
Gastro-enteritis	2				
Gastric ulcer	4				
Oesophageal bleeding	2				
Others	30				
Subtotal	83		49		23
Grand total					155

* E.c.i. = of unknown origin; ** COPD = Chronic Obstructive Pulmonary Disease

Table 2

Number of new complications occurring in 155 patients on a non-surgical ward during hospital stay. In 74 patient no complications occurred. One patient could have more than one complication.

Infectious complications		Non-infectious complications			
		Severe		Less severe	
Severe		Fever (not bacterial)	18	Vomiting	11
Pneumonia	7	Intestinal bleeding	6	Dermatosis	10
Septicaemia	5	Dehydration	4	Diarrhoea	9
Abdominal abscess	2	Kidney failure	4	Obstipation	9
Subtotal	14	Decubitus ulcer	3	Phlebitis	9
		Heart failure	3	Anaemia	6
Less severe:		Hemoptysis	2	Mild intestinal bleeding	4
Cystitis	5	Venous thrombosis	2	Oedema	4
Local candidiasis	4	TIA	2	Hyper/hypoglycaemia	4
Wound infection	3	Pancreatitis	2	Delayed wound healing	4
Conjunctivitis	2	Fistula	2	Rhinorrhagia	3
Laryngitis	1	Vaso-vagal collapse	2	Arthralgia	3
Onychia	1	Lung embolism	2	Atelectases	3
Furuncle	1	CVA	2	Thrombo- and/or leucopenia	3
Cholangitis	1	Epileptic insult	1	Oral mucosal defects	2
Subtotal	18	Ileus	1	Minor decubitus ulcer	2
		Cutaneous ulcus	1	Mild cardiac arrhythmias	2
		Liver decompensation	1	Otorrhoea	2
		Pleural fluid	1	Muscle cramps	2
				Mild metabolic deterioration	2
				Others	8
Total	32		59		104
Grand total: all complications					195

eating and is limited in his/her daily activities. Category III: the patient is completely dependent on assistance for his/her personal hygiene and eating.

A complication was defined as: a state in which a disease or accident is added to an existing illness without being related specifically to this illness (23). Complications were

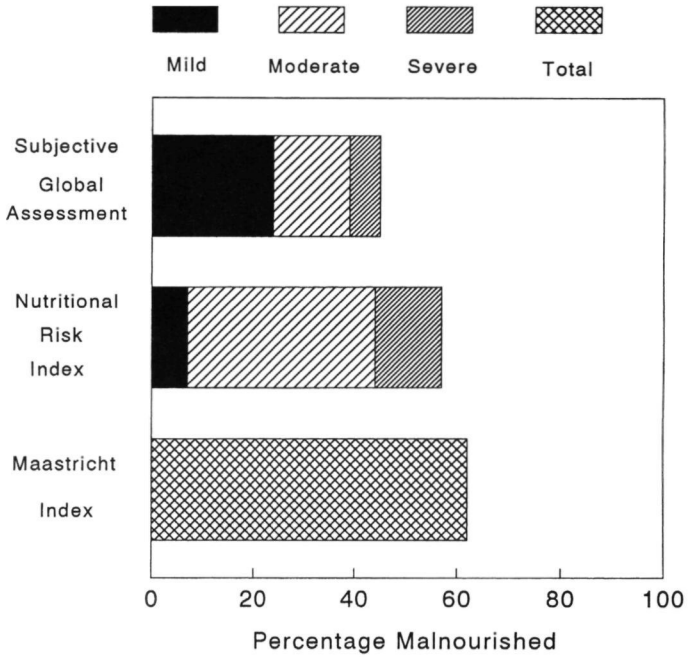

Figure 2 - Prevalence of malnutrition in 155 non-surgical hospitalized patients at admission. The Maastricht Index does not specify grades of malnutrition

divided into 'mild' and 'severe' and also into 'infectious' and 'non-infectious'. A list of relevant complications had been compiled before the start of the study (table 2). Physicians and nurses were instructed to record all new complications in the patients' files. The results of the assessment of the nutritional status were kept hidden from attending physicians and nurses, so as not to influence the treatment of the patient. The occurrence, type and severity of complications which occurred after admission were derived from the patients' files after discharge.

Data analysis

A Chi-square test was used to compare the results of the various indices. To analyze the association between the complications and the nutritional status graded for severity the non-parametric Spearman test and the Kruskal-Wallis test were used, because the group

Table 3

Characteristics (mean (SD)) of 155 patients on a ward for internal and gastrointestinal diseases, by nutritional status at admission. "Yes" is malnourished, "No" is not malnourished.

Characteristic	Presence of malnutrition at admission, by nutritional index							
	Subjective Global Assessment		Nutritional Risk Index		Maastricht Index		COMBI Index	
	Yes	No	Yes	No	Yes	No	Yes	No
n	70	85	88	67	93	62	90	65
Age (years)	57.3 (17.0)	57.0 (19.5)	59.3 (17.1)	53.4 (18.9)	57.1 (17.6)	56.4 (18.8)	58 (17.4)	54.9 (19.2)
Duration of hospital stay (days)	18.4* (13.1)	14.2 (11.3)	18.4** (14.4)	13 (9.3)	18.6* (14)	13.8 (9.4)	20*** (13.9)	12.6 (7.9)
Number of different drugs used (per day)	7.7*** (4.7)	5.1 (3.4)	7.2** (4.2)	5 (4)	7.1* (4.3)	5.5 (4)	7.6** (4.2)	5.1 (4)
Cancer # (% of patients)	21*	10	18	10	17	12	20*	9
Decreased functional capacity (% of patients)	60**	35	59***	26	51	37	60***	25
Surgery within 3 months before admission	12	6	12	5	10	8	12	6

Differences between means were tested using Student's t-test The variables expressed as percentages were tested using a Chi-square test (*p<0 05, **p<0 01, ***p<0 001) Decreased functional capacity refers to categories II and III combined (see methods) # = No differences were seen between disease categories gastrointestinal and non-gastrointestinal

size in some groups was too small for an individual comparison between all separate groups. Odds Ratio's with 95% confidence intervals were calculated for the development of complications in malnourished versus well-nourished patients. Multivariate logistic regression analysis with backwards variable exclusion was used to adjust for confounding factors (SAS Procedure (24)). Dichotomous variables were coded as 0 or 1.

During the total catchment period of 5.5 months 330 patients were admitted. We excluded 93 patients who were admitted exclusively for observation after endoscopic treatment and who were discharged within 3 days, and 13 patients who were unconscious or clinically unstable and thus unable to give their informed consent, or to answer the questions of the questionnaire (see appendix). Another eighteen patients refused to participate. In 51 patients nutritional status could not be assessed within 24 hours after admission. Most of these were acute admissions over the weekend; their nutritional status may therefore have been worse than that of the patients who did enter the study. The remaining 155 patients, 65 female and 90 male, with a mean age of 57.1 years (range 21-93 years, SD 18.2), were included in the study. Table 1 shows the diagnoses of these 155 patients. The mean duration of hospital stay was 16.1 days.

The frequency of any degree of malnutrition at hospital admission varied from 45% as assessed by the Subjective Global Assessment to 62% for the Maastricht Index (Figure 2). The severity of malnutrition diverged between indices, with the Subjective Global

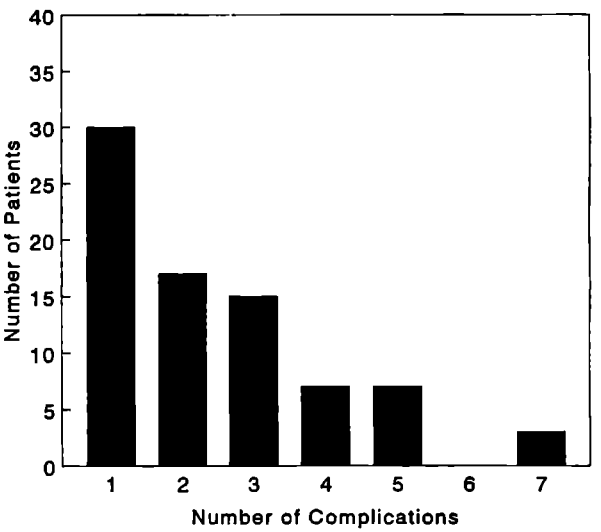

Figure 3 - Number of complications per patient occurring in 81 of the 155 patients at a non-surgical ward, during an average hospital stay of 16 days. In the 74 other patients no complications occurred

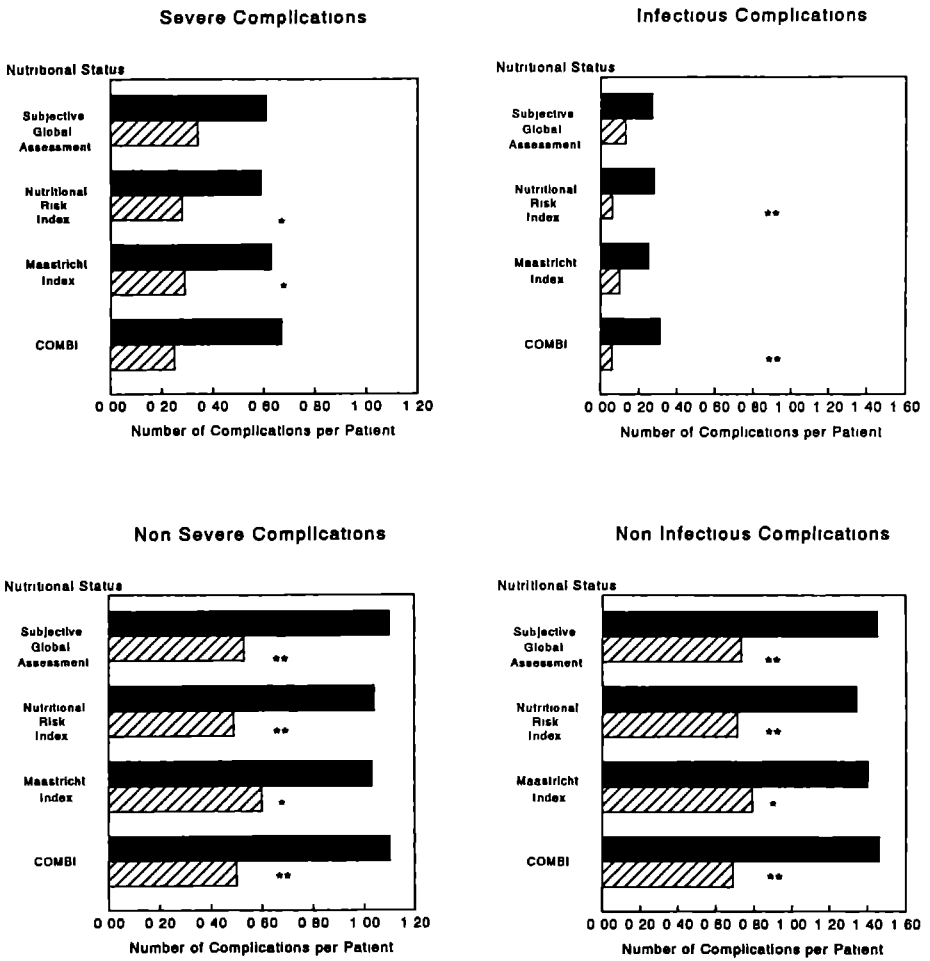

Figure 4 - Mean number of complications per patient during hospital stay in patients malnourished (solid bar) or well-nourished (shaded bar) at entry according to various methods of nutritional assessment Upper left severe complications, lower left non-severe complications, upper right infectious complications, lower right non-infectious complications The x-axis on the left hand panels runs from 0 to 1 2 and that on the right hand from 0 to 1 6 (p<0 05, ** p<0 01, *** p<0 001)*

Assessment scoring most cases as "mild" while the Nutritional Risk Index scored most cases as "moderate" or "severe"

During the first 4-month period data were gathered on ninety patients both at admis-

sion and at discharge. The nutritional status showed a small but significant improvement during hospital stay (64% malnourishment at admission, 53% at discharge, $p<0.05$) according to the Maastricht Index. The Subjective Global Assessment and the Nutritional Risk Index did not show a significant changes (41% versus 51% and 52% versus 49%, respectively). According to the Subjective Global Assessment more of the gastrointestinal patients were malnourished than of the internal medicine patients (61% versus 30%). As the other methods did not reveal a significant difference (Nutritional Risk Index 59% versus 47%, Maastricht Index 65% versus 64%), the results of the patients at the two wards were taken together. In the second period the Subjective Global Assessment was assessed by a different physician, but the percentage of patients malnourished at admission was similar to that in the first part of the study namely 46% versus 41%.

The severity of malnutrition was related with the diagnosis. According to the Subjective Global assessment, malnutrition was present in 54% of the patients with inflammatory bowel disease; 23% of them was moderately malnourished and 15% was severely malnourished. According to the Nutritional Risk Index, malnutrition was present in 77% of these patients; 31 % were moderately and 46% were severely malnourished. According to the Maastricht Index 90% of the patients with active inflammatory bowel disease were malnourished. A tendency towards more severe malnutrition was seen in cancer patients. Malnourished patients also differed from well-nourished patients in duration of hospital stay, number of different drugs used, and functional capacity (Table 3).

No complications occurred in 74 of the 155 patients; 32 patients suffered 3 or more complications (Figure 3). Two patients died during hospital stay. Figure 4 shows the mean number of complications per patient for the various groups of complications for well nourished and malnourished patients. A significantly higher number of complications was seen in malnourished than in well-nourished patients. The patients with cancer were more at risk of developing complications than non-cancer patients (Figure 5). Significant differences were observed in the total number of complications and in non-severe non-infectious complications between patients with cancer and without cancer ($p < 0.05$, for both). Figure 6 shows the mean number of complications for the two indices that yielded a grading in severity of malnutrition. Is was not possible to compare the separate groups because some of the group sizes were too small, but the Spearman correlation coefficient was significantly different from 0 both for the Subjective Global Assessment and the Nutritional Risk Index ($R=0.30$ and $R=0.24$, respectively). The Kruskal-Wallis test yielded similar results.

Table 4 shows the crude Odds Ratio's for the association between malnutrition and

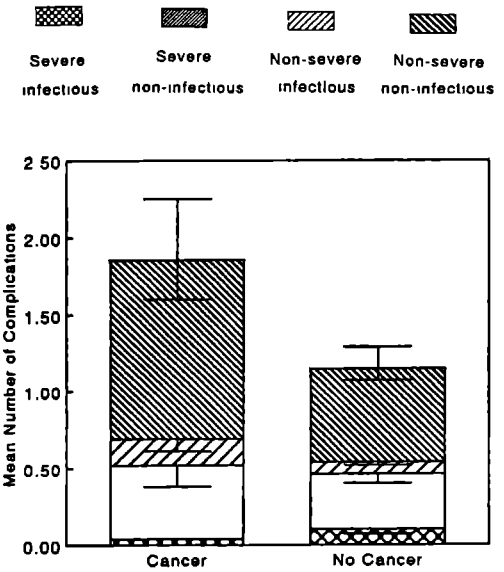

Figure 5 - Mean number of complications occurring per patient during hospital stay, as a function of the presence or absence of cancer (error bars indicate one SEM)

the occurrence of complications. The risk of complications was increased in malnourished patients in all methods of assessment of the nutritional status.

A number of variables could confound this relation by causing both malnutrition prior to admission and complications later on during hospital stay. The major potential confounder is the severity of disease. We entered the presence of cancer and of non-malignant diseases, divided into gastrointestinal and non-gastrointestinal, as independent variables in a multivariate analysis, and added 'number of drugs used', 'duration of hospital stay' and 'functional capacity' as proxies for the severity of the disease. Adjustment for these potential confounding factors lowered the Odds Ratio's for the risk of complications in malnourished patients (Table 5). However, all Odds Ratio's remained elevated, and that for the Maastricht Index remained significantly higher than 1 for 'All Complications'.

Prevalence of malnutrition

We found that at least 40 % of the patients in a ward for non-surgical patients were malnourished at admission, and that the risk of subsequent complications was higher in malnourished patients. The frequency of malnutrition was as high as or higher than that reported in surgical patients (1-10). This figure of at least 40% malnutrition can be an underestimation because patients were excluded if nutritional status could not be assessed within 24 hours after admission. This mostly involved admissions during the weekend of patients with acute conditions, who probably had a more severe illness. The validity of the indices used was confirmed in a study of the prevalence of apparent malnutrition in healthy volunteers (22). That study was performed in parallel with the present study, and it demonstrated 1.9 % apparent malnutrition in healthy volunteers according to the Nutritional Risk Index , and 3.8% according to the Maastricht Index. However, the

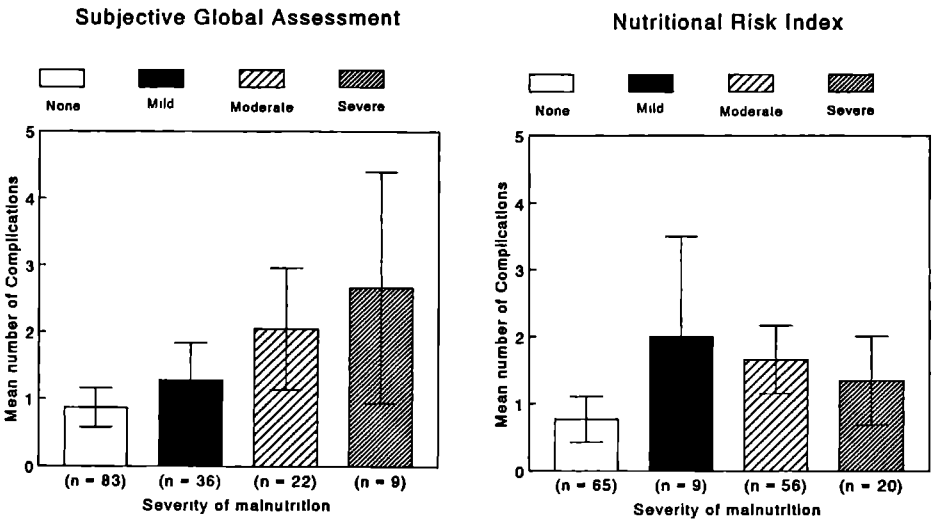

Figure 6 - Mean number of complications during hospital stay, as a function of the grade of malnutrition at admission. The left section presents malnutrition assessed by the Subjective global assessment. The right section shows the results of the Nutritional Risk Index. The Maastricht Index does not subdivide the malnourished patients in severity of malnourishment, and is not depicted. Error bars indicate one SEM.

Maastricht Index overestimated the prevalence of malnutrition in elderly volunteers (22). This probably had only a limited influence on the results of the present study, because only 16% of the patients were aged over 70 years.

One could argue that the group of patients was very heterogeneous and that it might have been preferable to study one disease in detail. We deliberately studied this heterogeneous population because our aim was to study the relation between nutritional status and complications in patients at a non-surgical ward. If a correlation could be demonstrated in this heterogeneous population, this would strengthen the need for active treatment of malnutrition.

The high prevalence of malnutrition in cancer patients, especially according to the Subjective Global Assessment, may suggest that the presence of cancer weighed heavily in the diagnosis of malnutrition made by a subjective method, even though cancer is not by itself diagnostic of malnutrition. However the physician assessing nutritional status had no

Table 4

Crude Odds Ratio's (with 95% confidence intervals) for risk of complications during hospital stay, in patients malnourished at entry compared with well-nourished patients on a non-surgical ward.

Type of complication (number of occurrences) *	Risk in malnourished patients, by method of assessment			
	Subjective Global Assessment	Nutritional Index Index	Maastricht Index	COMBI Index
Severe	2.5	2.7	2.4	3.5
(73)	(1.2-5.2)	(1.2-5.9)	(1.0-5.4)	(1.4-8.5)
Non-severe	2.7	2.5	2.6	2.9
(122)	(1.4-5.3)	(1.3-4.9)	(1.3-5.5	(1.4-6.2)
Infectious	3.1	3.8	1.9	4.3
(32)	(1.2-8.2)	(1.2-11.0)	(0.6-5.6)	(1.2-15.7)
Non-infectious	2.7	2.6	2.9	3.2
(163)	(1.4-5.2)	(1.3-4.0)	(1.4-6.1)	(1.5- 6.6)
All complications	2.7	2.8	3.1	3.3
(195)	(1.4-5.3)	(1.5-5.5)	(1.5-6.4)	(1.6- 7.1)

* The 155 patients developed 195 complications; one patient could suffer more than one complication.

knowledge of the medical history or the diagnosis.

Malnutrition and complications

Nutritional status, the occurrence of complications, and the underlying diseases constitute a triangle in which it is unclear what causes what (Figure 1). One could argue that malnutrition is not the cause of complications but that both malnutrition and complications are the result of the underlying disease or of other factors. The Nutritional Risk Index and the Maastricht Index use serum concentrations of proteins which are influenced by the nutritional status but also by inflammatory stress due to a disease. This is why we also used the Subjective Global Assessment, which is not influenced by these serum proteins.

Patients who were malnourished at admission developed more complications during hospital stay. Patients who were more severely malnourished were more at risk than to less malnourished patients. The crude Odds Ratio's for the risk of complications in malnou-

Table 5

Multivariate Odds Ratio's (with 95% confidence intervals) for occurrence of complications in malnourished patients compared with well-nourished patients after adjustment for confounding variables *.

Type of complication (number of occurrences)	Risk in malnourished patients, by method of assessment			
	Subjective Global Assessment	Nutritional Index Index	Maastricht Index	COMBI Index
Severe	1.2[a,b,c]	1.2[a,b,c]	1.5[a,c]	1.3[a,b]
(73)	(0.4-3.3)	(0.4-3.2)	(0.6-3.9)	(0.4-3.6)
Non-severe	1.9[a,c,e]	1.6[a,c]	2.2[c]	2.3[c]
(122)	(0.9-4.0)	(0.8-3.5)	(1.0-4.8)	(1.0-5.0)
Infectious	1.5[a,c]	1.0[a,c]	1.4[a,b,d]	1.4[a,c]
(32)	(0.5-4.8)	(0.3-4.1)	(0.3-6.9)	(0.3-7.5)
Non-infectious	1.7[a,c,e]	1.4[a,c,e]	2.2[a,c,d]	1.5[a,c,d]
(163)	(0.8-3.6)	(0.6-2.0)	(1.0-5.0)	(0.6-3.6)
All complications	1.7[a,c,e]	1.6[a,c,e]	2.4[a,c,d]	1.7[a,c,d]
(195)	(0.8-3.6)	(0.7-3.3)	(1.1-5.4)	(0.7-4.0)

* Adjusted for [a] functional capacity, [b] number of drugs used, [c] duration of hospital stay, [d] disease category, and [e] surgery within 3 months before admission.

rished versus well-nourished patients varied between 1.9 and 4.3. Larsson (9) reported crude Odds Ratio's of 1.9 and Robinson (13) of 2.6. The increased risk of complications in malnourished patients could be due to confounders such as age, underlying disease, or severity of the disease. Therefore we adjusted the crude Odds Ratio's for these variables. Because a general index for the severity of diseases does not exist we used proxy variables such as 'number of drugs used', 'duration of hospital stay' and 'functional capacity'. Such adjustment decreased the Odds Ratio's to values between 1.0 and 2.4. Presence of cancer was an especially important confounder. However, Odds Ratio's for the risk of complications in malnourished versus well-nourished patients still remained elevated after multivariate adjustment, and the Maastricht Index was still significantly higher than 1. One might argue that these values are inflated because of residual confounding; this is a well-known problem when confounding variables are measured with insufficient precision, as was probably the case here. On the other hand, one may also argue that we overadjusted the crude Odds Ratio's by including confounders such as functional capacity which may itself have been an end result of malnutrition. If this is the case then the adjusted Odds Ratio's underestimate the independent impact of malnutrition.

Although the disease category strongly predicted the occurrence of complications, treatment of the disease is not always possible or successful, and nutritional intervention would still be valuable if it reduced the occurrence of complications. Therefore the effect of nutritional intervention on the rate of disease-specific complications in non-surgical patients merits study.

APPENDIX

Subjective Global Assessment

Questionnaire

What was your usual weight 6 months ago ?

Did you lose weight during the past year ?

Did you lose appetite ?

Did you use food supplements ?

Are there complaints of

- nausea
- vomiting
- diarrhoea
- dizziness when risin
- swollen feet
- swollen abdomen
- loss of physical capacity

Physical examination

Extent of loss of subcutaneous fat upon physical examination

Extent of loss of muscular mass upon physical examination

Presence and extent of ascites

Presence and extent of oedema

Presence and extent of dehydration

Correction of weight for ascites, oedema and dehydration

Conclusion regarding nutritional status not, mild, moderately or severely malnourished.

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CHAPTER 4

SPECIFICITY OF INDICES OF MALNUTRITION WHEN APPLIED TO APPARENT HEALTHY PEOPLE: THE EFFECT OF AGE

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ABSTRACT

Protein-energy malnutrition is thought to be widespread in hospitalized patients. However, the specificity of indices used to assess malnutrition is uncertain. We therefore assessed the rate of false-positive diagnoses of malnutrition when biochemical-anthropometrical indices were applied to healthy subjects. Nutritional status was assessed in 175 healthy blood donors (aged 44.2 ± 13.4 years) and 34 highly fit elderly volunteers in the Nijmegen Four Days Walking March (aged 74.7 ± 3.6 years). We investigated both the Nutritional Risk Index (1.5 x albumin + 42 x present/usual weight) and the Maastricht Index (21 - 0.24 x albumin - 19 x transthyretin (pre-albumin) - 1.9 x lymphocytes - 0.04 x ideal weight). Earlier we found 52% - 64% of non-surgical hospital patients to be malnourished according to these indices. Apparent malnutrition was present in 1.9 % of the 209 volunteers according to the Nutrition Risk Index, and in 3.8 % according to the Maastricht Index. The prevalence of apparent malnutrition in the elderly volunteers was 5.9% and 20.6%, respectively. The rate of false-positive diagnoses was acceptably low below the age of 70 years for both the Nutritional Risk Index and the Maastricht Index, and will not cause a clinically significant bias in the assessment of the prevalence of malnutrition in hospitals. The high percentage of spurious malnutrition in elderly limits the use of the Maastricht Index to subject under the age of 70 years.

INTRODUCTION

Various methods have been developed to assess protein-energy malnutrition in hospital patients. In 20 published studies the observed frequency of malnutrition varied from 23 to 62%, with an overall mean of 38% (Table 1). In a previous study (1) in non-surgical hospital patients we found a prevalence of malnutrition of 52% using the Nutritional Risk Index and 64% using the Nutrition Index or Maastricht Index. The Nutrition Index was developed by workers in Maastricht (3); to avoid confusion with similarly named other indices we will designate it the Maastricht Index in this paper. In malnourished surgical patients as assessed by these indices, nutritional intervention has proven to reduce the number of post-operative complications (2-4). If the prevalence of malnutrition is truly high in non-surgical patients, a considerable percentage of these patients might also benefit from nutritional support. However, the specificity of current instruments for assessing malnutrition has not been properly assessed. The Nutritional Risk Index was developed by

Table 1

Published figures for the prevalence of malnutrition in random samples of hospitalized patients.

Patients	Reference	N	Prevalence
Surgical			
	Postma (15)	422	23%
	Hall (16)	367	29%
	Pettigrew (17)	198	32%
	McWhirter (18)	200	33%
	Bistrian (19)	131	40%
	Detsky (20)	202	44%
	Reilly (21)	406	48%
	Buzby (22)	100	62%
	Mean		41%
Geriatric			
	Larsson (23)	500	29%
	Füllöp (24)	552	34%
	Constans (25)	324	37%
	Sullivan (26)	110	38%
	Mean		33%
General Medicine			
	Larsson * (27)	382	29%
	Willard * (28)	200	32%
	Coats (29)	228	38%
	McWhirter (18)	300	45%
	Weinsier (30)	134	48%
	Robinson (31)	100	56%
	Reilly (21)	365	59%
	Bistrian (32)	251	44%
	Mean		44%

* = part of these samples were surgical patients.

calculating the association of various nutritional parameters with post-operative complications (5,6), and it has not been checked in healthy subjects. The Maastricht Index (7) has been developed by comparing 'objective nutritional parameters' in 50 patients selected for parenteral nutrition with the same parameters in 38 patients selected for elective minor surgical procedures. Neither of these indices have been validated in healthy subjects. We were concerned whether the high prevalence of protein-energy malnutrition that we observed in non-surgical hospitalized patients could originate from false-positive misclassification. We therefore investigated the prevalence of apparent malnutrition in a healthy population, in which the prevalence of true malnutrition should be low.

METHODS

Volunteers

The study was performed after permission had been obtained from the local ethical committee. We selected 175 blood donors, and 34 healthy elderly people. In the Netherlands blood donors give blood at least once or twice a year as an act of charity without financial compensation. All donors are registered and receive medical and laboratory check-ups at regular intervals. Only volunteers who were completely healthy, according to the most recent medical and laboratory check-up and who were free from active or chronic diseases, were selected for this study. Their mean age was 44.2 (SD 13.4) years and their body mass index 25.6 (SD 4.1) kg/m^2.

In the Netherlands persons aged 70 years or over are excluded from blood donorship. We therefore approached elderly people who had completed the 1993 Nijmegen Four Days Long Distance March, a sports event in which volunteers walk 30 km (19 miles) each day during four days (table 2). We excluded elderly persons suffering from a chronic disease, thereby reducing the risk of including malnourished persons. The remaining 34 elderly had a mean age of 74.7 (SD 3.6) years and a body mass index of 24.1 (SD 2.3) kg/m^2. The age distribution of subjects was similar to that of the hospitalized patients which we studied previously (1) (Table 2).

Table 2

Characteristics of 209 healthy Dutch volunteers investigated for the presence of apparent malnutrition

Age range (Years)	Women (n)	Men (n)	Height (cm) (SD)	Weight (kg) (SD)
21 - 29	18	17	176	74.7
			(8.5)	(12.2)
30 - 39	17	18	173	73.1
			(9.6)	(12.3)
40 - 49	17	18	172	77.0
			(8.4)	(15)
50 - 59	19	16	169	79.2
			(8.1)	(11.8)
60 - 69	16	21	169	74.3
			(9.3)	(11.4)
> 69	11	21	168	69.1
			(8.2)	(10.4)
Total	98	111	171.2	74.6
			(8.7)	(12.2)

Assessment of nutritional status

We used two methods to determine nutritional status (Table 3). The Nutritional Risk Index (5,6) is based on serum albumin and current versus usual weight; a value of 100 or less indicates malnutrition. The Maastricht Index (3) uses serum albumin, serum pre-albumin, blood lymphocyte count and percentage of ideal weight; a positive value indicates malnutrition. For the ideal weight the tables of the Metropolitan Life Insurance Company were used (8).

Current weight was determined using a Krupps or a Seca balance, and height using a Seca or Stanley 04-116 microtoise. The usual weight for blood donors was the weight registered by the blood bank 6 months prior to the assessment of the nutritional status; the usual weight was also ascertained in all subjects by means of a questionnaire. The weight reported by the blood donors and the weight recorded by the blood bank 6 month prior to assessment were highly similar (75.0 ± 12.2 and 75.4 ± 12.8 kg respectively).

Table 3

Indices used to determine the nutritional status in 209 healthy volunteers

Nutritional Risk Index (5,6) = 1.489 x serum albumin (g/L) + 41.7 x (present weight/ usual weight)

Nutritional Risk Index > 100: not malnourished;

100 ≥ Nutritional Risk Index ≥ 97.5: mild malnourishment;

97.5 > Nutritional Risk Index ≥ 83.5: moderate malnourishment;

Nutritional Risk Index < 83.5: severe malnourishment.

Maastricht Index (7) = 20.68 - 0.24 x serum albumin (g/L) - 19.21 x serum transthyretin (pre-albumin, g/L) - 1.86 x lymphocytes (10^6/L) - 4 x present weight/ideal weight*

Maastricht Index ≤ 0: not malnourished;

Maastricht Index > 0: malnourished.

* For the ideal weight the tables of the Metropolitan Life Insurance Company were used (8)

Laboratory methods

Blood levels of albumin and other analyses are influenced by body posture (9). Therefore blood sampling was performed with subjects in a semi-recumbent position identical to that used in patients in our clinical study and in most hospitals (4). In the elderly people blood was drawn before or at least 2 weeks after the four-days march. Training was performed about 5 hours per week. To exclude influences of the training on the blood levels of albumin and other analyses, the elderly volunteers were not to performing any training at least 2 days before the blood sampling.

Serum albumin concentrations were determined by spectrophotometry with bromcresol green on a BM/Hitachi 747 (Hitachi, Tokyo, Japan), transthyretin (pre-albumin) by immunonephelometry on a Cobas Fara II (Hoffmann-La Roche, Basel, Switzerland) using a rabbit anti-human pre-albumin anti-serum (DAKO, Copenhagen, Denmark). Transthyretin (pre-albumin) was calibrated against the CRM-470 international reference serum of the International Federation of Clinical Chemistry. Total number of blood lymphocytes was determined by an automatic cell counter (Sysmex NE 8000, TOA Med. Electron., Kobe, Japan).

We made the assumption that in a healthy population the true prevalence of malnutrition is zero percent. The observed percentage of malnourished persons in our sample therefore equals the percentage that is misclassified. The upper limit of the 95% confidence interval for the proportion malnourished was calculated as

$p + Z_\alpha \times SD(p)$, with $SD(p) = \sqrt{[p(1-p)/n]}$

in which p is the observed proportion malnourished, n is the sample size, and Z_α is 1.96. For the subgroup of elderly subjects 95% confidence intervals were calculated according to tables for small samples (10).

All calculations were done with the statistical program SAS (11).

RESULTS

The mean value of the Nutritional Risk Index for the total sample was 111.1 (SD 4.9). Four out of the 209 volunteers were classified as mildly malnourished (97.5<NRI<100) according to the Nutritional Risk Index (Figure 1a). Two were men and two women, and three of the four were over 50. The apparent percentage of malnutrition was thus 1.9% (upper 95% confidence limit 3.8%). Figure 1b shows the mean value of the Nutritional Risk Index by age. Three of the subjects were classified as malnourished because of a minor decrease of serum albumin. However, the weight of these 3 persons was above 100% of their usual weight. The mean present weight was 171.2 (SD 8.7, table 2) kg; in 52% of subjects it was lower and in 48 % higher than their usual weight. One male weighted 87% of his usual weight and was classified as malnourished by the Nutritional Risk Index; nevertheless his actual weight was higher than his ideal weight according to the Metropolitan Life Insurance Company (8). This person was the fourth one categorized as malnourished according to the Nutritional Risk Index. The outcome for the age categories of 60-69 and above 69 years was significantly lower than that for ages 21-29, indicating an increased apparent risk at a higher age. The mean prevalence of apparent malnutrition in the blood donors aged 21-69 years was 1.1 % (95% upper confidence limit 2.7%), as opposed to 6% (95% upper confidence limit 20%) in the elderly hikers who were aged over 69. No differences were observed between sexes.

The mean value of the Maastricht Index for the total sample was -3.63 (SD 2.25). Nine subjects, or 4.3% (upper 95% confidence limit: 7.0 %), were classified as malnou-

rished according to the Maastricht Index; these seven were males, and all nine were over 60 years of age (Figure 2a). The reasons for apparent malnutrition according to the Maastricht Index were a combination of factors in all cases. A significantly higher -i e. poorer- score for this index, was seen in the age categories 60-69 and above 69 years compared with the reference age category of 21-29 years, again indicating a higher probability of spurious malnutrition at a higher age (Figure 2b). The mean prevalence in the blood donors was 1 1 % (95% upper confidence limit 1.2%), and in the elderly marching competitors 20 6% (95% upper confidence 38%). No differences were seen between sexes.

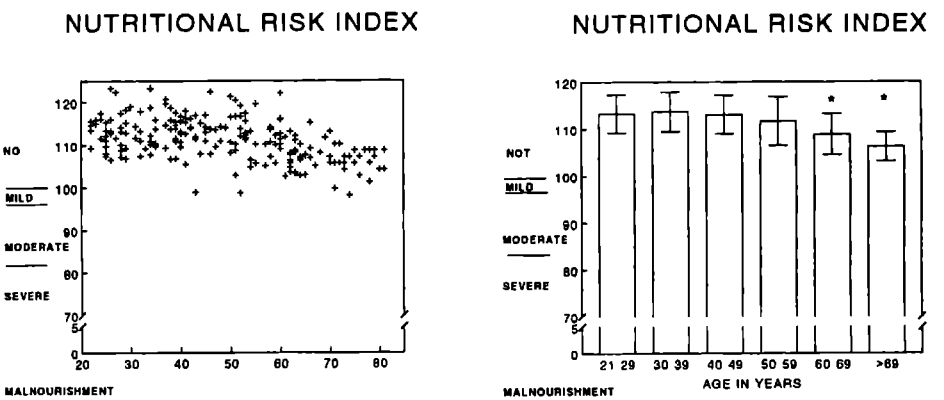

*Figure 1 - Figure 1a the left figure shows the individual value of the Nutritional Risk Index by age in 209 apparently healthy subjects Figure 1b the right figure shows the mean Nutritional Risk Index and the standard deviation by age category in 209 apparently healthy subjects, * P<0 05 compared with age category 21-29*

DISCUSSION

We applied two biochemical-anthropometric indices for assessment of clinical malnutrition to two groups of healthy subjects, and found that the rate of apparent malnutrition was low and of no clinical significance in the group of subjects aged less than 70 years; however, 6 to 21% of our group of highly fit, active elderly subjects were classified as malnourished.

We assumed that the volunteers were not malnourished. This is made plausible by process of the selection of these volunteers: a medical and laboratory examination brought out no abnormalities, and volunteers with chronic diseases were excluded. In the Netherlands blood donors are unpaid, and donorship is seen as an act of charity. The volunteers were socially well adjusted, with a regular income, and they were of normal body weight according to the Body Mass. In our group of elderly volunteers the BMI was not different from that in younger volunteers. It is well known that unselected elderly subjects can be malnourished concerning protein (12) and micronutrients. In most previous studies concerning malnutrition in elderly persons, subjects with (chronic) diseases were not excluded. If they were excluded the proportion of malnourished elderly persons was less than 10% (13). The group of elderly volunteers included in our study were physically active and their average nutrient intake was close to the Dutch recommendations (14). However, we can not exclude that a small percentage of the elderly persons was malnourished.

One could argue that some of the variables used in these indices are derived from laboratory methods which have been calibrated to yield normal values in a normal healthy population, so that the outcome is biased to normal values. However, the coefficients and constants that are used to calculate the value of each index are fixed and not calibrated per

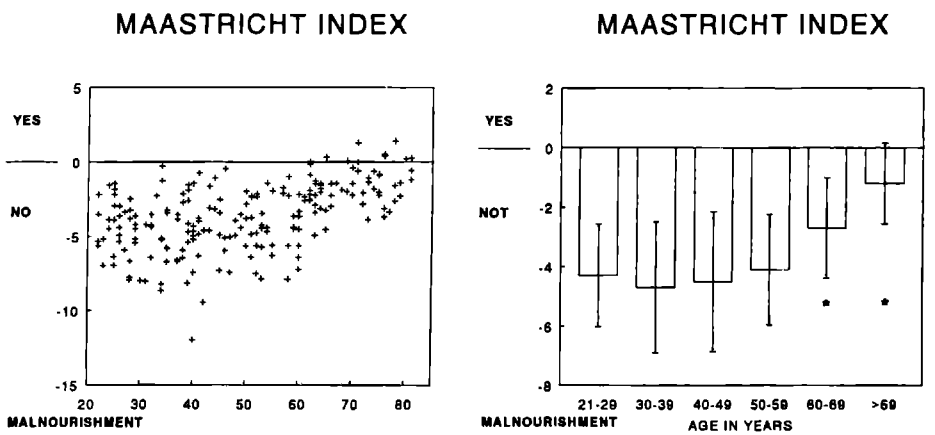

*Figure 2 - Figure 2a: the left figure shows the individual value of the Maastricht Index by age in 209 apparently healthy subjects. Figure 2b: the right figure shows the mean value of the Maastricht Index and the standard deviation by age category in 209 apparently healthy subjects, * : P<0.05 compared with the first age category.*

laboratory. The published reference ranges could be inapplicable, and outcome of the index could be erroneous if the coefficients and constants used are inappropriate for the population studied. Therefore, apparent prevalence of malnutrition in healthy volunteers could be considerable. This is illustrated by the discrepancy in frequency between the two indices. The less favourable values seen in otherwise highly fit elderly subjects could point to cases of malnutrition that were included in our sample in spite of our various precautions. However, it could also suggest that the risk of a misdiagnosis of malnutrition is appreciable in elderly patients. Using the Nutritional Risk Index, the percentage of apparent malnutrition in our highly fit elderly subjects aged ≥70 was 6% with an upper limit of the 95% confidence interval of 20%; using the Maastricht Index it was 21% with an upper confidence level of 38%. These figures put into question the clinical significance of nutritional indices applied to elderly subjects. A false diagnosis of malnutrition could cause a patient to receive unnecessary enteral or parenteral nutrition. This would be expensive and could induce complications.

The two elderly volunteers categorized as malnourished in the Nutritional Risk index were both classified as mildly malnourished. Obviously the risk of a false-positive diagnosis is highest in those subjects classified as mildly malnourished. The Veterans Affairs Total Parenteral Nutrition Group study (2) has demonstrated that perioperative parenteral nutrition was ineffective in such mildly malnourished patients, and was effective only in more severely malnourished patients. Therefore the consequence of misclassification in elderly patients using this index is limited. The Maastricht Index does not include grades of malnutrition. Application of this index in elderly patients results in 21% or more of the elderly patients being treated mistakenly as malnourished. However, the number of elderly subjects in our study precludes definitive conclusions.

Our results indicate that the high proportion of hospitalized patients diagnosed as malnourished is not the result of positive misclassification, except possibly when elderly patients are involved.

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CHAPTER 5

ZINC DEFICIENCY IN CROHN'S DISEASE

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ABSTRACT

The serum zinc concentration is frequently applied for the assessment of zinc deficiency, but this concentration is also influenced by other factors. The aim of this study was to compare various methods of assessing the zinc status in patients with Crohn's disease.
Serum zinc, serum alkaline phosphatase activity, and zinc in various types of cells were related to factors potentially inducing zinc deficiency: the number of liquid stools, weight loss, bowel resection and the extent and severity of inflammation. Thirty-one patients with more or less active Crohn's disease were included. In 68 % of these patients the serum zinc concentration was below the reference level, and it was correlated with the extent of bowel resection and the van Hees Index, but not with the Crohn's Disease Activity Index. Serum alkaline phosphatase activity was correlated with bowel resection. Zinc in blood cells was poorly correlated with factors inducing zinc deficiency.
A decrease of serum zinc concentration is frequently seen in active Crohn's disease. This study suggests that the determination of zinc in blood cells is not superior to the determination of the serum zinc concentration and serum alkaline phosphatase activity.

INTRODUCTION

Zinc is one of the most prominent trace elements in humans. It has been known to be essential for mammals since 1934 (1). Its deficiency causes a variety of symptoms (2). In patients with Crohn's disease, symptoms of zinc deficiency are observed occasionally (3, 4, 5). Cachectic patients with Crohn's disease are particularly at risk of clinical manifestations of zinc deficiency in the post-operative anabolic period, because in this period there is a great need for zinc and the quantity available in the zinc depots is limited. We call the zinc status in patients with symptoms of zinc deficiency in the post-operative period and without clinical symptoms of zinc deficiency in the pre-operative period subclinical zinc deficiency

In most studies concerning the zinc status in patients with Crohn's disease, only serum or plasma zinc is studied to determine the zinc status (table 1). Serum zinc is frequently decreased in patients with Crohn's disease, but this does not always indicate zinc deficiency. Serum zinc is also decreased in case of inflammatory stress (6) as a result of a decrease of serum albumin (the most important zinc transporting protein in blood) and redistribution of zinc into the liver. As an alternative to serum zinc, serum alkaline phos-

phatase activity has been suggested as a parameter of zinc deficiency because alkaline phosphatase activity is influenced by the zinc status (7). In some studies the determination of the zinc concentration in blood cells is used for the assessment of the zinc status, as an alternative to serum zinc. The zinc concentration in blood cells of patients with Crohn's disease was compared to the zinc concentration in blood cells of healthy volunteers. In most of the studies no differences were observed. In none of these studies this method was disputed as an insufficient method (table 1). Although the results of these studies are disappointing, determination of zinc in blood cells is still applied in clinical studies (8).

The question arises if the method in which patients with active Crohn's disease are compared to healthy volunteers is adequate. As in patients with subclinical zinc deficiency the serum zinc concentration is nearly normal, it is difficult to demonstrate a significant difference between patients with Crohn's disease and healthy volunteers.

We hypothesized that serum zinc, serum alkaline phosphatase and the zinc concentrations in blood cells are correlated with factors that are potentially related to zinc deficiency: the activity of Crohn's disease, weight loss, the extent of the inflammation, the extent of the resection or the number of liquid stools. We have chosen for these factors, potentially causing zinc deficiency, because no gold standard for the zinc status, applicable in clinical practice, is available. The activity of Crohn's disease has been suggested as a cause of zinc deficiency (9, 10, 11). Weight loss can be a result of malnutrition, which may also result in a decrease of zinc status. Valberg et al. (12) observed a decrease of zinc absorption in undernourished patients with moderate or severe disease activity. Diarrhoea results in an increase of zinc loss. Therefore, we selected the extent of inflammation, the extent of the resection and the number of liquid stools as potential causes of a decrease of zinc status. We postulate that the parameters for the zinc status, most frequently correlated with potential causes of zinc deficiency, are of the greatest value for the determination of the zinc status.

METHODS

Patients

This study was approved by the Committee for Ethics and Research in Humans of the University Hospital Nijmegen, in accordance with the Declaration of Helsinki.

The activity of Crohn's disease was determined using the CDAI (normal value <150

Table 1

Zinc deficiency in Crohn's disease, a literature survey

Author (reference)	Nr. of patients	Plasma or serum Zn* (μmol/L, patients/ controls)	Zinc in blood cells **						Alkaline Phos-phatase Activity
			Erythro cytes	Leuco cytes	Lympho cytes	MNC	Mono	PMNC	
Bro (21)	10	↓ (11.6/14.9)	.	.	.	N	.	.	N
Nakamura (25)	10	↓ (8.7/14.5)	.			.		.	
Ainley (26)	31	↓ (13.5/15.4)	N	.		N		N	
Hinks (27)	20	N (12.7/12.7)	.	N					
Sjogren (28)	30	↓ (12.2/14.0)	↓	.					
Valberg (12)	17	N (14.0/14.0)	.	N					
Deflandre (29)	29	↓ (13.4/16.8)	↓						
Kruis (30)	50	↓ (12.7)							
Schoelmerich (9)	54	↓ (11.9/14.5)							
Hessov (31)	87	↓ (11.0)							
Brignola (32)	27	↓	.						
Ringstad (33)	47	N (♂:14.4/12.7) (♀:13.5/12.9)	.						
Terwolbeck (36)	26	↓ (11.3/13.1)	.		↓	.		.	
Ainley (35)	32	↓ (13.5/15.4)	N		N	N	.	N	
Goode (36)	34	↓ (11.3/12.7)			N		↓	N	
Fernandez-Banarez (11)	14	↓ (14.4/15.6)							N
Sturniolo (37)	33	↓ (14.9/20.8)							
Fleming (38)	63	↓ (16.0/16.6)							
McClain (3)	52	↓							
Mills (39)	20	N (13.7/15.6)	.	.	.	.	.	.	.

* Mean plasma or serum zinc concentration in patients with Crohn's disease and in a control population ** Cell type according to the authors. N = normal compared to a control population, ↓ = decreased compared to a control population, . = not determined

(13)) and the van Hees Index (normal value <100 (14)). Only patients with a serum albumin less than 37 g/L at the last visit to the out-patient clinic were included to ensure that a significant part of the patients had active Crohn's disease on the day of investigation. The site of the inflammation was determined by X-ray and/or endoscopy. Medical treatment was recorded. Blood was taken in a fasting state for the determination of serum zinc, serum alkaline phosphatase activity and the zinc concentration in erythrocytes, MNC and PMNC. Blood was drawn using stainless steel needles and plastic syringes with 10 μL of preservative-free heparin (50 IU).

Procedures

Separation of blood cells performed by a modified discontinuous Percoll gradient.

The method of determination of the cellular zinc concentration was adapted from Milne (15), but partly modified (16). Blood was diluted 1:3 with GPB (13.5 mmol/L Na_2HPO_4, 3.3 mmol/L KH_2PO_4, 5.4 mmol/L KCl, 133 mmol/L NaCl and 5 mmol/L glucose in ion-free water and, if necessary, adjusted to pH 7.4 with HCO_3-). Three solutions of Percoll of different densities (1.060, 1.075 and 1.095 kg/L respectively) were prepared by dilution with Hanks' balanced salts solution (without Ca and Mg) and were kept at 4°C to prevent clumping and zinc exchange by the cells (17). Six mL of a heavier solution was layered in a 50 mL tube underneath the lighter solution using a stainless steel needle of 15 cm and a plastic syringe. Subsequently the tube was centrifuged for 20 min., at 500 x g at 4°C in a swing-out rotor without brake (Heraeus Crist Minifuge GL, Osterode am Harz, Germany). This resulted in separated layers of platelets, MNC, PMNC and erythrocytes. The MNC, PMNC and erythrocytes were harvested using a pipet and resuspended in 5 mL GPB in a 15 mL tube. Erythrocyte contamination of the MNC and PMNC fractions was removed by resuspending the cells twice for 10 min. at 4°C in 5 mL of erythrocyte lysing buffer (155 mmol/L NH_4Cl and 10 mmol/L $KHCO_3$ in ion-free water, pH adjusted to 7.4 with HCO_3-) and subsequent washing steps with 5 mL of GPB. After these steps the tube was centrifuged for 10 min., 200 x g, 4°C with brake. Cells were resuspended in 1 mL of GPB for cell counting and zinc determination. Cytospin preparations (Shandon; Astmoor, UK) were made at random to check purity of the cell suspensions. The cell suspensions contained less than 10% contamination by other cells. The cell suspensions were centrifuged, supernatants were aspirated and the cells were digested by the use of undiluted nitric acid. Erythrocytes were destroyed by lysis with de-ionized water.

Finally zinc content was determined in the samples. Albumin and alkaline phosphatase activity were measured using standard clinical procedures. The normal values for serum alkaline phosphatase activity, albumin, and zinc were determined using blood of 200 healthy blood donors (Serum albumin: 37-50 g/L; serum alkaline phosphatase activity: 25-120 U/L; serum zinc: 10-17 μmol/L). Alkaline phosphatase activity (Boehringer, Mannheim, Germany) was determination according to IFCC recommendations (18). Serum albumin was determined using a chemical method (19).

Determination of zinc

Zinc was determined by atomic absorption spectrophotometry (AAS)(Perkin Elmer 5000; Norwalk, Connecticut, USA). The lower limit of detection was 0.28 μmol/L and the response was linear to at least 15 μmol/L. The normal range of serum zinc in healthy volunteers was 10-17 μmol/L. Standard reference solution (ZnCl2, Titrisol, Merck, Darmstadt, Germany) was used in the concentrations expected to be found in the samples. Cells were counted by a Coulter counter Model ZM (Luton, Bedfordshire, England), adjusted to the size of the cells to prevent counting of cell fragments.

No detectable zinc concentrations could be determined in the solutions and in ion-free water put into tubes and bottles apart from a low zinc concentration in diluted Percoll (2,56 μmol/L).

Materials

Stainless steel needles and plastic syringes (Monoject, Sherwood, USA) were used. All solutions were prepared with de-ionized distilled water (resistance > 15 M Ω), processed through a Millipore system (Bedford, Massachusetts, USA). All materials were acid washed.

Only polypropylene pipet tips, plastic tubes (15 mL polystyrene, Greiner, Solingen, Germany and 50 mL polypropylene, Falcon, Becton Dickinson and Company, Lincoln Park, New Jersey, USA) and bottles (0.8 L, NUNC, Roskilde, Denmark) were used. All chemicals were of analytical grade (Merck, Darmstadt, Germany). Percoll was supplied by Pharmacia (Uppsala, Sweden). Hanks' balanced salts solution (without Ca and Mg) was obtained from Flow Laboratories (Rickmansworth, Herts, U.K.).

Spearman correlation coefficients between the parameters were determined by regression analysis and the null hypothesis, that correlation was zero, was examined. The number of significant p-values was an indication of the relation between a parameter for the zinc status and the various factors potentially inducing zinc deficiency. The significance of the difference in serum zinc concentration in patients with various localisations of Crohn's disease, and in patients with various medical treatments was calculated using an ANOVA analysis.

Table 2

Correlations between suggested factors inducing zinc deficiency and parameters to determine the zincstatus in patients with more or less active Crohn's disease (*R*, p).

	Serum Zinc Concentration	Serum Alkaline Phosphatase Activity	Zinc in blood cells		
			Erythro cytes	MNC	PMNC
CDAI	*-0.2*	*-0.14*	*0.2*	*-0.35*	*-0.3*
	0.28	0.43	0.29	0.08	0.15
van Hees Index	*-0.5*	*+0.5*	*-0.14*	*0.14*	*-0.05*
	0.01	0.01	0.38	0.48	0.95
Extent of inflammation	*-0.29*	*-0.33*	*-0.1*	*0.05*	*-0.3*
	0.16	0.04	0.55	0.84	0.14
Extent of resection	*-0.5*	*-0.39*	*0.05*	*0.1*	*0.02*
	0.01	0.01	0.85	0.74	0.96
Changes in body weight	*0.3*	*0.14*	*-0.28*	*0.32*	*0.4*
	0.11	0.41	0.09	0.55	0.03
Number of liquid stools	*-0.1*	*-0.1*	*-0.1*	*-0.3*	*-0.4*
	0.6	0.56	0.54	0.06	0.03

Population characteristics

Thirteen males and 18 females were studied, with a mean age of 37 years (22 - 62 years). The CDAI ranged from 49 to 298 with a mean of 157, and the van Hees Index from 104 to 310 with a mean of 177. The extent of the inflammation ranged from 2 to 180 cm (mean 58 cm). In 16 patients small bowel resection had been performed previously, ranging from 5 to 300 cm (mean 94 cm).

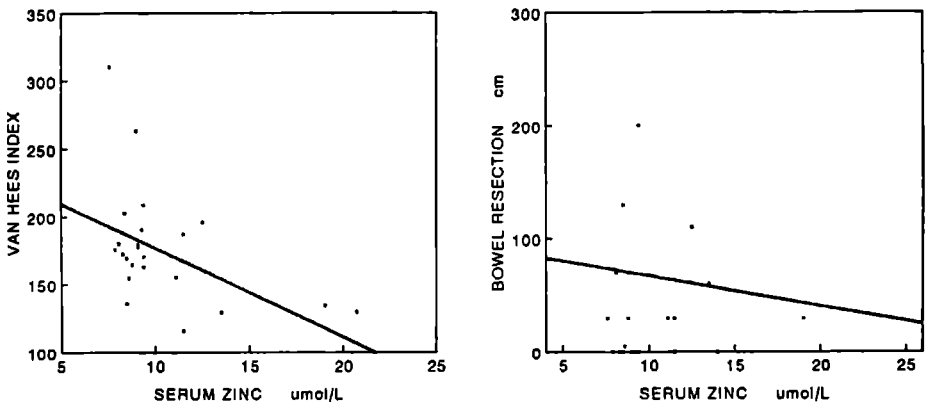

Figure 1 - Correlation of the serum zinc concentration and inflammatory activity of Crohn's disease
a. Left figure: serum zinc concentration and van Hees Index, R = -0.5, p = 0.01
b. Right figure: serum zinc concentration and the extent of bowel resection. R = -0.5, p = 0.01

Correlation between the parameters to determine the zinc status and probable factors inducing zinc deficiency

The correlations between parameters for the zinc status and factors potentially inducing a negative zinc balance are given in table 2.

In 68% of the patients with more or less active Crohn's disease, serum zinc was below the normal range (10-17 μmol/L). A distinct correlation was found between the serum zinc

concentration and both the van Hees Index (Figure 1a, P=0.01) and the extent of resection (Figure 1b, p=0.01). No correlation was found between the serum zinc concentration and the CDAI, the extent of inflammation, weight loss or number of liquid stools. A nearly significant difference (p=0.06) in serum zinc concentration was seen in patients with active Crohn's disease in both the small bowel and colon compared to inflammation in only the colon or small bowel (Figure 2a). There were no significant differences in serum zinc concentrations between the various medical treatments (Figure 2b).

The serum alkaline phosphatase activity was positively correlated with the van Hees index (Figure 3a, p=0.01) and negatively with the extent of resection (Figure 3b, p=0.01).

Zinc concentrations in erythrocytes, MNC and PMNC were not correlated with the Crohn's activity indices, the extent of inflammation or resection. The changes in body weight (Figure 4) showed a negative tendency towards a correlation with zinc in erythrocytes (p=0.09), but they showed a positive correlation with zinc in PMNC (p=0.03). The number of liquid stools showed a nearly significant negative correlation with the zinc content in MNC (p=0.06) and reached significance with zinc in PMNC (p=0.03) (Figure 5).

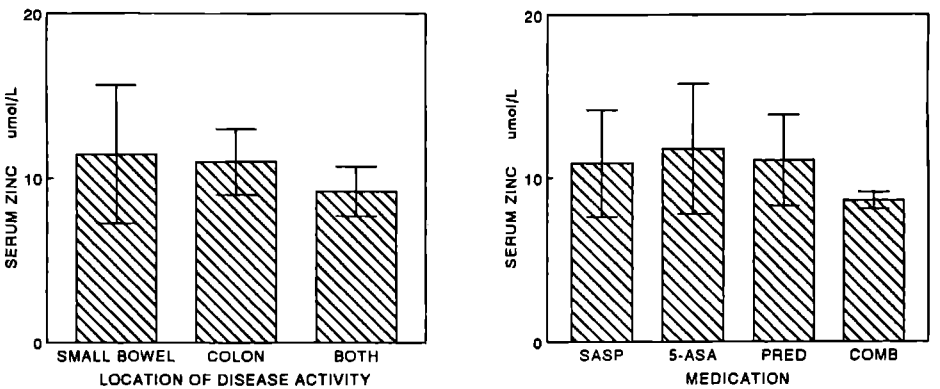

Figure 2 - Correlation of the serum zinc concentration and clinical variables

a. Left figure: serum zinc and location of disease activity. A lower zinc concentration was observed in patients with active Crohn's disease in small and large bowel compared to inflammation in only the colon or small bowel, which was nearly significant (p = 0.06)

b. Right figure: serum zinc and medical treatment. No significant differences were observed

Interrelations between the parameters to determine zinc deficiency and interrelations between factors related to zinc deficiency:

The interrelations between parameters for the zinc status are shown in Table 3. The serum zinc concentration was correlated with serum albumin (R=0.56, P=0.003). Zinc in MNC was correlated with serum alkaline phosphatase activity (p=0.03). Zinc in PMNC was correlated with both zinc in erythrocytes (p=0.03) and zinc in MNC (p=0.007). The zinc concentration in blood cells showed no correlation with the serum zinc concentration. The serum alkaline phosphatase activity was not significantly correlated with the serum zinc concentration (p=0.1).

The van Hees Index was correlated with the extent of inflammation (p=0.04). The van Hees Index and the CDAI were not significantly correlated.

DISCUSSION

The relative value of various methods to determine the zinc status in patients was studied by systematically investigating the relation with factors potentially inducing a decre-

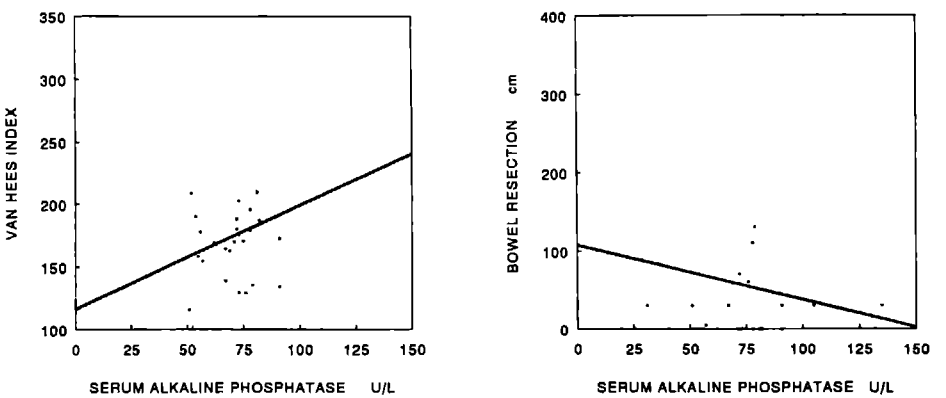

Figure 3 - Correlation of serum alkaline phosphatase activity and factors suggested to induce zinc deficiency

a. Left figure: serum alkaline phosphatase activity and the van Hees Index. R = + 0.5, p = 0.01

b. Right figure: alkaline phosphatase activity and the extent of resection. R = - 0.39, p = 0.01

ase of zinc status. This study design was used because a gold standard for the assessment of the zinc status, which can be applied in clinical practice, is not available.

Serum zinc concentration

In 68% of the patients with more or less active Crohn's disease we demonstrated a serum zinc concentration below the reference level. The serum zinc concentration was correlated with serum albumin concentration, bowel resection and the van Hees Index. In most studies a low serum zinc concentration was frequently seen in patients with active Crohn's disease (Table 1).

This decrease may be caused by a real zinc deficiency, but it can also be an apparent zinc deficiency owing to the inflammation: inflammation causes a decrease of serum albumin and because zinc is transported in part by albumin this decrease of serum albumin will result in a decrease of serum zinc. In addition, during inflammation zinc is redistributed from the serum into the liver. We call a decrease of serum zinc resulting from inflammation, apparent zinc deficiency, because no symptoms of zinc deficiency occur during inflammatory stress. The high prevalence of a decreased serum zinc level could partly be explained by a decrease of serum albumin, because the serum zinc concentration was correlated with serum albumin. In some of these patients with a low serum zinc concentration there will be a real decrease of zinc status, but their number is unknown. Therefore, we also correlated the serum zinc concentration with factors potentially inducing zinc deficiency. Serum zinc was correlated with the extent of bowel resection. Because the extent of bowel resection was not correlated with the severity of inflammation, this correlation cannot be explained by apparent zinc deficiency due to inflammation. This may indicate that in some of these patients with Crohn's disease with extensive bowel resection the zinc status was really decreased.

A correlation was seen with the van Hees Index, but albumin is one of the nine parameters in the van Hees Index, so the serum zinc concentration and the van Hees Index are not completely independent variables. No significant correlation was demonstrated with the CDAI. The studies of Schoelmerich (9) and Piroli (10) showed a correlation between serum zinc and the CDAI, but this could not be confirmed by McLain (3). Fernandez-Banarez (11) also demonstrated a correlation between the serum zinc concentration and the van Hees Index, but did not mention the partial relationship of the van Hees Index and serum zinc.

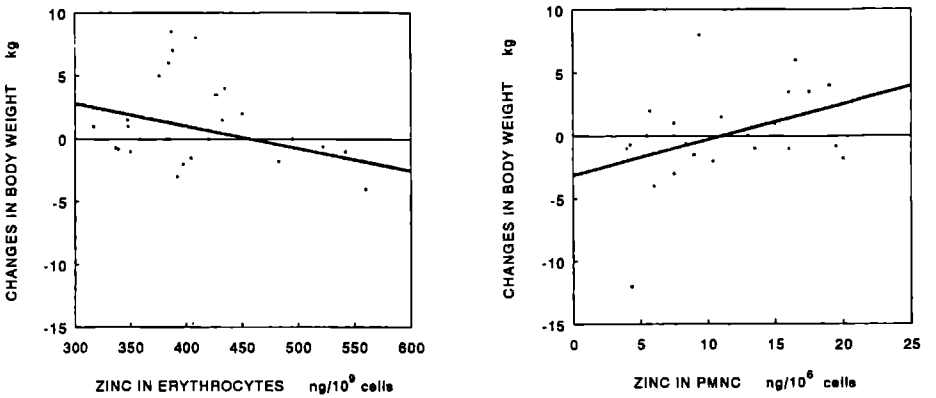

Figure 4 - Correlation of the zinc concentration in blood cells and changes in body weight
a. Left figure: zinc in erythrocytes and changes in body weight. R = - 0.28, p = 0.09
b. Right figure: zinc in PMNC and changes in body weight. R = + 0.40, p = 0.03

The serum zinc concentration was lower in patients with Crohn's disease located both in the colon and small bowel. In the study by Schoelmerich (9) no difference in serum zinc concentration was seen between groups of patients with different localizations of disease.

Serum alkaline phosphatase activity

The alkaline phosphatase activity is correlated with the extent of resection, the van Hees Index and the extent of inflammation.

Serum alkaline phosphatase activity has been suggested as a parameter of zinc deficiency because enzyme activity is influenced by the zinc status. Zinc is a component of more than 200 enzymes (20). Alkaline phosphatase is one of these zinc-dependent metallo-enzymes. In case of zinc deficiency, zinc-containing metallo-enzymes are not functioning optimally. Alkaline phosphatase activity is not only influenced by the serum zinc concentration, but also by other factors like bone turn-over and liver function. These factors induce variations in the serum concentration of the enzyme.

The negative correlation between serum alkaline phosphatase activity and the extent of bowel resection can be explained by a lower serum zinc concentration in these patients.

As alkaline phosphatase activity is not directly related with one of the parameters of

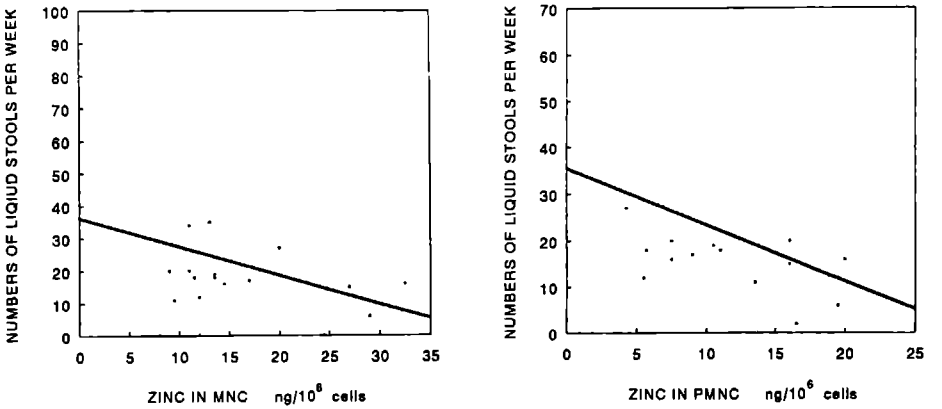

Figure 5 - Correlation of the zinc concentration in blood cells and frequency of liquid stools
a. Left figure: zinc in MNC and numbers of liquid stools. R = - 0.30, p = 0.06
b. Right figure: zinc in PMNC and numbers of liquid stools. R = - 0.40, p = 0.03

the van Hees Index, the correlation we demonstrated between alkaline phosphatase activity and disease activity could be of clinical significance for the determination of the zinc status. However, an increase of Crohn's activity was correlated with an increase of alkaline phosphatase activity. One would expect that in case of an increase of disease activity, serum zinc and subsequently serum alkaline phosphatase activity would decrease. The positive correlation between alkaline phosphatase activity and the van Hees Index probably does not indicate a correlation with the zinc status. In the studies by both Bro et al. (21) and Fernandez-Banarez et al. (11) the serum alkaline phosphatase activity in patients with Crohn's disease was increased compared to healthy volunteers, and this difference reached statistical significance in the study by Fernandez-Banarez for patients with active colitis (p=0.02). Serum alkaline phosphatase activity is influenced not only by the serum zinc concentration, but by other factors as well, like liver function disorders, especially in more active Crohn's disease. This may be the explanation why an increase of serum alkaline phosphatase activity was positively correlated with an increase of activity of Crohn's disease.

Zinc concentrations in blood cells

The small number of significant correlations (2 of the 18 tested) between the zinc concentrations in the various blood cells and factors potentially inducing zinc deficiency,

Table 3

Mutual correlation between parameters for the zinc status (*R*, p)

	Serum Zinc Concentration	Serum Alkaline Phosphatase Activity	Zinc in blood cells		
			Erythrocytes	MNC	PMNC
Serum Alkaline Phosphatase Activity	*0.33*				
	0.08				
Zinc in blood cells					
Erythrocytes	*0.2*	*0.17*			
	0.34	0.32			
MNC	*0.16*	*0.49*	*0.2*		
	0.52	0.03	0.3		
PMNC	*0.26*	*0.25*	*0.48*	*0.57*	
	0.26	0.73	0.03	0.007	

was unexpected. Besides the number of significant correlations, the mean p-value of a specific parameter also provides an indication of the significance of this specific method for the determination of the zinc status. The mean p-values were higher for blood cells (erythrocytes 0.45, MNC 0.46, PMNC 0.37) compared to the mean p-value for serum zinc (0.2) and serum alkaline phosphatase activity (0.25). Probably only the zinc concentration in PMNC was of clinical importance because of a significant positive correlation with body weight loss and a negative correlation with the number of liquid stools. Wolman et al. (22) demonstrated zinc loss to be correlated with the weight of the stools per day.

Zinc in blood cells has frequently been used to determine the zinc status, as an alternative to serum zinc, although the data to validate this method are conflicting. Controversy exists about the question whether zinc deficiency leads to an increase (12) or a decrease (23) in intracellular zinc concentrations. After zinc supplementation even a decrease of granulocyte zinc was observed (11). In most of the studies in which the zinc concentrations in blood cells of patients with active Crohn's disease were compared to those of healthy volunteers, no differences were observed (Table 1). But the conclusion was not drawn that this method has only limited value for the assessment of the zinc status.

The reasons for the observed low correlation between zinc concentrations in blood

cells and factors potentially inducing zinc deficiency (2 out of the 18 correlations), and also the frequent reports in the literature of normal zinc concentrations in blood cells of patients with Crohn's disease, could be explained by one of the following three hypotheses.
Firstly: the zinc status is not influenced by the factors that have been proposed as possible causes of zinc deficiency. This is not likely since zinc deficiency does occur in patients with Crohn's disease, and there are strong indications of subclinical zinc deficiency in patients with active Crohn's disease, because in these patients clinical symptoms of zinc deficiency emerge in the post-operative anabolic period. The correlation we demonstrated in this study between the serum zinc concentration and the serum alkaline phosphatase activity on the one hand and the extent of bowel resection on the other, supports the hypothesis that in these patients with Crohn's disease the zinc status is decreased.
Secondly: the risk of zinc contamination of the cell suspension is high, which makes the determination of zinc in blood cells difficult (24). In our experiments we could not demonstrate zinc contamination (see Methods). Because there is a correlation between zinc in PMNC and zinc in MNC as well as in erythrocytes, it is also unlikely that zinc contamination is the cause of this low correlation.
Thirdly: the zinc concentration in blood cells is very low and the exchangeable zinc pool in these cells is even smaller. Using 10 mL of blood and with a cell recovery of 50%, the final zinc concentration of the cell suspension is about 1.0 μmol/L when resuspended in 0.5 mL GPB, which is near the lower limit of detection of an AAS determination. In a previous study we demonstrated that the exchangeable zinc pool was 3% in erythrocytes, 24% in MNC and 26% in PMNC (17). This indicates that determination of the zinc concentration in blood cells is unsuitable for this purpose because of the low zinc concentrations in the final cell suspensions and the small exchangeable zinc pool of these cells.

In addition to the present study we performed animal experiments in which rats were made zinc-deficient. Hair loss was observed in the zinc-deficient rats. After four weeks the serum zinc concentration in zinc-deficient rats was 40% of that in control animals. No differences were observed between zinc concentrations in erythrocytes, MNC and PMNC taken from individual zinc-deficient rats and control rats. The zinc levels of the final cell suspensions were low in these samples and therefore an identical experiment was performed in which the blood samples of the various rats were pooled. No differences in zinc concentrations between blood cells of zinc-deficient rats and those of control rats were observed in this experiment either. Milne et al. (24) studied the zinc content of lymph nodes and spleen of zinc-deficient rats. Because of the larger number of cells the zinc concentration of the concentrated cell suspension taken from lymph nodes and spleen was

even higher than in our experiments. Milne's animal experiments revealed no differences either. These observations support the third hypothesis mentioned.

In conclusion: determination of zinc in blood cells is no better method than determination of serum zinc and serum alkaline phosphatase activity for the assessment of the zinc status in patients with active Crohn's disease. This is probable due to the low zinc concentrations in the cell suspensions and the small exchangeable zinc pool of these cells.

Since in the present study a clear correlation was shown between the extent of bowel resection on the one hand, and both serum zinc concentration and serum alkaline phosphatase activity on the other, a decrease of zinc status was likely in the patients with Crohn's disease as a group. However, it is not possible to determine the zinc status in individual patients with active Crohn's disease by these methods because of the interference of other factors that influence the serum zinc concentration and serum alkaline phosphatase activity. Better methods are needed. They should be applicable in clinical practice, and they should be able to detect subclinical zinc deficiency and distinguish real zinc deficiency from a decrease of serum zinc resulting from other factors.

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IN VIVO ZINC EXCHANGE BY BLOOD CELLS AND TISSUES DURING ZINC DEFICIENCY AND INFLAMMATORY STRESS

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ABSTRACT

During zinc deficiency and inflammation serum zinc concentrations are decreased. However, during inflammation no symptoms of zinc deficiency are present. This limits the use of serum zinc as an indicator of zinc deficiency, for instance in malnourished patients with an inflammation. There is a need for a method that can differentiate between both conditions. The physiologic changes in zinc metabolism during zinc deficiency and inflammatory stress were investigated and related to zinc exchange by blood cells.

In rats zinc deficiency was induced by a deficient diet and inflammation by turpentine injection. To study the in-vivo uptake and exchange of zinc by tissues and blood cells, ^{65}Zn was injected intraperitoneally.

Both in zinc-deficient rats and in rats with inflammatory stress serum zinc concentrations were decreased. In contrast the uptake in ^{65}Zn by erythrocytes, mononuclear cells, liver and bone marrow was increased in zinc-deficient rats, but not in rats with inflammatory stress. The uptake of ^{65}Zn by bone cortex was very slow in the zinc deficient rats and only half of that in control rats. In rats with stress the activity of ^{65}Zn was increased in liver and bone marrow, and to a lesser degree in muscle and skin. The observed differences in ^{65}Zn uptake by blood cells in Zn deficiency and inflammatory stress, make it possible to distinguish real zinc deficiency from a decrease of serum zinc caused by an inflammation. These differences provide a physiologic basis for new methods to determine zinc status.

INTRODUCTION

Zinc (Zn) is one of the major trace elements. It is part of many metallo-enzymes, cell structures and zinc fingers, which are components of the cell nucleus. Zn deficiency results in a variety of symptoms (1). Although various methods have been proposed to determine Zn deficiency, no single method can be used to determine Zn deficiency and most methods fail to reveal an apparent Zn deficiency. A status of apparent zinc deficiency may occur due to inflammation resulting in redistribution of Zn from serum to the liver and to a decrease of serum albumin (2), the most important Zn-transporting protein in blood. Apparent Zn deficiency can be observed in patients with inflammatory stress, for instance patients with active Crohn's disease. In these patients the serum Zn concentration may be decreased. The methods currently available to determine Zn deficiency, cannot distinguish between real and apparent Zn deficiency.

The aim of the present study was to investigate the physiologic reaction of Zn exchange by tissues and blood cells (as a kind of tissue) after induction of Zn deficiency and inflammatory stress. Special attention was given to the behaviour of injected ^{65}Zn as a tracer for the distribution and redistribution of Zn during a Zn-deficient state and during inflammatory stress. Animal models were used to induce a Zn-deficient state and an inflammatory stress state to differentiate between real and apparent Zn deficiency. Animals were used because it is almost impossible to find groups of patients with pure inflammatory stress or zinc deficiency in whom Zn might be determined in a variety of tissues.

METHODS

Animals

Male Wistar rats weighing 200 g on arrival (raised at the central animal laboratory, WU strain), were housed separately in stainless steel metabolic cages. The day-night rhythm was 12-12 hours and the temperature was 20°C. The humidity of the animal laboratory was 60%. The diet on which the rats were raised (SMRA) contained a variable and often excessive amount of Zn. The rats had free access to tap water ad libitum. To adapt the animals to the diet and the cages (separate metal metabolic cages for each animal with wire mesh bases to reduce coprophagy) and to reduce the excess of Zn in their depots, a diet (IRI-OB) (table 1 (3)) containing 12 mg/kg Zn was given to the rats for a period of 2 weeks before the start of the experiment. This Zn concentration is considered sufficient to maintain a normal Zn status (4). During this period demineralized water was provided to the rats. The guidelines for the care and use of laboratory animals in the Netherlands were followed. The experiments were approved by the animal welfare officer of the University of Nijmegen, and they were performed by specialized personnel.

Diets

The diet (IRI-OB) was produced by Hope Farms (Woerden, the Netherlands) using components low in Zn (table 1,(3)) resulting in a Zn concentration of less than 2 mg/kg. In the Zn-sufficient food Zn was added as sulphate to a final Zn concentration of 12 mg/kg. Sufficient quantities of other trace elements were added. The copper (Cu) concen-

tration in both versions of the diet was 9 mg/kg. The commercial diet SMRA (Hope Farms, Woerden, the Netherlands) contained 92 mg/kg Zn and 25 mg/kg Cu.

Materials

Solutions

Glucose Phosphate Buffer (GPB):

13.5 mmol/L Na_2HPO_4, 3.3 mmol/L KH_2PO_4, 5.4 mmol/L KCl, 133 mmol/L NaCl and 5 mmol/L glucose in ion-free water and, if necessary, adjusted to pH 7.4 with HCO_3^-.

Erythrocyte Lysing Buffer:

155 mmol/L NH_4Cl and 10 mmol/L $KHCO_3$ in ion-free water, pH adjusted to 7.4 with HCO_3^-.

Discontinuous Gradient of Percoll

Three solutions of Percoll of different density (1.060, 1.075 and 1.095 kg/L respectively) (5) were prepared by dilution with Hanks' balanced salts solution without Ca and Mg.

All solutions were prepared with deionized distilled water (resistance > 15 M Ω), processed through a Millipore system (Bedford, Massachusetts, USA). Only polypropylene pipet tips, plastic tubes (15 mL polystyrene, Greiner, Solingen, Germany and 50 mL polypropylene, Falcon, Becton Dickinson and Company, Lincoln Park, New Jersey, USA) and bottles (0.8 L, NUNC, Roskilde, Denmark) were used. All chemicals were of analytical grade (Merck, Darmstadt, Germany). Percoll was supplied by Pharmacia (Uppsala, Sweden). Hepes was obtained from Sigma (St Louis, MO, USA), albumin from Behring (Marburg, Germany), Hanks' balanced salts without Ca and Mg from Flow Laboratories (Rickmansworth, Herts, U.K.) and the radiotracer ^{65}Zn from Amersham International (Amersham, Buckinghamshire, U.K., code ZAS 2 , 3.7-37 GBq per mg of Zn as chloride in 0.1 mol/L HCl). There was no detectable Zn in solutions or in ion-free water put into tubes and bottles apart from a low Zn concentration in diluted Percoll (2,56 μmol/L). Stainless steel needles and plastic syringes (Monoject, Sherwood, USA) with 10 μL of preservative-free heparin (50 IU) were used.

Table 1

Composition of the IRI-OB diet

Ingredients	Concentration (g/100g)
Glucose	50.65
Corn starch	15.0
Ovalbumin	20.0
Sun flower seed oil	4.0
Fibre (cellulose)	5.0
Choline chloride	0.3
Mineral mix	
Sodium diphosphate	1.5
Potassium chloride	0.7
Calcium carbonate	1.0
Magnesium sulphate	0.5
Sodium meta-silicate	0.25
Trace element salt mix	0.1
Vitamin mix in glucose	1.0

Trace element salt mix	Concentration (mg/kg)
Mn	55
Fe	66
Zn	various conc.
Cu	9.3
Ni	2.4
Cr	1.8
Mo	0.2
Se	0.36
As	0.13
Co	0.2

Analytical Procedures

Zinc determination

Zn was determined by atomic absorption spectrophotometry (AAS)(Perkin Elmer 5000; Norwalk, Connecticut, USA). The lower limit of detection was 0.28 μmol/L and

the response was linear to at least 15 μmol/L. Standard reference containing $ZnCl_2$ (Titrisol, Merck, Darmstadt, Germany) was used in the concentrations expected to be found in the samples.

Zinc was determination in tissues in duplicate. A sample of the tissue (of about 50 mg) was weighed precisely. Tissue was destroyed in borosilicate tubes, washed in 8M HNO_3 (Suprapure, Merck, Darmstadt, Germany). Destruction was performed in three steps: with 1 mL and twice with 0.1 mL of HNO_3, with drying procedures at 95°C in between. The final residue was dissolved in 1 mL 0.5 M HNO_3 and the Zn concentration was determined. A standard reference of Bovine Liver SRM 1577a, processed in the same way, was used as control. For the determination of zinc in tissues, bone, muscle and liver are especially important, because these organs contain most of the total body zinc content (5).

Isolation procedure of the blood cells

Blood was drawn from anaesthetized rats by heart puncture using stainless steel needles and plastic syringes with 10 μL of preservative-free heparin (50 IU). Cell separation was performed using a discontinuous Percoll gradient (6). All solutions were kept at 4°C to prevent clumping and Zn exchange by the cells. Blood was diluted with GPB 1:3. Six mL of a heavier solution was layered in a 50 mL tube underneath the lighter solution using a stainless steel needle of 15 cm and a plastic syringe. Subsequently the tube was centrifuged for 20 min., at 500 x g at 4°C in a swing-out rotor without brake (Heraeus Crist Minifuge GL, Osterode am Harz, Germany). This resulted in separated layers of platelets, mononuclear cells (MNC), polymorphonuclear cells (PMNC) and erythrocytes. The MNC, PMNC and erythrocytes were harvested using a pipet and resuspended in 5 mL GPB in a 15 mL tube. Erythrocyte contamination of the MNC and PMNC fractions was removed by resuspending the cells twice for 10 min. at 4°C in 5 mL of erythrocyte lysing buffer and subsequent washing steps with 5 mL of GPB. After lysing and washing steps the tube was centrifuged for 10 min., 200 x g, 4°C with brake. Cells were resuspended in 1 mL of GPB for cell counting. The erythrocytes were washed with GPB twice and centrifuged for 10 min., 200 x g, 4°C with brake. Random Cytospin preparations (Shandon; Astmoor, UK) were made to check purity of the cell suspensions. The cell suspensions contained less than 10% contamination with other cells. The volume was determined from the weight difference of the tube. Numbers of cells and radioactivity were counted. The whole procedure was performed in one day.

Cells were counted using a Coulter counter Model ZM (Luton, Beds., England), adjusted to the size of the cells to prevent counting of cell fragments.

Radioactivity

^{65}Zn was measured by means of a NaI crystal with an automatic sample changer (LKB, Wallac 1282 Compugamma; Turku, Finland). The counting time was adjusted to ensure a statistical error of < 0.3 %. A tube with medium but without blood cells, incubated and washed according to the procedure of the cell-containing tubes, served as a blank.

Total body radioactivity of the rats was counted with a shadow shield counter consisting of two Tl-activated NaI crystals (4 x 6"), both at a distance of 45 cm from the rat, connected to a Nuclear Data multi-channel analyzer N.D. 60 A (Nuclear Data; Schaumburg, Illinois, USA). In the zinc-deficiency experiment the first determination of the ^{65}Zn whole-body retention was performed four hours after the intraperitoneal administration of ^{65}Zn. This first determination was taken as 100%. In the inflammatory stress experiment the results of the ^{65}Zn whole-body retention on the successive days were expressed as % of the activity of t = -6, one day after the ^{65}Zn injection.

Outline of the two experiments

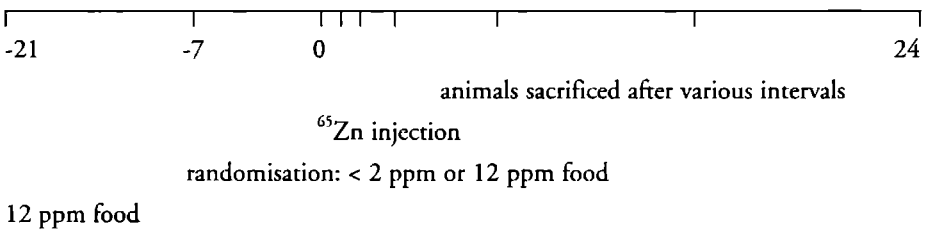
Experiment 1: zinc-deficiency experiment.

At the end of the adaptation period (day -7) the rats were divided into two groups of twelve rats each in such a way that the body weight distributions of the animals were similar. One group remained on the control diet and the other group was switched to the Zn-deficient diet containing < 2mg Zn/kg. The control group received pair-feeding throughout the rest of the experiment. At day 0, when the rats who had been given a diet low in Zn were in a mild Zn-deficient state, 6 μCi ^{65}Zn was injected intraperitoneally. After 3 hours and on days 1, 3, 7, 14, and 24, three to five mL blood were taken under inhalation of ether anaesthesia by heart puncture in 2 of the rats in each group. The serum was analyzed for albumin and Zn by AAS. The various blood cells were isolated. The rats were sacrificed after blood sampling and liver, muscle from the front legs, bone and bone marrow had been taken out after determination of total body retention of ^{65}Zn. Whole-body counting was performed after 4 hours and on days 1, 3, 7, 14 and 24. Whole-body counting was performed on the sacrificed animals of that sample day (before removal of the organs and tissues), as well as on all of the animals still alive on that day. Bone marrow was separated from the bone cortex using of a plastic syringe filled with air to blow the marrow out of the bone. During the experiment most of the zinc-deficient animals showed loss of hair.

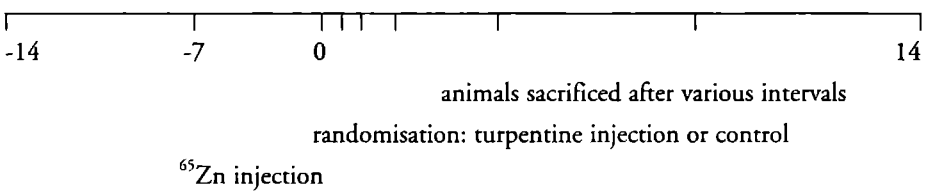
Experiment 2: inflammatory stress experiment.

Fourteen days prior to induction of inflammatory stress 64 rats were switched from the commercial diet to the purified diet (IRI-OB) and put into the metabolic cages to adapt the animals (see Animals and experiment 1). Throughout the experiment the rats received the IRI-OB food containing 12 ppm of Zn. On day -7 3 μCi ^{65}Zn was injected intraperitoneally. On day 0 the rats were divided into two groups of 32 rats in such a way that the body weight distributions of the rats were similar. Thirty-two rats were injected intramuscularly in the hind legs with 0.5 mL turpentine. Immediately before turpentine injection (t=0), after 4 hours, and on days 1, 2, 3, 4, 7, and 14, three to five mL blood was withdrawn from four rats of each group for blood cells. Serum was used to measure Zn and Cu concentrations. Rats were sacrificed under anaesthesia by breaking the neck. Total body ^{65}Zn retention was determined and liver, muscle, skin, bone and bone marrow were taken for determination of radioactivity. As ^{65}Zn was given 7 days prior to the induction of inflammatory stress, the fast changes in ^{65}Zn seen immediately after ^{65}Zn injection did not interfere with the changes in zinc concentrations seen directly after turpentine injection, as demonstrated in the control group of the zinc-deficiency experiment and by the study of Weigant and Kirchgessner (7). This injection of radioactive zinc seven days prior to the induction of inflammation resulted in an approximation of an equilibrium of ^{65}Zn with cold Zn.

Zinc-deficiency experiment



Inflammatory stress experiment



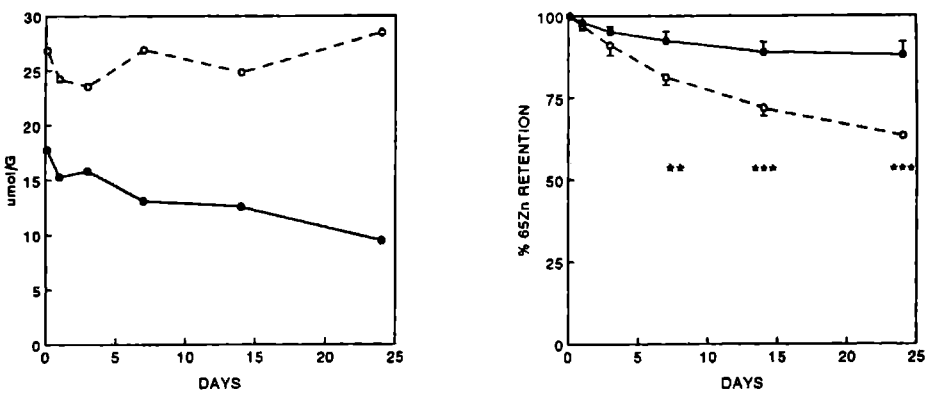


Figure 1: zinc deficiency experiment

Zinc concentration in serum (a, left figure) and whole-body retention of ^{65}Zn (b, right figure) in zinc-deficient (closed dots) and control rats (open dots). At the start of the curve the rats on a diet low in zinc were already mildly zinc-deficient. During zinc deficiency serum zinc is decreased. The whole-body retention of ^{65}Zn indicated that zinc-deficien t rats retained more ^{65}Zn than control rats. The error bars indicate standard deviations ($p=<0.05$; ** $p=<0.01$; *** $p=<0.001$).*

Calculations

Statistics:

A Wilcoxon Rank Sum test was performed to compare the data of the two groups throughout the total experiment. The Student t-test was performed for individual sample points, if enough samples were available. Differences between two groups were considered significant if $p < 0.05$ (two tails).

Biological half-life:

When plotting the natural logarithm of the whole-body data versus time, a linear relation was found from day 7 onwards. From the slope (γ) of this line the biological half-life of the ^{65}Zn was calculated using $T_{1/2} = \ln 2 / \gamma$, expressed as % of the activity at t = 3h.

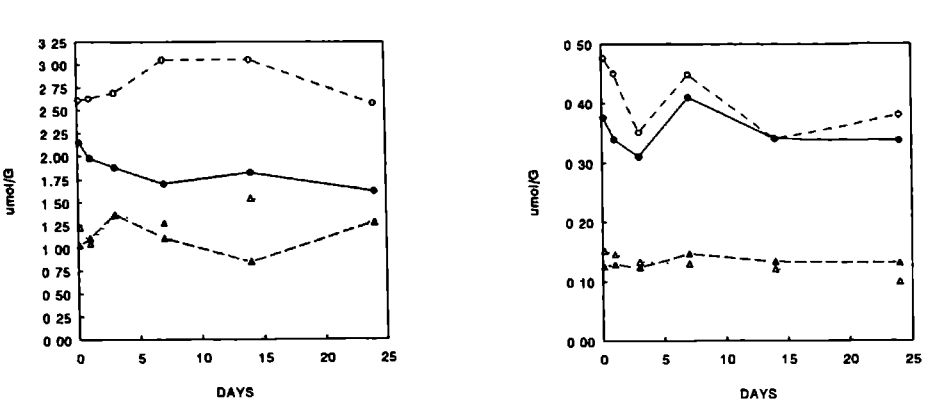


Figure 2: zinc deficiency experiment

2a: Left figure: zinc concentration of bone cortex (dots) and bone marrow (triangles) in zinc-deficient (closed dots , closed triangles respectively) and control rats (open dots, open triangles respectively). During zinc deficiency the concentration of zinc is severely decreased in bone cortex, but not in bone marrow.

2b: Right figure: zinc concentration of liver (dots) and muscle (triangles) in zinc-deficient (closed dots, closed triangles respectively) and control rats (open dots, open triangles respectively). This figure indicates that using a Wilcoxon Rank Sum test there is a very small but statistically significant decrease of zinc concentration in the liver, but not in muscle.

RESULTS

Zinc deficiency experiment

Serum Zn concentrations were significantly decreased after 7 days on the Zn deficient diet (day 0 in Figure 1a, 3 hours after the ^{65}Zn injection 15.15 versus 26.85 μmol/L) and after that, for a period of 30 days (day 24 in Figure 1a, 9.5 versus 28.5 μmol/L, Wilcoxon rank sum test $p = 0.0025$). In rats on the Zn-deficient diet Zn concentrations in bone cortex and liver were significantly decreased ($p < 0.01$ and <0.05 respectively), but not those in bone marrow and muscle ($p = 0.25$ and 0.9 respectively, Figure 2).

Radiotracer study: Zn-deficient rats retained more of the administered ^{65}Zn than control rats: the biological half-life was significantly increased (41 versus 154 days, $p < 0.001$, Figure 1b) compared to rats fed with adequate amounts of Zn. In Zn-deficient rats the

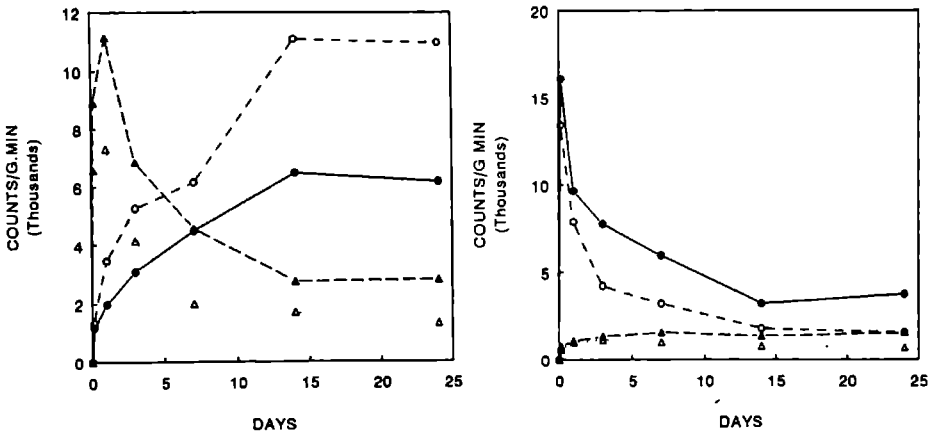


Figure 3: zinc deficiency experiment

3a: Left figure: the ^{65}Zn uptake and exchange of bone cortex (dots) and of bone marrow (triangles) in zinc-deficient rats (closed dots, closed triangles respectively) and control rats (open dots, open triangles respectively). During zinc deficiency the uptake of zinc by bone marrow is increased, but the uptake by bone cortex decreased compared to control rats.

3b: Right figure: the ^{65}Zn uptake and exchange of liver (dots) and of muscle (triangles) in zinc-deficient rats (closed dots, closed triangles respectively) and control rats (open dots, open triangles respectively). During zinc deficiency the uptake of zinc by liver and muscle is significantly increased compared to control rats.

accumulation of ^{65}Zn was higher in liver and bone marrow ($p = 0.0025$, both), and a higher retention was observed. This was also observed in muscle, but less pronounced ($p=0.005$). In contrast, in zinc-deficient rats the ^{65}Zn accumulation was reduced in bone cortex (Figure 3). Erythrocytes and MNC of Zn deficient rats accumulated and retained more ^{65}Zn ($p = 0.01$ and 0.05 respectively, Figures 4a and 4b), and the same picture - although less clear - was observed for PMNC for which the difference did not reach significance ($p = 0.2$, Figure 4c). All cell types showed a maximum value 3-5 days after ^{65}Zn administration, and they also showed an obviously slower release.

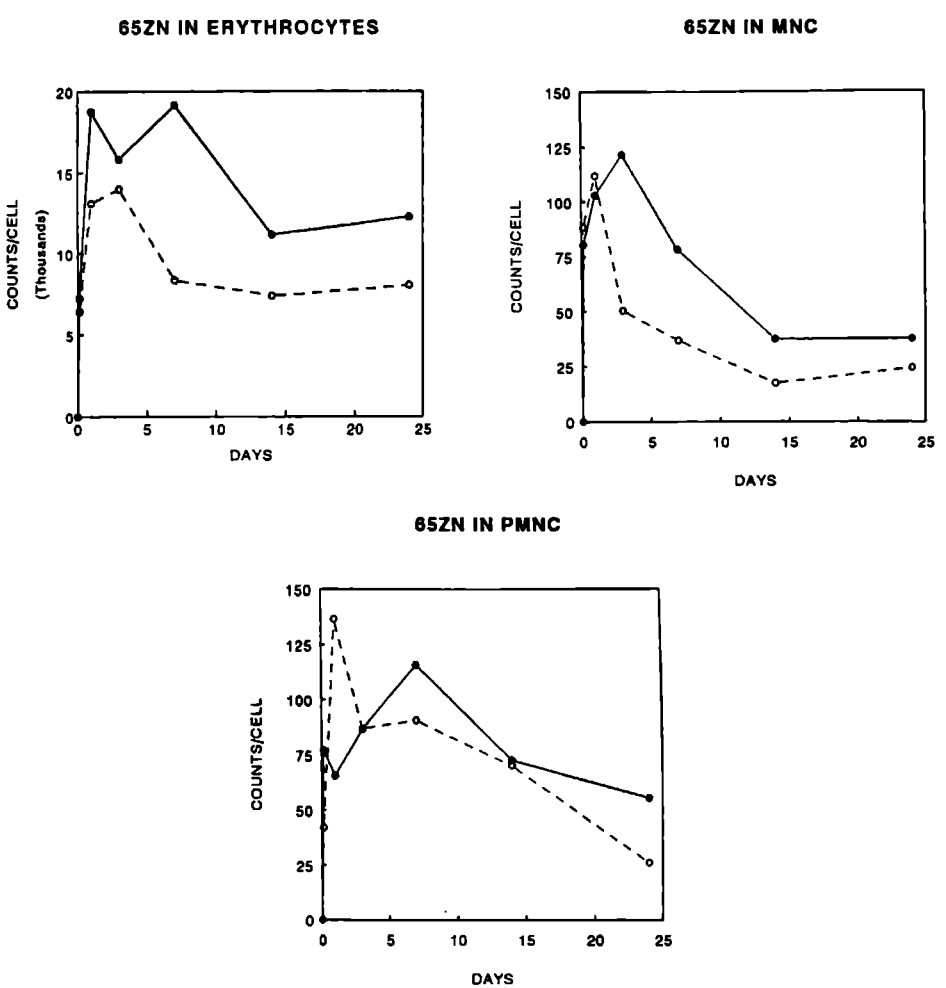


Figure 4: zinc deficiency experiment

4a: Upper left figure: the ^{65}Zn uptake and exchange of erythrocytes in zinc-deficient (closed dots) and control rats (open dots). During zinc deficiency the uptake of zinc by erythrocytes is significantly increased compared to control rats.

4b: Upper right figure: the ^{65}Zn uptake and exchange of MNC in zinc-deficient (closed dots) and control rats (open dots). During zinc deficiency the uptake of zinc by MNC is significantly increased compared to control rats.

4c: Bottom figure: the ^{65}Zn uptake and exchange of PMNC in zinc-deficient (closed dots) and control rats (open dots). During zinc deficiency the uptake of zinc by PMNC shows a tendency towards an increase compared to control rats.

Inflammatory stress experiment

In the animals with induced inflammatory stress, serum Zn concentrations decreased dramatically after the first day (Figure 5a), and a (significant although) less pronounced difference still existed later on. In the animals with induced inflammatory stress, the serum Cu concentrations increased significantly during the first few days reaching a maximum 7 days after administration of turpentine, which persisted throughout the experiment (Figure 5b).

No (significant) difference in whole-body retention of ^{65}Zn between the two groups (Figure 5c) was seen at any individual point or for the total period. The serum Zn concentration decreased during the experiment after turpentine injection.

In contrast, ^{65}Zn content was significantly increased in the liver until day 3 after turpentine injection, after which it decreased again (Figure 6a). In other compartments, like muscle and bone marrow (Figure 6b, 7b respectively), higher ^{65}Zn contents persisted longer, although in skin they did not remain so high for so long in skin (Figure 6c). On day 1 and day 7 the content of bone cortex was lower than in control rats (Figure 7a). No clear differences were observed for erythrocytes and MNC (Figures 8a and 8b), and in PMNC a tendency towards a lower content of ^{65}Zn was seen in the first four days after turpentine injection (Figure 8c).

DISCUSSION

Design of the experiments

The present experiments were designed to investigate the influence of Zn deficiency and inflammatory stress on the Zn exchange among various tissues and blood cells, by means of administration of a radioactive tracer: ^{65}Zn.

In experiment 1 a status of Zn deficiency could be induced by giving diets low in Zn (3). The zinc-deficient rats showed loss of hair. In experiment 2 a status of a decrease of serum zinc concentration not accompanied by symptoms of zinc deficiency was induced by administration of turpentine. By using the radio-active tracer ^{65}Zn we intended to measure the exchange of Zn in the various tissues and blood cells. We expected rapid changes of Zn in serum, and probably also of Zn in blood cells and other tissues, after the

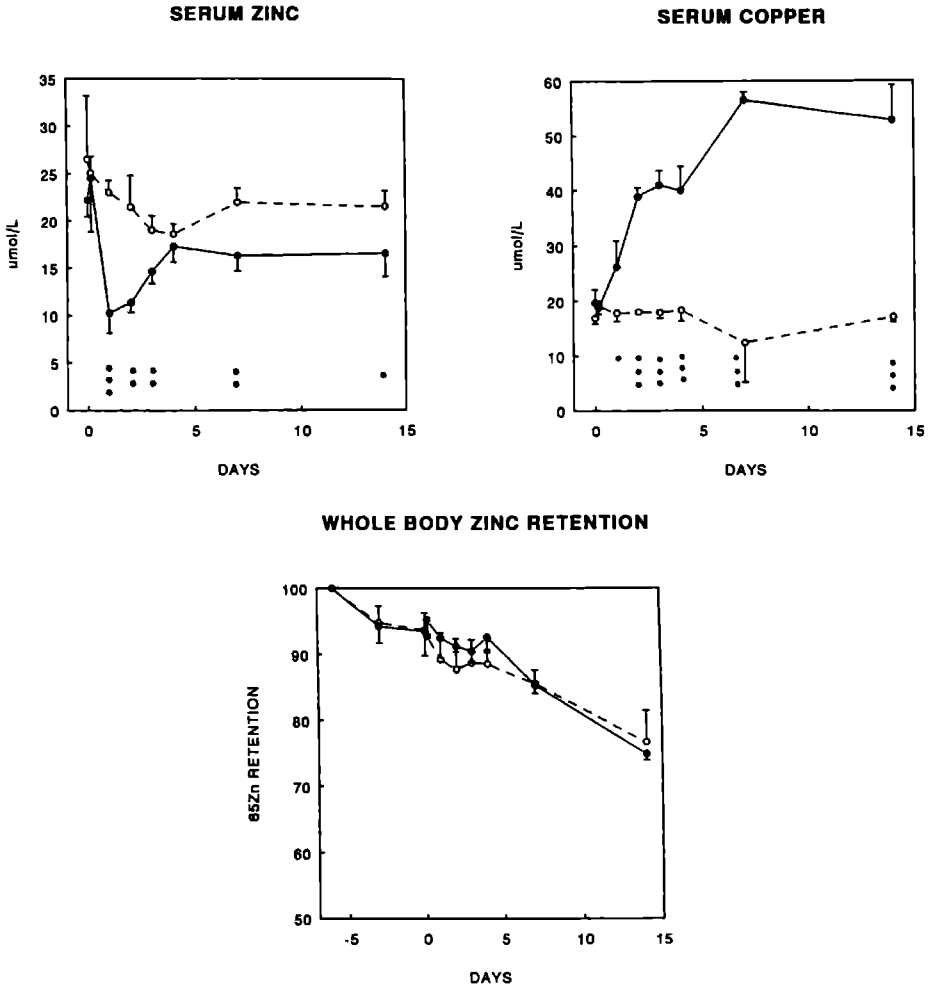


Figure 5: inflammatory stress experiment

5a : Upper left figure: serum zinc in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05;** p=< 0.01; *** p=<0.001). During inflammatory stress serum zinc is severely decreased after the first day. Later on this difference is less pronounced.*

5b : Upper right figure: serum Cu in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05; *** p=<0.001). Serum copper is increased during inflammatory stress.*

5c : Bottom figure: whole-body retention of ^{65}Zn in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations. No significant difference in whole-body retention is observed during inflammatory stress.

induction of inflammatory stress in contrast to slower shifts after induction of dietary Zn deficiency. The differences in physiologic response were demonstrated by the change in serum Zn concentration: in the Zn deficiency experiment serum Zn reached the lowest level after 33 days: 9.5 μmol/L. After turpentine injection the lowest Zn level was reached after only one day: 10.3 μmol/L. The intention was to provide ^{65}Zn when the serum zinc concentration was decreased. Therefore we used different time schedules of radiotracer administration in the Zn deficiency and the inflammatory stress experiments. In the Zn deficiency experiment ^{65}Zn was injected 7 days after induction of a deficient state. In the inflammatory stress experiment the injections of ^{65}Zn and turpentine could not be given simultaneously. The first days after an injection of ^{65}Zn rapid changes in ^{65}Zn content of the various body compartments were observed. We chose to administer ^{65}Zn prior to the induction of inflammatory stress.

By using this design the goals of this study could be attained as closely as possible.

Zinc uptake and retention in blood cells

The main objective of this study was to validate the observations made in vitro: the uptake of Zn by blood cells drawn from zinc-deficient rats is increased, contrary to blood cells drawn from rats with inflammatory stress. In the present study the in-vivo uptake of ^{65}Zn by blood cells in rats with Zn deficiency and in rats with inflammatory stress was measured. The uptake and retention of Zn by erythrocytes and MNC were increased in case of real Zn deficiency compared to control rats. No such difference could be demonstrated in case of inflammatory stress. The pattern of uptake of ^{65}Zn by blood cells of zinc-deficient rats suggested that a maximum is reached later than in control rats. This can be explained by the simultaneous rapid and enormous uptake of ^{65}Zn by liver and bone marrow. Following the time-course of the inflammatory experiment, in which serum Zn was severely depressed, no differences were observed between erythrocytes and MNC of rats with inflammatory stress and those of control rats. Even less ^{65}Zn was retained in PMNC isolated from rats with inflammatory stress. As far as we know, this is the first report of findings concerning the in-vivo exchange of ^{65}Zn by blood cells.

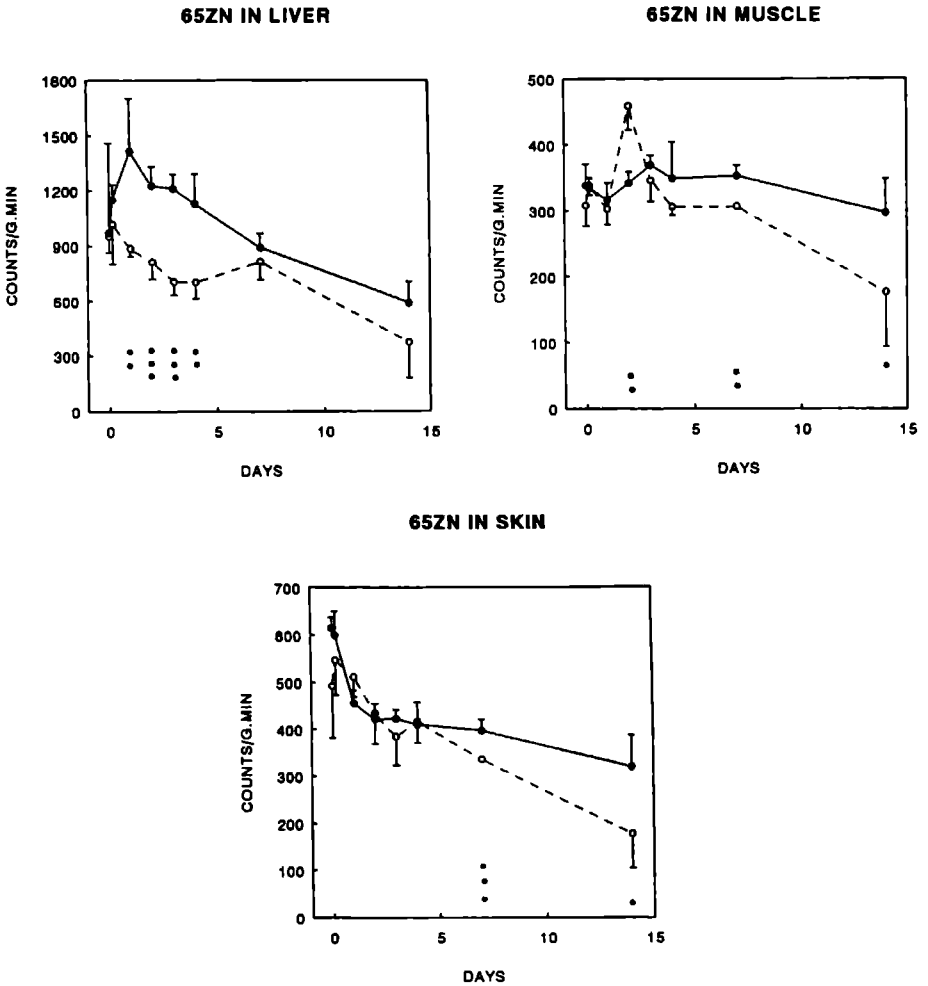


Figure 6: inflammatory stress experiment

*6a: Upper left figure: the ^{65}Zn activity of liver in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (** p=< 0.01; *** p=<0.001). During the first days of inflammatory stress the ^{65}Zn content of the liver is significantly increased.*

6b: Upper right figure: the ^{65}Zn activity of muscle in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05;** p=< 0.01). In muscle the ^{65}Zn content is significantly increased during inflammatory stress.*

6c: Bottom figure: the ^{65}Zn activity of skin in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05; *** p=<0.001). After the first days of inflammatory stress the ^{65}Zn content of the skin is significantly increased.*

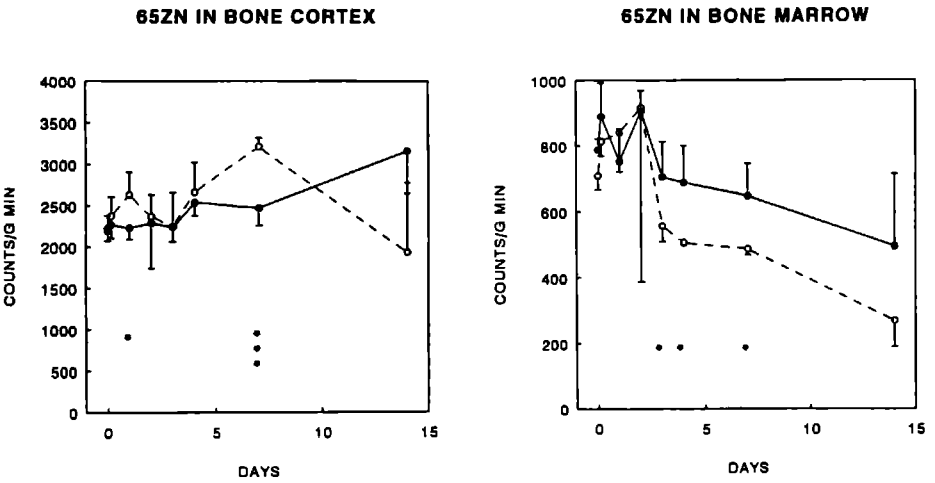


Figure 7: inflammatory stress experiment
7a: Left figure: the ^{65}Zn activity of bone cortex in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05; *** p=<0.001). On day 1 and day 7 the ^{65}Zn content of bone cortex is decreased.*
7b: Right figure: the ^{65}Zn activity of bone marrow in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05). In bone marrow the ^{65}Zn content is significantly increased after three days.*

Whole-body zinc retention

The higher ^{65}Zn retention in Zn-deficient rats indicated that the rats were Zn deficient. These rats showed loss of hair, one of the classic symptoms of Zn deficiency (1). In the induced inflammatory stress experiment, no difference in ^{65}Zn retention was seen between rats with stress and control rats. In these stressed rats no signs of Zn deficiency were observed, although the serum Zn concentration was severely depressed. The absence of difference in total body ^{65}Zn retention and of classic signs of Zn deficiency in the rats with inflammatory stress both indicated that in these rats only apparent Zn deficiency was present.

Serum copper concentration

In rats with induced inflammatory stress serum Cu concentrations were elevated.

This phenomenon, which is known to be caused by an increase of the Cu-containing protein ceruloplasmin, has been reported before (8). The long-persisting increase of serum Cu validated our inflammatory stress model, and it did indeed indicated that the experimental model resulted in a long-lasting inflammation, although after 3 days the serum Zn concentration partially recovered.

Zinc uptake and exchange by tissues

Concentration of zinc in bone, muscle and liver are the main parameters, because these organs account for the greater part of the total body zinc content (9). Bone marrow, liver and muscle retained more ^{65}Zn, unlike bone cortex in which less ^{65}Zn was retained in Zn-deficient rats. We could demonstrate that bone cortex and bone marrow should be separated before Zn determination in Zn deficiency experiments because of their different behaviour during Zn deficiency. This was also demonstrated after measurement of total Zn concentrations in tissues. During Zn deficiency, the Zn concentrations in bone cortex decreased, although no clear changes were observed in bone marrow, muscle or liver, indicating that especially bone marrow - and also muscle - are spared the effect of Zn deficiency for a long period. Bone cortex is the principal source of Zn in case of zinc deficiency. Changes in concentrations of total Zn in various tissues during Zn deficiency are described in some studies (9,10,11,12), but in all of these studies only once and on a fixed day (9,11,12) or in a special situation (signs of severe zinc deficiency (10)) tissue samples were collected. Prospective studies in which consecutive tissue samples are collected, have not been performed. In all studies the concentrations are decreased in serum (plasma) and in bone, but not in other tissues. For example: O'Dell et al (10) found no decrease of Zn in muscle, liver, skin and brain; Giugliano et al (11) reported unchanged zinc concentrations in muscle, spleen and thymus and lowered zinc concentrations in liver, kidney, testis, intestine and especially plasma and bone; Roth and Kirchgessner (12) reported a decrease of 72% in serum, 65% in femur, 34% in pancreas, 23% in muscle, 11% in testis and 7% in brain. Van de Wouwe et al (9) determined the zinc content of tissues after 21 days and showed a decrease in pancreas, tibia and muscle, and an increase in spleen but no changes in liver, kidney, cerebrum and cerebellum. Gupta et al (13) demonstrated a decrease of Zn in liver, testis and kidney. Van de Wouwe et al (14) demonstrated a decrease of Zn only in liver and bone, but the control rats were injected with saline which will also induce a stress reaction.

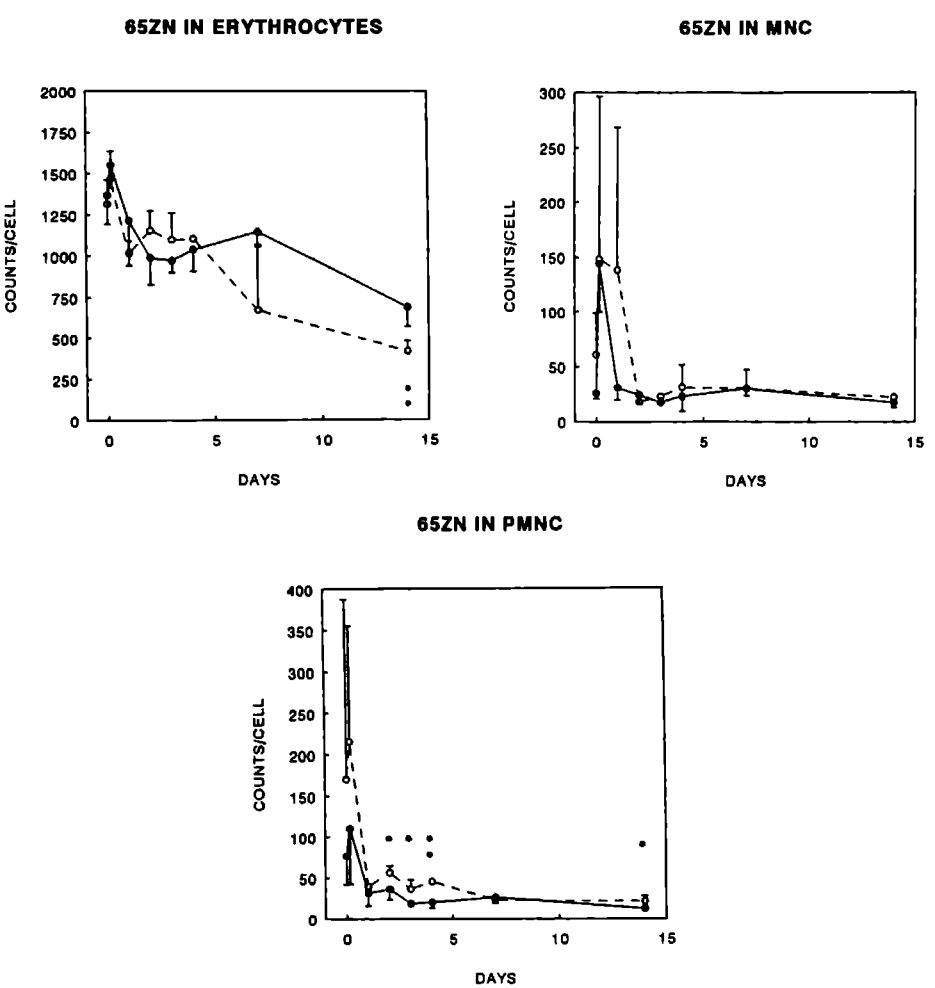


Figure 8: inflammatory stress experiment

*8a: Upper left figure: the ^{65}Zn activity of erythrocytes in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (** p=<0.01). Only on day 14 a higher ^{65}Zn content was observed in erythrocytes of rats with inflammatory stress, but the Wilcoxon Rank Sum test did not demonstrate a difference for the total period.*

8b: Upper right figure: the ^{65}Zn activity of MNC in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations. No differences in ^{65}Zn content were observed.

8c: Bottom figure: the ^{65}Zn activity of PMNC in rats with inflammation (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=<0.05; ** p=<0.01). In PMNC the ^{65}Zn content was decreased in rats with inflammatory stress on days 2, 3, 4, and 14.*

In contrast to the situation of zinc deficiency, ^{65}Zn was given 7 days prior to the induction of stress, in other words ^{65}Zn was assumed to be more or less in equilibrium with total (cold) Zn. Therefore, changes in ^{65}Zn activity in tissues represent redistribution of Zn. During inflammatory stress we measured a redistribution of ^{65}Zn activity to the liver. Later on during the experiment a tendency toward increase of ^{65}Zn retention in muscle, bone marrow and skin was observed. No such increase was found in bone cortex. It might be hypothesized that during inflammatory stress a partial recovery of the serum Zn concentration is due to (a combined action of) an initial but brief redistribution of zinc to the liver and a persistent decrease of serum albumin, the main zinc-transporting protein (16). This hypothesis is supported by the present experiment in which the decrease of serum zinc occurred simultaneously with the decrease of the activity of ^{65}Zn in the liver. Later on higher ^{65}Zn activity was measured in muscle and skin. Therefore, the smaller decrease after day 3 was probably due to a combination of a decrease of serum albumin and redistribution to other tissues like muscle and skin. A limited number of studies have been performed during inflammatory stress and most of these were limited to short-term effects (14,15). Tufft and Nockels (16) and Klasing (17) demonstrated an increase of weight and Zn concentration of the liver and spleen during a longer-lasting bacterial infection. In an acute inflammatory model Pakarek et al (15) demonstrated an increase of zinc content in liver and a decrease in kidney after intraperitoneal injection of leucocytic endogenous mediator (LEM). No significant changes were observed in the GI tract, pancreas, heart, lungs or spleen. Van de Wouwe (14) demonstrated only marginal differences after endotoxin exposure, but the control rats were also injected - with saline -, which will induce an acute stress reaction.

Conclusions

The present in-vivo studies demonstrate that the zinc metabolism of the tissues is different during inflammation and during zinc deficiency, probably to adapt to the different needs of these tissues in both situations. This is also demonstrated by the increase of zinc uptake by blood cells during zinc deficiency, which was not demonstrated during inflammation. By using blood cells, it is possible to distinguish between situations with real and with apparent Zn deficiency.

We also demonstrated that during inflammation the serum Cu concentration was increased. In theory the distinction between real and apparent Zn deficiency can also be

made by the combined determination of serum Zn and Cu: in real Zn deficiency serum Zn concentrations are decreased and serum Cu is normal; in inflammatory stress serum Zn concentrations are decreased, and serum Cu concentrations increased. In clinical practice a combination of both conditions is frequently present: for instance in patients with active Crohn's disease the decrease of serum Zn is partly explained by an increase of zinc loss, but also partly by a decrease due to inflammatory stress. In case serum Cu is increased and serum Zn decreased, this indicates that the decrease of serum Zn is partly caused by inflammatory stress. But these results do not provide an answer to the question whether a real Zn deficiency partly causes the decrease of serum zinc.

Therefore, it is necessary to find a method that demonstrates real zinc deficiency. The present experiments support and provide a physiologic basis for earlier studies. If in experiments could be demonstrated that the **in vitro** ^{65}Zn uptake by blood cells of Zn-deficient rats is also increased and not in rats with an inflammation, this would be of value to determine real zinc deficiency.

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IN VITRO ZINC UPTAKE BY BLOOD CELLS IN RATS WITH ZINC DEFICIENCY

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ABSTRACT

Several methods are used to determine the zinc status; all these methods have their limitations. The serum zinc concentration is influenced by the zinc status, but also by the serum albumin concentration and inflammatory stress. The results of studies on the zinc concentration of blood cells in zinc deficiency are conflicting, probably in part because of technical and analytical problems.

The aim of this study was to investigate, under standard conditions, the uptake of ^{65}Zn-labeled zinc by blood cells, taken from rats with alimentary zinc deficiency and, as a pilot experiment, from rats in which an inflammation is induced. In both conditions the serum zinc concentration is reduced. In clinical practice this makes it difficult to determine whether the decrease of serum zinc is the result of a real or an apparent zinc deficiency. In stress, like in inflammatory disease, the decrease of zinc reflects an apparent zinc deficiency resulting from redistribution of serum zinc into the liver and because of decrease of serum albumin concentration. Over 70% of the serum zinc is bound to albumin. Blood cells from zinc-deficient and control rats were isolated using a discontinuous Percoll gradient and incubated under nearly physiologic conditions in a ^{65}Zn containing medium. A significant increase of the in vitro uptake of ^{65}Zn labeled zinc by the blood cells of zinc-deficient rats was seen: erythrocytes 1.3, mononuclear cells 2.0 and polymorphonuclear cells 2.6 times the control values. After induction of inflammation blood was drawn only after 2 days as a pilot experiment. No change in ^{65}Zn labeled zinc uptake by erythrocytes and mononuclear cells was demonstrated , although the serum zinc and albumin concentrations were decreased but a small but significant increase of zinc uptake by polymorphonuclear cells was observed. This study of ^{65}Zn uptake in vitro under standard conditions may prove of value for distinguishing in patients real zinc deficiency from apparent zinc deficiency owing to, e.g., stress, although additional experiments should be performed.

INTRODUCTION

Zinc is one of the most prominent trace elements in humans. It is known to be essential for mammals since 1934 (1). Its deficiency causes hair loss, acrodermatitis enteropathica and other skin lesions (2), delayed wound healing (3), dysfunction of the immune system (4,5) and growth retardation (6). Zinc deficiency is a complication in many pathological conditions. However, no good method is available for detection of real zinc deficien-

cy. One of the best parameters is the zinc concentration in various tissues, but this method is not suitable in medical practice. Neither is whole-body retention of ^{65}Zn as a function of time. Blood cells are a kind of tissue and are easy to obtain. Attempts have been made to determine the zinc concentration in blood cells, but much blood is needed to be able to determine the zinc concentration and the risk of zinc contamination is great. Therefore, it is not surprising that the results are conflicting. Controversy exists about the question of whether zinc deficiency leads to an the increase (7) or decrease (8) in intracellular zinc concentrations. A decrease of granulocyte zinc has been observed after zinc supplementation (9).

The aim of this study was to investigate the in vitro uptake of ^{65}Zn labeled zinc by various blood cells in physiological conditions in rats. This method was applied to cells (obtained by discontinuous Percoll gradient) of zinc-deficient rats in comparison to those of control rats. The induction of dietary zinc deficiency in the rats was monitored by the whole-body retention of ^{65}Zn and by serum zinc concentration. In various forms of stress, serum zinc concentration is low as a result of redistribution of zinc from serum to the liver rather than of deficiency. In separate experiments, we therefore studied the zinc uptake of blood cells taken from rats in which an inflammation in the hind legs was induced and compared the results with those found in real zinc deficiency.

The method of measurement of the in vitro uptake of zinc by blood cells is a combination of a modified cell separation method described by Milne et al (10) and a modification of methods to determine the in vitro uptake of ^{65}Zn by erythrocytes or whole blood (11,12,13,14).

METHODS

Animals

Male Wistar rats of 200 g were housed separately in stainless-steel metabolic cages. The diet on which the rats were raised (SMRA) contains a variable and sometimes an excessive amount of zinc. Therefore, in experiments 1, 2 and 3 a diet (IRI-OB) (table 1 (15)), containing 12 mg/kg zinc was given to the rats during the 2 wk before the experiment to adapt the animals to this diet and to reduce the excess of zinc in their depots. This zinc concentration is considered sufficient to maintain a normal zinc status (16). The guidelines for the care and use of laboratory animals in the Netherlands were followed.

Table 1

Composition of the IRI-OB diet

Ingredients	Concentration (g/100g)
Glucose	50.65
Corn starch	15.0
Ovalbumin	20.0
Sun flower seed oil	4.0
Fibre (cellulose)	5.0
Choline chloride	0.3
Mineral mix	
Sodium diphosphate	1.5
Potassium chloride	0.7
Calcium carbonate	1.0
Magnesium sulphate	0.5
Sodium meta-silicate	0.25
Trace element salt mix	0.1
Vitamin mix in glucose	1.0

Trace element salt mix	Concentration (mg/kg)
Mn	55
Fe	66
Zn	various conc.
Cu	9.3
Ni	2.4
Cr	1.8
Mo	0.2
Se	0.36
As	0.13
Co	0.2

Materials

The diet (IRI-OB) was produced by Hope Farms (Woerden, the Netherlands) using components low in zinc (table 1,(15)). In the zinc-sufficient food zinc was added as sulphate to a final zinc concentration of 12 mg/kg. A sufficient quantity of other trace elements

was added. The Cu concentration in both versions of the diet was 9 mg/kg. The commercial diet SMRA (Hope Farms, Woerden, the Netherlands) contained 92 mg/kg Zn and 25 mg/kg Cu.

Stainless-steel needles and plastic syringes (Monoject, Sherwood, USA) with 10 μL of preservative-free heparin (50 IU) were used. All solutions were prepared with deionized distilled water (resistance > 15 MΩ), processed through a Millipore system (Bedford, Massachusetts, USA).

Only polypropylene pipet tips, plastic tubes (15 mL polystyrene, Greiner, Solingen, Germany), 50 mL polypropylene (Falcon, Becton Dickinson and Company, Lincoln Park, New Jersey, USA) and bottles (0.8 L, NUNC, Roskilde, Denmark) were used. All chemicals were of analytical grade (Merck, Darmstadt, Germany). Percoll was supplied by Pharmacia (Uppsala, Sweden). Hepes was obtained from Sigma (St Louis, MO, USA), albumin from Behring (Marburg, Germany), Hanks' balanced salts without Ca and Mg from Flow Laboratories (Rickmansworth, Herts, U.K.) and the radiotracer ^{65}Zn from Amersham International (Amersham, Buckinghamshire, U.K., code ZAS 2 , 3.7-37 GBq mg of Zn as chloride in 0.1 mol/L HCl). No detectable zinc could be determined in solutions and in ion-free water put into tubes and bottles apart from a low zinc concentration in diluted Percoll (2.56 μmol/L).

Zinc was determined by atomic absorption spectrophotometry (AAS, Perkin Elmer 5000; Norwalk, Connecticut, USA). The lower limit of detection was 0.28 μmol/L, and the response was linear to at least 15 μmol/L. Standard reference containing $ZnCl_2$ (Tritisol, Merck, Darmstadt, Germany)

was used in the concentrations expected to be found in the samples. Cells were counted by a Coulter counter Model ZM (Luton, Beds., UK), adjusted to the size of the cells to prevent counting of cell fragments. ^{65}Zn was measured with an NaI crystal with an automatic sample changer (LKB, Wallac 1282 Compugamma; Turku, Finland). The counting time was adjusted to assure a statistical error of less than 0.3%. A tube with medium but without blood cells, incubated and washed according the procedure of the cell containing tubes, served as a blank.

Total body radioactivity of the rats was counted with a shadow shield counter consisting of two Tl-activated NaI crystals (4 x 6 inch), both at a distance of 45 cm from the rat, connected to a Nuclear Data multichannel analyzer N.D. 60 A (Nuclear Data; Schaumburg, Illinois, USA). The first determination of the ^{65}Zn whole-body retention was performed 4 h after the ip injection of ^{65}Zn, and the second after 24 h. The results of the

^{65}Zn whole-body retention at the successive days were expressed as percent of the activity after 24 h rather than after 4 h because the losses during the first 24 h were rather erratic.

Solutions

Glucose Phosphate Buffer (GPB):
13.5 mmol/L Na_2HPO_4, 3.3 mmol/L KH_2PO_4, 5.4 mmol/L KCl, 133 mmol/L NaCl and 5 mmol/L glucose in ion-free water and if necessary adjusted to pH 7.4 with HCO_3^-.

Erythrocyte lysing buffer:
155 mmol/L NH_4Cl and 10 mmol/L $KHCO_3$ in ion-free water, pH adjusted to 7.4 with HCO_3^-.

Incubation Medium:
145 mmol/L NaCl, 6 mmol/L KCl, 2 mmol/L $MgCl_2$, 2.4 mmol/L $CaCl_2$, 7.6 μmol/L $ZnCl_2$, 5.6 mmol/L glucose, 10 mmol/L Hepes and albumin 40 g/l in ion-free water tracered with ^{65}Zn and adjusted to pH 7.4 with CO_2.

Procedures

Discontinuous Gradient of Percoll

Three solutions of Percoll of different density (1.060, 1.075, and 1.095 kg/L, respectively) (10) were prepared by dilution with Hanks' balanced salts solution without Ca and Mg. Six millilitres of a heavier solution were layered in a 50-mL tube underneath the lighter solution using a stainless-steel needle of 15 cm and a plastic syringe.

Isolation procedure of the blood cells

Cell separation was performed using a discontinuous Percoll gradient. All solutions were kept at 4°C to prevent clumping and zinc exchange by the cells. The method described by Milne et all (10) was modified, because blood cells of rats clot easily. Blood was drawn from anaesthetized rats by heart puncture using stainless-steel needles and plastic syringes with 10 μL of preservative-free heparin (50 IU). Blood was diluted 1:3 with GPB. The Percoll solutions of different densities were layered underneath the diluted blood.

Subsequently, the tube was centrifuged for 20 min, at 500 x g at 4°C in a swing-out rotor without brake (Heraeus Crist Minifuge GL, Osterode am Harz, West Germany). This resulted in separated layers of platelets, mononuclear cells (MNC), polymorphonuclear cells (PMNC) and erythrocytes. The MNC, PMNC and erythrocytes were harvested using a pipet and resuspended in 5 mL GPB in a 15 mL tube. Erythrocyte contamination of the MNC and PMNC fractions was removed by twice resuspending the cells for 10 min. at 4°C in 5 mL of erythrocyte lysing buffer and subsequent washing steps with 5 mL of GPB After lysing and washing steps the tube was centrifuged for 10 min., 200 x g, 4°C with brake. Cells were resuspended in 1 mL of GPB for cell counting. The erythrocytes were washed twice and centrifuged for 10 min, 200 x g, 4°C with brake. At random, Cytospin preparations (Shandon, Astmoor, UK) were made to check purity of the cell suspensions. The cell suspensions contained less than 10% contamination of other cells.

Incubation procedure of the blood cells

The incubation was performed in physiologic conditions. Volumes corresponding to 3.75×10^6 MNC and 1.25×10^6 PMNC (the mean concentration of blood cells in 1 mL of blood in rat) were taken and centrifuged. Supernatant was discarded and 1 mL of incubation medium added. In case of erythrocytes, 0.4 mL of the concentrated suspension was added to 0.6 mL of the incubation medium. Incubation was carried out in a shaking bath for 1 h at 37°C and was stopped by adding 5 mL of cold (4°C) NaCl 0.9%. The cells were centrifuged and resuspended four times using cold NaCl 0.9%. The last time the cells were resuspended in 1 mL of GPB The volume was determined from the weight difference of the tube. Number of cells and radioactivity were counted. The whole procedure was performed in 1 d.

Calculations and statistics

Calculation of the uptake of zinc:

Since the zinc concentration (Zn_m : 7.6 μM) and the radioactivity (R_m) of the incubation medium per millilitre are known, and the number of cells (N_c) and the radioactivity (R_c) of a cell sample are measured, the zinc uptake of the cells (Zn_c) can be calculated using the expression:

$$\frac{R_c}{R_m} \times 7.6 \times \frac{10^6 \text{ or } 10^9}{N_c} = Zn_c \text{ in nmol Zn/ } 10^6 \text{ or } 10^9 \text{ cells}$$

Statistics:

The Student's t-test was performed; differences between two groups were considered significant if $p < 0.05$ (two tails).

Calculation of the biological half-life:

When plotting the natural logarithm of the whole-body data versus time, a linear relation was found from day 7 on. From the slope (γ) of this line the biological half-life of the ^{65}Zn was calculated using $T_{1/2} = \ln 2 / \gamma$.

Design of the experiments

Experiment 1: ^{65}Zn whole-body retention in zinc deficiency.

After adaptation (see Animals), 16 rats were injected ip with 74 kBq (2 μCi) ^{65}Zn (day 0). Eight rats received the Zn-adequate food (IRI-OB; 12 mg/kg Zn), and eight others Zn-deficient food (<2 mg/kg Zn). The control group received pair feeding throughout the experiment.

Whole-body counting was performed on days 0, 1, 2, 5, 9, 14, 21 and 28. Three mL of blood were taken under anaesthesia by heart puncture on day 0, 7, 14, 21, and 28. The serum was analyzed for albumin and Zn by AAS.

Experiment 2: ^{65}Zn uptake by MNC, PMNC and erythrocytes in zinc deficiency.

After adaptation (see Animals), four rats were fed the IRI-OB food, whereas four others received the Zn deficient variant of this food. Blood samples were taken as above on days 0, 10, and 31, and used for isolation of blood cells that were used for incubation.

Experiment 3: ^{65}Zn uptake by erythrocytes in zinc deficiency.

After adaptation (see Animals), eight rats were fed the IRI-OB food, and eight others the Zn-deficient variant. Blood samples, now 1.5 mL, were taken as above on days 0, 6, 13, 20, and 27, and used for isolation of erythrocytes only. These cells were also used for incubation with ^{65}Zn.

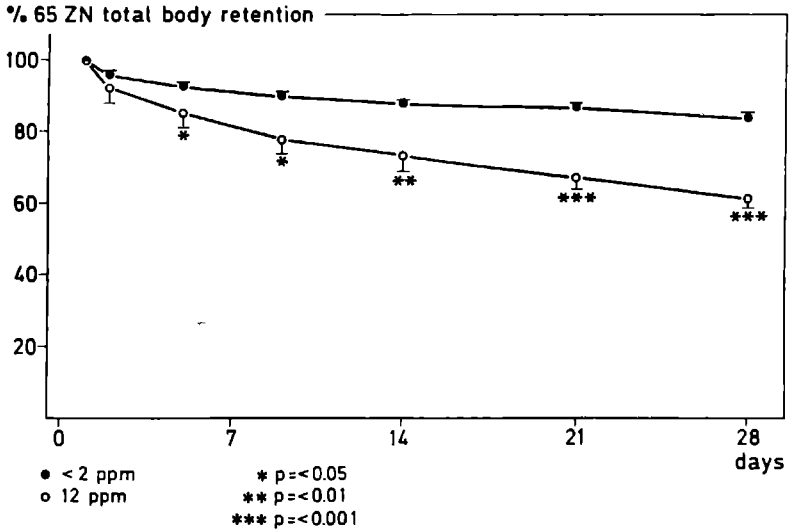

Figure 1 : Whole-body retention of ^{65}Zn in zinc-deficient (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). The whole-body retention of ^{65}Zn 1 d after ip injection was taken as 100%. The error bars indicate SD (p=<0.05; ** p=< 0.01; *** p=<0.001).*

Experiment 4: ^{65}Zn uptake by blood cells in stress.

Eight rats (not adapted to the IRI-OB food) received the commercial diet during the experiment. On day 0, four rats were injected im in the hind legs with 0.5 mL turpentine. Four rats served as controls. On day 2, 3 mL of blood were withdrawn for blood cells and in vitro ^{65}Zn uptake. Serum was used to measure zinc and albumin concentrations. This experiment was performed twice.

RESULTS

The whole-body retention of ^{65}Zn is normally calculated as a percentage of injected dose immediately after injection. Because two rats lost a significantly greater quantity of ^{65}Zn (52% and 36%) compared to the other rats (5.55% sd 4.42%) in faeces and urine (not separately collected) during the first 24 hours, the whole-body retention at day one is taken as 100%. Probably part of the ^{65}Zn was injected in the gut. In experiment 1 zinc-deficient rats retained more ^{65}Zn compared to control rats (Figure 1, $T_{1/2}$ = 176 d versus

$T_{1/2}$ = 51 d). The ^{65}Zn whole-body retention confirmed the difference in zinc status between the rats. Serum zinc decreased gradually (Figure 2). On day 28 the serum zinc concentration was 40% of that of control rats. No change was seen in the serum albumin concentration in the zinc-deficient rats.

The results of experiment 2 and 3 are presented in Figures 3, 4, and 5. In all types of blood cells of zinc-deficient rats the in vitro zinc uptake was increased. In erythrocytes and PMNC, significant differences were seen after 13 and 10 d respectively. The zinc uptake by erythrocytes in experiment 3 confirmed the results of experiment 2 (results not shown). In MNC, differences were seen at 31 d. After 4 wk the increase of the zinc uptake by PMNC, MNC, and erythrocytes reached peaks of 2.6, 2.0, and 1.3 times the control respectively.

Subsequently the influence of stress was tested (experiment 4). Serum zinc was reduced by 47% and serum albumin by 18% (Figure 6). The uptake of zinc by erythrocytes and MNC did not show any difference (Figure 7), but a significant difference was seen in the uptake of zinc by PMNC: in the case of inflammation 14.8 ± 2.0 pmol/10^6 cells and in control rats 8.8 ± 1.9 pmol/10^6 cells. These results were confirmed in a second experiment.

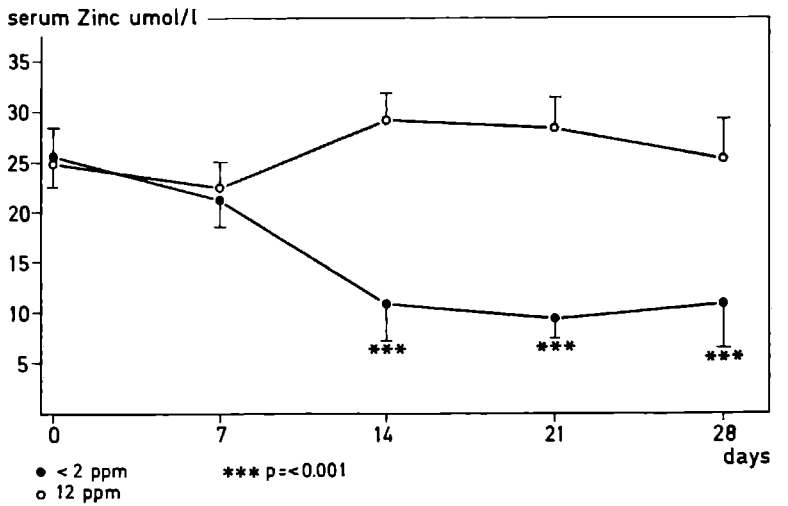

*Figure 2 : Serum zinc in zinc-deficient (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). In zinc-deficient rats serum zinc declined; a significant difference was reached after 14 d. The error bars indicate SD (*** p=<0.001).*

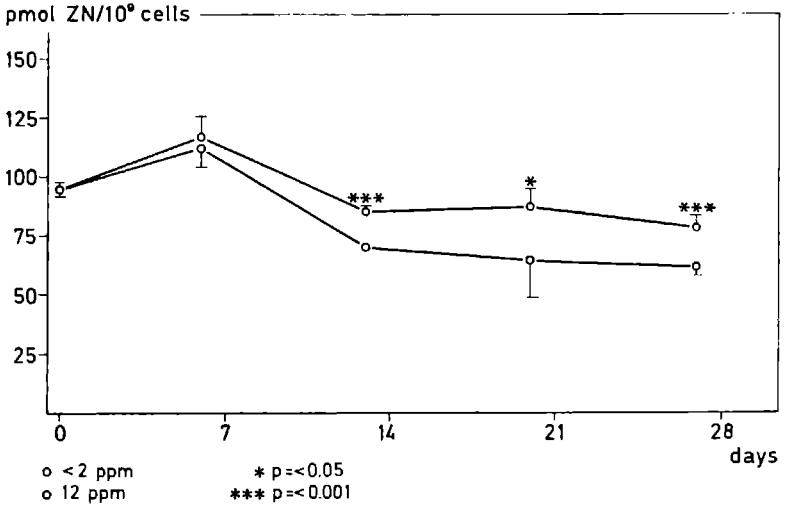

Figure 3 : Zinc uptake by erythrocytes of zinc-deficient (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). In vitro the erythrocytes drawn from zinc-deficient rats take up more zinc after 14 d. Approximately 0.08 pmol Zn/10^6 cells was taken up by erythrocytes. The error bars indicate SD (p=<0.05; *** p=<0.001).*

DISCUSSION

The zinc concentrations in blood cells in cases of zinc deficiency have been determined, but the results are conflicting. Some authors describe a decrease (7) and others an increase (8) in the intracellular zinc concentration. Also, a decrease of the intracellular zinc concentration after zinc supplementation has been described (9). One of the reasons for these conflicting results is that it is not clear if these persons were really zinc deficient. Another explanation is that zinc concentrations in MNC and PMNC suspensions are low. Using 3 mL of blood and with a cell recovery of 50%, the final zinc concentration of the cell suspension is about 0.3 μmol/L when resuspended in 0.5 mL GPB, which is near the lower limit of detection of an AAS determination. Also the risk of zinc contamination of the suspension must be considered. Therefore, determination of zinc in blood cells is difficult (17). To our knowledge, a decrease of the concentration of zinc in blood cells in zinc-deficient animals has never been demonstrated. We therefore studied the in vitro uptake

of ^{65}Zn-labeled zinc by blood cells taken from zinc-deficient rats and rats with an inflammation to determine the need for zinc.

The reason why we studied both zinc-deficient rats and rats with an inflammation is that, in both conditions, the serum zinc concentration is decreased. This makes it difficult to determine whether the decrease of serum zinc concentration is the result of a real or an apparent zinc deficiency. In stress, like in inflammatory disease, the decrease of serum zinc (18,19) reflects an apparent zinc deficiency, because zinc is redistributed from the serum to the liver and because the serum albumin concentration decreases. Over 70% of serum zinc is bound to albumin (20), and therefore, a decrease of the serum albumin concentration will result in a decrease of the serum zinc concentration.

In our experiments, whole-body retention of ^{65}Zn as a function of time served as an indicator of the zinc status. A significant difference in whole-body ^{65}Zn retention was seen between the rats on a diet with less than 2 mg/kg zinc and rats consuming food containing

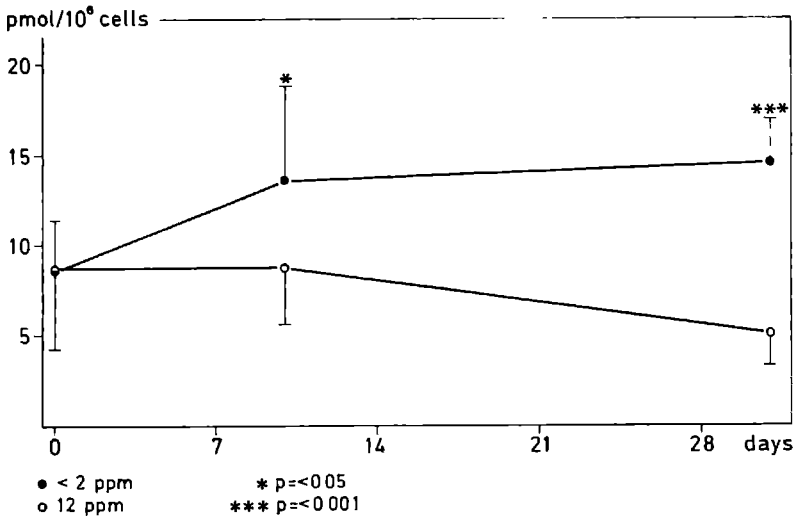

*Figure 4 : Zinc uptake by polymorphonuclear cells of zinc-deficient (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). At day 31 the PMN cells drawn from zinc-deficient rats took up 260% of the zinc taken up by cells drawn from rats in the control group. Approximately 10 pmol Zn/10^6 cells is taken up by PMN cells. The error bars indicate SD (*p=<0.05; ***p=<0.001).*

12 mg/kg zinc, showing that, indeed, the experimental animals had become zinc deficient (Figure 1). Significant differences between the two groups of rats were also found in serum zinc (Figure 2) and in the in vitro uptake of zinc in the three cell types tested (Figure 3, 4, and 5), but particularly in the PMN cells.

The uptake of ^{65}Zn labelled zinc by blood cells has been studied before (11, 12, 13, 21, 22), and the partly preliminary results of these studies point in the same direction as our study. However, the in vitro uptake of zinc by the three blood cell types in a standard medium as in our experiment has never been studied. Two of the studies are only published in abstract without necessary details (13,22). Moreover, In one of these studies only the in vivo uptake was studied, and in the other only the results of the uptake of zinc by erythrocytes was described. The in vitro uptake of ^{65}Zn by whole blood has been examined by Berry et al. (11) and Chester and Will (21). The zinc uptake by blood cells of zinc-deficient rats was higher than in zinc-sufficient rats, but the increase of ^{65}Zn uptake may have been owing in part to a decrease of serum zinc concentration: a higher specific acti-

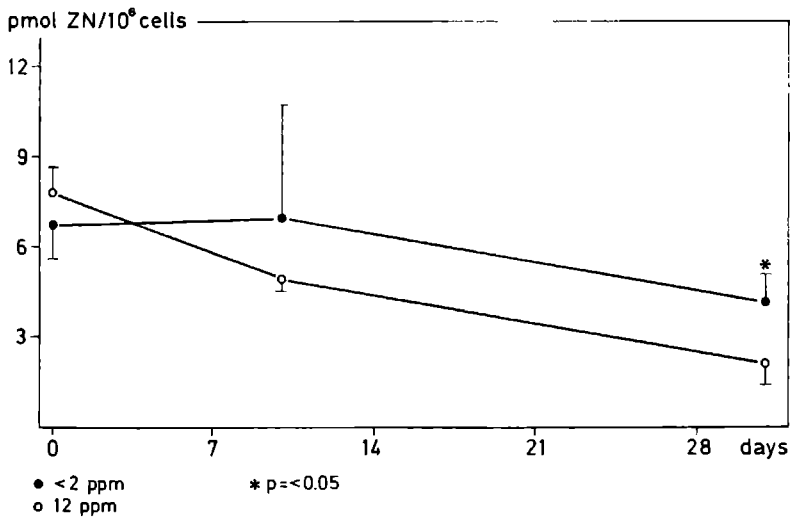

Figure 5 : Zinc uptake by mononuclear cells of zinc-deficient and control rats. Approximately 8 pmol Zn/10^6 cells were taken up by MNC. Closed dots represent rats with <2 mg/kg in the diet and the open dots represent the zinc-sufficient group (diet with 12 mg/kg). The error bars indicate SD (p=<0.05).*

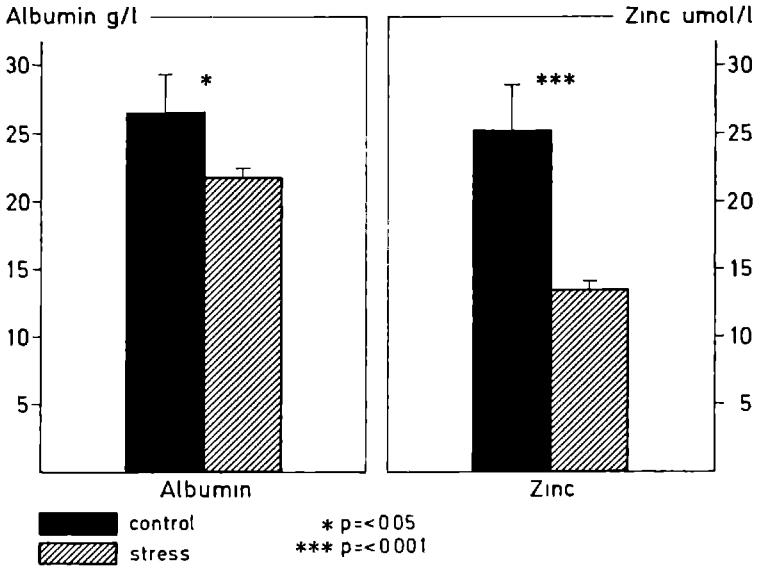

Figure 6 : Serum albumin and serum zinc in turpentine induced stress after 2 d. The serum albumin and zinc concentration was decreased by 18% respectively 47% 2 d after inducing the inflammation. The error bars indicate SD. The closed bars indicate control group and the striped bars the stress group. The error bars indicate SD (p=<0.05; *** p=<0.001).*

vity of ^{65}Zn would cause an increased ^{65}Zn uptake, even when the uptake of total zinc is the same. The ^{65}Zn uptake should therefore be performed in a standard medium with fixed conditions, as in the present paper.

There is an apparent contradiction between our results indicating a higher uptake of ^{65}Zn by blood cells in zinc deficiency and the finding that, in experimental zinc deficiency in animals no significant decrease of zinc concentration in leucocytes can be demonstrated (17). The explanation is that changes in the intracellular zinc concentrations are to small to be determined by the AAS method. Theoretically there is also a second mechanism. Cells formed during a period of zinc deficiency may have (as a compensatory mechanism) more transport carriers for zinc on their membrane (23), causing a faster zinc transport across the membrane. When the efflux would increase of the same amount as the influx, the zinc concentration would not be influenced. A third explanation would be that, by zinc deficiency the in- and outflow of zinc are increased because the integrity of the cell membrane is decreased.

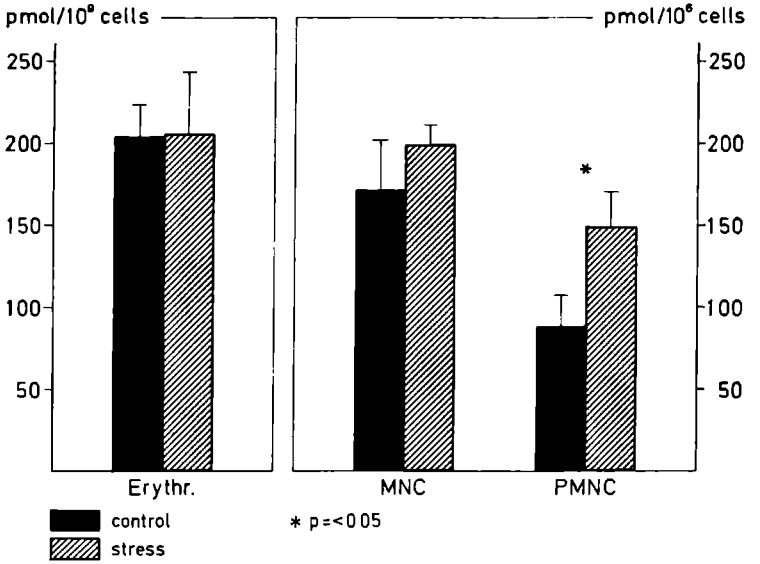

Figure 7 : Zinc uptake by blood cells of rats in stress (striped bars) and control rats (closed bars) after 2 d. No significant differences were seen in zinc uptake by erythrocytes and MNC. A small but significant increase of zinc uptake was demonstrated in PMN cells. The error bars indicate SD ($p=<0.05$).*

The relatively high zinc concentration of the Percoll gradient didn't have a significant effect on the in vitro uptake of zinc. By performing the isolation procedure at 4°C, zinc exchange was largely prevented (own experience).

In general, a decrease of serum zinc concentration caused by stress is not regarded as zinc deficiency. In stress zinc is redistributed from several tissues into the liver. Also, the serum albumin concentration is decreased and because 70% of the serum zinc is transported by albumin a decrease of serum albumin will result in a decrease of serum zinc concentration.

In our stress experiments no difference was seen in the in vitro uptake of zinc by erythrocytes and MNC after 2 days although serum zinc and serum albumin were reduced in these animals (Figure 6). Part of the decrease of serum zinc concentration is the result of the decrease of serum albumin. A small but significant increase of uptake of zinc was demonstrated by PMNC.

Chester and Will (21) found a higher uptake of ^{65}Zn by whole blood of animals that had received an ip endotoxin injection (short term experiment). However, by addition of ^{65}Zn to whole blood the incubation is performed in a medium with a variable albumin and trace element concentration. The in vitro uptake of ^{65}Zn by blood cells is inversely related to the albumin concentration in the medium (14) and consequently, the uptake of ^{65}Zn by blood cells, drawn from animals in stress, is higher if it is performed in its own serum, as was mentioned before. Therefore the increase of zinc uptake by blood cells in stress as described by Chester and Will can be the result of the decrease of serum albumin concentration. Also, the higher specific activity of ^{65}Zn would cause an increased ^{65}Zn uptake like mentioned before.

In the experiment described by Sasser et al (12) stress was induced by a skin injury from ß radiation. The long term effects of zinc deficiency and stress were studied (10 mo). Both in zinc deficiency and in stress they found an increase of zinc uptake by blood cells in vitro. For a considerable part the increase of zinc uptake in stress was the result of changes in the plasma protein composition, but there were some intrinsic changes. However, these changes could be the result of a true zinc deficiency. ß radiation resulted in a complete epilation and accumulation of plasma exudate over 12% of the body surface. Large quantities of zinc can be lost by skin lesions (24). Also no pair feeding was used. The absence of the results of a zinc balance study or the results of a ^{65}Zn total body counting in their long-term experiment makes the interpretation of this experiment difficult. Also in these long term experiments the higher specific activity of ^{65}Zn owing to a decrease of serum zinc can account for the increase of ^{65}Zn uptake.

In our study the cells that showed the greatest increase of zinc uptake in zinc deficiency (PMNC) were the only cells that showed a small, but significant increase of zinc uptake in case of inflammation. This can be the result of the higher sensitivity of these cells for low serum zinc concentrations or the shorter half-life of the PMN cells compared to the other blood cells, if the change resulting from zinc deficiency is built in during the formation of the cells. A longer exposure to zinc deficiency or a shorter half-life of the cells would result in more affected cells. A long-term stress experiment would probably give more insight in this problem.

Several methods are used to determine the zinc status, e.g., serum zinc (25), alkaline phosphatase activity levels (26), and zinc concentrations in urine (27) and hair (28). All these methods have their limitations. Serum zinc is influenced by the zinc status, but also by the serum albumin concentration and stress (18). The zinc concentration in urine

shows no correlation with the degree of zinc deficiency (29). The concentration of zinc in hair is decreased in the early phase of zinc deficiency, but because zinc deficiency can slow down the hair growth, the zinc concentration may return to normal (30). The whole-body retention of a dose of ^{65}Zn as a function of time seems to reflects the zinc status (16). It may be a suitable tool for research, but not for daily practice (31). The activity of zinc depended enzymes, e.g., serum alkaline phosphatase is influenced by many other factors such as liver function and bone turnover. Determination of tissue zinc concentrations are probably a more appropriate method to determine the zinc status, but tissue specimens are generally difficult to obtain. Blood cells are a kind of tissue and are more easily obtained. However, as previously explained, determination of zinc in blood cells is difficult (17) and validity of this method has never been demonstrated in animals. In our opinion, it is essential to validate a method to determine zinc deficiency in an animal experiment, because it is very difficult to perform an experiment in humans with proven zinc deficiency or stress as the only factors.

The results of this study point to a possible application of measuring ^{65}Zn uptake by blood cells as an indicator of the zinc status. The determination of ^{65}Zn activity in blood cells is not as difficult as the determination of a low-zinc concentration. This is the reason why we have studied the in vitro uptake of zinc by blood cells. Our experiments demonstrate that, in the case of a low serum zinc concentration, the in vitro uptake of zinc by MNC and erythrocytes can discriminate between true zinc deficiency and the effect of a stress factor after 2 d. This opens the possibility of distinguishing between true and apparent zinc deficiency. The results of our experiments should be confirmed in humans. Additional experiments concerning the effect of variables influencing the incubation, e.g., incubation time, incubation temperature, the effect of various diets, and the effect of long-term inflammation are needed.

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THE IN VITRO UPTAKE OF ZINC BY BLOOD CELLS IN RATS WITH LONG TERM INFLAMMATORY STRESS

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Serum zinc (Zn) is generally used to detect zinc deficiency, but it is influenced by several factors, e g , inflammation In clinical practice this makes it difficult to determine whether the decrease of serum zinc is the result of a real or an apparent zinc deficiency Other methods to determine the zinc status have been tested but have disadvantages In previous studies we have demonstrated that the in vitro uptake of zinc by blood cells, under standard conditions, is increased in zinc-deficient rats The uptake of zinc by blood cells has been studied before, but because we used a new method, the results of the present study differ from others The aim of the present study was to investigate the in vitro uptake of zinc during inflammatory stress

After the first day of inflammation, when a major decrease of serum zinc concentration was observed, a smaller difference persisted until the end of the experiment From one day after turpentine injection a decrease of serum albumin concentration was observed in the rats with an inflammation When the serum zinc concentration was corrected for the decrease of serum albumin a small, mostly non-significant decrease of serum zinc was observed in the stress group after the first few days This suggests that after the fourth day the decrease of serum zinc was mainly due to a decrease of serum albumin No increase of the in vitro zinc uptake by blood cells, especially erythrocytes, was observed in case of inflammation, this in contrast to the findings in case of real zinc deficiency In mononuclear cells (MNC) and polymorphonuclear cells (PMNC) a small increase of zinc uptake was observed only during the first three days Because after the fourth day the decrease of serum zinc was mainly the result of a decrease of serum albumin, these results indicate that the uptake of zinc by MNC and PMNC was not influenced by an apparent zinc deficiency due to a decrease of serum albumin Consequently this method can probably differentiate between an apparent zinc deficiency resulting from inflammatory stress and a real zinc deficiency Additional experiments to validate this method should be performed

INTRODUCTION

Zinc deficiency is a complication of many pathologic conditions and manifests itself in many symptoms, such as growth retardation (1), lack of appetite, skin lesions and loss of hair (2), impaired wound healing (3), dysfunction of the immune system (4,5), decreased protein synthesis and through a decrease of retinol binding protein, night blindness

However, no adequate method is available for detection of real zinc deficiency. Generally the serum zinc concentration is used for this purpose, but serum zinc is influenced by several other factors apart from zinc deficiency, e.g., hydration, albumin concentration influenced by inflammatory diseases, drugs like steroids, etc. We have demonstrated that the in vitro uptake of ^{65}Zn labelled zinc by various blood cells, drawn from zinc-deficient rats, was increased (6). In the present study the same method was applied to rats in which inflammatory stress was induced by an intramuscular injection of turpentine.

The aim of the present study was to investigate a) the physiologic process of the in vitro uptake of zinc by the various blood cells during long term inflammatory stress; b) the zinc status during inflammation and c) the suitability of this method to differentiate between a real and an apparent zinc deficiency.

METHODS

General remarks concerning the design of the study

Turpentine (0.25 mL) was injected intramuscularly, to induce a long-standing inflammation causing stress. An identical group of rats served as controls. At variable times blood was taken by heart puncture from 4 rats of the stress group and 4 rats of the control group for determination of the in vitro uptake of zinc by blood cells. The serum zinc and serum albumin concentrations were evaluated as well.

The various types of blood cells were separated and incubated as described before (6). The total body retention of intraperitoneally injected ^{65}Zn served as a control to determine if zinc deficiency occurred. This was studied in a separate experiment, because the ^{65}Zn was given in vivo. The uptake of ^{65}Zn by the cells in vivo would influence the measurements of the uptake of ^{65}Zn by the blood cells in vitro. Wherever the word 'stress' is used in this study, it refers to 'inflammatory stress induced by an inflammation caused by a turpentine injection'.

Animals

Male Wistar rats of 200 g were housed separately in stainless steel metabolic cages from 7 days before the start of the experiment to adapt the animals to these cages. The rats

were raised on a commercial diet (SMRA)(6). The guidelines for the care and use of laboratory animals in the Netherlands were followed.

Materials

Details concerning materials and procedures were published previously (6). Only a summary of the procedures and the information relevant to this study are given.

Zinc was determined by atomic absorption spectrophotometry (Perkin Elmer 5000; Norwalk, Connecticut, USA). Cells were counted by a Coulter counter Model ZM (Luton, Beds., UK).

^{65}Zn was measured with a NaI crystal with an automatic sample changer (LKB, Wallac 1282 Compugamma; Turku, Finland). Total body radioactivity of the rats was counted with a shadow shield counter consisting of two Tl-activated NaI crystals (4 x 6") connected to a Nuclear Data multi-channel analyzer N.D. 60 A (Nuclear Data; Schaumburg, Illinois, USA).

Procedures

Isolation of the blood cells

Blood was drawn from anaesthetized rats by heart puncture. Cell separation was performed using a discontinuous Percoll gradient using three solutions of Percoll of different densities (1.060, 1.075 and 1.095 kg/L respectively) (7). All solutions were kept at 4°C to prevent clumping and zinc exchange by the cells. The method described by Milne (7) was modified because blood cells of rats clot easily. Blood was diluted 1:3 with glucose-phosphate buffer. The Percoll solutions of different densities were layered underneath the diluted blood. Subsequently the tube was centrifuged. This resulted in separate layers of platelets, mononuclear cells (MNC), polymorphonuclear cells (PMNC) and erythrocytes. The MNC, PMNC and erythrocytes were harvested. Erythrocyte contamination of the MNC and PMNC fractions was removed by using an erythrocyte-lysing buffer containing NH4Cl. Cells were resuspended in a glucose phosphate buffer for cell counting. Cytospin preparations (Shandon; Astmoor, UK) were made at random to check the purity of the cell suspensions. The cell suspensions contained less than 10% contamination of other cells.

Incubation procedure of the blood cells

The incubation was performed under nearly physiological conditions (6). Volumes corresponding to 3.75×10^6 MNC and 1.25×10^6 PMNC (the mean number of blood cells in 1 mL of blood in rat) were taken and the cells incubated in an incubation medium containing albumin. Incubation was carried out in a shaking bath for 1 hour at 37°C and was stopped by adding 5 mL of cold (4°C) NaCl 0,9%. The cells were washed and the numbers of cells and radioactivity of the cells were determined.

Calculations

Biological half-life

The biological half-life was calculation of from the total body retention, measured as a function of time as follows:

When plotting the natural logarithm of the whole-body data versus time, a linear relation was found from day 7 on. From the slope (γ) of this line the biological half-life ($T_{1/2}$) of the ^{65}Zn was calculated using $T_{1/2} = \ln 2 / \gamma$.

Calculation of the uptake of zinc

Since the zinc concentration (Zn_m: 11.6 μM) and the radioactivity (R_m) of the incubation medium per mL are known and the number of cells (N_c) and the radioactivity (R_c) of a cell sample are measured, the zinc uptake of the cells (Zn_c) can be calculated using the equation:

$$\frac{R_c}{R_m} \times Zn_m \times \frac{10^6 \text{ or } 10^9}{N_c} = Zn_c \text{ in nmol Zn per } 10^6 \text{ or } 10^9 \text{ cells}$$

Estimation of the corrected serum zinc concentration in rats with stress:

Serum zinc is mainly transported by albumin (alb) and alpha-1-macroglobulin (non alb). Alpha-1-macroglobulin is not an acute phase protein. In case of inflammation the serum zinc concentration is decreased because of redistribution of serum zinc into the liver and because of a decrease of serum albumin concentration. Because in normal situations about 75% of the serum zinc is transported by serum albumin (8) an estimation of the serum zinc concentration of the inflammation group can be made.

The total serum zinc concentration in the control rats is divided into two parts.

$$Zn\ tot = Zn\ non\ alb + Zn\ alb$$

or

$$Zn\ tot\ (control) = 0.25 \times Zn\ tot\ (control) + 0.75 \times Zn\ tot\ (control)$$

We hypothesize that the binding of zinc to the binding proteins (the number of zinc molecules transported by one protein molecule), in the normal situation and in case of an inflammation will be similar. The factor 0.25 represents the fraction of the serum zinc concentration not bound to albumin in the control rats. This fraction is mainly bound to alpha-1-macroglobulin. The concentration of this protein is not significantly changed in stress, so the number of zinc molecules transported by alpha-1-macroglobulin in the inflammation group will be equal to that of the control group.

In the rats with an inflammation the fraction of serum zinc transported by albumin is decreased resulting from the decrease of serum albumin. If the serum level of albumin in the inflammation group will be increased to the level of the control group, the corrected serum zinc concentration can be calculated by the equation:

$$Corr\ Zn\ tot\ (inflam) = 0.25 \times Zn\ tot\ (control) + \frac{Alb\ control}{Alb\ inflam} \times ((Zn\ tot\ (inflam)) - (0.25 \times Zn\ tot\ (control)))$$

Zn tot (control):	total serum zinc concentration of the control group
Corr Zn tot (inflam):	corrected serum zinc concentration of the inflammation group
Alb control:	serum albumin of the control group
Alb inflam:	serum albumin of the inflammation group
Zn tot (inflam):	total serum zinc concentration of the inflammation group

If the decrease of serum albumin is the main cause of the decrease of serum zinc, the corrected serum zinc concentration of the inflammatory group should be identical to the serum zinc concentration of the control group. If a difference is still observed between the corrected serum zinc concentration and the serum zinc concentration of the control group, this difference will be the result of other causes, e.g., redistribution.

This equation is only used to explain in part the observed decrease and subsequent increase of serum zinc concentration during the experiments; it should not be used in cli-

nical practice to correct the serum zinc concentration for the decrease of serum albumin, because we made the assumption that the binding of zinc to the binding proteins will not change in case of stress, and because the concentration of alpha-1-macroglobulin is not measured.

Statistics:

A Wilcoxon rank sum test was performed for comparison of the independent results of both groups of rats on all the data collected during the experiment or during a period of the experiment.

The Student t-test was performed on the data collected at one moment; differences between two groups were considered significant if $p < 0.05$ (two tails). In case of repeated measurements and only one significant difference between the two groups the Bonferonni correction was applied.

Design of the experiments

Experiment 1: in vitro uptake of ^{65}Zn by MNC, PMNC and erythrocytes during long term inflammation.

After an adaptation period of 7 days in which 64 rats were housed separately in stainless steel metabolic cages, the rats were divided into two groups of equal mean weight and numbers: 32 rats in the inflammation and in a control group. Before turpentine was injected in the rats of the inflammation group, blood was collected from 4 rats of the inflammation group and 4 rats of the control group. Turpentine was injected intramuscularly in a dose of 0.25 mL into both hind legs. Four hours, 1 day, 2, 3, 4, 7 and 14 days after the turpentine injection 3 ML blood was withdrawn under anaesthesia by heart puncture from 4 rats of the inflammation group and 4 rats of the control group, for collection of blood cells. Also, 2 mL was drawn for determination of the serum zinc and albumin concentrations. The control group was pair fed throughout the experiment.

Experiment 2: ^{65}Zn whole-body retention in zinc deficiency.

The design of the experiment was the same as in experiment one. All the rats were injected intraperitoneally with 74 kBq (2 μCi) ^{65}Zn seven days before the turpentine injection. The whole-body activity at the time of the turpentine injection was taken as 100%. During the first 7 days after the ^{65}Zn injection the stress group was of course identical with

the control group. Whole-body counting was performed after 4 hours and on days 1, 2, 3, 4, 7, and 14. The serum was analyzed for albumin and zinc concentrations.

RESULTS

During experiment two the whole-body retention of ^{65}Zn (Figure 1) showed no difference between the groups, indicating absence of zinc deficiency (inflammation group $T_{1/2}$=34 days, control group 31.5 days). The serum zinc and serum album concentrations were comparable to the concentrations found in experiment one.

In experiment one the serum albumin concentration decreased during the first day (Figure 2). Thereafter a constant difference persisted (about 7 g/L). Using a Wilcoxon rank sum test a highly significant difference was calculated if all the data of both groups were compared ($p<0.0001$).

During the first day a considerable decrease of serum zinc concentration was seen in the rats with stress (day one: inflammation group 12.5 μmol/L and control group 20.4 μmol/L, $p=0.002$) (Figure 3). After that a smaller difference persisted (of about 4 μmol/L). A Wilcoxon rank sum test was performed for comparison of the independent results of both groups of rats on all the data collected during the experiment. A highly significant

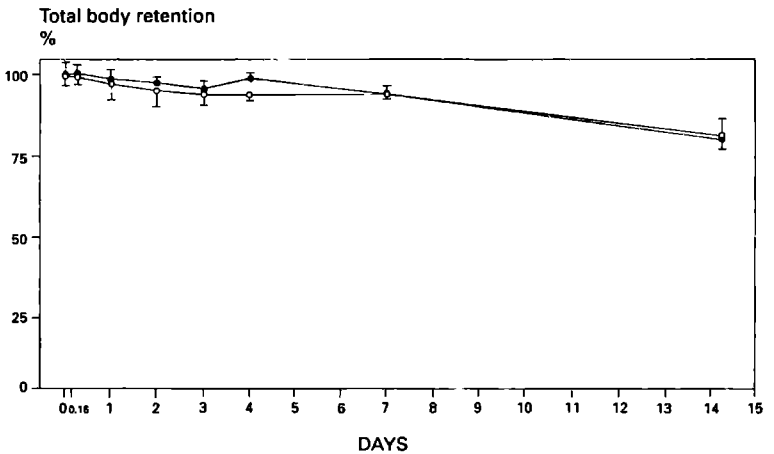

Figure 1: Total body retention of ^{65}Zn in rats with an inflammation (closed dots) and control rats (open dots) during the experiment

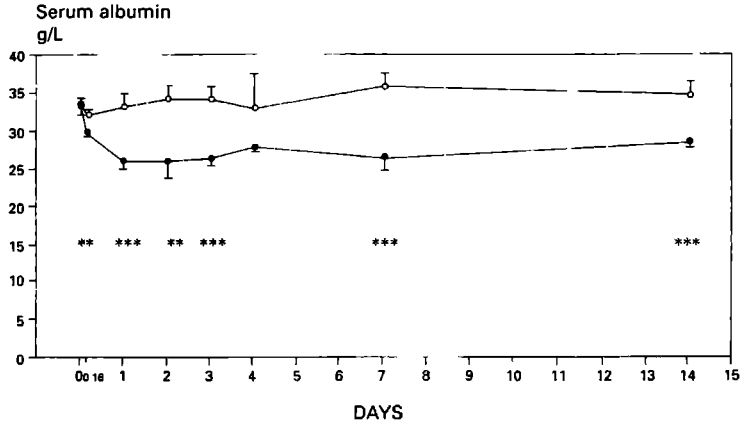

*Figure 2 : Serum albumin concentration in rats with an inflammation (closed dots) and control rats (open dots) during the experiment (** $p<0.01$; *** $p<0.001$).*

difference was calculated ($p<0.0001$).

When the serum zinc concentration of the rats with an inflammation was corrected for the decrease of serum albumin using the equation described in Methods, the differences between the groups were smaller, especially after the third day.

No difference in the in vitro uptake of zinc by erythrocytes was present except for a decrease of uptake by the erythrocytes of the inflammation group on day 7 ($p<0.04$) (Figure 4a). Because repeated measurements were performed on independent variables a Bonferroni correction for repeated measurements was applied. After the Bonferroni correction no significant increase of uptake of zinc by the erythrocytes of the rats of the inflammation group was observed. Using the Wilcoxon rank sum test no difference observed either during the first three days ($p=0.39$), or when the data of the total experimental period were compared ($p=0.42$). On day two the PMNC showed an increase of uptake of zinc compared with the control group (5.5 versus 3.9 pmol/L, $p=0.02$) (Figure 4b). When all the data of the first days of both groups were compared, no significant difference was observed ($p=0.08$). The same was seen when all the data of the total experimental period were compared ($p=0.87$). In MNC an increase of uptake of zinc was seen during the first three days (day one 6.1 versus 4.5 pmol/L/10^6 cells, $p<0.001$; Wilcoxon test during the first three days: $p=0.0007$) (Figure 4c). When all the data were compared a significant difference was calculated ($p=0.02$), but after the third day no difference was observed between the groups ($p=0.78$).

The serum zinc concentration is often used to determine the zinc status (9) but it is influenced by several factors such as time elapsed after the meal, the serum albumin and stress. This makes it difficult to determine whether a decrease of serum zinc concentration is the result of a real or an apparent zinc deficiency. In stress, like in inflammatory disease, the decrease of serum zinc (10,11) is due to an apparent zinc deficiency caused by a redistribution of zinc from the serum to the liver and by a decreased serum albumin concentration, but the total body zinc content is believed to remain unchanged.

Several other methods have been used to determine the zinc status, e.g., alkaline phosphatase activity (12), zinc concentration in urine (13) and zinc concentration in hair (14), but all these methods have limitations. The zinc concentration in urine shows no correlation with the degree of zinc deficiency (15). The concentration of zinc in hair is decreased in the early phase of zinc deficiency but because zinc deficiency can slow down hair growth, it may return to normal (16). The whole-body retention of a dose of ^{65}Zn as a function of time reflects the zinc status (17). It may be a suitable tool for research but not for daily practice (18). The activity of zinc-dependent enzymes, e.g., serum alkaline phosphatase, is influenced by many other factors such as liver function and bone turnover. One of the best parameters for determination of the zinc status is the zinc concentration in body tissues, but this method is not suitable for medical practice either. Blood cells are a kind of tissue and easy to obtain. Attempts have been made to determine the zinc concentration in blood cells but this requires much blood and the results are conflicting: controversy exists about the question whether zinc deficiency leads to an the increase (19) or decrease (20) in intracellular zinc concentrations. A decrease of the zinc concentration of granulocytes has even been observed after zinc supplementation (21). One explanation of these conflicting results is that it is not always clear if the subjects were really zinc deficient. Another is that the concentration of zinc in MNC and PMNC suspensions is small (6), increasing the risk of contaminating the suspension with extraneous zinc. Determination of zinc in blood cells is therefore difficult. To our knowledge a decrease in the concentration of zinc in blood cells in zinc-deficient animals has never been demonstrated although attempts have been made (22). Inducing a zinc deficiency in an animal model, is important to validate a method because it is very difficult to perform an experiment in humans with proven zinc deficiency or stress as the only factor.

Previously we studied the in vitro uptake of ^{65}Zn labelled zinc by blood cells, taken from zinc-deficient rats, to see whether this uptake is an indicator of the zinc status. The uptake of zinc by all cells investigated was increased: after four weeks in PMNC the uptake 2.6, in MNC 2.0 and in erythrocytes 1.3 times the uptake of the control group (6).

In the present study we examined the uptake of zinc, labelled with ^{65}Zn, by blood cells in case of inflammatory stress (Figure 1). The whole-body retention of ^{65}Zn as a function of time served as an indication for the zinc status.
No difference in zinc retention was observed indicating that in case of long term inflammation no true zinc deficiency was present despite a marked decrease of serum zinc (Figure 3). The in vitro uptake of zinc by erythrocytes was not influenced by inflammatory stress (Figure 4a). The in vitro uptake of zinc by MNC and PMNC (Figure 4b and 4c) was increased in case of severe inflammation. After the first days after the turpentine injection the zinc uptake of these cells is normal, although the serum zinc is still decreased, resulting from the decreased albumin. Thus after the first days the apparent zinc deficiency did not have an influence on the uptake in zinc by PMNC and MNC. These results conform those of an earlier performed pilot experiment (6) in which inflammation was also induced by

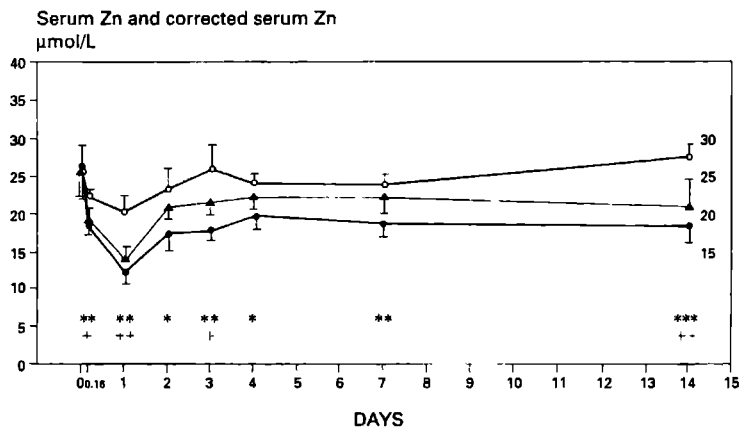

Figure 3 : Serum zinc concentration in rats with an inflammation (closed dots), control rats (open dots) and serum zinc concentration corrected for the decrease of serum albumin (closed triangle) during the experiment. The error bars indicate SD ($p<0.05$; ** $p<0.01$; *** $p<0.001$ for serum zinc in rats with an inflammation compared with control rats. + $p<0.05$; ++ $p<0.01$ for corrected serum zinc in rats with an inflammation compared with control rats)*

turpentine. Only the uptake of zinc by blood cells after 2 days was studied. No difference was seen in the in vitro uptake of zinc by erythrocytes after 2 days although serum zinc and serum albumin were reduced in these animals. In MNC a small, non-significant increase was demonstrated. A significant increase of in vitro uptake of zinc in case of inflammation was observed in PMNC. The results of the present study confirm these results.

The uptake of ^{65}Zn labelled zinc by erythrocytes or total blood cells during zinc deficiency or stress has been studied before (23,24,25,26). The in vitro uptake of zinc by the three separate types of blood cell in a standard medium as in our experiment has never been reported before. The partly preliminary results of these studies concerning the zinc uptake by blood cells during inflammation do not point in the same direction as our study. However, these studies had certain shortcomings. Two of the studies were only published in abstract form, lacking necessary details (25, 27). Moreover, in one of these only the in vivo uptake was studied and in the other only the results of the uptake of zinc by erythrocytes was described. It is only in two paper that the results in case of stress are presented. Contrary to our results Chester and Will (26) found a higher uptake of ^{65}Zn by whole blood of animals which had received an intraperitoneal endotoxin injection (short term experiment). However, by addition of ^{65}Zn to whole blood the incubation takes place in a environment with a variable albumin and trace element concentrations. The in vitro uptake of ^{65}Zn by erythrocytes is inversely related to the albumin concentration in the medium (28) and consequently the uptake of ^{65}Zn by blood cells drawn from animals in stress is higher when it is tested in the animals own serum. Therefore the increase of zinc uptake by blood cells in stress as described by Chester and Will (26) may be the result of the decrease of serum albumin concentration. Also, the higher specific activity of ^{65}Zn will cause an increased ^{65}Zn uptake, even when the uptake of total zinc (cold zinc and tracer ^{65}Zn) is the same. In the experiment described by Sasser et al (24) stress was induced by a skin injury from beta radiation. The long term effects of zinc deficiency and stress were studied (10 months). Both in zinc deficiency and in stress they found an increase of zinc uptake by blood cells in vitro. For a considerable part the increase of zinc uptake in stress was the result of "changes in the plasma protein composition, but there were some intrinsic changes". However, these changes could be due to a true zinc deficiency. Beta radiation resulted in a complete epilation and accumulation of plasma exudate over 12% of the body surface. Large quantities of zinc can be lost via skin lesions (29). No pair feeding was used. The absence of a zinc balance study and the absence of a ^{65}Zn total body counting in their long term experiment make the interpretation of this experiment difficult. Furthermore in

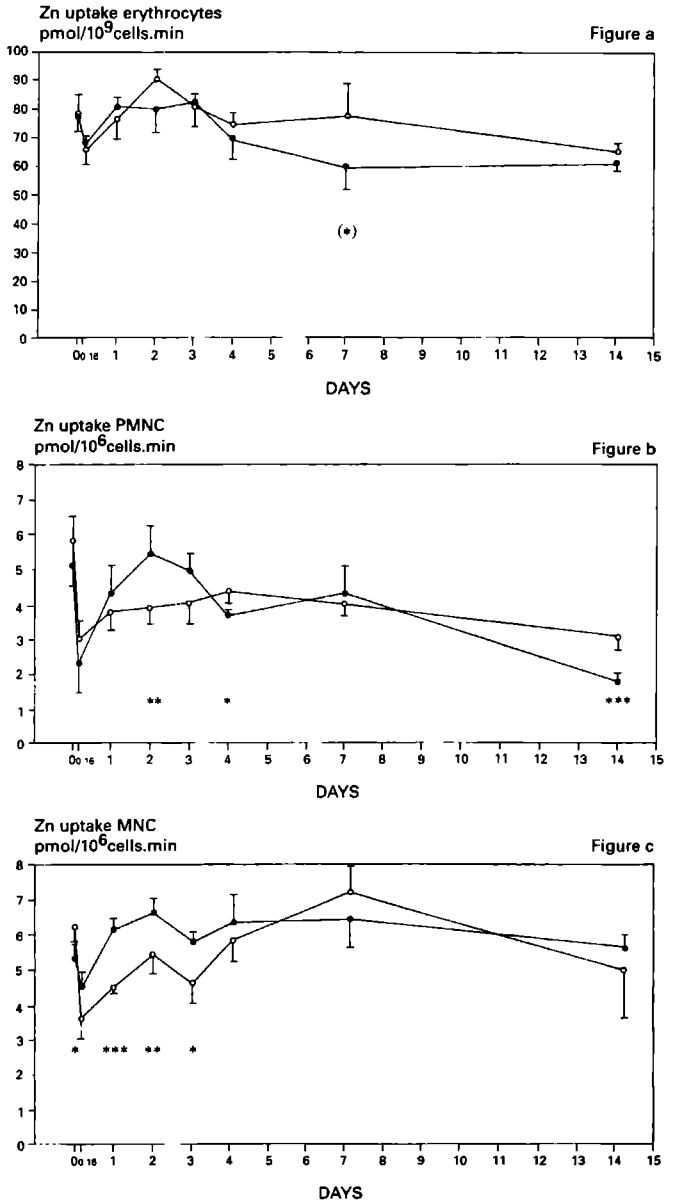

Figure 4 : In vitro uptake of zinc by blood cells in rats with an inflammation (closed dots) and control rats (open dots) during the experiment. Figure 4a (upper figure): uptake by erythrocytes; figure 4b (middle figure): uptake of zinc by polymorphonuclear cells; figure 4c (bottom figure): uptake of zinc by mononuclear cells. The error bars indicate SD ($p<0.05$; ** $p<0.01$; *** $p<0.001$; (*) not significant if a Bonferroni correction of the t-test for repeated measurements was used).*

the long term experiments performed by Sasser et al. the higher specific activity of ^{65}Zn resulting from a decrease of serum zinc can account for the increase of ^{65}Zn uptake. The uptake of zinc by blood cells should therefore be studied in a standard medium under fixed conditions, as in the present paper.

During the first few days of the present experiment the serum zinc concentration was markedly reduced. The serum zinc concentration of the rats with stress was hardly reduced, compared to the concentration in the control group, after correction of the serum zinc concentration of the stress group for the reduction in serum albumin concentration after the third day. This indicates that after the third day the decrease of serum zinc was mainly due to a decrease of serum albumin. During the first few days, together with a decrease of serum albumin, serum zinc was also reduced because of an increase of zinc uptake by the liver, probably the result of an increase of metallothionin in the liver. After stabilisation of metallothionin in the liver this redistribution process gradually ceases and the serum zinc is from then on mainly influenced by the serum albumin concentration.

The equation that is used to explain the observed decrease and subsequent increase of serum zinc concentration during the experiments, can be used in clinical practice to correct the serum zinc concentration for the decrease of serum albumin if all the hypothesis are true. The assumption that the binding of zinc to the binding proteins will not change in case of stress, should be confirmed. The concentrations of serum albumin and alpha-2-macroglobulin in (human) subjects with stress should be measured and the concentrations of albumin and alpha-2-macroglobulin in subjects without stress should be known.

Evaluating the aims of this study we may conclude that the results of these experiments indicate that the reduction in serum zinc concentration is probably the result of the physiologic process of reduction in serum albumin concentration and, during the first few days, a short term increase of zinc uptake by the liver (redistribution process). These experiments stress the fact that the total body retention of ^{65}Zn is a reference for the zinc status. Probably the in vitro uptake of zinc by erythrocytes can differentiate between an apparent zinc deficiency due to an inflammatory stress and a true zinc deficiency. The results of this study point to a possible application of measuring ^{65}Zn uptake by blood cells as indicator of the zinc status.

The results of our experiments should be confirmed in humans. Additional experiments concerning the effect of variables influencing the incubation, such as incubation time, incubation temperature and the effect of various diets are needed.

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ZINC EXCHANGE BY BLOOD CELLS IN NEARLY PHYSIOLOGIC STANDARD CONDITIONS

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ABSTRACT

Determination of zinc concentrations in white blood cells has been used to establish zinc deficiency. During pathological conditions changes in zinc concentrations in these blood cells were observed. However, these investigations were hampered by the low amount of zinc in this form per mL blood. Earlier we demonstrated that, in the case of zinc deficiency, the uptake of zinc was increased, using the in vitro exchange of zinc by the various blood cells with extracellular zinc labeled with ^{65}Zn in fairly physiologic conditions. In case of inflammation, no increase of zinc uptake by erythrocytes was seen, indicating that this method probably can be used to differentiate real from apparent zinc deficiency. Only during the first days of the inflammatory process, probably representing the redistribution phase during which zinc moves from the serum to the liver, a small increase of in vitro zinc uptake was seen in mononuclear cells (MNC) and polymorphonuclear cells (PMNC).
Earlier papers raised some questions; e.g., is the uptake part of an exchange process and can the efflux of zinc by the cells be measured by the same method; what is the influence of time on the process of zinc uptake; what is the magnitude of the uptake of zinc by the cells compared to the zinc concentration in the cells; and, what is the influence of temperature on the uptake of zinc?
In the present study, the influence of incubation time and temperature on the uptake of zinc by human and rat blood cells and on the release of zinc by rat blood cells was studied. By varying the incubation time, at least three phases of uptake of zinc in the various cells were found, a fast phase during the first half hour, probably caused by an aspecific binding of zinc on or in the cell membrane; a second fast uptake between 60 - 330 min, probably caused by an influx of zinc in the cell as part of the exchange process of zinc; and a slow third phase after 5.5 hours, in which probably the in- and efflux of the rapidly exchangeable intracellular pool is more or less equilibrated). For mononuclear cells, polymorphonuclear cells, and erythrocytes of rats the rapidly exchangeable intracellular pool is resp 40%, 53% and 10%, respectively, of the total zinc content of the cells. This study is also performed in human cells; in human cells the exchangeable pool of mononuclear cells and erythrocytes is 17% and 3.5% of the total zinc content of the cells respectively. The efflux of zinc by blood cells can be measured by the same method. Both the uptake and the loss of zinc by blood cells of rats were compared and are of the same magnitude, indicating that the in vitro uptake of zinc we described elsewhere, is part of an exchange process. Increasing temperature during incubation procedures results in an increase of zinc uptake by human blood cells, even at high temperatures of 41°C although there are gradual diffe-

rences between the various blood cells. Both the in- and efflux of zinc by blood cells are very small at 4°C.

INTRODUCTION

Zinc is one of the most prominent trace elements in humans. Zinc deficiency causes hair loss, skin lesions (1), delayed wound healing (2), and growth retardation (3). Although in one study an improved functioning of polymorphonuclear leucocytes in vitro is described in case of zinc deficiency is described (4), other studies have found an impaired function of blood cells (5,6). The zinc content of blood cells has been proposed as a method for judging the zinc status although in animal experiments this method has never been validated to our knowledge. Measurement of the in vitro uptake of zinc by blood cells in nearly physiological standard conditions is probably a method to determine the zinc status (7, 8). Earlier raised some questions; e.g., is the uptake part of an exchange process, and can the efflux of zinc by the cells be measured by the same method; what is the influence of time on the process of zinc uptake; what is the magnitude of the uptake of zinc by the cells compared to the zinc concentration in the cells; and what is the influence of temperature on the uptake of zinc?

The aim of this study was to investigate the physiologic process of zinc uptake and efflux by the various blood cells by the measurement of the influence of incubation time and incubation temperature on the influx of zinc by blood cells. Also, the efflux of zinc by the cells was determined.

MATERIALS AND METHODS

Design of the experiments

In both the first and fourth experiments we used rats so we would be in a the position to compare the influx and efflux of zinc from the various types of blood cells. For the study of the efflux of zinc by blood cells, rats had to be injected with a considerable amount of ^{65}Zn in order to incorporate enough ^{65}Zn in the blood cells. This experiment was therefore not possible in humans. In the second experiment also we studied the influence of the incubation time on the uptake of zinc by blood cells, and employed human

cells taken from four volunteers. The third experiment, also performed with cells from human blood, was meant to determine the influence of temperature on the uptake of zinc.

Experiment 1: Influence of incubation time on the influx of zinc by blood cells taken from rats.

After an adaptation period of 2 weeks, in which all rats were fed the IRI-OB food containing 12 mg/kg of zinc (see Animals), 5 mL of blood were drawn by heart puncture under anaesthesia from each of four rats. The blood of the four rats was pooled, mixed, and blood cells were isolated. All procedures were performed at 4°C, except during the incubation period. MNC, PMNC, and erythrocytes were incubated at 37°C for 12, 30, 60, 120, 330, 1020, and 1440 minutes in the incubation medium containing ^{65}Zn. The cells were washed and the number of cells and their ^{65}Zn content were determined.

Experiment 2: Influence of incubation time on the influx of zinc by blood cells taken from human volunteers.

Blood samples, now 20 mL, were taken from each of four fasting healthy volunteers. Because it was difficult to perform the cell isolation procedure on 20 mL of blood, the blood was separated into four portions and the isolation procedure was performed on each of the four portions. After the isolation procedure the various cell types of each volunteer were combined and the incubation procedure of the various cell types was performed as described in experiment 1.

Experiment 3: Influence of incubation temperature on the influx of zinc.

Twenty mL of blood were taken by vena puncture from two healthy volunteers, as described in experiment 2. Blood was mixed and blood cells were isolated. The MNC, PMNC, and erythrocytes were incubated for 1 hour in ^{65}Zn containing incubation medium at 4°C, 20°C, 37°C, and 41°C. The cells were washed according to the described procedures. Number of cells, volume, and radioactivity were determined.

Experiment 4: Zinc efflux during isolation and incubation procedure.

After adaptation (see Animals) four rats were injected ip with 740 kBq (20 μCi) ^{65}Zn on day 1 and 2. From each of these four rats 5 mL of blood were taken under anaesthesia by heart puncture on day 4. The blood of these four rats was mixed and the blood cells were isolated at 4°C and incubated at 37°C for 1 hour in the incubation medium according to the procedures described. The incubation medium did not contain ^{65}Zn.

Table 1

Composition of the IRI-OB diet

Ingredients	Concentration (g/100g)
Glucose	50.65
Corn starch	15.0
Ovalbumin	20.0
Sun flower seed oil	4.0
Fibre (cellulose)	5.0
Choline chloride	0.3
Mineral mix	
Sodium diphosphate	1.5
Potassium chloride	0.7
Calcium carbonate	1.0
Magnesium sulphate	0.5
Sodium meta-silicate	0.25
Trace element salt mix	0.1
Vitamin mix in glucose	1.0

Trace element salt mix	Concentration (mg/kg)
Mn	55
Fe	66
Zn	various conc.
Cu	9.3
Ni	2.4
Cr	1.8
Mo	0.2
Se	0.36
As	0.13
Co	0.2

Subsequent washing was performed at 4°C. After each consecutive step of the separation, lysation, washing, or incubation solutions were stored and the radioactivity, volume, and number of cells in each were determined. In order to avoid a rise in temperature caused by the manipulation of the suspension and therefore continuation of the exchange process, the radioactivity of the cell suspensions was determined at the beginning and at the end of

the separation and incubation procedure only, and not after each consecutive step. Using the radioactivity, the volume, and the number of cells of the last cell suspension and of the preserved solutions (all separation, lysation, washing, and incubation solutions used were stored), the efflux of zinc by the various blood cells could be calculated (see Calculations).

Animals and human volunteers

Male Wistar rats, 200 g, were housed separately in stainless steel metabolic cages. The commercial diet (SMRA) on which the rats were raised contains a considerable and variable amount of zinc (about 92 mg/kg) and copper (about 25 mg/kg). Therefore, in experiments 1 and 4 a diet (IRI-OB; table 1 (9)), containing 12 mg/kg zinc and 9.3 mg/kg copper, was given to the rats during the 2 weeks before the experiment, to adapt the animals to this diet and to reduce the excess of zinc in their bodies. This zinc concentration is considered sufficient to maintain normal zinc status (10). Blood was taken from healthy volunteers (35-40 years in age).
The guidelines for informed consent of the volunteers and for the care and use of laboratory animals in The Netherlands were followed.

Materials and methods

The diets (IRI-OB and SMRA; Table 1 (9)) were produced by Hope Farms (Woerden, the Netherlands), table 1 (9). Stainless steel needles and plastic syringes (Monoject, Sherwood, USA) with 10 μL of preservative-free heparin (50 IU) were used. All solutions were prepared with deionized distilled water, processed through a Millipore system (Bedford, Massachusetts, USA) (resistance >15 MΩ). Only polypropylene pipet tips, plastic tubes (15 mL polystyrene, Greiner, Solingen, Germany and 50 mL polypropylene, Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) and bottles (0.8 L, NUNC, Roskilde, Denmark) were used. All chemicals were of analytical grade (Merck, Darmstadt, Germany). Percoll was supplied by Pharmacia (Uppsala, Sweden). Hepes was obtained from Sigma (St Louis, MO, USA), human albumin from Behring (Marburg, Germany), Hanks' balanced salts without Ca and Mg from Flow Laboratories (Rickmansworth, Herts, UK) and the radio tracer ^{65}Zn from Amersham International (Amersham, Buckinghamshire, UK), code ZAS 2, 3.7-37 GBq/mg $ZnCl_2$ in 0.1 mol/L

HCl. The concentrations of zinc in the solutions and in ion- free water, after having been in contact with tubes and bottles, were below the lower limit of detection of the atomic absorption spectrophotometer, except for a low zinc concentration in diluted Percoll (2.56 μmol/L).

Zinc was determined by atomic absorption spectrophotometry (Perkin Elmer 5000; Norwalk, Connecticut, USA). The lower limit of detection was 0.28 μmol/L and the response was linear to at least 15 μmol/L. Standard reference containing $ZnCl_2$ (Titrisol, Merck, Darmstadt, Germany) was used in the concentrations expected in the samples. Cells were counted by a Coulter counter Model ZM (Luton, Bedforshire, UK), adjusted to the size of the cells to prevent counting of cell fragments. ^{65}Zn in the tubes was measured with a NaI crystal using an automatic sample changer (LKB, Wallac 1282 Compugamma; Turku, Finland). The counting time was adjusted to assure a statistical error of <0.3%. A tube with medium but without blood cells, incubated and washed according to the procedure of the cell containing tubes, served as a blank.

Solutions

Glucose Phosphate Buffer (GPB): 13.5 mmol/L Na_2HPO_4, 3.3 mmol/L KH^2PO_a, 5.4 mmol/L KCl, 133 mmol/L NaCl, and 5 mmol/L glucose in ion-free water, pH adjusted to 7.4 with HCO_3.

Erythrocyte Lysing Buffer: 155 mmol/L NH_4Cl and 10 mmol/L $KHCO_3$ in ion-free water, pH adjusted to 7.4 with HCO_3.

Incubation Medium: 145 mmol/L NaCl, 6 mmol/L KCl, 2 mmol/L $MgCl_2$, 2.4 mmol/L $CaCl_2$, 7.6 μmol/L $ZnCl_2$, 5.6 mmol/L glucose, 10 mmol/L Hepes and 40 g/L albumin in ion-free water tracered with about 10 MBq ^{65}Zn/L and adjusted to pH 7.4 with CO_2. In experiment 4 no ^{65}Zn was added.

Methods

Discontinuous Gradient of Percoll

Three solutions of Percoll with different density (1.060, 1.075 and 1.095 kg/L respectively) (11) were prepared by dilution with Hanks' balanced salts solution without

Ca and Mg. Six mL of a heavier solution were layered in a 50 mL tube underneath the lighter solution by using a stainless steel needle of 15 cm and a plastic syringe.

Isolation procedure of the blood cells

Cell separation was performed using a discontinuous Percoll gradient. All cell suspensions were kept at 4°C to prevent clumping and zinc exchange by the cells. The method described by Milne (11) was modified because rat blood cells clot easily. Blood was drawn from anaesthetized rats by heart puncture using stainless steel needles and plastic syringes with 10 μL of preservative-free heparin (50 IU). Blood was diluted with 2 volumes GPB. The Percoll solutions of different densities were layered underneath the diluted blood. Subsequently, the tube was centrifuged for 20 min., at 500 x g at 4°C in a swing-out rotor without brake (Heraeus Crist Minifuge GL, Osterode am Harz, Germany). This resulted in separate layers of platelets, mononuclear cells (MNC), polymorphonuclear cells (PMNC), and erythrocytes. The MNC, PMNC and erythrocytes were harvested by using a pipet and resuspended in 5 mL GPB in a 15 mL tube. Erythrocyte contamination of the MNC and PMNC fractions was removed by twice resuspending the cells for 10 min at 4°C in 5 mL of erythrocyte lysing buffer and subsequent washing steps with 5 mL of GPB. After the lysing and washing steps the tube was centrifuged for 10 min, 200 x g, 4°C with brake. Cells were resuspended in 1 mL of GPB for cell counting. The erythrocytes were washed twice and centrifuged for 10 min., 200 x g, 4°C with brake. At random Cytospin preparations (Shandon; Astmoor, UK) were performed to check the purity of the cell suspensions. The cell suspensions contained less than 10% contamination of other cells.

Incubation procedure of the blood cells

The incubation was performed in physiologic conditions (e.g., albumin containing medium, incubation at 37°C, number of cells identical to the number in blood). Volumes corresponding to 3.75 x 10^6 MNC and 1.25 x 10^6 PMNC (the mean concentration of blood cells in 1 mL of blood in rat), or in the case of human cells, to 1.25 x 10^6 MNC and 3.75 x 10^6 PMNC (the mean concentration of blood cells in 1 mL of human blood) were taken and centrifuged. Supernatant was discarded and 1 mL of incubation medium added. In the case of erythrocytes 0.4 mL of the concentrated suspension was added to 0.6 mL of the incubation medium. Incubation was carried out in a shaking bath for 1 h at 37°C and was stopped by adding 5 mL of cold GPB. The cells were centrifuged and resuspended four times using cold GPB. The last time the cells were resuspended in 1 mL of GPB. The volume was determined and number of cells and radioactivity were counted.

Calculation of the uptake of zinc by the use of ^{65}Zn containing incubation medium: Since the zinc concentration (Zn_m : 7.6 μM) and the radioactivity (R_m) of the incubation medium per mL are known and the number of cells (N_c) and the radioactivity (R_c) of a cell sample are measured, the zinc uptake of the cells (Zn_c) can be calculated using the expression:

$$\frac{R_c}{R_m} \times Zn_m \times \frac{10^6}{N_c} = Zn_c \text{ in nmol Zn per } 10^6 \text{ or } 10^9 \text{ cells}$$

Mathematic formula fitting the curves of experiments 1 and 2:
The curves expressing the results of experiments 1 and 2 showed three phases. The various parts of the curves were approximated by the expression:

$$Y = a(1 - e^{-a'X}) + b(1 - e^{-b'(X-d)}) + cX$$

The slope of the curves is expressed by a' and b'. The intercept of the extrapolation of the curve between t = 5.5h and t = 24h to time 0 (the y-axis) has than the value a + b. In this formula it is assumed that X-d can only be equal or more than 0. 'd' is the lag-time of the second part of the curve. The rate of zinc uptake was calculated by the use of two points in the calculated period.

Calculation of the efflux of zinc (experiment 4):

Using the ^{65}Zn content of the last cell suspension and the preserved solutions the ^{65}Zn radioactivity of the intermediate cell suspensions, between the beginning and at the end of the separation and incubation procedure, was calculated. Because the activity of the last cell suspension was known, the activity of the preceding cell suspension can be calculated and subsequently the activity per 10^6 or 10^9 cells in these suspensions.

$A_{z-1} = A_z + A_{wash}$ and

$A_z = R_z \times C_z$

$A_{wash} = A_{cells} + A_{lysis} + A_{efflux}$

in which

A_z = total activity in cells of final suspension

R_z = activity per 10^6 or 10^9 cells in the final cell suspension

C_z = total number of cells in the final cell suspension divided by 10^6 or 10^9

A_{z-1} = total activity in cells of the one-but final suspension

A_{wash} = total activity in cells in the wash of the one but final suspension

A_{cells} = total activity in cells loosed in the wash; the activity per cell is identical to Rz

A_{lysis} = activity originating from damaged cells. The number of cells involved is calculated as the difference between the number of cells in the final suspension and in the one but final suspension + the last wash. Knowing the ^{65}Zn activity in these cells, the amount due to lysis can be calculated.

A_{efflux} = activity released from the cells while washing the cells of the one but last suspension.

The efflux can be calculated from the decrease of ^{65}Zn content of the blood cells during the consecutive steps and by the A_{efflux} of the successive steps. For this calculation it is assumed that on day 4 of the experiment the specific activity of the ^{65}Zn was constant throughout the cells.

Statistics

The mean and the standard deviation were calculated if the same data of several subjects were collected at the same moment (experiment 2).

RESULTS

In the first experiment the influence of incubation time on the uptake of zinc by blood cells, drawn from rats, was studied (Figure 1). During the first 5.5 hours a fast uptake of zinc was seen (MNC 0.07 pmol Zn/10^6 cells.min, PMNC 0.077 pmol Zn/10^6 cells.min, erythrocytes 0.003 pmol Zn/10^6 cells.min). In 5 out of 6 curves (experiments 1 and 2) a shoulder can be observed from the period of 30 - 60 min.; so probably there are two rapid phases in the uptake of zinc by blood cells (0 - 30 min. and 60 - 330 min.). In the third period, 5.5 - 24 h, only a very small increase of zinc uptake was seen (MNC 0.0025 pmol Zn/10^6 cells.min, PMNC 0.0032 pmol Zn/10^6 cells.min, erythrocytes 0.00017 pmol Zn/10^6 cells.min).

The point of intersection of the extrapolation of the curve of the third part of the curve with the Y-axis in the figures probably represents the amount of the maximum upta-

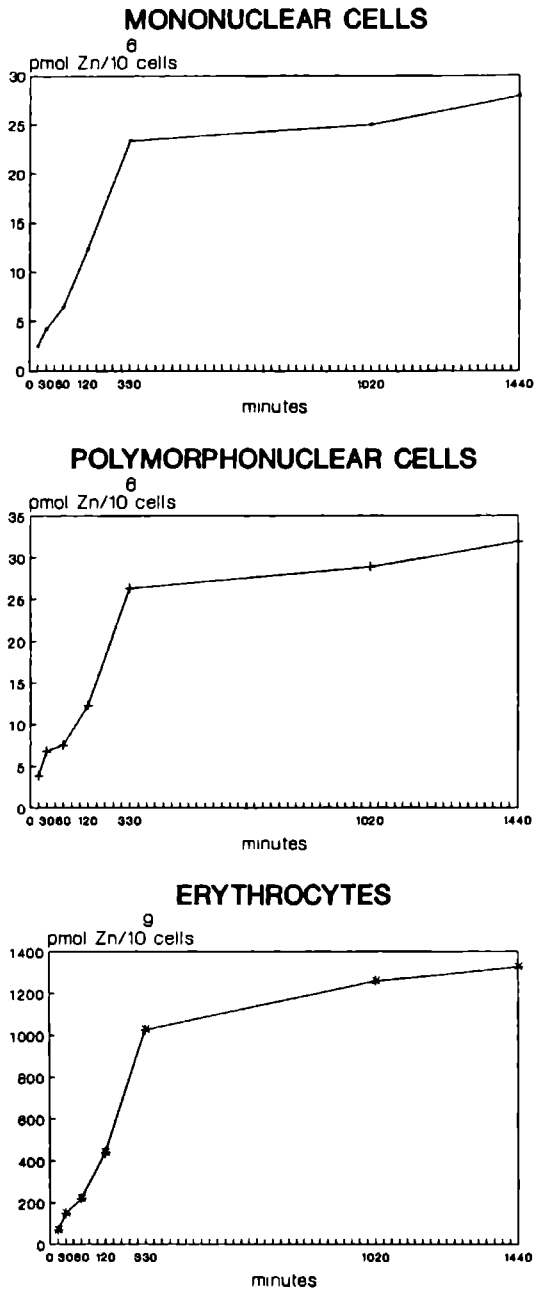

Figure 1 - The influence of incubation time (X-axis) on the uptake of zinc by blood cells (Y-axis), drawn from rats (experiment 1). The upper figure represents the uptake by MNC; the figure in the middle the uptake by PMNC and the bottom figure the uptake by erythrocytes.

ke of the cells and, in consequence, represents the exchangeable pool of zinc. The values in tables 2 and 3 represent the size of the exchangeable pool of the various cells and the total intracellular zinc content in rats and humans, respectively.

Table 2
Zinc uptake by blood cells of rats

Type of cells	estimation of the maximum zinc uptake by the cell membrane pmol Zn/10^6 cells	size of the exchangeable pool* pmol Zn/10^6 cells	total cellular zinc content (13) pmol Zn/10^6 cells
MNC	4.9 (8%)	22 (36%)	60
PMNC	7.1 (14%)	24 (53%)	51
Erythrocytes	0.17 (1.5%)	0.92 (10%)	11

* after 24 hours, including the membrane

The figures of experiments 1 and 2 show a tendency toward a shoulder after 30 min. In experiment 2, standard deviations were calculated. In MNC of humans, this shoulder was significant if the curves of the shoulder were extrapolated for the period 60-330 min, and the standard deviations were taken in account. The differences in zinc uptake in the first 5.5 hours were not significant for PMNCs and erythrocytes, if the standard deviation was taken into account (Figure 2) although the similarity of the curves is striking (table 3). The point of intersection of the extrapolation of the shoulder of the curve with the Y-axis probably gives an indication of the maximum uptake of zinc by the cell surface. In rats the point of intersection was in MNC approximately 4.9 pmol Zn/10^6 cells, PMNC 7.1 pmol/10^6 cells, and erythrocytes 0.17 pmol/10^6 cells.

In experiment 2 the influence of incubation time on the uptake of zinc by blood cells from healthy volunteers was studied (Figure 2). In erythrocytes and MNC, the uptake of zinc resembles the same process as in the various cells taken from rats. During the first 5.5 h, a fast uptake was observed especially in MNC and erythrocytes (0.075 pmol/10^6 cells.min and 0.0019 pmol/10^6 cells.min respectively). After the first 5.5 hours the uptake of zinc decreased (0.027 pmol/10^6 cells.min and 0.00018 pmol/10^6 cells.min respectively). The point of intersection of the extrapolation of third phases with the Y-axis was 19 pmol/10^6 cells and 0.66 pmol/10^6 cells in MNC and erythrocytes respectively (table 3). In

Table 3

Zinc uptake by blood cells of healthy volunteers

Type of cells	estimation of the maximum zinc uptake by the cell membrane pmol Zn/10^6 cells	size of the exchangeable pool* pmol Zn/10^6 cells	total cellular zinc content (13) pmol Zn/10^6 cells
MNC	3.26 (3.9%)	19 (17%)	113
PMNC	-	-	78
Erythrocytes	0.022 (0.13%)	0.61 (3.5%)	17

* after 24 hours, including the membrane

PMNC the difference in zinc uptake between the first 5.5 h and the third part was small: 0.024 pmol/10^6 cells.min and 0.02 pmol/10^6 cells.min indicating that the point of intersection of the second part can not to be calculated reliably.

In human cells, especially in MNC and erythrocytes, a tendency towards a shoulder after 12 and 30 min respectively, can be observed. The point of intersection of the extrapolation of the shoulder of the curve with the Y-axis was 3.3 pmol/10^6 cells and 0.022 pmol/10^6 cells in MNC and erythrocytes respectively (table 3).

The average percentage of the standard deviation (standard deviation compared to the value of the zinc uptake at that moment) was 26.1%, 24.2%, and 22.1% in MNC, PMNC and erythrocytes, respectively. This standard deviation is a combination of the inter-individual and the intra-individual standard deviation)

In experiment 3 the influence of the temperature during the incubation period on the influx of zinc was studied (Figure 3). With the rise of the incubation temperature more zinc is taken up by the blood cells. The uptake of zinc at 4°C is low (MNC 3.5 pmol Zn/10^6 cells, PMNC 0.2 pmol Zn/10^6 cells, erythrocytes 0.001 pmol Zn/10^6 cells 35%, 11% and 12.5% of the uptake at 37°C respectively). A further increase of zinc uptake is seen if incubation temperature is increased from 37°C to 41°C. These experiments have been performed with human blood cells.

In experiment 4 the efflux of zinc from blood cells was determined (Figure 4). After an adaptation period of 2 weeks ^{65}Zn was administered ip on two subsequent days. At the fourth day the ^{65}Zn in MNC, PMNC, and erythrocytes had reached equilibration with the

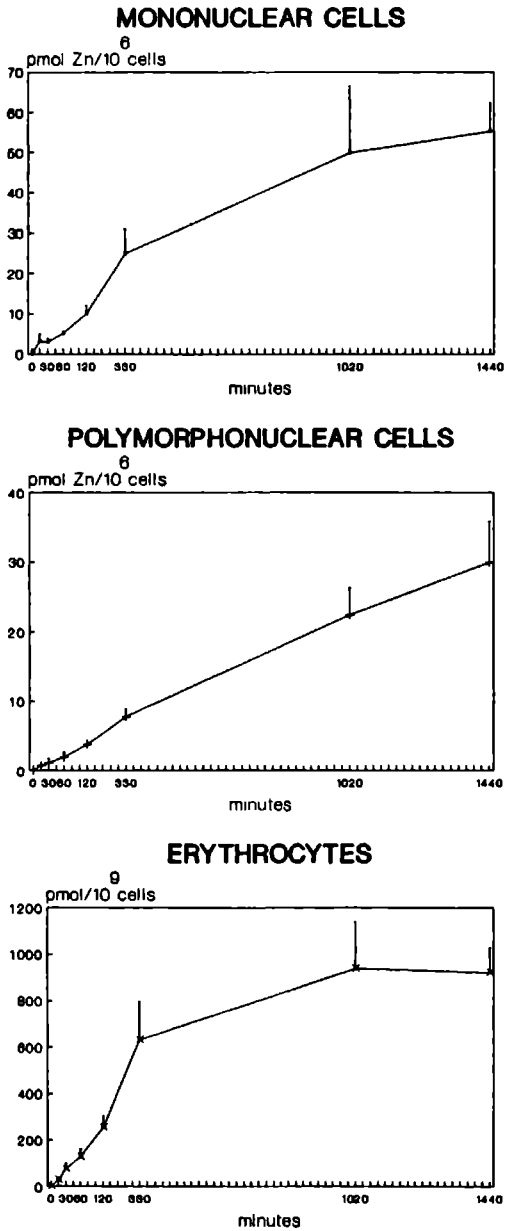

Figure 2 - The influence of incubation time (X-axis) on the uptake of zinc by blood cells (Y-axis), drawn from healthy volunteers (experiment 2). The upper figure represents the uptake by MNC; the figure in the middle the uptake by PMNC and the bottom figure the uptake by erythrocytes. The error bars indicate the standard deviation

intracellular zinc pools of the blood cells (unpublished data, own observation). This observation was supported by the results of the first experiment, which indicate that zinc in the blood cells in vitro reaches equilibration with their environment after 5.5 - 24 h (Figure 1) although in vitro the milieu for the cells is constant and in vivo the environment of the cells is in constant interaction with the tissues. Blood was drawn on day 4.

The efflux can be calculated by the loss of activity in the cells of the cell suspensions (Ax see Design of the experiments). A considerable decrease of activity is observed after the first lysation procedure, especially in the PMNC cell suspension, compared to the loss of ^{65}Zn during the rest of the whole procedure. This is because of loss of polluting cells like erythrocytes in the PMNC cell suspension and platelets in the MNC cell suspension, cells that are not counted in the Coulter counter but contain considerable amounts of ^{65}Zn. The total loss during the rest of the procedure was 39%, 24% and 28% (MNC, PMNC, and erythrocytes, respectively) of the total ^{65}Zn radioactivity in the cells calculated, after the separation and first lysation step. Also during the second lysation and washing steps polluting cells, not counted in the Coulter counter, are loosed. During all incubation and washing steps after the Percoll isolation of the various blood cells, MNC, PMNC and erythrocytes, lost 17 %, 19 %, and 20 % of their ^{65}Zn respectively; 75%, 76 % and 82%, respectively, of this efflux of ^{65}Zn was lost during incubation and the first washing step after the incubation period.

The efflux of ^{65}Zn from the blood cells can be calculated not only from the loss of radioactivity of the blood cells in the cell suspensions after each step (A_x), but also from the A_{efflux}, the activity released from the cells while washing the cells and determined in the various lysation, incubation, and washing solutions (see Design of the experiments). A slightly lower efflux is the result: 12%, 10%, and 19%. Probably the A_{efflux} is less reliable because the A_{efflux} is calculated as the difference between the total loss of activity and the loss of activity owing to loss of cells.

Figure 4 represents the total number of blood cells of the successive steps and consequently the loss of cells during the whole procedure. Only the cells counted by the Coulter counter are represented, not the polluting cells like platelets or polluting erythrocytes in the MNC and PMNC suspensions.

DISCUSSION

Determination of zinc concentrations in blood cells has been used for the determination of the zinc status. Changes in concentrations in blood cells have been demonstrated in case of zinc deficiency (12). Determination of zinc in blood cells is difficult, however (13), because the zinc concentrations in MNC and PMNC suspensions are low. Using 3 mL of blood and assuming a cell recovery of 50% (see also the results of experiment 4) the final zinc concentration of the cell suspension is about 0.3 μmol/L when resuspended in 0.5 mL GPB, which is near the lower limit of detection of an AAS determination. There is also a risk of zinc contamination of the suspension.

Using a medium containing ^{65}Zn we demonstrated that the in vitro uptake of zinc by blood cells is increased in the case of zinc deficiency (7). In inflammatory stress, in which a decrease of serum zinc concentration also can be demonstrated, no increase of zinc uptake by erythrocytes was observed. In MNC and PMNC, a minor increase of zinc uptake was demonstrated only during the first days of inflammatory stress (8). Probably this method can differentiate between a real and an apparent zinc deficiency caused by inflammatory stress. The method is a combination of a modified blood cell separation procedure (11) and the in vitro uptake of ^{65}Zn by erythrocytes (14). In the present study, we investigated the influence of incubation time and incubation temperature on the in vitro uptake of zinc by blood cells. Also, the in vitro efflux of zinc from blood cells during the in vitro isolation and incubation steps in nearly physiologic conditions was studied.

In experiments 1 and 2, we studied the influence of incubation time on the uptake of zinc by three types of blood cells. All three types of cells showed more or less the same response. During the first 5.5 h zinc was taken up fast. After five and a half hours the ^{65}Zn concentration increased only slowly.

Five out of six curves (experiments 1 and 2) show a similar pattern that - idealized - can be represented as shown in Figure 5. This curve can de regarded as the summation of three curves, e.g.,

1) A rapid section (within the first half hour),

2) A second rapid phase (ending at about t = 5.5 h); and

3) A slow, quasilinear process, visible after t = 5.5 h.

These three phases can be explained as follows: during the first phase a rapid uptake in (or exchange on) the cell membrane takes place, a process that ends, c.q. reaches equilibrium, within the first half hour. During the second phase, a part of the intracellular zinc exchanges with the ^{65}Zn of the medium. This is also a rapid process that, however, does not start

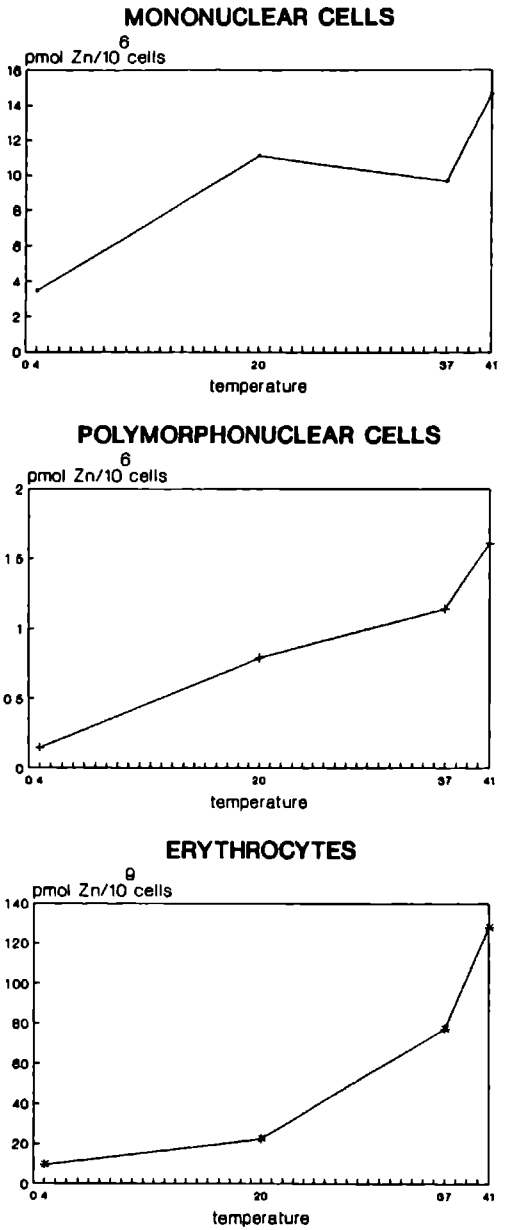

Figure 3 - Influence of incubation temperature in °C (X-axis) on the uptake of zinc by human blood cells (Y-axis) (experiment 3). The upper figure represents the uptake by MNC; the figure in the middle the uptake by PMNC and the bottom figure the uptake by erythrocytes. The incubation time was 1 h.

at time 0 but at about t = 0.5 h (Figure 5). This lag-time could be explained by assuming that the slope of the second curve is proportional to the y-value of the first curve. We believe however, that the reason why the two processes can be separated is the result of a special circumstance: the cells were not immediately at the proper temperature, i.e., at 37°C, because the temperature used at the isolation procedure was 4°C. The lag-time could therefore be caused by the time required to warm up the cells. The first phase, a physical process, would hardly be affected by the temperature difference and therefore shows no lag-time. The third phase is probably an uptake in or exchange with a Zn compartment (either structural or chemical) that is much slower than the effect of the second phase. It therefore shows as a quasi linear phase (c.X in the expression in 'Calculations and Statistics'). The phenomenon of a shoulder, probably the result of the way the isolation and incubation is performed, provides the opportunity to study the successive phases in zinc uptake by blood cells.

The first phase is not more than a shoulder. When taking in consideration the standard deviation of the points up to 5.5 h (only possible in experiment 2), the shoulder is only significant in MNC and not in PMNC and erythrocytes. However, taking into account the striking similarity between the curves, we believe this feature is real, especially since a plausible explanation can be given. Also Jones et al. (15) studied the uptake of zinc by blood cells during 45 minutes and found a rapid uptake during the first 15 min, a slower uptake between 15 - 30 minutes and a gradual increase in uptake after 30 min. These details were not discussed in their article. Because we cannot give exact numerical value for the shape of the curve (values a and d in the equation); we have to settle for the rough estimates given in tables 2 and 3.

The combination of the inter-individual and the intra-individual standard deviation was about 25% for all cells. It is noteworthy that the standard deviation increases with a longer incubation period and larger uptake of zinc by the cells, indicating that the increase in standard deviation is mainly owing to the biological variation between the cells of the various persons (interindividual standard deviation), and, to a minor extent, owing to variations as a result of the method (intraindividual standard deviation). This was confirmed by other experiments. In other experiments with human cells (to be published separately) the intraindividual standard deviation in MNC, PMNC, and erythrocytes was 11%, 15%, and 5%, respectively.

The point of intersection of the extrapolation of the curve at the shoulder (t = 30 - 60 min) with the Y-axis probably gives an indication of the maximum uptake of zinc by the cell surface, although no complete equilibration has been reached. In experiment 1 the

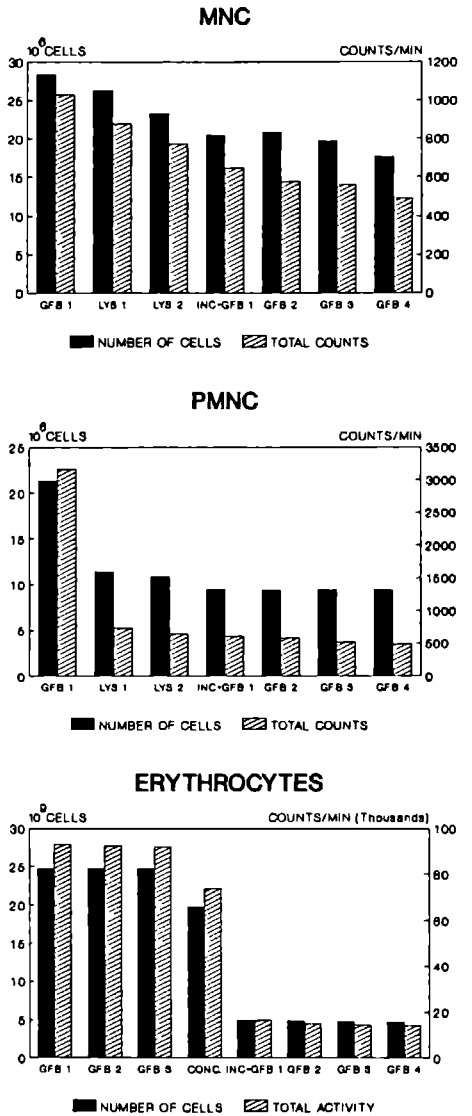

Figure 4 - Quantity of the cellular ^{65}Zn concentration and the total number of blood cells during washing and incubation procedures and consequently the loss of cells during the successive steps (experiment 4). MNC (upper figure), PMNC (figure in the middle) and erythrocytes (bottom figure). Along the X-axis the successive cell suspensions are indicated(GFB1, LYS1, LYS2, INC+GFB1 and so on, represents the successive steps during the isolation and incubation procedures; LYS represents the lysation and the consecutive washing step). The Y-axis shows the number of cells (left) and the counts/10^6 cells.min (right).

quantity of zinc that was taken up by the surface of the cells was in MNC, PMNC, and erythrocytes 8%, 14%, and 1.5%, respectively, of the total quantity of zinc in the cells.

The point of intersection of the extrapolation of the curve after 330 min (the end part of the curve) with the Y-axis in the figures probably represents the maximum zinc uptake by the rapidly exchangeable zinc intracellular pools, both the membrane and the cytoplasm (a and b, respectively, in the section Calculations and Statistics). The values in table 2 represent the size of the pool that is probably labeled by exchange with intracellular zinc not tightly bound to proteins, peptides, and amino acids. By comparison of the rapidly exchangeable pool as indicated in table 2 with the total cellular zinc content of the various blood cells (table 2 (13)), the size of the readily exchangeable pool is estimated to be 40%, 53% and 10%, respectively, for MNC, PMNC, and erythrocytes, of the total cellular zinc content. There is probably a gradual difference between rapidly exchangeable and slowly exchangeable bound zinc (second category of zinc binders that do exchange with ^{65}Zn in the medium at a slow rate or do not exchange at all), which probably explains the small slope after t = 5.5 h. In cells, zinc is tightly bound to metallothioneine, among others.

The amount of the exchangeable pool can be calculated by comparison of the exchangeable pool with the cold zinc concentrations of the various blood cells mentioned in the literature. The PMNC showed the fastest uptake and the greatest exchangeable pool of zinc. The uptake of zinc by leucocytes after the first hour was about 34 times the uptake by erythrocytes, and during the third section the velocity of the uptake was about 24 times the uptake by erythrocytes.

The process of zinc uptake of blood cells was studied in experiment 2, in human cells. Because more blood could be taken, we were also able to study the variations in results between individual persons. The results in human cells confirmed the results in cells taken from rats, although the size of the exchangeable pool and the maximum zinc uptake by the cell membrane was smaller compared to the cells taken from rats. The size of the exchangeable pool of intracellular zinc in erythrocytes found in our study shows a remarkable resemblance to the results found by Simons (16), total intracellular zinc 135.7 $\mu mol/10^{13}$ cells and an exchangeable component of 6.7 $\mu mol/10^{13}$ cells (4.9%). The standard deviation at the first hour of the experiment was very small compared to the quantity of zinc uptake after 24 h, indicating that the method has a small intraindividual standard deviation and that the standard deviation after 24 h was a result of the interindividual variation. The percentage of the standard deviation at a particular moment compared to the value at that particular moment was constant.

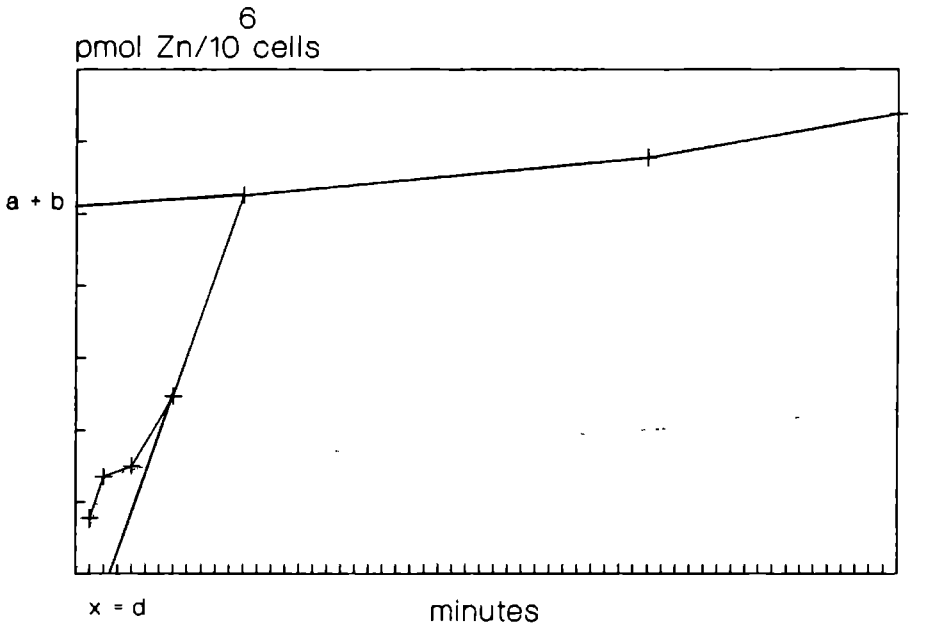

Figure 5 - Theoretical model to explain the phases in the uptake of zinc by blood cells. The dotted line represents a fast uptake by the cell membrane, the interrupted line represents the uptake by the intracellular pool and the uninterrupted line represents the sum of both lines and approximates the curves drawn in figure 1 and 2

The in vitro uptake of zinc by various blood cells has been studied before in whole blood (17, 18), in erythrocytes (14,19,20) and in leucocytes (6,15,17,21,22). To our knowledge no study has been performed in which the uptake of zinc by the various blood cells in one experiment has been studied in a nearly physiologic standard medium. Moreover the results of these studies are not unanimous, because of variations in study design and incubation media. The in vitro uptake of ^{65}Zn by whole blood has been examined by e.g. Berry et al (18) and Chester and Will (17), however, no physiologic standard incubation medium was used. Freshly drawn heparinized whole blood was incubated with ^{65}Zn. The in vitro uptake of ^{65}Zn by erythrocytes is inversely related to the albumin concentration in the medium (14), and consequently the uptake of ^{65}Zn by these cells is influenced by the albumin concentration of the serum when incubation is performed in whole blood. Also, the higher specific activity of ^{65}Zn owing to a decrease of serum zinc can in part account for the increase of ^{65}Zn uptake.

The uptake of zinc by erythrocytes was described by Sasser et al (19), both in plasma

and in saline during a period of 10 h. The rate of uptake was 0.6% of the total intracellular zinc concentration per hour for erythrocytes in plasma. This is in the same range as in our study. Also van Wouwe et al (14) described the zinc uptake by erythrocytes. The uptake of zinc decreased after 10 h; a maximum uptake was reached after 24 to 30 h. In the study by Dennis et al (22) the in vitro and in vivo uptake of zinc by leucocytes was examined. The uptake in vivo and in vitro were parallel but in vitro the uptake was 1/10 of the in vivo effect. However, in this study it was not clear if the albumin concentration and specific activity of ^{65}Zn in vivo and in vitro were identical. Contrary to our results, Chvapil et al (6) found that zinc uptake reached equilibration after 8 min but in his study the incubation was performed in a synthetic medium with an unphysiologic high Zn concentration (82 μmol/L $ZnCl_2$).

In experiment 3, the influence of the incubation temperature on the uptake of zinc was studied. With higher temperatures the velocity of the uptake of zinc was higher. At 4°C only a small uptake was observed possibly because of a nonspecific binding of zinc on the cell membrane (see experiments 1 and 2). Higher uptake was seen in these human blood cells with temperatures increasing above 37°C. This suggests an active process for the uptake of zinc by blood cells (Figure 3), like that suggested by Torrubia (20). Probably fever is increasing the uptake of zinc, supporting the function of these blood cells. A considerable uptake of zinc at 20°C by leucocytes compared to erythrocytes was observed, suggesting that zinc uptake by leucocytes is less influenced by a decrease of temperature. If the uptake after 1 h, at 4°C in experiment 3 is compared with the results of experiment 2, especially the point of intersection of the extrapolation of the curve at the shoulder (t = 30 - 60 min) with the Y-axis (which probably gives an indication of the maximum uptake of zinc by the cell surface) we observe a great similarity, although the numerical figures of experiment 3 are only a rough estimation. In MNC the uptake in experiment 2 is 3.26 pmol/10^6 cells and in experiment 3 (after 1 h of incubation) 3.47 pmol/10^6 cells. In erythrocytes the uptake was 22.3 pmol/10^9 cells in experiment 2 (with a maximum uptake after 24 h of 937 pmol/10^9 cells!) and 9.5 pmol/10^9 cells in experiment 3. These results strengthen the hypothesis concerning the process of the uptake of zinc by blood cells explained in the discussion of experiment 1.

The efflux of zinc during the procedure was studied in experiment 4. During the first separation and lysation procedure considerable activity was lost which was mostly owing to the loss of polluting cells, because the decrease of the intracellular zinc concentration

during the washing steps after incubation was small. Therefore, the washing steps have only a minor effect on the estimation of the size of the exchangeable pool. The separation and washing procedures were performed at 4°C. A larger but still small loss of activity was observed during incubation (Figure 2). This procedure was performed at 37°C. If one assumes that the ^{65}Zn activity in cell suspensions represents the cellular zinc concentration of MNC, PMNC and erythrocytes (see Materials and Methods), the loss of ^{65}Zn represented the efflux of zinc in 1 h. In 1 h of incubation, MNC, PMNC, and erythrocytes lost 17%, 19%, and 20%, respectively, of the intracellular zinc concentration. These results can be compared with the magnitude of the uptake of zinc during the first hour demonstrated in experiment 1 (23%, 23% and 16.4%, respectively), so in- and efflux are at the same magnitude. The uptake of zinc by blood cells is part of an exchange process, rather than just zinc uptake. At low temperatures the efflux of zinc was very small during the washing procedures.

The efflux of zinc by (red) blood cells was investigated in several studies (6, 15, 22 - 24), partly with in vitro incubated blood cells. The incubation time varied between 7 min (6) and 2 h (24). In one study, an in vivo labeling was performed (22); the time between the ^{65}Zn injection and blood collection was 1 h. The interpretation of the results of these zinc efflux studies are difficult because owing to the short incubation period, probably most of the ^{65}Zn was only bound to the cell membrane. It is not surprising that a rapid wash out of zinc was found (50%) in 1 h (22).

The efflux during procedures at 4°C was small (washing procedures). So, the amount of efflux of zinc by blood cells is not only influenced by the time of incubation of the cells and the composition of the medium (15) but also by the temperature of the medium.

The questions that have been raised concerning the uptake of zinc by blood cells can be answered by the studies described in this paper: the uptake of zinc is part of an exchange process of which the magnitude has been described; the process of the uptake of zinc can be divided in three phases and is influenced by the temperature. The magnitude of the interindividual and intra-individual standard deviation of the method has been described. Together with the physiologic importance of this study the results of this study, should be considered in case the in vitro uptake by blood cells is used as a method to determine the zinc status.

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SERUM ALKALINE PHOSPHATASE ACTIVITY DURING ZINC DEFICIENCY AND LONG-TERM INFLAMMATORY STRESS

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ABSTRACT

A decrease of serum zinc can be caused by a real zinc deficiency but can also be caused by an apparent zinc deficiency, like in inflammatory stress. The aim of this study was to evaluate the diagnostic power of serum alkaline phosphatase activity in the discrimination between pathophysiologic states of 'real' and 'apparent' zinc deficiency. A decrease of serum zinc was induced in growing and adult rats, by providing a diet low in zinc and by causing inflammatory stress. Alkaline phosphatase activity was determined using reagents low and enriched in zinc. Serum alkaline phosphatase was decreased in zinc-deficient adult rats ($p<0.01$). In zinc-deficient growing rats alkaline phosphatase activity was not different from normal rats but alkaline phosphatase activity decreased rapidly. In the same growing rats a significant difference was found in alkaline phosphatase activity determined using a buffer low and enriched in zinc ($p<0.001$). After inducing inflammatory stress a decrease of alkaline phosphatase activity ($p<0.01$) and serum zinc ($p<0.001$ between both groups of rats) was seen during the first few days. After the initial phase of inflammation alkaline phosphatase activity normalized, serum zinc showed a rise which and after correction for the decrease of serum albumin reached the level of the control rats. A difference in alkaline phosphatase activity using a buffer low and enriched in zinc was observed, only during the first days after induction of inflammatory stress ($p<0.001$). Probably the method of measurement of the difference in enzyme activity, using buffers low and rich in zinc, can be used as an indication for zinc deficiency in situations with changing alkaline phosphatase enzyme concentrations. Alkaline phosphatase activity is decreased during the initial phase of inflammatory stress resulting from a decrease of serum zinc.

INTRODUCTION

Zinc deficiency is a serious disorder expressed by a variety of symptoms like delayed wound healing, dysfunction of the immune system, anorexia, decreased protein synthesis, night blindness, skin diseases, hair loss, and growth retardation (1).

Usually the serum zinc concentration is used to determine the zinc status (2), but the serum zinc concentration is not only decreased in case of real zinc deficiency, but also in case of stress (3). During stress serum zinc is redistributed from the serum into the liver. In addition, the serum albumin concentration will decrease and since serum zinc is bound to albumin for about 75% (4), a decrease of serum albumin will result in a decrease of

serum zinc. Because the decrease of serum zinc during stressful events does not result in symptoms, characteristic of real zinc deficiency, this situation is called apparent zinc deficiency. In conclusion serum zinc is not the perfect parameter for the zinc status. Several other methods are used to determine the zinc status, e.g. determination of zinc concentrations in urine (5), in hair (6), and in blood cells (7). All of these methods have disadvantages.

Zinc is a component of more than 200 enzymes (8). In case of zinc deficiency, zinc containing metallo-enzymes often do not function optimally. Alkaline phosphatase (AP) is one of these zinc dependent metallo-enzymes. Serum AP activity may be used to determine the zinc status (9), but AP activity is also influenced by other factors, for instance growth. The aim of this study was to investigate the influence of a physiologically induced decrease of the serum zinc concentration, like in case of dietary induced zinc deficiency and inflammatory stress, on serum AP activity in growing and adult rats. The concentration of zinc in the reagents is of influence on the determination of AP activity (10-12). In general for complete expression of serum AP activity a reagent with a high concentration of zinc is used. We made the hypothesis that in case of real zinc deficiency the difference in AP activity using buffers low and rich in zinc is greater than in normal situations.

MATERIAL AND METHODS

Animals

In all experiments male Wistar rats were used. The rats were housed separately in stainless steel metabolic cages. Before the adaptation period the rats were fed commercial chow. The diet on which the rats were raised contained variable amounts of zinc (< 2, 12 or 92 mg/kg (IRI-OB, (13)). In experiments 1, 2, and 3, a diet containing 12 mg/kg zinc was given from 2 weeks before the start of the experiments, to adapt the animals to the diet and the metabolic cages. The zinc concentration of 12 mg/kg is considered sufficient to maintain a normal zinc status (14). All blood samples were taken under ether anaesthesia by heart puncture. The guidelines for the care and use of laboratory animals in the Netherlands were followed.

Design of the experiments

Five experiments were performed to study the influence of physiologically induced variations in zinc concentration on the AP activity. In the first 2 experiments zinc deficiency was induced in adult and, respectively, growing rats. The AP activity was studied. In the first experiment also the ^{65}Zn retention was determined as a gold standard for the zinc status. In the third experiment adult rats were made zinc deficient and the AP activity was determined in buffers low and enriched in zinc. Because in the first experiment the effect of zinc supplementation in zinc deficiency was investigated, the fourth experiment was designed to study the influence of zinc supplementation in rats on a diet with a normal or a high zinc content. In the fifth experiment inflammatory stress was induced.

Experiment 1

Zinc deficiency in adult rats, AP activity and the effects of zinc supplementation.

After adaptation (see above) 16 male rats (300 g) were injected intraperitoneally with 74 kBq (2 μCi) of ^{65}Zn (day 0). Eight rats received a diet with an adequate amount of zinc (IRI-OB; 12 mg/kg Zn), 8 others were fed a zinc-deficient diet (<2 mg/kg Zn). After 1 month all rats received a diet with an adequate amount of zinc (12 mg/kg). The control group received pair feeding throughout the experiment. Three mL blood were taken under ether anaesthesia by heart puncture on days 0, 7, 14, 21, 28 and 36. The serum was analyzed for zinc and magnesium concentration, albumin and for AP activity, in a buffer enriched in zinc. The total body retention of i.p. administered ^{65}Zn served as reference for the degree of zinc deficiency. Total body counting was performed after 4 hours and on days 0, 1, 2, 5, 9, 14, 21, 28 and 35. The total body retention at day one was taken as 100%.

Experiment 2

Zinc deficiency in growing rats, AP activity using buffers low and rich in zinc.

This experiment was identical to experiment 1, but it was performed in young rats with a body weight of about 100 grams at the start of the adaptation period. Blood samples were taken at days 0, 17, 24, 37, 51, 65 for serum zinc and albumin assay, and for AP activity determination with reagents low or enriched in zinc.

Experiment 3

Zinc deficiency in adult rats, AP activity using buffers low and enriched in zinc.

The difference was that serum AP activity was determined with reagents low or rich in zinc content. Blood was withdrawn on days 0, 3, 7, 14, 21, and 28.

Experiment 4:

Zinc supplementation in normal rats, AP activity.

After an adaptation period of 2 weeks, 32 Wistar rats (250 g) were divided into 2 groups. Sixteen rats received a diet with an adequate amount of zinc (IRI-OB; 12 mg/kg Zn). Sixteen others received a diet rich in zinc (92 mg/kg Zn). After the first day eight rats in both groups received a supplementation of 10 mg/kg of zinc (as zinc acetate) in the diet. Three mL blood were taken under anaesthesia by heart puncture on days 0, 7, and 14. The serum was analyzed for zinc, magnesium and albumin concentration and for AP activity determined with reagents low or enriched in zinc.

Experiment 5:

Inflammatory stress, AP activity using buffers low and enriched in zinc.

After an adaptation period of 7 days in which 64 rats, with a mean body weight of 200 g, were housed separately in stainless steel metabolic cages and raised on a standard IRI-OB diet, rats were divided into two groups of 32 animals of equal mean body weight. To induce stress in one of these groups turpentine was injected intramuscularly in a dose of 0.25 mL into both hind legs to induce a long-standing inflammation. Before turpentine was injected, blood was collected from 4 rats in the inflammation group and 4 rats in the control group. Four hours, 1 day, 2, 3, 4, 7 and 14 days after the turpentine injection 2 mL of blood was taken under ether anaesthesia by heart puncture from 4 rats in the inflammation group and from 4 rats in the control group, for determination of serum zinc, magnesium and albumin concentrations and determination of AP activity with reagents low or enriched in zinc. The control group was pair fed throughout the experiment. The total body retention of i.p. injected ^{65}Zn served as a control to determine if zinc deficiency occurred. All the rats were injected i.p. with 74 kBq (2 μCi) ^{65}Zn seven days before the turpentine injection. The total body activity at the time of the turpentine injection was taken as 100%. Total body counting was performed after 4 hours and on days 1, 2, 3, 4, 7, and 14.

Materials

The diet (IRI-OB) was produced by Hope Farms (Woerden, the Netherlands) using components low in zinc (<2 mg/kg Zn, (13)). In the zinc-sufficient diet zinc was added as sulphate to a final zinc concentration of 12 mg/kg. Sufficient quantities of other trace ele-

ments were also added. The Cu concentration in both versions of the diet was 9 mg/kg.

Stainless steel needles and plastic syringes (Monoject, Sherwood, USA) were used. All solutions were prepared with deionized distilled water (resistance > 15 MΩ), processed through a Millipore system (Bedford, Massachusetts, USA).

Only polypropylene pipet tips, plastic tubes (15 mL polystyrene, Greiner, Solingen, Germany and 50 mL polypropylene, Falcon, Becton Dickinson and Company, Lincoln Park, New Jersey, USA) and bottles (0.8 L, NUNC, Roskilde, Denmark) were used. All these material were free of zinc contamination. All chemicals were of analytical grade (Merck, Darmstadt, Germany).

Determination of zinc

All the determinations of zinc and AP activity were performed in a blinded fashion. Zinc was determined by flame atomic absorption spectrophotometry (AAS)(Perkin Elmer 5000; Norwalk, Connecticut, USA). The lower limit of detection was 0.28 μmol/L and the response was linear till at least 15 μmol/L. Standard reference containing $ZnCl_2$ (Tritisol, Merck, Darmstadt, Germany) was used in the concentrations expected to be found in the samples. No detectable zinc could be found in solutions and in deionized water used for the analytical experiments.

Determination of alkaline phosphatase activity

AP activity was determined at 30°C according to recommendations of the IFCC (15), except for the modification of the zinc concentration. A Multistat III centrifuged analyzer (Instrumentation Laboratory, IJsselstein, the Netherlands) was used. 4-nitrophenylphosphate and 2-amino-2-methyl-1-propanol (AMP) were provided by Instruchemie (Hilversum, the Netherlands). The AMP contained a relatively low concentration of zinc (1.6 μmol/L) and is used in clinical practice. Appropriate zinc suppletion for the zinc rich modification of the AP activity determination resulted in a zinc concentration of 1.0 mmol/L in the test assay mixture.

AMP-buffers contain a chelator (16), which inhibits the activity of AP by removing zinc. By addition of zinc, this inactivation can be inhibited. In case high concentrations of

the chelator are present in the buffer, more zinc should be added to compensate for the zinc inactivating properties of the chelator. So the increase of activity of AP by zinc is in part due to the inhibition of the chelator and in part because zinc is a structural component in the enzyme. In the buffer used in the present experiments only a slight pollution was found using the method described by Derks et al. (17), implying that the low zinc concentration of the AMP-buffer was valid. A buffer with a low zinc concentration was used, to be able to detect small variations in serum zinc concentration by variations in the activity of the enzyme. In case high concentrations of zinc were present in the buffer small variations in the zinc concentration of the serum would not result in a variation in enzyme activity.

Final concentrations of the other components in the test mixture were: AMP 350 mmol/L, 4-nitrophenylphosphate 16 mmol/L, Na 140 mmol/L, Cl 160 mmol/L, Phosphate 0.4 mmol/L, pH 10.3, magnesium as magnesium acetate 2.0 mmol/L.

Total body counting

The radio tracer ^{65}Zn was supplied by Amersham International (Amersham, Buckinghamshire, U.K., code ZAS 2 , 3.7-37 GBq per mg of Zn as chloride in 0.1 mol/L HCl). Total body radioactivity of the rats was counted with a shadow shield counter consisting of two Tl-activated NaI crystals (4 x 6") connected to a Nuclear Data multi-channel analyzer N.D. 60 A (Nuclear Data; Schaumburg, Illinois, USA).

Calculations

Biological half-life of the ^{65}Zn total body retention

The biological half-life ($T_{1/2}$) of ^{65}Zn was calculated from the total body retention in % measured as a function of time.

From the slope (γ) of the linear part of the line, which is calculated by the least square method from the natural logarithm of the total body data, the biological half-life ($T_{1/2}$) of the ^{65}Zn was calculated using $T_{1/2} = \ln 2/\gamma$.

Estimation of the corrected serum zinc concentration in serum during stress.

In case of inflammation the serum zinc concentration can be corrected for the decrease of serum albumin by increasing that part of the serum zinc concentration that is bound to albumin, to the same extent as the concentration of albumin in the inflammation group has to be increased to reach the level of the control group. That part of the total serum zinc, that is transported by albumin, should be increased by the factor:

Alb control/ Alb inflammation.

The corrected serum zinc concentration can be calculated by the equation:

$$\text{Corr Zn tot (inflam)} = 0.25 \times \text{Zn tot (control)} + \frac{\text{Alb control}}{\text{Alb inflam}} \times ((\text{Zn tot (inflam)} - (0.25 \times \text{Zn tot (control)}))$$

Where

Zn tot (control)	= total serum zinc concentration of the control group;
Corr Zn tot (inflam)	= corrected serum zinc concentration of the inflammationgroup;
Alb control	= serum albumin of the control group;
Alb inflam	= serum albumin of the inflammation group;
Zn tot (inflam)	= total serum zinc concentration of the inflammation group.

The factor 0.25 represents the fraction of the serum zinc concentration that is mainly bound to the stable fraction of alpha macroglobulin, in rat alpha-1-macroglobulin (18) and in human alpha-2-macroglobulin. The concentration of this protein is not significantly changed by stress (18,19) and affinity of zinc to this protein is high (20), so that the number of zinc molecules transported by alpha-1-macroglobulin in the inflammation group will be equal to that of the control group. Using this equation we postulate that the binding of zinc to the binding proteins (the number of zinc molecules transported by one protein molecule), in the normal situation and in case of an inflammation will be similar, and that zinc bound to alpha-1-macroglobulin is not affected by inflammation. In case a difference still remains between the corrected serum zinc concentration and the serum zinc concentration of the control group, this will be the result of other causes, e.g. redistribution of serum zinc into the liver.

Correction of alkaline phosphatase activity

Although standard reference solutions were used for the determination of AP activity a difference was seen in AP activity in the serum of rats as determined with buffers low and high in zinc content. The results are provided without correction unless stated otherwise. To calculate the difference between both methods the assumption was made that the AP activities should be identical on day 0. The AP activity determined using a buffer low in zinc was corrected by using the following equation for each serum sample:

$$\text{Corr AP} = \frac{\text{mean AP high Zn at day 0}}{\text{mean AP low Zn at day 0}} \times \text{AP low Zn}$$

Statistics:

Wilcoxon rank sum test was used to compare the results in both groups of rats during a period of the experiment.

The Student t-test was performed on the data collected at time point; differences between two groups were considered significant if $p < 0.05$ (two tails).

RESULTS

Experiment 1:

Normally in adult rats the total body retention of ^{65}Zn is calculated as a percentage of injected dose immediately after administration. Because two rats lost a significantly larger quantity of ^{65}Zn (52% and 36%) compared to the other rats (5.55% sd 4.42%) during the first 24 hours, the total body retention at day one is taken as 100%. Probably this greater loss can be explained by a partial injection of the ^{65}Zn into the gut. When plotting the natural logarithm of the total body data of experiment 1 versus time, a linear relation was found from day 7 on. Zinc-deficient rats retained more ^{65}Zn compared to control rats ($T_{1/2}$ = 176 days versus $T_{1/2}$ = 51 days; Figure 1a), confirming that these animals indeed had become Zn deficient.

Serum zinc and AP activity decreased gradually in the zinc-deficient rats (zinc: 11.7 versus 29.3 μmol/L, $p<0.001$ on day 21, Figure 1b and AP: 60 versus 77 U/L, $p<0.01$, Figure 1c). On day 28 the serum zinc concentration was 40% of that of control rats. No

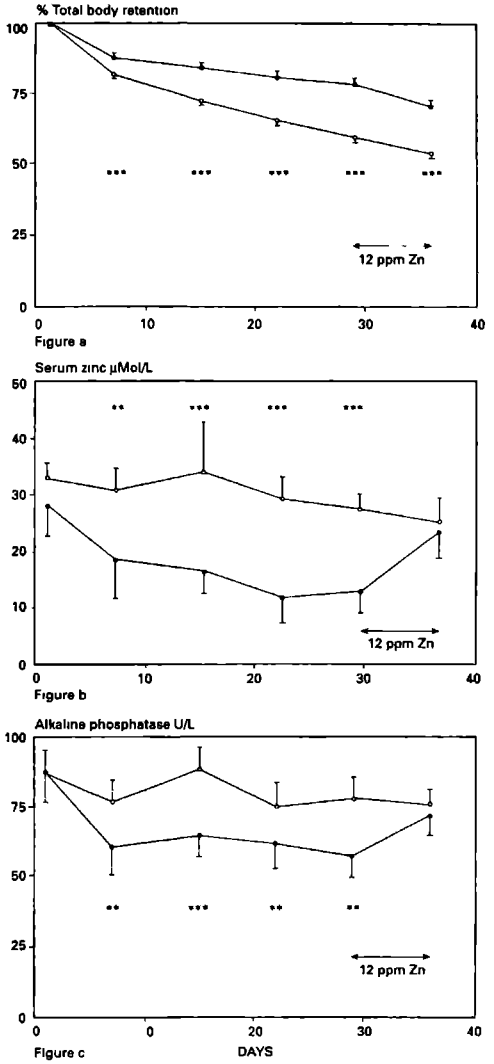
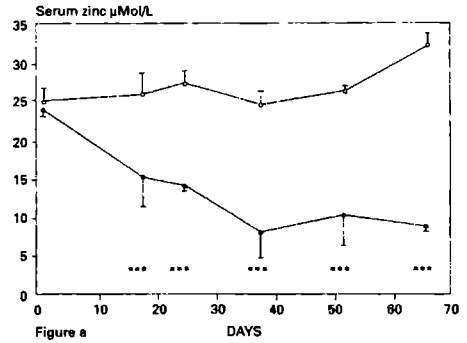


Figure 1 - zinc deficiency experiment in adult rats

*a: Total body retention of ^{65}Zn in zinc-deficient adult rats (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). The total body retention of ^{65}Zn one day after i.p. injection was taken as 100%. The error bars indicate standard deviations (*** p=<0.001).*

*b: Serum zinc in zinc-deficient adult rats (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots; ** p=< 0.01;*** p=<0.001).*

*c: Serum alkaline phosphatase activity in zinc-deficient adult rats (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots, ** p=< 0.01; *** p=<0.001).*



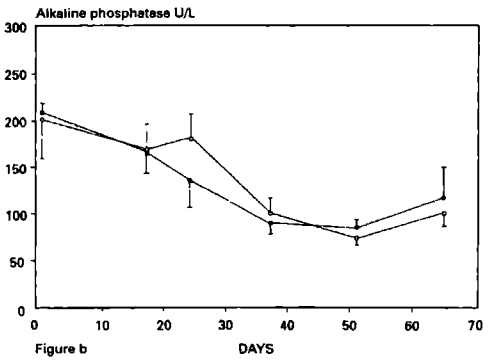


Figure 2 - zinc deficiency experiment in growing rats.
*a: Serum zinc in zinc-deficient growing rats (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots). The error bars indicate standard deviations (*** p=<0.001).*
b: Serum alkaline phosphatase activity in zinc-deficient and control growing rats (diet < 2 mg/kg; closed dots) and control rats (12 mg/kg; open dots).

change was seen in the serum albumin concentration in the zinc-deficient rats. After 1 month zinc was added to the diet. Serum zinc and AP activity were normalized after 1 week.

Experiment 2:

In growing rats on a Zn-deficient diet, serum zinc gradually decreased to 8.0 µmol/l after 37 days (control group 24.7 µmol/l, p<0.001, Figure 2a). No significant difference was observed in the activity of AP between normal and zinc-deficient rats (Figure 2b), but

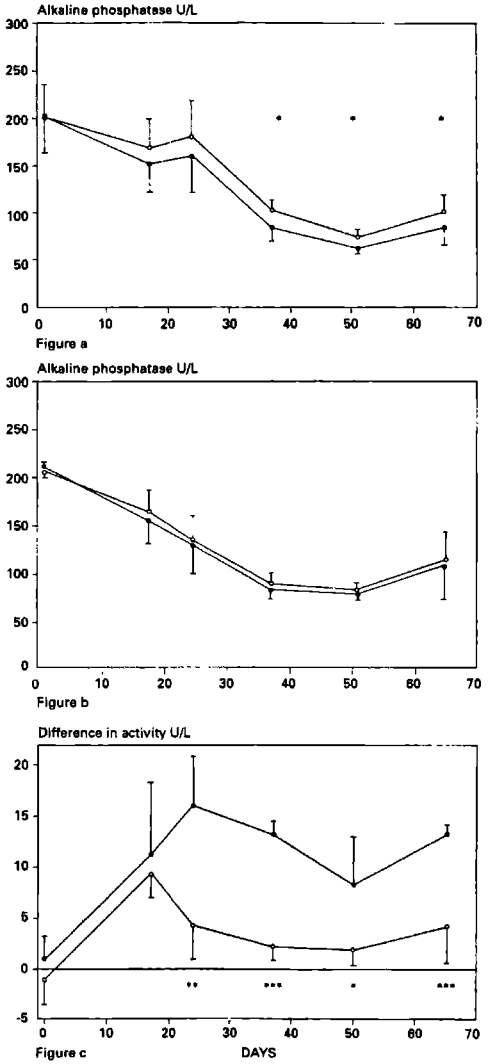


Figure 3 - zinc deficiency experiment in growing rats.

a: Serum alkaline phosphatase activity in zinc-deficient rats, using buffers low (closed dots) and rich (open dots) in zinc. The error bars indicate standard deviations (p=< 0.05).*

b: Serum alkaline phosphatase activity in control rats, using buffers low (closed dots) and rich (open dots) in zinc.

c: Difference in alkaline phosphatase activity by using buffers low and rich in zinc, between zinc-deficient rats (closed dots) and control rats (open dots) (p=< 0.05, ** p=< 0.01; *** p=<0.001).*

the activity decreased rapidly in both groups. In the zinc-deficient rats the lowest value was reached later.

Also the activity was determined using a buffer low and enriched in zinc. At the first day (day 0) the difference between the AP activity using buffers with a low and an enriched zinc content was, in the zinc-deficient group 21 U/L, and in the control group 24 U/L. Serum AP activity using both methods should by definition be identical at day 0. Using the equation mentioned in 'Calculations', the AP activity using a buffer low in zinc was corrected.

The AP activity in zinc-deficient rats, determined using a buffer low in zinc and a buffer high in zinc is shown (Figure 3a). The reagents low in zinc result in a lower AP activity during the study. In Figure 3b the same was shown for the control rats. Comparing Figure 3a and 3b the difference between both methods was greater in zinc-deficient rats than control rats. In Figure 3c this difference is shown. This difference was highly significant on individual point after inducing zinc deficiency and for the total period using the Wilcoxon rank sum test ($p < 0.001$).

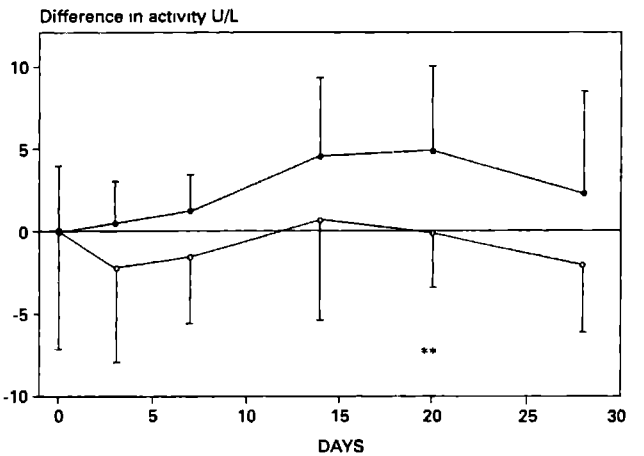
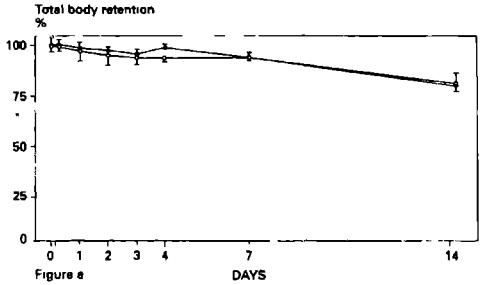
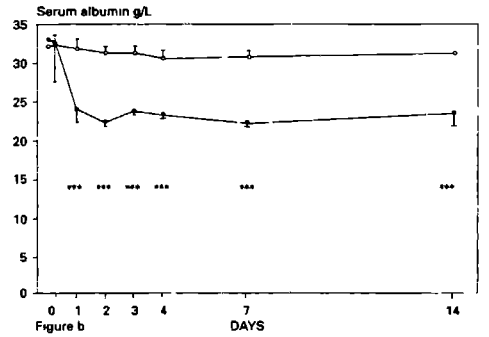


Figure 4: zinc deficiency experiment in adult rats
*Difference in alkaline phosphatase activity by using buffers low and rich in zinc, between zinc-deficient adult rats (closed dots) and control rats (open dots). A significant difference was observed using a Wilcoxon rank sum test, and on day 20 using a Student t-test. The error bars indicate standard deviations (** p=< 0.01).*





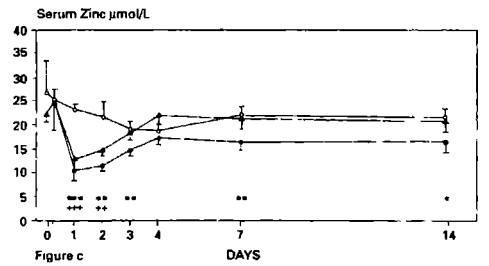


Figure 5: inflammatory stress experiment

a: Total body retention of ^{65}Zn in rats with an inflammation (closed dots) and control rats (open dots) during the experiment.

*b: Serum albumin concentrations in rats with an inflammation (closed dots) and control rats (open dots) during the experiment (*** $p<0.001$).*

c: Serum zinc concentrations in rats with an inflammation (closed dots) and control rats (open dots) and serum zinc concentrations corrected for the decrease of serum albumin (closed triangle) during the experiment. The error bars indicate standard deviations ($p=< 0.05$; ** $p<0.01$; *** $p<0.001$, for serum zinc in rats with an inflammation compared with control rats, and ++ $p<0.05$; +++ $p<0.01$ for corrected serum zinc in rats with an inflammation compared with control rats).*

Experiment 3:

Because of the results in experiment 2 the difference in AP activity using buffers low and high in zinc was also studied in adult rats. The results of this experiment confirmed the results in experiment 1, especially the serum zinc and AP activity.
After correction for the difference in AP activity with the reagents low and enriched in zinc at day 0, a significant difference was observed in AP activity for the total period using the Wilcoxon rank sum test ($p = 0.01$) and for individual sample times on day 20 (Figure 4).

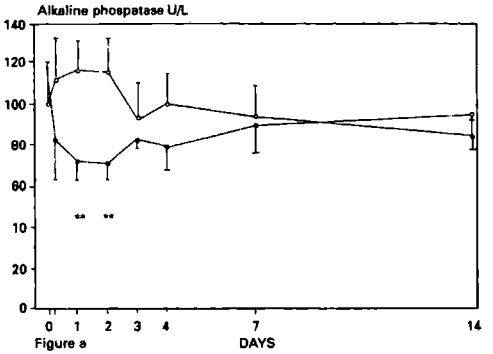
Experiment 4:

In this experiment the results of zinc supplementation in normal quantities was studied in rats raised on a diet with sufficient zinc and on a diet rich in zinc. No change in time or difference between the group supplemented by extra zinc and the control group was observed in serum zinc concentration and in AP activity.

Experiment 5:

The total body retention of ^{65}Zn (Figure 5a) showed no difference between the groups, indicating that no zinc deficiency did occur during inflammatory stress (inflammation group $T_{1/2}$=34 days, control group $T_{1/2}$=31.5 days). None of the rats showed symptoms of zinc deficiency like loss of hair. The serum albumin concentration decreased during the first day.

Thereafter a constant difference persisted (23.8 versus 31.3 g/L, $p<0.0005$, Figure 5b)). Using a Wilcoxon rank sum test a highly significant difference was calculated if all the data of both groups were compared ($p<0.0001$). Simultaneously during the first day a considerable decrease of serum zinc concentration was seen in the rats with stress (day one: inflammation group 10.3 μmol/L and control group 23.0 μmol/L, $p=0.002$) (Figure 5c). After the first few days a smaller difference persisted. A Wilcoxon rank sum test was performed for comparison of the independent results of both groups of rats on all the data collected during the experiment. A highly significant difference was calculated ($p<0.0001$). When the serum zinc concentration of the rats with an inflammation was corrected for the decrease of serum albumin using the equation described in 'Methods', the differences between the groups largely disappeared, especially after the third day (Figure 5c). A decrease of AP activity was only seen on the first two days (71 versus 115 U/L, $p<0.05$; Figure 6a). Using the same method described in 'Calculations' and applied in experiments 2 and experiment 3, a significant difference was also observed in AP activity determined using a buffer low and high in zinc. This difference largely disappeared after the fourth day (Figure



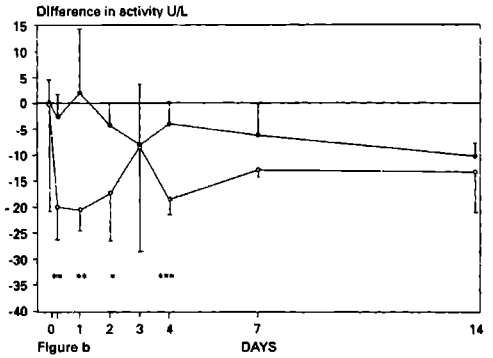


Figure 6: inflammatory stress experiment
*a: Serum alkaline phosphatase activity in rats with an inflammation (closed dots) and control rats (open dots) during the experiment (** p<0.01).*
b: Difference in alkaline phosphatase activity by using buffers low and high in rich, between rats with inflammatory stress (closed dots) and control rats (open dots). The error bars indicate standard deviations (p=< 0.05; ** p=< 0.01; *** p=<0.001).*

6b). During these experiments no changes in serum magnesium concentrations were demonstrated.

The total body retention of ^{65}Zn as a function of time served as an indication of the zinc status. In experiment 1, in which adult rats were made zinc deficient, the ^{65}Zn total body retention confirmed that the animals had become zinc deficient. To detect eventual changes in zinc status during the first hours and days of the inflammatory stress experiment (experiment 5), we preferred a linear decrease of the total body retention, so ^{65}Zn was injected 7 days before the turpentine injection. During the inflammatory stress experiment no difference in ^{65}Zn retention was observed between the rats with inflammatory stress and the control rats, indicating that no zinc deficiency occurred.

After introduction of a zinc-deficient diet the serum zinc concentration fell in adult and more impressively in growing rats. Normalization of the diet resulted in an increase of serum zinc concentration. Supplementation of the same quantity of zinc in the diet of control rats did not influence the serum zinc concentrations.

After induction of inflammatory stress the serum zinc concentration decreases rapidly. This decrease continued during the first 3 days, after which a smaller difference persists. The serum albumin concentration decreased within one day to a level which remained stable throughout the experiment. After correction of the serum zinc concentration for the decrease of serum albumin no difference in serum zinc concentration was observed after the second day. This indicates that after the initial phase of inflammatory stress the decrease of serum zinc is mainly resulting from a decrease of serum albumin. Probably the initial rapid decrease of serum zinc is caused by an increase of zinc uptake by the liver, an observation that has been demonstrated by Solomons et al. (22).

The determination of activity of zinc dependent enzymes has been used as a method to determine the zinc status, e.g. serum AP activity (21). The of AP activity is limited by the influence liver function and bone turnover, factors that influence the concentration of AP enzyme. The advantage of using AP activity as a method to determine the zinc status is that the determination of AP activity is possible in each clinical laboratory. Serum AP activity is the result of two factors: the protein concentration of the enzyme in the serum and the activity of the enzymatic molecules. The former factor is influenced by release of the enzyme by AP containing tissues, mainly liver and bone. The latter factor is influenced by the number of zinc and magnesium atoms in the AP enzyme molecule (10-12). During the experiments no changes in magnesium concentration were observed. The zinc deficiency experiments in adult rats confirmed that serum AP activity is decreased in zinc deficiency. Sandstead (23) demonstrated an increase of serum zinc concentration and AP

activity during zinc deprivation in marginally zinc-deficient men. This was not confirmed in the present experiments. The increase of AP activity after zinc supplementation may be an indication of a preceding zinc deficiency (experiment 1). Our experiments confirmed studies in animals (24-26) and in patients (27,28). No increase was demonstrated in case of zinc sufficiency (experiment 4). Kasarkis (25) proposed the AP ratio (the magnitude of the increase of AP activity after zinc supplementation) as an indicator of the zinc status. Some criticism can be addressed to this method. The results of experiment 4 demonstrated that this method, the AP ratio, cannot be used to differentiate between rats receiving food with a marginal but sufficient quantity of zinc and rats receiving a diet rich in zinc.

In growing rats (experiment 2), no difference was observed between normal and zinc-deficient rats. In a study in infants the same problem was observed; no correlation was demonstrated between serum zinc and AP activity (29). During growth the AP activity declines because of a decrease of concentration of the enzyme. In case of zinc deficiency growth is retarded. Maybe the concentration of the AP enzyme in the serum remains high in the zinc-deficient rats, which counteracts the decrease of activity of the enzyme due to a decrease of zinc concentration. In the zinc-deficient rats the lowest AP activity value was reached later.

In the inflammatory stress experiment AP activity was decreased during the initial phase of inflammation, showing that AP activity is influenced by inflammatory stress. This is not known from clinical practice (30) although in physiological stress experiments published in veterinary journals, a decrease of AP activity has been observed (31) (without giving an explanation). After the initial phase no difference in AP activity was observed in the present experiment, although serum zinc was still decreased. The normalisation of the serum zinc concentration after correction for the decrease of serum albumin, indicates that after the second day the decrease of serum zinc was mainly the result of a decrease of serum albumin. Probably AP activity is influenced by the shift of serum zinc into the liver during the first days after inducing inflammatory stress, but not by an apparent decrease of serum zinc due to a decrease of serum albumin in long lasting inflammation. AP activity probably represents the serum concentration of free zinc.

We postulated that in case of zinc deficiency the difference of AP activity, determined in a buffer low and a buffer rich in zinc, would be greater than in zinc sufficiency. This method would largely bypass the influence of the concentration of the enzyme on the assessment of AP activity and the influence of other minerals like magnesium and offers the opportunity of having a instantaneous monitor of the zinc status. This would be the case if the difference in activity would be greater than the difference in enzyme concentra-

tion, because of liver or bone disorders. In our experiments the concentrations in the buffer were chosen within the range of zinc concentrations used in laboratory practice because it is well-known that very low (see 'Methods') and high concentrations in the AMP-buffer (several times higher than the concentrations used in clinical practice) added in vitro, will decrease serum AP activity (11,26,32). The hypothesis was confirmed by experiment 2 in zinc-deficient growing rats, experiment 5 in rats with inflammatory stress, and in experiment 3 in zinc-deficient adult rats. On individual sample times this was highly significant in growing rats and rats with inflammatory stress. In zinc-deficient adult rats this difference was smaller resulting from considerable standard deviations caused by physiological differences and the limited number of animals. The method could be improved by increasing the difference of the AP activity. Essential for this method is the use of an AMP-buffer with a low concentration of chelator and consequently low zinc concentration. To our knowledge only one study has suggested that manipulation of the method of determination of AP activity could be used for the diagnosis of zinc deficiency (41). In this study a method was described in which inactivated AP was re-activated by serum. Also in this study a greater difference was observed in case of zinc deficiency.

We may conclude that the experiments demonstrate that, in case of a low serum zinc concentration, the difference in AP activity probably can differentiate between a true zinc deficiency and an apparent zinc deficiency resulting from a decrease of serum albumin; even if AP enzyme levels are changing. This method should be confirmed in humans.

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CHAPTER 11

SUMMARY

Malnutrition is a status resulting from a (relative) shortage of nutrients which results in a decrease of biological functions (**chapter 2**) This suggests that malnutrition is caused by insufficient nutritional intake Also the disease process itself plays an important roll in malnutrition, therefore the expression nutritional depletion, in stead of malnutrition, corresponds better with the clinical condition of a patient The disease process exerts multiple effects on the nutritional status, especially by changing the metabolism Nutritional depletion can be divided into a syndrome resulting from depletion of energy or a syndrome resulting from depletion of proteins, resembling nutritional depletion of energy or proteins in tropical medicine marasmus and kwashiorkor, respectively Both conditions have their own causes and clinical outcome, but in patients often a combination of both is seen protein-caloric nutritional depletion This condition carries a high mortality risk

Not only macronutrients (carbohydrates, fat and proteins), but also micronutrients, like zinc, can be depleted Zinc is active as part of many metallo-enzymes Zinc deficiency will cause a decrease of enzyme function A decrease of function of metallo-enzymes will result in symptoms of zinc deficiency A low serum zinc concentration may reflect real zinc deficiency but also inflammatory stress during inflammation the zinc transporting protein will decrease and subsequently total serum zinc concentration In addition there is a shift of zinc from the serum into the liver This decrease of serum zinc concentration does not result in classical symptoms of zinc deficiency, like hair loss and skin lesions This condition is called apparent zinc deficiency A decrease of serum zinc can be caused by real zinc deficiency or by inflammatory stress There is a need for a method that can distinguish between the two conditions

The aim of this thesis was to study the prevalence of malnutrition and to study the validity of the methods to assess malnutrition in patients

In **chapter 3** we studied the prevalence of malnutrition in non-surgical hospitalized patients and its association with disease complications In a population of 155 non-surgical hospitalized patients the prevalence of energy-protein nutritional depletion, the association with the disease process, and complications arising during the course of the disease were studied Three methods for the assessment of the nutritional status were used a subjective assessment based on the clinical picture the Subjective Global Assessment, and two methods based on weight and laboratory values the Nutritional Risk Index and the Maastricht Index According to these criteria the prevalence of nutritional depletion ranged from 45% to 62% at admission In the first 90 patients the nutritional status at dis-

charge was also studied. The Maastricht Index showed a significant improvement during admission in nutritional status, from 64% to 53%. At least 50% of the patients with active inflammatory bowel disease were malnourished. Patients with nutritional depletion demonstrated a higher incidence of complications, with an Odds Ratio of about 3 (2.7-3.1). A multivariate correction for the disease category and for the severity of the disease decreased the Odds Ratio to 1.6 (Nutritional Risk Index) and 2.4 (Maastricht Index). Because the parameters for severity of the disease are also influenced by the nutritional status the real Odds Ratio's will be in between these figures.

Because of the high prevalence of nutritional depletion the question was raised of a potential false positive misclassification. This was studied in 175 healthy blood donors and 34 elderly volunteers attending the Nijmegen Four Days Walking Event (**chapter 4**). These volunteers were considered to be healthy on the basis of a questionnaire and simple medical examination. For the assessment of the nutritional status the objective parameters: the Nutritional Risk Index and the Maastricht Index, were applied. The figures for the prevalence of nutritional depletion for the whole population were 1.9% and 3.8% respectively, but in volunteers older than 70 years they were much higher: 5.9 and 20.6%, respectively. In general the risk of false positive misclassification was very low, but in elderly persons the risk was high if the Maastricht Index was used, which limits the value of the Maastricht Index in these persons.

Not only the prevalence of nutritional depletion of macronutrients (protein-energy malnutrition) in patients with chronic inflammatory bowel disease was studied (**chapter 5**), but also the question of the prevalence of nutritional depletion of micronutrients especially of zinc, in patients with Crohn's disease. Various methods are used for the assessment of zinc deficiency. A "Gold Standard" for the assessment of the zinc status, which can be applied in clinical practice is not available. To compare the relative value of various frequently used methods for assessment of zinc deficiency, these methods were correlated with the presence of possible factors inducing zinc deficiency: number of liquid stools, weight loss, bowel resection and the extent and severity of inflammation (Crohn's Disease Activity Index, van Hees Index).

In 31 patients with more or less active Crohn's disease the zinc status was assessed by the serum zinc concentration, serum alkaline phosphatase activity and the zinc content of

blood cells. In 68% of the patients the serum zinc concentration was below normal. The serum zinc concentration was correlated with the extent of bowel resection and activity of the disease according to the van Hees Index. Serum alkaline phosphatase activity was also correlated with bowel resection. Zinc in erythrocytes and mononuclear blood cells was poorly correlated with factors inducing zinc deficiency. Only zinc in polymorphonuclear cells correlated with weight loss and the number of liquid stools. Because of these disappointing results using the zinc content of blood cells this method was applied in an experiment in which zinc-deficient and control rats were studied. No significant differences in the zinc content of blood cells, between zinc-deficient rats and control rats were observed. Determination of zinc in blood cells has no additional value for the assessment of the zinc status in individual patients.

The previous study emphasizes the need for better methods to assess the zinc status. This method should be applicable in clinical practice and should distinguish real zinc deficiency from apparent zinc deficiency caused by inflammation.

In **chapter 6** the changes in zinc metabolism during zinc deficiency and inflammatorion were studied in zinc-deficient rats and rats with a chronic inflammation. In both situations serum zinc concentrations are decreased but inflammation results in an apparent zinc-deficient status since no symptoms of zinc deficiency are present.

In the first experiment a group of rats were fed a zinc-deficient diet and in the second experiment, inflammatory stress was induced by an intramuscular turpentine injection. In both studies, control rats were used. ^{65}Zn was injected intraperitoneally to study the in vivo uptake and exchange of zinc in tissues by determination of the activity of ^{65}Zn in blood cells and tissues. The ^{65}Zn total body retention was determined to measure total body zinc changes and used as a reference. In the zinc deficiency experiment concentrations of cold zinc were determined, as well.

Both in zinc-deficient rats and in rats with inflammatory stress serum zinc concentrations were decreased. The total body ^{65}Zn retention was increased in case of zinc deficiency, but no differences were observed between rats with stress and control rats. The uptake in ^{65}Zn by erythrocytes and mononuclear cells **in vivo** was increased in zinc-deficient rats, but not in rats with inflammatory stress. The uptake of ^{65}Zn by cortical bone was very slow in zinc-deficient rats, and less in control rats. In zinc-deficient rats a decrease of concentration of cold zinc was only seen in bone cortex. The uptake of zinc by the liver was increased during the initial phase of inflammation.

Bone appears to be an important source of zinc, in case of zinc deficiency, and could be used to determine the zinc status. However, this is impractical in clinical practice. Contrary to the findings in zinc deficiency, no differences were observed in ^{65}Zn uptake **in vivo** in blood cells in inflammatory stress, which offers an opportunity to distinguish real and apparent zinc deficiency.

The results of the previous study could be used to develop new methods to determine the zinc status.

In **chapter 7** the **in vitro** uptake of ^{65}Zn by blood cells was studied in zinc-deficient rats in chapter 7. Serum zinc decreased in zinc-deficient rats. A significant increase of uptake of zinc by blood cells of zinc-deficient rats in vitro was observed. This was demonstrated in successive blood samples in a period of 4 weeks, and was confirmed in a second experiment. This suggests that the **in vitro** uptake of zinc by blood cells could be used for determination of the zinc status.

In a pilot experiment inflammation was induced in rats. After 2 days serum zinc was markedly decreased, but no difference was demonstrated in zinc uptake by erythrocytes and mononuclear cells.

The previous study suggested that by the employment of the in vitro uptake of zinc by blood cells, real and apparent zinc deficiency could be distinguished. In **chapter 8** the in vitro zinc uptake by blood cells in rats with inflammatory stress was studied. To validate this hypothesis consecutive blood samples were taken from rats with chronic inflammation. Serum zinc was markedly depressed during the first few days after induction of the inflammation. Later on this decrease became less and attributable to a decrease of serum albumin, the zinc transporting protein. The in vitro uptake of zinc by erythrocytes was not increased in rats with inflammation. This study confirmed the previous experiment, and strengthened the hypothesis that by measuring the in vitro uptake of zinc by blood cells, zinc deficiency could be established, and real zinc deficiency distinguish from apparent zinc deficiency.

The methodology of the assessment of the zinc status by measuring the in vitro uptake of zinc by blood cells was studied in detail in **chapter 9**. The influence of incubation time and

temperature revealed the physiological process of the uptake of zinc by (blood) cells. At least three phases were recognized in zinc uptake: a brief fast phase probably representing aspecific binding of zinc, a second slower phase probably representing the active uptake of zinc by the cell, and a slow phase in which the in- and efflux of zinc were more or less equilibrated. In mononuclear and polymorphonuclear cells the exchangeable intracellular zinc pool was 40 and 53% respectively in rats. In erythrocytes this pool was much smaller: 10%. The efflux of zinc from (blood) cells was also studied. The efflux of zinc was of the same magnitude as the influx. Both the influx and the efflux of zinc in blood cells were very small at 4°C, and of the same magnitude as in the first phase in the process of zinc uptake. This suggests that the first phase of zinc uptake is probably due to non-specific binding.

The method of measuring the in vitro uptake of zinc by blood cells is an important new method to determine the zinc status but this method is rather time consuming and therefore especially of interest for research.

Zinc deficiency will result in symptoms that are caused by suboptimal functioning of many metallo-enzymes. The function of metallo-enzymes is of interest, because it can probably be used as a method to determine the zinc status, and because if a suboptimal enzyme function due to zinc deficiency is demonstrated, this dysfunction is also directly related to the mechanism by which symptoms of zinc deficiency are caused: malfunction of metallo-enzymes. Determination of the serum alkaline phosphatase activity is a daily routine in every clinical laboratory. Alkaline phosphatase is a zinc dependent metallo-enzyme. Therefore the influence of zinc deficiency, growth and inflammation on the serum alkaline phosphatase activity were studied (**chapter 10**). In zinc-deficient rats serum alkaline phosphatase activity was decreased. In zinc-deficient growing rats no decrease could be observed, probably because the decrease of alkaline phosphatase activity is compensated by an increase of alkaline phosphatase enzyme due to growth retardation in the zinc-deficient rats.

Serum alkaline phosphatase activity was also determined in a buffer low and a buffer enriched in zinc. The difference in activity was greater in zinc-deficient growing rats than in the control rats. Serum alkaline phosphatase activity was decreased by inflammatory stress but only during the initial phase. This could be explained by the decrease of serum zinc concentration due to redistribution of zinc from serum into the liver. In the same initial period an increase of difference in alkaline phosphatase activity determined in a buffer low in zinc and a buffer enriched in zinc was demonstrated. So changes in serum alkaline

phosphatase activity in time, can be an indication for changes in zinc status. The difference in alkaline phosphatase activity, using buffers low and enriched in zinc, can be an method that can instantaneously be used, and may differentiate between real zinc deficiency and a decrease of serum zinc caused by an chronic inflammatory process.

CONCLUDING REMARKS

The aim of this thesis was the assessment of malnutrition, both of macronutrients and micronutrients, in patients.

The following questions were derived from the aim of this thesis:

- What is the prevalence of nutritional depletion?
- Can the currently available methods to determine nutritional deficiency distinguish between nutritional depletion and a normal nutritional status?
- Can the currently available methods to determine nutritional deficiency distinguish nutritional status from disease activity?

In the first part of this thesis the association between the disease process and malnutrition of **macronutrients** was studied. In the second part the influence of a specific disease process, inflammation, and a reduced intake of one of the **micronutrients**, zinc, was investigated. For both macronutrients and the micronutrient zinc, methods for the assessment of malnutrition were studied and some showed to be applicable and reasonably specific for malnutrition, and in part independent of the disease process.

Which answers can be drawn from the previous studies?

Nutritional depletion of **macronutrients** is frequent, not only in surgical patients but also in non-surgical patients. Malnourished patients suffer a higher risk of complications, and this risk can be explained in part by the disease activity. Nutritional depletion assessed by the Maastricht Index showed the highest risk of complications even after correction for disease. These methods can at least in part distinguish nutritional status from disease activity.

The methods to determine malnutrition do not lead to over-estimation of nutritional depletion by false positive misclassification, except for the Maastricht index in elderly subjects, so these methods can distinguish between nutritional depletion and a normal nutritional status.

In patients with active Crohn's disease a decrease of serum zinc concentration was also frequently observed. This suggests that malnutrition of the micronutrient zinc is frequent.

This decrease could be explained by real zinc deficiency but also by apparent zinc deficiency induced by the inflammatory process. In this study, it was not clear, by which method zinc deficiency should be established. There is a need for a method that reflects the zinc status and can distinguish between real and apparent zinc deficiency. The in vitro uptake of zinc by blood cells can be used to diagnose zinc deficiency so this method can distinguish between nutritional depletion and a normal nutritional status. By the use of erythrocytes real zinc deficiency can be distinguished from apparent zinc deficiency.

Serum alkaline phosphatase activity is influenced the zinc status but also by the concentration of the enzyme. Partially this can be reconciled by the assessment of the difference in serum alkaline phosphatase activity using two buffers, one low and one enriched in zinc. The difference in serum alkaline phosphatase activity is greater in case of zinc deficiency.

Serum alkaline phosphatase activity, and also the difference in alkaline phosphatase activity, is decreased during the initial phase of inflammation, but not in the phase of chronic inflammation in which serum zinc is mainly decreased due to a decrease of serum albumin, so this method can distinguish nutritional status from disease activity.

The results of the previous studies give rise to new questions and subsequent aims for studies in the future.

The effect of nutritional intervention on the complication risk should be studied in malnourished non-surgical patients. It would be of interest to study false negative misclassification by the methods used in the studies of chapters 2 and 3. The method of measuring in vitro uptake of zinc by erythrocytes should be studied in patients. Actually, at this moment several groups, engaged in zinc research, are using this method. The assessment of the zinc status by determination of serum alkaline phosphatase activity, using buffers low and enriched in zinc is also of interest, but the method should be improved by the development of buffers with even lower zinc concentrations.

BESTAAT ER IN NEDERLANDSE ZIEKENHUIZEN ONDERVOEDING?

Een samenvatting van dit proefschrift voor de geïnteresseerde leek

Ondervoeding wordt vaak geassocieerd met de problematiek van mensen in derdewereldlanden. Dat ondervoeding ook in Nederlandse ziekenhuizen voorkomt is nagenoeg onbekend bij de gemiddelde Nederlander. Wat de medicus betreft, die weet weliswaar dat een patiënt na een operatie ondervoed kan raken maar is zich toch ook onvoldoende bewust van ondervoeding bij niet-chirurgische patiënten. Tekenen van ondervoeding worden slechts gezien als een gevolg van de ziekte. Dat patiënten evenwel ook ondervoed kunnen raken door te weinig voedselinname of gebrekkige voedselopname door de darm wordt onvoldoende overwogen. Daarnaast verandert de ziekte ook vaak het stofwisselingsproces waardoor een patiënt uiterlijk een beeld kan vertonen dat sterk lijkt op dat van personen in derdewereldlanden met eiwitondervoeding.

Het is daarom dan ook van groot belang ondervoeding te kunnen herkennen en de omvang van het probleem van ondervoeding bij patiënten vast te leggen. In dit proefschrift is hiertoe een poging ondernomen, waarbij onderzoek is gedaan naar de bruikbaarheid van methoden om een dergelijke ondervoeding vast te stellen. Ondervoeding kan een tekort aan lichaamsmassa omvatten, veroorzaakt door een (relatief) tekort aan energie dragers als suikers en vetten alsmede de bouwstof eiwit, maar kan ook betrekking hebben op vitaminen, mineralen en sporenelementen.

Uit dit proefschrift blijkt dat minimaal 40% van de patiënten bij opname op een afdeling interne geneeskunde in meer of mindere mate ondervoed is en dat een kleine 10% zelfs ernstig ondervoed is. Indien een patiënt ondervoed is heeft hij een grotere kans op complicaties, nieuwe ziektebeelden of problemen tijdens het ziekenhuisverblijf. Zowel complicaties tijdens een ziekenhuisverblijf als ook ondervoeding worden door de artsen in het algemeen toegeschreven aan de primaire ziekte waarvoor de patiënt is opgenomen. Uit dit proefschrift blijkt dat de kans op complicaties maar ten dele door de ziekte zelf verklaard kan worden en dat deze ook beïnvloed wordt door de voedingstoestand. Een van de methoden om ondervoeding vast te stellen is de Maastricht Index. Van de toegepaste methoden om ondervoeding vast te stellen, voorspelt de Maastricht Index het beste de kans op complicaties, ook na correctie voor de ziekte. De observatie dat de kans op complicaties gecorreleerd is met ondervoeding, onderstreept de noodzaak om aandacht te besteden aan de voedingstoestand van de patiënt. Het door ons gevonden percentage ondervoeding bij opname in een ziekenhuis werd door velen als hoog beschouwd. Een van de verklaringen zou kunnen zijn dat patiënten ten onrechte als ondervoed werden beschouwd. Daarom werd met dezelfde methoden als waarmee de voedingstoestand bij de patiënten werd bepaald bij, in principe, gezonde vrijwilligers de voedingstoestand onderzocht. Voor de gehele groep gold dat bij minder dan 5% van de vrijwilligers ondervoeding werd gevon-

den. Dit betekent dat het gevonden percentage ondervoeding bij patiënten waarschijnlijk niet te hoog is. Bij de groep oudere vrijwilligers lag de situatie iets anders. Wanneer de voedingstoestand met de Maastricht Index bij ouderen werd bepaald, werd ruim 20% van de, in principe, gezonde ouderen als ondervoed beschouwd. Alhoewel de Maastricht Index het beste, onafhankelijk van de ziekte, toekomstige complicaties voorspelt, dient deze methode niet gebruikt te worden in studies waarin een aanzienlijk deel van de patiënten 70 jaar of ouder is. Bij ouderen zou de bepaling van de voedingstoestand dan ook beter niet met behulp van de Maastricht Index moeten worden uitgevoerd. Patiënten met chronische darmontstekingen (ziekte van Crohn en colitis ulcerosa), bleken bij opneming in het ziekenhuis in meer dan 50% ondervoed.

Alhoewel in onze studies de beschreven objectieve methoden om ondervoeding vast te stellen (waarvan de Maastricht Index er een is) goed bruikbaar bleken voor onderzoeksdoeleinden, betekent dit niet dat men deze methoden ook voor de bepaling van de voedingstoestand van een individuele patiënt kan gebruiken. Men zal dan altijd de laboratoriumwaarden die deel uit maken van deze index, als ook het gewicht, moeten relateren aan het klinisch beeld van de patiënt. Het gewicht van de patiënt wordt bijvoorbeeld mede beïnvloed door een tekort of een overmaat aan vocht in het lichaam van de patiënt. De subjectieve klinische blik blijkt volgens ons onderzoek ook bruikbaar.

Bij de ziekte van Crohn werden naast ondervoeding van macro-nutriënten (de hoofdbestanddelen van het voedsel die zorgen voor de energie en de opbouw van het lichaam) ook tekorten aan micro-nutriënten (de voedingsmiddelen die in geringe mate in de voeding aanwezig zijn en verantwoordelijk zijn voor het goed functioneren van het lichaam) vastgesteld. Zo bleek de zinkconcentratie in het serum bij ruim tweederde van de patiënten met een meer of minder actieve ziekte van Crohn verlaagd. Deze verlaging van de zinkconcentratie kan enerzijds verklaard worden door een reëel tekort aan zink, maar anderzijds ook door een verlaagde concentratie van het eiwit dat zink in het bloed transporteert: albumine. Bij ontstekingen daalt namelijk de albumineconcentratie in het bloed. Daarnaast is bekend dat zink bij ontstekingen, van het bloed naar de lever wordt verplaatst. Wij noemen deze situatie waarbij de serum zinkconcentratie verlaagd is, maar er geen verschijnselen van zinktekort zijn, een schijnbare zinkdeficiëntie. Al met al is het onduidelijk hoe groot het percentage patiënten met een echte zinkdeficiëntie is. Er bestaat daarom behoefte aan een methode die zinktekort zou kunnen vaststellen en die een reëel zinktekort zou kunnen onderscheiden van een schijnbaar zinktekort. In dit proefschrift zijn een aantal methoden op hun bruikbaarheid getest. Zo wordt de concentratie van zink in weefsels, zoals spieren of bot, wel als maat gebruikt om de zinkstatus vast te stellen. Het is ech-

ter erg belastend voor een patiënt om door een arts een stukje spier of bot uit het lichaam te laten nemen. Weefsels bestaan uit cellen. In het bloed zitten ook cellen. Men zou het bloed als een soort weefsel kunnen zien. Wellicht zou de concentratie van zink in deze cellen de zinkstatus kunnen representeren. Omdat er geen 'Gouden Standaard' voor de zinkstatus van deze patiënten bestaat werd in het bovenstaande onderzoek bij patiënten met de ziekte van Crohn de methoden om een zinktekort vast te stellen gecorreleerd aan factoren die een reëel zinktekort zouden kunnen veroorzaken zoals diarree en vermagering. De concentratie van zink in bloedcellen bleek geen sterk verband met deze factoren te hebben. Dit suggereerde dat de bepaling van de zinkconcentratie in bloedcellen onvoldoende gevoelig zou zijn om zinktekort vast te stellen. Ter bevestiging van dit vermoeden werden ratten zinkdeficiënt gemaakt. De zinkconcentratie in bloedcellen van deze ratten werd onderzocht en deze bleek niet te verschillen van de concentratie in bloedcellen van normale ratten. Geconcludeerd kan worden dat bepaling van zink in bloedcellen geen aanvullende waarde heeft op de normale methode om de zinkstatus vast te stellen: de serum of plasma zinkconcentratie.

Wij moesten daarom op zoek naar andere methoden om een zinktekort vast te stellen. Daartoe werden ratten zinkdeficiënt gemaakt. De concentratie van zink in de weefsels werd bepaald. Hierbij bleek dat een daling van de zinkconcentratie lang niet in alle weefsels kon worden waargenomen. De zinkconcentratie in bot leek nog het beste te correleren met de verminderde zink-inname en het ontstaan van verschijnselen van zinktekort zoals haaruitval. Bot is echter in de klinische praktijk niet makkelijk te verkrijgen. In het lichaam circulerende bloedcellen, met name rode bloedcellen en mononucleaire bloedcellen, bleken in geval van een zinktekort juist een sterk verhoogde opname van zink te vertonen. Dit werd niet gezien bij ratten met een chronische ontsteking. Een verhoogde zinkopname door bloedcellen lijkt dus specifiek voor zinktekort. Een ontstekingsproces zorgt niet voor een fout-positieve uitslag.

Bij het bovenstaande experiment werd de zinkopname door bloedcellen gemeten terwijl deze cellen zich in het lichaam bevonden. De vraag was nu of deze bloedcellen van zinkdeficiënte ratten buiten het lichaam een zelfde verhoogde opname van zink zouden laten zien. Indien dit het geval was, dan zou dit wellicht een methode kunnen zijn om een reëel zinktekort vast te stellen. Wederom werden ratten zink deficiënt gemaakt. De bloedcellen werden uit het lichaam gehaald en de opname van zink door deze cellen, buiten het lichaam, werd gemeten. Eenzelfde verhoogde opname van zink door de bloedcellen werd vastgesteld. Ter controle van deze methode werd een chronische ontsteking bij ratten opge-

wekt, om de invloed van een schijnbare zinkdeficiëntie te onderzoeken. Rode bloedcellen bleken geen verhoogde opname te vertonen, alhoewel de serum zinkconcentratie sterk daalde. Met deze methode kan blijkbaar worden vastgesteld dat er een zinktekort is en dat dit niet wordt beïnvloed door een ontsteking die een schijnbare ondervoeding veroorzaakt. De methode werd in detail onderzocht. Vastgesteld kon worden dat de opname van zink door bloedcellen een onderdeel was van een uitwisselingsproces van zink door deze cellen met hun omgeving. Verschillende stappen in dit opnameproces konden worden onderscheiden. Er werden aanwijzingen gevonden voor zowel een aspecifieke temperatuur onafhankelijke binding van zink aan de cellen, alsmede een temperatuur afhankelijk actief opname proces. De aspecifieke binding was echter van beperkte omvang. De uitwisseling van zink door bloedcellen kon bij 40C vrijwel volledig worden stopgezet.

De methode om de zink status vast te stellen door middel van de verhoogde opname van zink door bloedcellen lijkt veelbelovend. Een nadeel is echter de bewerkelijkheid van de procedure: het vergt ongeveer een dag werk. Dit hoeft geen bezwaar te zijn bij research maar is in de klinisch praktijk niet optimaal. Daarom is door ons ook onderzoek gedaan naar een andere methode, die beter aansluit bij de routinematige laboratoriumbepalingen. De bepaling van de activiteit van het enzym alkalische fosfatase wordt dagelijks verricht op een klinisch chemisch laboratorium. Alkalische fosfatase is een enzym dat zink nodig heeft om optimaal te werken. Het is bekend dat het enzym bij een lage zinkspiegel minder actief is. Men zou dus de activiteit van dit enzym als maat voor de zinkstatus kunnen gebruiken. Daarom werd onderzoek gedaan naar de toepasbaarheid van serum alkalische fosfatase activiteit als maat voor de zinkstatus. Daarbij moeten wij ons realiseren dat de activiteit van dit enzym, zoals dit in een klinische chemisch laboratorium wordt gemeten, het resultaat is van de activiteit van de individuele enzymmoleculen en de concentratie van het enzym. Wederom werden ratten zinkdeficiënt gemaakt. Tevens werd in een andere groep ratten een chronische ontsteking opgewekt. De alkalische fosfatase activiteit bleek te worden beïnvloed door de zinkstatus, maar ook door de concentratie van het enzym zelf: de activiteit van het enzym nam, bij ratten die in de groei waren, in de loop van de tijd af. Het is bekend dat de botaanmaak op later leeftijd afneemt en daarmee de concentratie van dit enzym. Om de invloed van de concentratie van het enzym op de gemeten activiteit te verminderen werd het verschil in activiteit bepaald wanneer er weinig en wanneer er veel zink aan het serum werd toegevoegd. Men zou verwachten dat indien in het serum zelf weinig zink aanwezig is het verschil in activiteit groter is dan wanneer reeds veel zink in het bloed serum aanwezig is. Het verschil in enzymactiviteit bleek inderdaad groter te zijn in bloed

van zinkdeficiënte ratten. Bij ratten met een ontsteking kon deze toename van het verschil in enzymactiviteit alleen gedurende de eerste dagen worden waargenomen. Dit is de fase waarin zink van het bloed naar de lever wordt getransporteerd. In deze fase werd naast een toename van het verschil in enzymactiviteit, een verlaging van de absolute enzymactiviteit waargenomen.

Samenvattend kunnen wij stellen dat ondervoeding frequent wordt gevonden bij ziekenhuispatiënten; dat ondervoeding gepaard gaat met een verhoogde kans op complicaties en dat deze verhoogde kans op complicaties maar gedeeltelijk door de ziekte zelf kan worden verklaard. In het algemeen zijn de methoden waarmee ondervoeding kan worden vastgesteld goed bruikbaar om tot een juiste classificatie van de voedingstoestand bij patiënten te komen. Bij een actieve vorm van de ziekte van Crohn komt niet alleen ondervoeding van macro-nutriënten maar ook een verlaagde serumconcentratie van het micro-nutriënt zink voor. Het vaststellen van een tekort aan zink is moeilijk. Zowel de opname van zink door rode bloedcellen als de bepaling van de serum alkalische fosfatase activiteit volgens een specifieke methode lijken veelbelovende nieuwe methoden om de zinkstatus vast te stellen.

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De laatste jaren is het onderzoek een nieuwe richting ingeslagen: onderzoek naar ondervoeding van de patiënt in het ziekenhuis. Veel van dit onderzoek is opgezet met de hulp van Prof. M. Katan. Beste Martijn, jij hebt mij geleerd om op een meer systematische manier wetenschappelijk onderzoek te verrichten. Verder dank ik je voor de grondige correcties van de artikelen die wij samen hebben geschreven. De uitvoering van deze studies is voor een belangrijk deel verricht door studenten. Fons, Angelika, Kristelle en Liesbeth, bedankt voor jullie hulp. In het bijzonder wil ik Tjard Schermer bedanken. Hij is nog steeds betrokken bij vervolgstudies die wij momenteel ontwerpen. Ook Hedwig van Heereveld, Brigit Bär, Gerard de Wild en Joanna Kruimel wil ik bedanken voor hun hulp bij deze studies. Beste Joanna, jouw onderzoek naar een nieuwe intraveneuze vetemulsie is inmiddels voltooid. Het schrijven van de artikelen heeft enige vertraging opgelopen door de grote hoeveelheid werk die je voor het nieuwe curriculum hebt moeten verzetten. Ik wens je de komende jaren meer tijd om je promotie te voltooien.

Dr. F.M. Nagengast, beste Fokko, wij zijn de enigen die nog overgebleven zijn uit de laatste jaren van het "van Tongeren Tijdperk". Wij hebben beiden gemerkt wat het betekent om onderzoek te doen op een afdeling die al een te krappe formatie heeft om alle klinische taken te verrichten, laat staan om zelf wetenschappelijk onderzoek in het laboratorium te bedrijven. Marie-Louise, ik heb bewondering voor het feit dat je het al die jaren bij mij op de kamer hebt uit gehouden. Het zal je af en toe veel moeite hebben gekost om de rommel die ik achter liet te accepteren.

Beste Pa en Ma, jullie zagen na mijn voltooiing van de middelbare school meer in een technische opleiding. Via de studie medicijnen ben ik toch in een biochemisch onderzoek verzeild geraakt. Uiteindelijk hadden jullie het toch bij het rechte eind.

Dr. M.W.S.M. Dooper, beste Marten, jou wil ik speciaal bedanken voor het onder de aandacht brengen van het ondervoedingsprobleem van de ziekenhuispatiënt in de lekenpers.

Beste Wil, beste Diny, dank voor het altijd weer bijspringen in ons huishouden op de meest onverwachtse tijden.

Zoals uit dit proefschrift blijkt, zijn deze studies niet zonder tegenslagen verlopen. Zonder een plaats waar ik alles kon relativeren zou dit onderzoek nooit zijn voltooid. Ine, we zijn

al vele jaren bij elkaar. We hebben elkaar gesteund tijdens studie en werk. Jij en onze kinderen, Floor en Sietze, vormen zo'n plaats waar ik afstand kon nemen van het werk.

CURRICULUM VITAE

Antonius Hermanus Josephus Naber werd geboren op 22 maart 1956 te Almelo. Na zijn lagere school op de St.Egbertus school te Almelo van augustus 1962 tot juni 1968, voltooide hij de middelbare school op het Pius X college te Almelo met het verkrijgen van het Atheneum B diploma in juni 1974.

De studie medicijnen werd van augustus 1974 tot september 1981 gevolgd op de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen heeft hij met lof afgelegd. Als student was hij lid van de onderwijscommissie van de medische faculteit. In deze periode werd een nieuw curriculum ontwikkeld, een curriculum dat in 1995 grondig is hervormd. Tijdens zijn co-schappen die hij liep tussen 1979 en 1981 was hij lid van de affiliatie kerngroep (een commissie die de kwaliteit van het onderwijs tijdens de co-schappen bewaakt). Na voltooiing van de studie medicijnen werd van oktober 1981 tot december 1984 de opleiding Inwendige Geneeskunde gevolgd in het Catharina Ziekenhuis te Eindhoven, met als opleider Dr. J.H. Flendrig (tot aan zijn vertrek naar de Rijks Universiteit Maastricht) en aansluitend Dr. H.F.P. Hillen. De opleiding Inwendige Geneeskunde werd voltooid in het Radboud Ziekenhuis onder de leiding van Prof. Dr. A. van 't Laar.

De opleiding gastroenterologie werd gevolgd in het Radboud Ziekenhuis tussen 1986 en 1989 onder de toenmalige leiding van Dr. J.H.M. van Tongeren. In deze periode werd een begin gemaakt met het beschreven onderzoek. Sinds april 1996 is de promovendus geregistreerd als gastroenteroloog. Op dit moment is hij werkzaam als staflid op de afdeling Maag-, darm- en leverziekten van het Radboudziekenhuis in Nijmegen.

Hij is lid van de doelgroep commissie voeding van het PAOG/NIKOG, een commissie die nascholing op het gebied van voeding initieert. Hij is als organisator en spreker betrokken geweest bij diverse nascholingscursussen en congressen. Hij was, mede met de apotheker drs. H. Bakker en de verpleegkundige P. Verbroekken, organisator van patiëntendagen voor de patiënten die thuis totale parenterale voeding ontvangen; een initiatief waaruit later de patiëntenvereniging voor patiënten met thuis parenterale voeding is voortgekomen. Hij was initiatiefnemer tot het oprichten van een voedingsteam in het Radboudziekenhuis te Nijmegen en was lid van de voorbereidingscommissie. Tevens is hij lid van het bestuur van de SVEM (Sectie Voeding En Metabolisme), een onderdeel van de Nederlandse Vereniging voor Gastroenterologie en is hij voorzitter van de Nederlands Voedingsteams Overleg, een overleg waaraan voedingsteams die werkzaam zijn in de Nederlandse ziekenhuizen, deelnemen. Hij is 'council member' voor Nederland bij het ESPEN (European Society of Parenteral and Enteral Nutrition). De promovendus geeft gastcolleges "enterale en paren-

terale voeding", alsmede colleges betreffende "voeding en stress", aan studenten van de studierichting voeding en gezondheid van de Landbouw Universiteit te Wageningen. Hij heeft de afgelopen 5 jaar een drietal subsidies verkregen waarop drie promovendi werkzaam zijn.

De promovendus is gehuwd, heeft twee kinderen en hoopt op dezelfde dag te promoveren als zijn vrouw.

STELLINGEN

behorende bij het proefschrift

The assessment of
MALNUTRITION IN NON-SURGICAL PATIENTS
from macronutrient to micronutrient,
with special emphasis on zinc

Ton Naber

Nijmegen, 15 september 1997

1. In Nederland komt ondervoeding frequent voor bij patiënten die opgenomen worden op een afdeling voor inwendige ziekten.
   Dit proefschrift

2. Een schijnbaar zinktekort veroorzaakt door een ontstekingsproces kan d.m.v. de bepaling van de in-vitro opname van zink door bloedcellen worden onderscheiden van een reëel zinktekort.
   Dit proefschrift

3. De serum alkalische fosfatase activiteit is verlaagd tijdens de initiële fase van een ontstekingsproces, hetgeen verklaard kan worden door een vermindering van de serum zinkconcentratie.
   Dit proefschrift

4. Promoveren na beoordeling van het proefschrift door een manuscriptcommissie is een archaïsche gewoonte indien men als voorwaarde voor goedkeuring van het proefschrift een tevoren vastgesteld aantal artikelen in internationale tijdschriften hanteert.

5. De kwaliteit van de vanuit het ziekenhuis naar de thuiszorg verplaatste zorg kan worden verbeterd indien deze zorg vanuit het ziekenhuis zou worden gecoördineerd, waardoor geldverslindende activiteiten kunnen worden voorkomen.

6. De clusterstructuur als organisatievorm van grote ziekenhuizen bemoeilijkt in sterke mate de invoering van een ziekenhuisbreed functionerend voedingsteam.

7. A short bowel is not equal to a short life.

8. Voor langdurige toediening van parenterale voeding in de thuissituatie is een arterio-veneuze shunt een veiliger toedieningsweg dan een centraal veneuze catheter.
   Naber AHJ, van Tongeren JHM.
   Clin Nutr 1989.

9. Bij hyperthyreoidie en een hoog TSH gehalte dient aan een verlaagde gevoeligheid van de hypofyse/hypothalamus voor het schildklierhormoon te worden gedacht.
   Hermus A, Ross H, van Liessum P, Naber A, et al,
   Neth J Med 1991.

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