ABSTRACT

ZINC IN CEREBROSPINAL FLUID AND SERUM IN SOME NEUROLOGICAL DISEASES

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The trace elements zinc and copper are essential components of many enzymes, some of which are of importance for the development and function of the central nervous system. Deficiency of the metals has been shown to lead to malformations and to the loss of myelin in animals. Earlier reports of zinc concentrations in the cerebrospinal fluid are few and the results variable. In multiple sclerosis and in epilepsy therapy with phenytoin there are varying reports of changes in serum concentrations of zinc and copper. A method was developed for the determination of zinc in cerebrospinal fluid by flame atomic absorption spectrophotometry utilizing a pulse nebulizer technique. Zinc and copper in serum were determined by flame atomic absorption spectrophotometry with continuous aspiration.

The normal concentrations of zinc in cerebrospinal fluid was 0.16±0.03 micromoles per litre (mean ± S.D.). The zinc concentrations were correlated with protein and albumin concentrations in the cerebrospinal fluid but not with the serum zinc levels. In the patients with increased protein concentrations in the cerebrospinal fluid or with subarachnoid haemorrhage increased zinc levels were found.

In 50 patients with multiple sclerosis lower serum concentrations of zinc were found compared to age and sex matched controls. In younger patients low serum levels of copper were also observed. There was no correlation between zinc and protein parameters in the cerebrospinal fluid of multiple sclerosis patients.

In untreated epileptic males low serum zinc concentrations were observed. During the first 72 hours of phenytoin therapy increased serum concentrations of zinc and copper were found. During long-term therapy with phenytoin alone or in combination with other antiepileptic drugs there was an increased serum concentration of copper and ceruloplasmin but no change in zinc concentration compared with controls.

Key words: zinc, copper, serum, cerebrospinal fluid, albumin, multiple sclerosis, epilepsy, phenytoin, blood brain barrier damage, subarachnoid haemorrhage.
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av

RAGNAR PALM
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Umeå 1982
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To
INGEGERD
ANNA ANDERS
OSCAR and BRITTA
This thesis is based upon the following papers which will be referred to in the text by their Roman numerals.

   Submitted for publication.

II. Palm R, Hallmans G. Zinc concentrations in the cerebrospinal fluid of normal adults and patients with neurological diseases.
    Submitted for publication.

     Submitted for publication.

IV. Palm R, Hallmans G. Zinc and copper metabolism in phenytoin therapy.
    Submitted for publication.
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Zinc (Zn) and copper (Cu) are trace elements and of importance for plants, animals and man. A trace element is an element that comprises less than 0.01% of the total mass of an organism (1).

Raulin (2) in 1869 found that Zn was necessary for the growth of Aspergillus niger. In 1934 Todd (3) showed that Zn was necessary for the optimum growth of rats. Zinc deficiency in man was first suspected in 1961 in Iranian males and later in Egyptians (4). Dwarfism, hypogonadism, hepatosplenomegaly, skin lesions and mental lethargy were found in zinc deficient people. A defective absorption of zinc is the underlying cause of acrodermatitis enteropathica (5). Low serum (S-) or plasma (P-) concentrations in man have been shown to occur in many situations, e.g. in patients with alcoholism, cirrhosis of the liver, malabsorption syndromes, during dialysis, inflammatory and infectious diseases and during therapy with chelating agents as penicillamine (6). In pregnant rats, zinc deficiency may cause malformations of the central nervous system (CNS) of the foetuses (7), and may also cause delayed myelinisation (8). In man zinc deficiency has been proposed as an explanation to the CNS symptoms in liver cirrhosis, fetal alcohol syndrome, malabsorption and acrodermatitis enteropathica (5). Low S-Zn concentrations during human pregnancy may be a sign of zinc deficiency, implying risks to mother and infant (9).

The zinc content of a 70 kg male is 1.5 - 2.0 g. The daily intake is 12 - 15 mg, of which 20 - 30% is absorbed from the gastrointestinal tract (5). The metal is excreted in the urine, about 0.5 mg/day, by the gastrointestinal tract, 1 - 2 mg/day or in sweat, 0.5 mg/day (5).

In the blood there is a high zinc content in the erythrocytes (10), where the metal is incorporated in the zinc metalloenzyme carbonic anhydrase (5). In serum or plasma about 80% of the zinc is bound to albumin and about 20% to $\alpha_2$-macroglobulin, (11). About 2 - 3% is bound to amino acids as histidine or cysteine (12) and a lesser amount
exists in ionic form (5). The S-Zn concentration is about 16% higher than P-Zn, mainly because zinc is released from disintegrating platelets during the clotting process (13). A low S- or P-Zn concentration may indicate a zinc deficiency or reflect a shift of metal from the blood to another body pool (6). The levels are however also influenced by age and sex (14), by diurnal variations (15), food intake (16), venous stasis (16), oral contraceptives and estrogens (17), pregnancy (9), corticosteroid (18) or ACTH therapy (19). In fertile females a variation in S-Zn levels is seen during the menstrual cycle (20).

Copper has been used in the treatment of mental and other diseases since the time of Hippocrates. In 1928 Hart et al (21) reported that Cu was an essential component of normal blood formation in the rat. Mills in 1930 (22) reported that copper supplementation accelerated haemoglobin synthesis in hypochromic anaemia in infants treated with iron salts. Bennets and Chapman (23) found that copper deficiency in ewes during the critical period of myelinisation of the lambs may result in loss of myelin and sway-back. In man, copper deficiency in infants has been described during malnutrition, infection and metabolic disorders (24).

The human body contains approximately 80 mg copper (25). The daily intake is 2 - 5 mg and about 30% is absorbed (24). The metal is excreted mainly in the bile, 0.5 - 1.3 mg/day, and only a small part via the urine, 0.01 - 0.06 mg/day (24). In the blood 50% of the copper content is found in the erythrocytes, mainly in the enzyme superoxide dismutase (24). In plasma or serum 93% of the copper is found incorporated in the enzyme ceruloplasmin (Cp) and the remaining bound to albumin and amino acids (24). Hypocupremia has been described in kwashiorkor and marasmus and in malabsorption states (24). Hypercupremia has been found in inflammatory and infectious diseases, haematologic disorders, neoplastic diseases, liver diseases, during pregnancy and estrogen therapy (24).

Zn and Cu are essential components of many metalloenzymes (26 - 27). In the CNS Zn is found in RNA and DNA polymerase and carbonic anhydrase,
while Cu is found in superoxide dismutase, cytochrome c oxidase and dopamin β-hydroxylase. In deficiency states reduced activities of Zn and Cu dependent enzymes have been found (5, 27).

In the brain the highest zinc concentrations are found in the hippocampus, hypothalamus and cerebellum (28). The highest copper levels are noted in the substantia nigra, locus ceruleus and putamen (29). Both Zn and Cu concentrations are higher in grey matter than in white.

Some studies have been tried to define the role of Zn and Cu in the etiology and pathogenesis of certain neurological disorders. Low P-Zn levels have been found in multiple sclerosis (M.S.) (30) and in chronic alcoholism (31), while high levels have been found in M.S. (32) and in Pick's disease (33).

Low S-Cu or P-Cu concentrations have been described in two genetically determined abnormalities of copper metabolism. In both of them lesions are seen in the CNS. In Wilson's disease Cu is accumulated in the brain, liver and other organs probably because of a deficient synthesis of Cp and a decreased excretion of the metal by the bile (24). In Menke's disease a reduced absorption of Cu leads to extensive lesions of the brain, convulsive seizures and mental retardation (24).

Interest of the role of Cu in the pathogenesis of M.S. has been sparked by the similarity of the CNS lesions in M.S. and in copper deficient lambs with swayback. Increased (34), decreased (35) and unchanged (36 - 38) concentrations of S-Cu or P-Cu have been observed in M.S. patients.

In epilepsy therapy with phenytoin (PHT) alone or in combination with other antiepileptic drugs, altered serum or plasma concentrations of Zn and Cu have also been reported. Zn concentrations have been reported variously as decreased (39), unchanged (40 - 41) or increased (42). The Cu concentrations have been reported unchanged (39) or increased (37, 40 - 46). S or P concentrations of Cp have been reported unchanged (41, 43) or increased (44, 46). Very high P-Cu levels have been found in
patients clinically intoxicated with PHT (41). During epilepsy therapy with carbamazepin increased P-Zn and P-Cu concentrations have also been reported (42).

Zn concentrations of cerebrospinal fluid (CSF) have been analyzed in normals and patients with neurological diseases but the levels reported vary widely in different reports (I, Table 1). In neurological diseases low CSF-Zn concentrations were reported in patients with alcohol withdrawal seizures (31) and increased levels in patients with increased CSF-protein levels (47). Other authors have not found any relation between CSF-Zn and CSF-protein levels (48 - 50). Bogden et al (49) reported normal CSF-Zn levels in nine patients with subarachnoid haemorrhage (SAH).

PURPOSES

The studies were undertaken with the following goals in mind:
1. To develop a sensitive, clinically useful method for determination of Zn in CSF.
2. To determine the normal CSF-Zn concentrations.
3. To investigate the relationship between CSF-Zn and protein parameters in CSF.
4. To investigate the relationship between CSF-Zn and S-Zn concentrations.
5. To determine the CSF-Zn concentration in some neurological diseases and in patients with a low S-Zn level.
6. To investigate the Zn and Cu status of M.S. patients.
7. To investigate the Zn and Cu status of untreated epilepsy patients, during the first month of PHT therapy and in patients on long-term therapy with PHT alone or in combination with other antiepileptic drugs.
PATIENTS

Pooled CSF from six patients with different neurological diseases was used to determine background absorption, sensitivity, precision, between run variation and accuracy of the new method. The accuracy was also checked in another ten patients with neurological diseases.

Normal CSF-Zn (I + II) and serum samples (I - IV)

Normal CSF-Zn concentrations were determined in 18 healthy volunteers (I), nine from each sex, and in 34 patients (II), 14 males and 20 females, assumed to have a normal CSF. The patients suffered from dizziness, vasovagal syncope, headache, neuralgic pain and other pain syndromes or psychoneurosis. The neurologic examination did not reveal any objective signs of a lesion in the CNS. Their CSF was clear and uncoloured, had a leucocyte count of less than 5/μl and a CSF-protein concentration of less than 500 mg/l.

Normal serum samples were obtained from 75 healthy volunteers, 31 males and 44 females.

The normal patients and the volunteers had no clinical or laboratory signs of infection, liver disease or alcoholism. None of the females were pregnant. The volunteers were completely free from all pharmacotherapy including oral contraceptives, while the patients were not being treated with corticosteroids, ACTH, oral contraceptives, estrogens, PHT or other drugs known to influence Zn and Cu status in man.

From healthy volunteers and from patients assumed to have a normal CSF have been sampled age and sex matched controls to the CSF-samples from other patients. Control samples of serum have been taken only from healthy volunteers (II - IV).

Patients with pathologic CSF or low S-Zn concentrations (II, Table 3) CSF was obtained from patients with neurological diseases where a spinal tap was indicated for diagnostic reasons.
Guillain-Barré syndrome (GBS) (n = 7) and other causes of blood-brain barrier damage (BBBD) (n = 4) (CSF-protein ≥ 750 mg/l): Two of the patients with GBS were being treated with corticosteroids. Of the patients with other causes to increased CSF-protein levels two had myelopathies, one had a cervical disc herniation with a partial spinal block and one had experienced an intracerebral bleeding two years earlier.

Brain tumors (n = 12): Two patients had meningiomas, eight had malignant gliomas and two had brain metastases.

Zinc deficient patients (n = 5): This group included four patients with chronic alcoholism and one with a jejuno-ileal shunt because of obesity. All had a S-Zn concentration of less than 10.0 μmol/l.

Estrogen treated patients (n = 8): Six females were on oral contraceptives. Four of them had had transitory ischemic attacks, one had lumbago and one paresthesias. Two females were on estrogen therapy. One showed a torticollis and the other M.S.

Corticosteroid treated patients (n = 4): Two patients had chronic polyneuropathy and two had optic neuritis.

Subarachnoid haemorrhage (SAH) patients (n = 6): The patients had SAH from ruptured arterial aneurysms. Four of them were seriously ill and required parental nutrition and corticosteroid therapy.

Multiple sclerosis patients (III)
Fifty patients with clinically definite M.S. according to the criteria of McDonald and Halliday (51) are included. There were 21 males and 29 females. The median age for the males was 34 years (range 22 - 68) and for the females 39 years (range 23 - 76). CSF samples were obtained from 11 males and 18 females. No clinical or laboratory signs of infection, liver disease, malnutrition or pressure sores were observed. Blood Hb was more than 115 g/l and ESR below 25 mm/h in all patients. None of the women were pregnant. They were not being treated with ACTH, corticosteroids, estrogens, oral contraceptives or PHT.
Epilepsy patients (IV)

a) Therapeutic group
Thirteen patients with untreated epilepsy, eight males, median age 34 years (range 19 - 59) and five females, median age 53 years (range 18 - 58) are included. None had experienced seizures during the week prior to the start of therapy. They were followed during the first month of therapy with PHT as the sole antiepileptic drug. PHT was given as 100 mg tablets (Fenantoin™, ACO, Sweden), 200 mg at 08.00, 100 mg at 18.00. Serum samples were obtained before the start of therapy, 24, 48, 72 hours and one month after the first dose of PHT. In some patients CSF-samples were obtained before the therapy started and after one month of therapy and in some patients 24 hour urinary samples were collected during the first 72 hours and after one month of PHT therapy.

b) Long-term therapy group
This group included ten males and ten females with a median age of 41 years (range 19 - 72 years). They were being treated with PHT alone (n = 5) or combined with other antiepileptic drugs as carbamazepine, ethosuximide, valproate sodium or primidone.

In both patients groups there were no signs of infection, liver disease or alcoholism. They had no pharmacotherapy with corticosteroids, ACTH or oral contraceptives. None of the women were pregnant.

METHODS

In the M.S. patients (III) certain clinical parameters were recorded. The stage of the disease, duration, disability level and degree of malignancy were noted according to Johnson et al (52). The criteria for the parameters are described in (III).

CSF sampling. All samples except the pooled samples in (I) and the samples from the SAH patients (II) were obtained at 08.00 after overnight fasting. The lumbar punctures were performed in the lateral recumbent
position after 10 - 30 minutes rest. The skin was anaesthetised with one ml of XylocainR 10 mg/ml (Astra, Sweden) and the puncture performed with a sterile disposable hypodermic needle 0.9 x 90 mm (Mediplast, Sweden). The first ml was used for the cell count. In the healthy volunteers the CSF-samples from the 10th to the 17th ml were taken for analysis of zinc and proteins, in the patients samples from the 5th to the 20th ml were used for the analyses. CSF was allowed to drop directly into acid washed plastic tubes, which were immediately sealed with ParafilmR (American Can Company, USA) and frozen at -20°C until analyzed. The CSF-samples were not centrifuged or transferred to other tubes. CSF-samples with evidence of blood contamination were rejected.

In the patients with SAH, CSF was obtained through an intraventricular catheter. The samples were aspirated with a zinc free syringe. The first two ml were rejected and the following eight ml were transferred to an acid washed glass tube and centrifuged at 5000 r.p.m. for 15 minutes. The supernatant was then transferred to acid washed plastic tubes with Pasteur pipettes and frozen at -20°C. Neither fasting CSF samples nor serum samples were obtained from these patients.

Serum sampling. Blood samples were drawn from an antecubital vein after minimal stasis immediately following the lumbar puncture. No vacutainer system was used. When additional blood samples were drawn, the sample for Zn and Cu analysis was taken in the first tube. The blood was collected in acid washed glass tubes, allowed to clot for two hours, centrifuged at 5000 r.p.m., for ten minutes and the serum was then transferred to acid washed plastic tubes with Pasteur pipettes. The tubes were sealed with ParafilmR and frozen at -20°C until analysed.

Instrument. An AA-6DB Atomic Absorption Spectrophotometer (Varian Techtron Pty. Ltd. Melbourne, Australia) was used for zinc and copper determinations. It was equipped with a hollow-cathode lamp, a 10 cm air-acetylene burner and an adjustable tantalum nebulizer. For the CSF-
Zn determinations a teflon cone was used for analysis with pulse nebulizer technique (53). For the data read-out a strip chart recorder (Vitatron 2001, the Netherlands) for manual estimation of peak heights and a peak reader module PRM-6 with printer (L L Elektronik, Umeå, Sweden) permitting simultaneous digital registration of peak heights and peak areas were used (54). The further operative conditions are described in (I). The teflon cone was not used for the determination of Zn and Cu in serum or urine. Zn was measured at 213.9 nm and Cu at 324.7 nm.

Reagents. The preparation of the standards for the determination of Zn in CSF with the direct method developed and with the standard addition method is described in (I). Reference samples for Zn and Cu determinations in serum and urine were taken from solutions of the metals in 0.1 mol/l HCl.

Glass- and plastic wares. The pipettes and volumetric flasks were of borosilicate glass and the plastic tubes, 12 x 75 mm, were of polystyrene. All plastic tubes and the glass material used were acid washed and tested for Zn as described in (I).

CSF-Zn determinations

Direct method: The samples and standard solutions of 0, 0.1, 0.2 and 0.3 μmol Zn/l in 0.150 mol/l NaCl were injected into the cone and the absorbance values registered. In (II) standards with 0.6, 0.9 and 1.2 μmol Zn/l in 0.150 mol/l NaCl were also used. The standards were used to develop a standard curve by the least squares method. The CSF-Zn concentrations of the patient samples were calculated from the standard curve (I, Fig. 1).

Standard addition method: The samples were divided into four 1.0 ml fractions. To each was added 100 μl of a standard solution containing 0, 1, 2, or 3 μmol Zn/l. A blank solution of 1 ml 0.150 mol/l NaCl + 100 μl H2O was also prepared. Absorbance values were measured for the different fractions and a regression line was calculated by the least
squares method. The CSF-Zn concentration was read from the X-axis at the intercept of the absorbance of the blank (I, Fig. 1).

The sample injection procedure was the same for both methods. One hundred μl of the sample was injected into the cone with a Finnpipette® (Finn Pipette Oy, Finland). For each sample five injections were made each separated by two injections of 0.1 mol/l HCl and one of pure water. For the registration of absorbance values digital peak height read-out was used in all analyses. In (I) manual measurement and digital peak area read-out were additionally used to test the different read-out methods. The highest and lowest absorbance values obtained were rejected and the mean of the three remaining was used for the calculations. Background corrections with the hydrogen lamp were not made.

Zn and Cu analysis in serum and urine
The serum samples were diluted eleven times and the urine samples three times with 0.1 mol/l HCl. A continuous aspiration technique was utilized and the mean of two determinations was used for the calculations (55).

CSF-protein analysis
The concentrations were analyzed according to Lowry et al (56) with tyrosine as the standard.

Albumin, ceruloplasmin and α2-macroglobulin analysis
The levels were determined with electroimmuno assay according to Laurell (57).

PTH analysis
The serum concentrations of PHT were determined by the EMIT technique according to Kupferberg (58).

Statistics
All results are given as the mean ± S.D. unless is marked otherwise. The differences between group means for different variables were tested
using Student's t-test for paired or unpaired variables. The test was modified if the variances were significantly different \((p < 0.01; F\text{-test})\). Product moment correlation coefficients \((r)\) were calculated for selected variables and tested using Student's t-test. In (II) Wilcoxon matched-pairs signed-ranks test and Spearman correlation coefficient test were additionally used. The levels of statistical significance were chosen as follows: \(p <0.05^*, p <0.01^{**}\) and \(p <0.001^{***}\).

RESULTS

I.
No difference was found in the background absorption between CSF and 0.150 mol/l NaCl with the direct method (I, Table 2). The sensitivity of the method, i.e. the concentration absorbing 1% of the light, was estimated to 0.13 \(\mu\)mol/l. The precision was found to be somewhat better with the direct method compared to the standard addition method (I, Table 3). No significant difference in mean concentrations between two test runs was found. Of the three read-out methods tested no significant difference was found between the mean concentrations obtained. Digital peak area read-out showed a poorer precision than the manual measurement or the digital peak height read-out.

For the determination of the accuracy the direct method was compared with the standard addition method. In pooled CSF 91% and 84% of standard addition values were obtained during two different runs (I, Table 3) when digital peak height read-out was used and in ten patients in average 88% (I, Fig. 2).

Normal values for CSF-Zn in healthy males was \(0.16 \pm 0.04 \mu\)mol/l and in the females \(0.13 \pm 0.02 \mu\)mol/l (I, Table 4). The sex difference is not statistically significant. The CSF-Zn concentrations were found to be positively correlated with the CSF-protein and CSF-albumin concentrations and to the CSF/S albumin ratio (I, Table 5, Fig. 3).
II.
The healthy volunteers and the patients assumed to have a normal CSF were regarded as controls (II, Table 1, Fig. 1). No significant sex difference was found in the CSF-Zn, CSF-protein, CSF-albumin concentrations or in the CSF/S-albumin ratio. Higher S-Zn concentrations were found in the males \( (p = 0.024) \). Significant positive correlations were found in the controls between CSF-Zn and CSF-protein, CSF-Zn and CSF-albumin and CSF-Zn and CSF/S-albumin ratio (II, Table 2). The best correlation was registered between CSF-Zn and CSF-albumin (II, Table 2, Fig. 2). No correlation was found between CSF-Zn and S-Zn levels.

In patients with CSF protein concentrations over 750 mg/l increased CSF-Zn levels were recorded in GBS patients, in those with other causes of BBB damage and in patients with malignant brain tumors (II, Table 3, Fig. 3). Increased CSF-Zn concentrations were found in some of the patients with malignant brain tumors and a normal CSF-protein concentration. Two patients with meningiomas had normal CSF-Zn levels. Normal CSF-Zn concentrations were found in patients with zinc deficiency or undergoing estrogen or corticosteroid treatment. The patients with SAH had considerably increased CSF-Zn levels, on average ten-fold the normal CSF-Zn levels (II, Fig. 3). No correlation was found between CSF-Zn and CSF-protein or CSF-albumin concentrations in any of the patient groups.

III.
In the M.S. patients lower S-Zn concentrations were found compared with the controls (III, Table 1). For S-Cu slightly lower (not significant) levels were noted in patients and there was more variation in the values for the patients than for the controls. In the controls significantly higher S-Zn and lower S-Cu levels were found in the males compared to the females. This sex difference was not found in the M.S. patients. No difference in S-albumin concentrations were found in the M.S. patients compared to the controls (III, Table 1). Before the age of 45 significantly lower S-Zn and S-Cu levels were found in the males and lower S-Cu in the females with M.S. compared with the controls.
(III, Table 2). S-Zn was found to be negatively correlated with age in the control males but not in the male M.S. patients (III, Fig. 1). No significant correlations were found between age and the S-Zn or S-Cu levels in the female patients or controls (III, Fig. 2).

Lower S-Zn levels were found in the males with slowly progressing disease, moderate or severe disability levels, low disability score and a duration of more than six years (III, Table 3 - 6). In males with short duration and mild residual symptoms lower S-Cu concentrations were noted.

No significant differences were found in the CSF-Zn, CSF-protein or CSF-albumin concentrations in the M.S. patients compared with the controls (III, Table 7). The M.S. males had lower CSF/S-albumin ratio compared to the controls. Positive correlations were noted between CSF-Zn and the protein parameters in CSF in the controls but not in the M.S. patients (III, Table 8, Fig. 3).

IV.
Untreated epileptic males had lower S-Zn levels than the controls (IV, Table 1). The serum concentrations of Cu, Cp and albumin were within the normal limits in the untreated epileptics. At the start of PHT therapy the increases in the S-Zn and S-Cu concentrations noted after 24 hours, were maintained at 48 and 72 hours (IV, Fig. 1). After one month of therapy, S-Zn had returned to pre-therapy levels but S-Cu had increased even more. The serum levels of albumin and $\alpha_2$-macroglobulin were unchanged during the study and S-Cp was increased after one month of PHT therapy. The urinary excretion of Zn and Cu was unchanged during this period (IV, Table 2). The CSF-concentrations of Zn in the untreated epileptics were not different from those of the controls (IV, Table 3) and no changes were noted after one month of therapy. Significantly increased S-Cu and S-Cp concentrations were noted in patients on long-term therapy with PHT compared to the controls (IV, Table 4). No difference was found in the S-Zn levels. No correlations were found between S-PHT levels and the serum concentrations of Zn, Cu and Cp. The highest S-Cu levels were noted in a woman with PHT intoxication.
In the whole control material to studies (II - IV) significantly higher S-Zn and S-albumin and lower S-Cu concentrations were found in the males compared to the females (Table 1).

Table 1. Serum concentrations of Zn, Cu and albumin in healthy volunteers. The difference between males and females (t-test) is indicated.

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<td></td>
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<td>years</td>
<td>µmol/l</td>
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<td>MALES</td>
<td>31</td>
<td>36 + 12</td>
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<td>15.2 ± 2.5**</td>
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<td>FEMALES</td>
<td>44</td>
<td>38 + 11</td>
<td>13.0 ± 1.6</td>
<td>16.7 ± 2.4</td>
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<tr>
<td>MALES +</td>
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<td>37 + 11</td>
<td>13.7 ± 1.8</td>
<td>16.1 ± 2.6</td>
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DISCUSSION

The normal CSF-Zn levels obtained with the present method are lower than those in previous reports (I, Table 1). Only Kjellin (59) using neutron activation analysis reported CSF-Zn concentrations almost in the same range in five patients. That method has a high sensitivity and limited risks of contamination. Probable explanations to the differences include contamination problems, non-atomic absorption, and non-standardized sampling. Different analytical methods have also been used. The contamination problems are considerable in Zn analysis especially when measuring the very low concentrations in CSF (60). In some of the previous reports the analytical procedures have included the addition of reagents, ion exchange chromatography, centrifugation and the transferring of samples between different tubes. All these steps increase the contamination risk. To minimize the contamination the
samples were not centrifuged and were kept in the same tube until analysis. The proper washing of glass and plastic wares is very important since e.g. dust contains Zn. Zn from haemolysed erythrocytes, which have a high concentration of Zn (14 x 10^{-12} \mu mol Zn/erythrocyte) (10) is a potential source of contamination if a traumatic spinal tap occurs. CSF will be macroscopically clear and uncoloured when there are less than 500 erythrocytes/\mu l (61 - 62). A bleeding causing 700 erythrocytes/\mu l could theoretically add only 0.01 \mu mol Zn/l to the CSF if erythrocyte haemolysis is complete. Consequently, a macroscopically clear and uncoloured CSF has been considered not to contain significant amounts of erythrocytes to influence the CSF-Zn concentrations. The CSF samples for Zn analysis in these studies were taken late in the spinal tap when the risk of a traumatic bleeding is usually less.

The background absorption is another problem in Zn analysis. In CSF sodium is the main cation (63) and it has a pronounced absorption at the Zn resonance line (64). In previous reports there are few indications of how the correction for non-atomic absorption was performed. In the present study the background absorption could be reduced to a low and acceptable level by optimizing the instrument. Since 0.150 mol/l NaCl and CSF were found to have the same background absorption, NaCl was used to compensate for the background absorption. Non-standardized sampling may have caused discrepancies in previously reported results. Often significant details of the procedures were not given e.g. the position of the patient during the removal of CSF, the fraction of CSF analyzed for Zn, the time of day when samples were taken, if fasting samples were taken etc.

The pulse nebulizer technique used in the CSF-Zn determinations has some advantages compared with continuous aspiration technique. Smaller sample volumes are required and the system is less susceptible to clogging.

Lower CSF-Zn levels were found with the direct method than with the standard addition method. This difference may reflect differences in
nebulization between CSF and the standards. Assuming that the CSF-Zn concentrations obtained with the direct method are 88% of the standard addition values, the normal CSF-Zn concentration was $0.18 \pm 0.04 \, \mu\text{mol/l}$ for the males and $0.15 \pm 0.03$ for the females.

The healthy volunteers and the patients assumed to have a normal CSF, had CSF-Zn concentrations on the same range and no significant sex difference was found. The healthy volunteers and normal patients were used as controls for the other patients.

The CSF-protein and CSF-albumin concentrations and the CSF/S-albumin ratio in the controls were in the normal range (65 - 66). The CSF/S-albumin ratio is regarded as a reliable indicator of the blood-brain barrier function (65). No sex difference was found in the protein parameters in CSF as was described by Breebaart et al (66) but not by Tibbling et al (65). A positive correlation between age and CSF-protein parameters was described by some authors (65) but not by others (66). In the present study there was no significant correlation between age and protein parameters or Zn in CSF possibly because there were few patients in higher age groups.

It is not known if Zn in CSF is bound to proteins. The good correlation between CSF-Zn and CSF protein parameters in the controls, especially between CSF-Zn and CSF-albumin, indicates however that the metal is probably bound to proteins mainly albumin. Albumin in CSF is derived from S-albumin (67). There are proportionately more amino acids than albumin in CSF compared to serum, which may indicate other binding possibilities.

In patients with BBB damage as in GBS and brain tumors increased CSF-Zn concentrations were found. Only Kjellin (47) has found increased CSF-Zn levels in three patients with increased CSF-protein levels. There was however no significant correlation between Zn and the protein parameters in CSF.
In the SAH patients, CSF-Zn levels were increased almost tenfold, on average, which contrasts with the reports of Bogden et al (49), where in nine patients with SAH, CSF-Zn was normal.

The lack of correlation between CSF-Zn and S-Zn concentrations in the controls as well as in the zinc deficient patients are in accordance with some earlier reports (48 - 49). The increased CSF-Zn levels during estrogen therapy reported by Bogden and Troiano (31) could not be verified in the present study.

It is probable that the wide range of CSF-Zn levels found previously in normals and in various pathological conditions can be attributed to analytical methods which were too insensitive and had a poor accuracy.

The S-Zn and S-Cu levels obtained in the healthy volunteers were in the normal range (68). Higher S-Zn concentrations in the males has been reported by some authors (14, 16, 69) but not by others (70, 71). The higher S-Cu levels in the females was also found by Hartoma (69) but not by others (72). Decreasing S-Zn levels with increasing age was described by Lindeman et al (14) who found the age relation in both sexes. Decreasing S-Cu concentrations with increasing age have also been reported (72 - 73). Their findings were not confirmed by the present studies (III).

The earlier reports of the S-Zn and S-Cu concentrations in patients with M.S. or on PHT therapy are confusing. Non-standardized investigations may explain the ambiguities. It is necessary to draw samples at the same time of the day because of diurnal variations (15). Fasting samples ought to be obtained since a meal depresses the S-Zn levels by about 15% (16). Other explanations for the variable results include lack of age and sex matched controls, contamination problems and differences in the analytical methods used. Age and sex matched controls for the patients and standardized sampling are necessary because of the variations in the S-Zn and S-Cu levels with those parameters.
Malabsorption and structural abnormalities in the intestine were described in M.S. patients by some authors (74 - 75) but not by others (76 - 77). Low S-Zn and S-Cu concentrations have been described in malabsorption states (9, 78 - 79).

The decreased S-Zn levels found in the M.S. patients are in accordance with Wong et al (30) but contrast with the recent reports by Dore-Duffy et al (32). Malabsorption is one of the reasons for the low S-Zn concentrations in the patients with slowly progressive disease and moderate or severe disability. The depressed S-Cu levels in younger patients also support the malabsorption theory. There is an inverse relationship between S-Zn and S-Cu concentrations (80). The higher S-Cu levels with increasing age in the M.S. male patients, which were not found in the controls, may also be an indirect sign of a zinc deficiency in patients who have had the disease for a long time (III). The importance of the trace element changes in M.S. is still unknown.

Mickel (81) has proposed that lipid peroxidation may be an etiologic factor in M.S. An enteric inflammation would increase the absorption of lipid peroxides which affect the myelin. Zinc inhibits lipid peroxidation in vivo as well as in vitro (82 - 83). A zinc deficiency may theoretically further increase the postulated peroxidative damage in CNS. The value of zinc therapy in stopping or slowing the progress of M.S. remains to be evaluated.

The low S-Zn concentrations in the untreated epileptic males may indicate that they were zinc deficient but the etiology of the deficiency is not known. They were healthy in all other respects. It is suggested that Zn is a necessary factor in the metabolism of the inhibitory transmitter GABA (39). Epileptic seizures are common in chronic alcoholics, who are often zinc deficient. A zinc deficiency may play a role in the development of epileptic seizures. PHT administration had a marked effect on the serum concentrations of Zn and Cu at the start of the therapy. The drug may increase the absorption of the metals from the intestine. In rats high daily doses of PHT have been shown to in-
crease the absorption of $^{65}\text{Zn}$ (84). Another possibility is that PHT chelate binds Zn and Cu in the blood and a relative deficiency of the metals in the tissues may occur. The increased S-Cu concentrations in long-term therapy with PHT may be an indirect sign of a zinc deficiency. The symptoms in PHT intoxication resemble in part those in acute zinc deficiency (85). High S-Cu concentrations have been described in PHT intoxication (41). Peters et al (86) have reported good therapeutic effects with chelating agents on clinical symptoms during PHT intoxication. The existence of a zinc deficiency and/or a copper accumulation in the tissues during PHT treatment requires verification. Biochemically Zn as well as Cu inhibit the Na-K-dependent ATPase and the sodium pump, and in animals intraventricular injections of the metals lead to seizures. Copper accumulation in PHT therapy may theoretically oppose the membrane stabilizing effect of PHT by inhibition of Na-K-ATPase. Clinically, overdoses of the drug are known to activate seizures (87).

Zn and Cu are important for the development, function and protection of the nervous system in animals. We know little of the role of trace element metabolism in neurological diseases.

Further investigations in this field should include:
1. Determination of CFS-Zn concentrations in patients with cerebrovascular diseases. Is it possible to differentiate between small intracerebral bleedings and infarctions?
2. The study of CSF-Zn patients with SAH should be expanded. Can zinc determinations be a help in the diagnosis of rebleedings?
3. CSF-Zn, S-Zn and S-Cu determinations should be made in neurological diseases other than those described in this thesis.
4. Determinations of Zn and Cu concentrations in the tissues should be made during PHT treatment. Are there any signs of zinc deficiency and/or copper accumulation?
5. Copper and other trace elements in CSF should be measured in various neurological diseases.
GENERAL CONCLUSIONS

1. A method for the determination of zinc in CSF with atomic absorption spectrophotometry utilizing pulse nebulizer technique was developed, where the sampling technique was standardized, the risks of contamination with zinc were minimized and the background absorption was reduced and compensated for.

2. Normal CSF-Zn concentration with the present method was 0.14 ± 0.03 μmol/l and no significant sex difference was found.

3. In controls the CSF-Zn concentrations were correlated with the protein and albumin levels in CSF and to the CSF/S-albumin ratio.

4. The CSF-Zn levels were not correlated to the S-Zn concentrations.

5. In patients with increased CSF-protein concentrations increased CSF-Zn levels were found. The highest CSF-Zn levels were noted in patients with subarachnoid haemorrhage. In these patients no correlation was found between CSF-Zn and the protein parameters in CSF.

6. In zinc deficient patients (S-Zn < 10 μmol/l) a normal CSF-Zn concentration was found. Treatment with estrogens or corticosteroids did not influence the CSF-Zn.

7. M.S. patients have depressed S-Zn levels and in younger patients low S-Cu concentrations were found.

8. There was no correlation between CSF-Zn and protein parameters in CSF from M.S. patients.

9. Untreated epileptic males had low S-Zn concentrations. At the start of PHT therapy increased S-Zn and S-Cu concentrations were recorded.

10. In long-term therapy with PHT increased S-Cu and S-Cp levels were found.
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REFERENCES


64. Kahn HL, and Manning DC. Background correction in atomic absorption spectroscopy. Amer Lab 1972; 4: 51 - 56.


