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THE ROLE OF ZINC IN HUMAN FETAL DEVELOPMENT

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Thesis submitted in part-fulfillment for the degree of Master of Science (Biochemistry)

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SUMMARY

Zinc status in human pregnancy was investigated by measuring leucocyte and plasma zinc at 9±3, 20±3, 32±3 weeks of gestation and at delivery. Muscle zinc was measured in rectus muscle obtained at Caesarian section deliveries. Leucocytes and plasma zinc was measured concurrently in 31 women of childbearing age who acted as controls. In all, 175 pregnant women, attending The Ottawa General Hospital's obstetrics clinic, enrolled in the study. They provided a combination of longitudinal and cross-sectional data which was used to establish a profile of tissue and plasma zinc in pregnancies that resulted in fullterm, singleton, live births.

Leucocyte, but not plasma zinc correlated with muscle zinc at delivery ($r = 0.745$, $n = 13$, p 0.01). A significant difference (p 0.05) was found between leucocyte zinc at 9±3 weeks of gestation and that of non-pregnant controls, suggesting early changes take place in tissue zinc metabolism during pregnancy. The previously well documented decline in plasma zinc was confirmed, as was a report (Meadows et al. 1981) that leucocyte zinc falls between early pregnancy and term. The percentage fall was 5.2%.

Anthropometric measurements of neonates were correlated with maternal leucocyte and plasma zinc. Birthweight correlated negatively with plasma zinc in the first trimester ($p=0.01$). Infants were then allocated to one of three groups.

Those weighing >90th centile for gestational age were classified large for gestational age (LGA). Those weighing < 90th, but >10th centile were classified appropriate for gestational age (AGA) and those weighing < 10th centile for gestational age were considered small for gestational age (SGA). Mothers of LGA babies had significantly lower ($p < 0.05$) mean leucocyte zinc at 32 weeks gestation than did mothers of AGA or SGA babies. At 20 weeks, the difference between mean leucocyte zinc in mothers of AGA babies and that of both SGA and LGA babies, approached significance ($p = 0.06$). Contrary to the findings of Meadows et al. (1981) mothers of SGA babies did not have significantly lower leucocyte zinc levels than mothers of AGA babies at delivery. At 9 ± 3 weeks gestation, mothers of LGA babies had the highest leucocyte zinc levels and mothers of SGA babies had the lowest, but these differences were not significant. Nevertheless, it is important to note that the lowest leucocyte zinc recorded in early pregnancy was from a mother who subsequently produced a growth retarded male infant with multiple congenital malformations. No significant differences were found in plasma zinc between the three groups at any time in pregnancy.

Cord blood leucocyte and plasma zinc were significantly higher than maternal values but significant correlations with maternal levels were not found.

16 3-day diet records were evaluated for maternal zinc intake in the third trimester. Average daily intake from food was 30% higher than the Canadian Recommended Daily Nutrient Intake (RDNI) and when a prenatal supplement containing zinc was also consumed, intakes rose to almost 300% of the RDNI. These intakes did not prevent tissue zinc depletion which however, did not generally adversely affect pregnancy outcome, in this study.

INTRODUCTION

The hypothesis on which this thesis is based is that in humans, marginal zinc deficiency during pregnancy may retard fetal growth. Zinc status has been assessed by the serial measurement of leucocyte and plasma zinc, and the usefulness of these approaches to the diagnosis of sub-optimal zinc nutrition will be discussed.

Many species respond to even short periods of zinc deficiency by failing to grow and reproduce normally. In man too, there is evidence that adequate zinc is necessary for normal growth and development, both in and out of the uterus (Jameson 1976 a, Golden and Golden 1981). What remains to be determined is the role if any, of zinc status in the 75% of North American cases of intrauterine growth retardation (IUGR) where the etiology remains obscure (Crosby et al. 1977).

Retrospective and prospective studies have shown that generalised maternal malnutrition is associated with a significant lowering of birthweight (for review see Hurley 1980). Malnutrition is however a rather diffuse term and needs to be further sub-divided into its component parts, one of which is zinc.

Since the advent of atomic absorption spectroscopy, it has been possible to measure the micromolar quantities of zinc in body tissues and fluids. Abnormalities have been detected in many disease states (for review see Solomons 1979). Likewise, it has been possible to measure the zinc content of foods and experimental diets, and thus gain some insight into the requirements for zinc balance. Thus the role of zinc in animal and human nutrition has begun to unfold and its importance in animal reproduction has already been demonstrated.

The impetus for study of the role of zinc in human reproduction comes from numerous animal experiments which show that zinc deficiency in pregnancy results in offspring with congenital abnormalities, biochemical changes and IUGR (for review see Hurley 1980).

In humans, zinc deficiency is the etiological basis for the symptoms of Acrodermatitis Enteropathica, a rare inherited disorder of zinc absorption. The outcome of pregnancy in women with this rare disease used to be extremely poor, with a high incidence of congenital abnormalities and IUGR (Verburg et al. 1974, Hambidge et al. 1975). Maintenance of normal plasma zinc levels during pregnancy, by large dose of oral zinc, has recently resulted in two normal pregnancies in women with this unusual

condition (Brenton et al. 1981). This is compelling evidence that zinc can affect human fetal development, as well as that of experimental animals.

LITERATURE REVIEW

1. ANIMAL STUDIES OF ZINC AND FETAL DEVLEOPMENT

The importance of zinc in embryonic development was first demonstrated in poultry. Hens were made severely zinc deficient by feeding diet containing only 1ppm of zinc. 50% of the embryos were malformed (Blamberg et al. 1960). Under less severe conditions of zinc deprivation, it had previously been shown that chicks were small and weak and their survival was impaired (Turk et al. 1959).

The first report that zinc deficiency in the pregnant mammal resulted in failure of development of the fetus was published in 1966 (Hurley and Swenerton). Newborn rats from dams deprived of zinc in pregnancy (1ppm dietary zinc), showed a 90% incidence of congenital malformations. The full-term young weighed about half as much as the young of ad-libitum fed controls and significantly less than those of pair-fed controls. Severe zinc deficiency causes anorexia, therefore pair feeding experiments are essential.

Further work by Hurley's group demonstrated that zinc deficiency in the prenatal period need be only transitory to exert its deleterious effects (Hurley et al. 1971). When zinc was

omitted from the diets of pregnant rats for as little as three days, a significant number of young were deformed and important abnormalities of brain development occurred. So rapid is the effect of dietary zinc deprivation in the rat that when zinc was omitted from the diet for the first three days of gestation abnormal cell division in the pre-implantation embryo was observed. Thus Hurley suggested that zinc cannot be mobilized from body stores to meet even the short term needs of the fetus (Hurley and Shrader 1975).

Hickory et al. (1979) documented decreased calcification of bone, skeletal malformations and reduced birthweight in fetuses of zinc deficient rats. (1.3ppm dietary zinc). Reduced nitrogen retention in late pregnancy and growth retarded offspring were reported by Greeley et al. (1980), who fed pregnant rats a diet moderately low (6ppm) in zinc throughout pregnancy.

A moderate restriction of zinc intake (9ppm dietary zinc) in the pregnant rat was less teratogenic, but markedly decreased the litter size and the individual weights of the offspring. Slightly more than half of these marginally zinc deficient young survived to weaning, compared to the young of zinc adequate controls, in spite of cross-fostering to zinc sufficient mothers (Hurley 1977).

In rats, plasma zinc falls by 40% only 24 hours after the initiation of a zinc deficient diet. Hurley believes that the rat embryo is totally dependent on maternal plasma for its supply of zinc and that maternal plasma cannot be replenished from the zinc pool in bone, unless there is an accompanying calcium deficiency in which case zinc becomes available to the fetus secondary to bone demineralization. This was demonstrated by feeding a diet deficient in both calcium and zinc, showing it to be less teratogenic than a diet deficient in zinc alone. The zinc content of the fetuses was higher, and that of the maternal bones was lower, than from those which had received a solely zinc deficient diet. Furthermore, the release of zinc from bone did not occur in parathyroidectomised rats, providing evidence that zinc withdrawal from bone is secondary to the maintenance of calcium homeostasis (Hurley and Tao 1972).

Calcium supplements (500-2000mg/day) are sometimes prescribed in pregnancy to overcome suspected dietary deficiencies and prevent withdrawal of calcium from the mother's bones. In so doing, it would appear from the work of Hurley and Tao, that the pool of zinc and possibly other important minerals present in bone would be rendered unavailable to the fetus. In view of the demonstrated importance of zinc in animal fetal development and the marginal zinc intakes reported in human pregnancy (for review

see Dreosti 1982), the prescription of calcium supplements in pregnancy should be considered only in the context of overall mineral nutrition.

Continuing on the theme of pre-natal supplements, non-heme iron, which is universally present in such supplements inhibits the absorption of inorganically bound zinc (Solomons and Jacob 1981). The effect of iron on the absorption of organically bound zinc present in common foods remains to be studied. It is not surprising that in a recent review, Hurley et al. (1983) urge a cautious approach to mineral supplementation in pregnancy.

In animals, little is known about the transfer of zinc between other tissue pools than bone and plasma; while no homeostatic mechanism for plasma zinc itself has been detected, it is possible that metallothionein synthesis and degradation exerts some control over the various pools of body zinc (see page 35). It does seem teleologically unlikely that higher mammals, including man, who reproduce relatively infrequently and in small numbers, would have succeeded evolutionarily, if their fetuses were totally at the mercy of uncontrolled maternal plasma zinc levels.

The effects of zinc deficiency (1ppm dietary zinc) during the last third of pregnancy were investigated in rats by McKenzie et al. (1975). Compared to pair-fed controls, the full-term fetuses exhibited intrauterine growth failure. Examination of brain,

liver and placenta revealed marked differences in the way zinc deficiency affected the growth of these organs. Although the brains of zinc deficient fetuses were smaller than those of controls, brain and body were symmetrically growth retarded. However, their livers were 35% lighter when expressed as a percentage of total body weight. Placentas were only marginally affected. Interestingly, when the total DNA of the zinc depleted brains was measured, it was much reduced, while the concentration of protein, RNA, lipid and cholesterol was increased. Thus the total number of neurones was decreased. This is interesting, in view of the observed behaviour changes in adult rats deprived of zinc in utero (Halas and Sandstead 1975).

The role of zinc deficiency in the reproduction of primates was studied by Swenerton and Hurley (1980). They developed a zinc deficient diet for monkeys and observed its effect on reproductive performance. Zinc deficient diets (4ppm) were commenced when the monkeys had mated; skin lesions and hair loss developed very rapidly. Plasma zinc levels fell and were significantly lower than controls after one to two weeks. They continued to fall to less than one third of control values by the seventieth day. However there were no gross fetal abnormalities in the few pregnancies that occurred, although there was one abortion. The small number of pregnancies that were investigated made it impossible to draw conclusions.

When zinc deficiency was instituted before mating no pregnancies occurred and after prolonged zinc deficiency menstrual cycles and ovulation ceased.

In view of the effect of zinc deficiency on pre-implantation eggs (Hurley and Schrader 1975), one may speculate that a zinc deficient diet instituted at mating might abort a pregnancy so early that it was undetectable. Furthermore, in primates the zinc available to the fetus may not be entirely reflected in the maternal plasma zinc. In any case the fall in plasma zinc following dietary restriction is much less rapid in monkeys and man than in rats (Hurley and Schrader 1975). Recently Golub et al. (1984) working with monkeys have shown that moderate zinc deficiency (4ppm dietary zinc) has effects on maternal haematocrit and general health that could be expected to affect fetal growth and development. Reports on fetal outcome from this group are awaited.

To summarise: Species differences between primates and rats in the response to zinc deprivation, make it clear that it would be unwise to extrapolate from the extensive data obtained in rats to the possibility of effects in humans. In rats plasma zinc falls more rapidly in response to dietary deficiency than it does in primates. The relatively more rapid intrauterine growth rates

of rats would also tend to aggravate marginal zinc deficiency. The differences from the rat data found with the costly experiments on monkeys make it even more important to study human pregnancies directly.

2. ZINC IN HUMAN FETAL DEVELOPMENT

Investigation of the role of zinc in human fetal development has been hampered by lack of a reliable and easily applicable test for the measurement of zinc status. Further complications arise when consideration is given to the question of efficiency of transfer of maternal zinc to the fetus, and to the transfer of zinc between tissue pools in the mother.

Recent estimates of daily zinc intake for pregnant women consuming a western-type diet suggest that zinc intake may be insufficient to cover the zinc requirements for the products of conception and increased maternal tissue (Jameson 1982). He suggests a total extra zinc requirement of 350 to 400mg for pregnancy, to cover fetal and maternal tissue growth. If this is divided into a daily allowance for the last two trimesters, when most of the tissue growth is occurring, he is recommending an extra 5.5 to 7.3mg/day in the last six months of pregnancy, assuming a bio-availability of 30 to 35% (WHO 1983, Recommended Nutrient Intakes for Canadians 1982).

The Canadian Bureau of Nutritional Sciences currently recommends an extra zinc intake of 1mg/day in the second trimester and 2mg/day in the third trimester. This is considered sufficient to provide the zinc content (17.3mg/kg) of an average full-term

infant (Shaw, 1979) when bjo-availability and women with higher than average requirements are taken into account.

The American Recommended Dietary Allowance is 5mg/day extra throughout pregnancy, making the daily allowance for pregnancy exactly double the Canadian recommendation for the third trimester. In formulating the American allowance consideration was given to reports that sub-optimal zinc nutriture may be widespread in the United States (for review see Sanstead 1973), and to research that has shown that zinc deficiency causes reproductive failure in animals (for review see Hurley 1980).

These differences between dietary recommendations for zinc in pregnancy emphasize the confusion that exists in the field. The rationale for the various recommendations and how the recommendations might be applied to diets for pregnancy will be considered in the Discussion under the title of 'Dietary Zinc.'

Hambidge et al. (1983) found that middle-income pregnant North American women consumed less than two thirds of the recommended daily allowance (RDA) for zinc, and Jameson (1982) found essentially the same among Scandanavian women.

Extremely low plasma zinc levels and poor bio-availability of dietary zinc have been linked with a high incidence of anence-

phalic abortions in Turkey (Cavdar et al. 1980). Raised hair zinc levels have been found in mothers of infants with Spina Bifida (Bergman et al. 1980) and it was suggested that this might be indicative of abnormal maternal zinc metabolism. Vir et al. (1981b) recorded a decline in hair zinc as normal pregnancy advanced. The use of hair zinc as an indicator of zinc status is reviewed in Section 3 of the Literature Review.

Low plasma zinc levels of alcoholic mothers have been cited as being possibly related to the stunting and dsymorphogenesis seen in the Fetal Alcohol Syndrome (Flynn et al. 1981). However despite the plausibility of the hypothesis, their experimental data do not really support it (Editorial 1982). Birthweights of babies from alcoholics were not significantly lower than those from non-alcoholics. The incidence of birth defects was inversely related to plasma zinc levels at delivery, but no longitudinal study of plasma zinc in pregnancy was made. It is not sensible to relate plasma zinc level at delivery to dysmorphogenesis that is known to have occurred in the first trimester, particularly in view of the extreme lability of plasma zinc under conditions of stress during delivery. It is however interesting to note that zinc deficiency and brief acute ethanol intoxication have been reported to act synergistically to cause fetal abnormalities in rats (Russel and Goldsmith 1981).

Two third world studies of maternal serum zinc in relation to birthweight report conflicting relationships. Atinmo et al. (1980) found maternal and cord serum zinc to be lower for small-for-gestional age (SGA) babies than for appropriate-for-gestational age (AGA) ones, whereas Prema (1980) found mean maternal serum zinc levels in pregnancy to be higher, when the baby weighed less than 2.0 kg.

Metcoff (1980) found plasma zinc at mid-pregnancy correlated negatively with birth weight, whereas Meadows et al. (1981) found mean plasma zinc levels at delivery were not significantly different in mothers who delivered growth retarded babies, from those who had babies of normal size.

McMichael et al. (1982), using multiple regression analysis to control for five known independent variables (maternal age, gravidity, weight, cigarette smoking and duration of gestation), found no association between mid-pregnancy plasma zinc and birthweight. Intrauterine growth retardation (IUGR) was more common (7%) among infrequent eaters of meat, than among daily meater eaters (2%). Red meat is an important source of dietary zinc, but full diet records were not kept, and it is not likely that meat consumption was the only dietary variable involved.

Jameson (1976a) reported that women whose babies were malformed, dysmature (i.e. full-term but wasted), or who had abnormal deliveries had significantly lower serum zinc in pregnancy than women whose pregnancies and outcome were normal. Mothers whose babies were SGA by his definition, (i.e. stunted) had serum zincs that did not differ from normal. 234 women were enrolled in this study and the numbers of malformed, dysmature and SGA babies were 8, 13, and 4 respectively. Jameson based his conclusions on subjective clinical classification of the babies, rather than measurement of their growth characteristics, but this study was the first to associate a range of fetal growth abnormalities with maternal zinc status. Given the difficulties of measuring and defining zinc status, and of classifying growth disorders in neonates (see Section 3 and 4), it was a valuable contribution to emerging evidence that zinc plays an important role in human fetal development.

In a further paper (1976b) Jameson associated low serum zinc in early pregnancy with premature and post-mature labour and excessive blood loss at delivery. Jameson's papers present clinical evidence that warrants a further look at maternal zinc status and pregnancy outcome.

In a recent clinical trial of zinc supplementation during pregnancy, Jameson (1982) succeeded in reducing the incidence of

complications of pregnancy, but he did not investigate IUGR per se. 133 women whose serum zinc was less than 10uM/L in the 14th week of pregnancy were alternately assigned to a zinc supplemented group or a control group. The supplement was 45mg/day of elemental zinc and the study was not reported to be double-blind. The incidence of prolonged gestation or labour, post-maturity and Caesarian section was significantly lower in the zinc supplemented group. Unfortunately it is not reported how the length of gestation was determined, nor what the serum zinc levels were in the two groups as pregnancy advanced. He does report that in a different group of women who received no zinc supplement, the incidence of complications was lower in women who maintained a relatively constant serum zinc, than in those whose serum zinc declined in pregnancy. A double-blind trial, control over possible errors in assessing the length of gestation and objective measurements of the babies might have been more conclusive.

In a brief report in the Lancet, Meadows et al. (1981) reported that 44 mothers who gave birth to small-for-dates babies, had significantly reduced leucocyte zinc levels, while their plasma zinc levels did not differ from those of controls who had normal babies. Furthermore, the difference was most marked for those 22 mothers whose babies showed signs of long-term IUGR. This is an important report because it is the only report to date

of an investigation of maternal zinc status where an attempt was made to measure some other parameter of zinc status than plasma zinc and where a significant difference in pregnancy outcome was found, between a normal zinc status group and a low zinc status group. Although data on zinc status was obtained at intervals throughout pregnancy for women who produced AGA babies, zinc status was only measured at delivery for those who had SGA babies. Thus the profile of zinc status across pregnancy in the two groups could not be compared. Vir et al. (1981b) measured hair and plasma zinc in pregnancy in a small (60) group of pregnant women, none of whom gave birth to low birthweight babies. However of the three women with the lowest serum zinc, and highest hair zinc, two had spontaneous abortions.

The inconclusive nature of all of the foregoing investigations of zinc nutriture and human fetal development point to the need for a study design which can fulfill the following criteria:

1. Include another indicator of zinc status besides plasma zinc; preferably one which better reflects whole body zinc status.

2. A longitudinal study to document the changes in zinc status associated with abnormal and normal fetal development.

This idea is expanded in the section titled 'THE THESIS'.

3. CLINICALLY APPLICABLE MEASUREMENTS OF ZINC STATUS

INTRODUCTION

In searching for a clinically applicable measurement of zinc status serious methodological and conceptual problems arise. Defining zinc status involves nutritional, biochemical and physiological considerations that encompass the wide and still ill-defined functions of zinc in the human body. It is unlikely that any one clinically feasible measurement will be adequate to cover all aspects of zinc status. Nevertheless low plasma zinc is often still equated with zinc deficiency, and in many studies of the role of zinc in pregnancy, plasma zinc has been the only measure of maternal zinc status.

PLASMA ZINC AS AN INDICATOR OF ZINC STATUS

The most frequently used index of zinc status is plasma zinc, in spite of the fact that it represents as little as 3% of total body zinc, is extremely labile, and often does not correlate with other indices of zinc status. The inadequacies of plasma zinc as an indicator of total body zinc status have been emphasised by Solomons (1979). The following factors contribute to the lability of plasma zinc.

Plasma albumin concentration.

The largest fraction (66%) of plasma zinc is bound to albumin, the remainder is distributed between the 2-macroglobulin fraction (25%-30%) and high affinity zinc binding ligands such as histidine and cysteine (8%). In hypoalbuminemia due to disease (eg. chronic alcoholism) or physiological hypoalbuminemia such as occurs due to the hemodilution of pregnancy, plasma zinc is an indicator of albumin concentration, rather than a measure of the state of zinc nutriture.

Stress.

Infections and inflammatory processes produce dramatic falls in plasma zinc. ACTH (Falchuk 1977) and bacterial endotoxins (Pekarek et al. 1974) have been shown to mediate this fall, and production of a low molecular weight protein by leucocytes. Leucocyte Endogenous Mediator (LEM), is believed to result in redistribution of plasma zinc via the liver, possibly under the action of the pituitary-adrenocortical axis (Falchuk 1977). Thus under conditions of stress, plasma zinc becomes a totally mis-leading indicator of zinc status.

Diurnal and postprandial variations in plasma zinc.

Plasma zinc levels are highest in the morning and 20% reductions after meals are not uncommon. These variations can be

controlled by measuring plasma zinc fasting, and at a fixed time of day (Metland and Brubakk 1973).

Oral Contraceptives.

Women taking oral contraceptives have been reported to have reduced plasma zinc levels (Prasad 1975). This may be of some significance if conception occurs soon after cessation of oral contraception, since Jameson (1976 a&b) associated low plasma zinc in early pregnancy with complications of pregnancy and delivery.

Starvation and Re-feeding.

Anorexia raises plasma zinc (Golub et al. 1984). This rise may result from the catabolism of zinc-containing muscle tissue, before lipolysis and ketone body formation are established. If this is so, then with acute muscle wasting, a rise in plasma zinc occurs despite overall negative zinc balance. Conversely during recovery and anabolism, a low plasma zinc may occur despite total body positive balance (Golden and Golden 1979).

It is essential to be aware of these documented factors that are responsible for the lability of plasma zinc, in any study where plasma zinc is used as an indicator of zinc status. There is no doubt however, that under conditions of experimental zinc deficiency induced by a zinc deficient diet, a predictable fall in

plasma zinc occurs within three weeks (Prasad et al. 1978).

Plasma zinc in pregnancy.

Most studies report that plasma zinc declines to a variable extent throughout pregnancy (Table 1). Some decline in plasma zinc during pregnancy may be explained by the fall in plasma albumin due to hemodilution, since 65% of plasma zinc is loosely associated with albumin. Blood volume expansion reaches its maximum of 130% of normal at twenty-six weeks of gestation, but some researchers found plasma zinc continues to decline after this stage (see Table 1).

Estrogens have been shown to lower plasma zinc, but no correlation between estriol excretion and serum zinc was found by Jameson. This led him to propose that a decline in plasma zinc beyond 26 weeks of gestation represents a marginal zinc status (Jameson 1976a).

Hambidge et al. (1983) were unable to explain either the 17% fall in plasma zinc by the 9th week of gestation, or the continuous fall to term in their study. Zinc supplementation (15 mg/day) however caused only a temporary rise, after which the usual decline continued. This may suggest that either the decline was normal, or the supplement was too small to overcome the

TABLE 1

REPORTED VALUES FOR PLASMA ZINC IN PREGNANCY

Reference	Serum or plasma zinc (uM/L)		
	First half of pregnancy	Last half of pregnancy	At Term
Jameson 1976	16.5	15.0	15.0
Hunt et al. 1979	12.7 ± 0.006	12.7 ± 0.04	
Cavdar et al.* 1980	13.5 ± 0.04	12.2 ± 0.03	10.4 ± 0.04
Meadows et al. 1981	14.2 ± 0.05	9.6 ± 0.03	9.2 ± 0.05
McMichael et al. 1982	$11.2 \pm 0.3 \times 10^{-5}$	$11.2 \pm 0.3 \times 10^{-5}$	
Hambidge et al. 1983	10.9 ± 1.5	9.64 ± 1.7	9.76 ± 1.7

* Values are for malnourished women.

[Values have been converted to uM/L and S.E.M. has been calculated from given data where possible.]

decline in the later stages of pregnancy. Jameson (1983) gave a larger supplement (45mg/day) but unfortunately did not report the plasma zinc values that resulted, although the incidence of protracted labour, excessive blood loss and dysmaturity, in this group of women decreased.

HAIR ZINC AS AN INDICATOR OF ZINC STATUS

Since Prasad et al. (1966) demonstrated that hair zinc was low in zinc-deficient males and that with treatment there was clinical improvement with an associated increase in hair zinc, this particular measurement has been frequently used. Nevertheless the comments of Bradfield and Hambidge (1980) need to be reiterated. They stated that "the physiological significance of the trace element content of hair has yet to be defined." They went on to emphasize that this is particularly true of states of malnutrition where hair growth is very abnormal and expression of the results in terms of hair weight or hair length would give quite different values. A more optimistic and extensively referenced review of hair zinc has been published by Gibson 1980, but since hair growth abnormalities occur in many serious illnesses, as well as in malnutrition the use of hair zinc to assess zinc deficiency should be limited to conditions not associated with severe metabolic disturbances. The other major

problems with hair analysis are the adventitious sources of contamination from the environment, sweat, sebum, shampoos, etc. To avoid these problems, carefully standardised washing procedures are needed (Base 1966), usually involving a detergent, hexane-ethanol and acetone-ether plus a detergent. It is also important to standardize the collection procedures; for details the reader is referred to Gibson (1980). The advantages of hair are apparent: It is freely available, non-invasively obtained and can be repeatedly sampled.

Hair zinc has been measured longitudinally in pregnancy (Vir et al. 1981), (Hambidge et al. 1983), but in neither study did it differ from normal non-pregnant values. The normal range however is wide: [168.5 $\pm$ 47 ug/g = mean $\pm$ SEM, n=150 (Hambidge et al. 1983)]. Vir observed a significant fall from the second trimester; Hambidge did not, but his group obtained a significant rise in hair zinc following supplementation. While hair zinc levels of populations may be of interest, it seems unlikely that isolated measurements will be a sensitive indicator of an individual's current zinc status.

URINARY ZINC

Most zinc excretion occurs via the gastrointestinal tract

(Wolman et al. 1979); it is only a small fraction which appears in the urine. This fraction does diminish in some forms of zinc deficiency although it increases in others. It is invalid to assume that the finding of a high urinary zinc excretion is evidence of zinc deficiency. It is certainly evidence of unusual renal handling of zinc or other substances to which zinc is attached, but without measurements of absorption it is not possible to say whether zinc balance will be abnormal. Any prolonged urinary leak of zinc unmatched by increased absorption must eventually induce a total depletion. Urinary measurements cannot determine whether a new steady state has been achieved.

RED CELL ZINC

There have been some attempts to use erythrocytes to measure zinc status but although red cell zinc declines in experimental zinc deficiency (Prasad et al. 1979), the results in diseases such as cirrhosis (Versieck et al. 1974) have been at variance with other measurements (Keeling et al. 1980). It may be that as with potassium, the zinc content of the red cells varies with the hemoglobin content (Keitel 1957); certainly there are potential binding sites for zinc on the hemoglobin molecule.

THE ZINC TOLERANCE TEST

The administration of an oral pharmacological dose (25-50 mg of zinc, as zinc sulphate) and the serial measurement of plasma zinc in the following four hours constitutes a zinc tolerance test. The difficulty of interpreting the results is demonstrated in the following paragraph.

Zinc absorption in experimental animals is regulated by zinc status (Cousins 1979). Freeland-Graves et al. (1980) used a zinc tolerance test to demonstrate increased absorption of a pharmacological oral zinc load in healthy humans, after three weeks on a diet that was low in bio-available zinc. They suggested the increased absorption reflected zinc deficiency induced by the diet. In contrast, Crohn's disease patients with hypozincemia and clinical signs of zinc deficiency, exhibited decreased response to an oral zinc load. The authors (McClain et al. 1980) suggested decreased absorption resulting from intestinal pathology, caused the zinc deficiency. Clearly the zinc tolerance test cannot measure zinc status via zinc absorption if zinc absorption is dependent on factors other than zinc depletion.

Results of zinc tolerance tests therefore need careful interpretation (for review see Editorial 1981b).

ALKALINE PHOSPHATASE ASSAY

The activity of alkaline phosphatase unlike that of many zinc dependent enzymes, responds quickly to dietary zinc deprivation and repletion according to Prasad et al. (1978) but Baer and King (1978) could not confirm this. The ratio of the maximal post treatment to pretreatment serum alkaline phosphatase activity correlated inversely with pretreatment serum zinc level in subjects who received a zinc deficient diet initially, and later were supplemented with zinc (Kasarskis and Schuna 1980). Healthy pregnant women who received 15mg/day of zinc as zinc sulphate showed increased serum alkaline phosphatase activity compared to an unsupplemented group, although plasma zinc did not differ in the two groups (Hambidge et al. 1983). The author suggested this may indicate the zinc status of the non-supplemented group may not have been optimal. This is possible but increasing the activity of a zinc dependent enzyme by increasing available zinc only shows that the enzyme is regulated by the concentration of zinc, unless abnormalities associated with the lower level of activity can be demonstrated.

LEUCOCYTE ZINC

Zinc is primarily an intracellular cation, so in attempting to assess zinc status it is logical to measure zinc in cells that can be easily sampled. Erythrocytes and leucocytes are readily available from blood samples, but leucocytes are more representative of lean tissue, since they have a nucleus and mitochondria and are not specialised for the transport of one particular protein. Lean tissue contains some 40% of total body zinc, and 90% of whole body zinc turnover has been claimed to be accounted for by this pool (Babcock et al. 1982). The zinc content of muscle then would be expected to yield useful information about zinc status. Unfortunately sampling of muscle for routine investigation of zinc status is neither ethically nor practically feasible. However it has been shown that in normal subjects and patients with liver disease (Jones et al. 1981) there is a positive relationship between muscle zinc and leucocyte zinc, so that leucocyte zinc may be a very useful indicator of zinc status. This relationship was confirmed by Meadows et al. (1981) in pregnant women.

4. THE TRANSFER OF ZINC BETWEEN TISSUE POOLS

INTRODUCTION

While the measurement of zinc status remains a problem in clinical medicine as a whole, in obstetrics there is another aspect of zinc metabolism to consider. The transfer of zinc between maternal tissue pools, between these pools and plasma, and most importantly, across the placenta, will determine the adequacy of the fetal zinc supply. It is reasonable to expect that a low maternal zinc status would reduce the zinc available to the fetus, but it is important to remember that we do not know how zinc is transported across the human placenta, nor what controls the movement of zinc between zinc pools in pregnancy.

EXCHANGE OF CELLULAR ZINC IN VITRO

Isotope studies demonstrate considerable rates of movement of zinc between cells and their external medium. Thus in leucocytes, zinc influx and efflux proceed at a rate that transfers the equivalent of total intracellular zinc every hour (Jones et al. 1980). Transfer of zinc depends not only on the intracellular concentration of zinc, but also on the affinity of zinc binding ligands in the extracellular medium (Jones et al. 1980).

EXCHANGE OF BODY ZINC IN VIVO

Analysis of the whole body retention curve for ^{65}Zn demonstrated the presence of body zinc pools with different turnover rates (Aamodt 1982). One representing 32% of total body zinc had a biological half-life of 18.2 days; another turned over more slowly, had a half-life of 380 days, and accounted for the remainder of the body pool. Part of the rapidly turning over pool was identified as muscle zinc by Babcock et al. (1982), who postulated that zinc pools in muscle respond rapidly to changes in zinc status.

TISSUE LEVELS OF ZINC IN ZINC DEFICIENCY

A study from Jackson, Jones and Edwards (1982) showed that in growing rats deprived of zinc, muscle zinc did not decrease appreciably while bone zinc decreased by almost 60%, testicular zinc by over 50% and liver zinc by 20%, compared to zinc adequate controls. These reflections of zinc nutriture are informative of the incorporation of zinc into growing tissue under conditions of zinc deprivation. They may suggest no more than that bone can continue to grow whilst incorporating less zinc than normal, while muscle cannot, so that the result is normal muscle zinc levels in a much reduced muscle mass. Certainly the zinc deprived rats

weighed less than the controls. Hurley and Tao (1972) showed that zinc, once incorporated into bone is not released when zinc deficiency occurs unless there is a simultaneous calcium deficiency.

METALLOTHIONEINS

The high cysteine content (30-33%) of zinc metallothioneins (MT) provides intracellular binding sites for zinc and some other metals (Kagi et al. 1974).

Zinc induces MT synthesis by regulating the expression of the thionein gene. The nascent thionein then binds the zinc that initiated its production (Menard et al. 1981).

Zinc-MT can function as a reservoir of zinc, activating zinc-dependent apo-enzymes in vitro (Udom and Brady 1980). During zinc depletion, the half-life of Zn-MT in rat liver and kidney is reduced (Oh et al. 1978) and ten hours after re-feeding zinc to zinc depleted rats a 450% increase in liver MT synthesis occurred (McCormick et al. 1981). Thus, in vivo, liver MT exerts a homeostatic effect on other body pools of zinc.

A role for MT in the control of zinc absorption was suggested

by Richards and Cousins (1977), who found that zinc administration induced mucosal cell synthesis of MT. It was suggested that MT sequestered excess dietary zinc and prevented its transfer to the circulation.

High neonatal levels of MT have been found in the liver and other tissues in man and several other animals (Bakka and Webb 1981, Brady et al. 1982). Brady has suggested that high levels of MT are present in order to supply zinc to rapidly developing tissues for nucleic acid and protein synthesis and other metabolic processes (Brady 1982).

PLACENTAL TRANSFER OF ZINC

Transfer of zinc can occur in both directions across the placenta of rabbits (Terry and Boyd 1960). Meadows et al. (1980) point out that if this situation also exists in man, the possibility of loss of zinc from the fetus to the mother arises if maternal zinc deficiency develops.

5. INTRAUTERINE GROWTH RETARDATION

INTRODUCTION

Infants whose intrauterine growth has been retarded frequently have the growth characteristics of premature babies, even though they have spent the normal length of time in the uterus (Usher and McLean 1974). Public health statistics group together all infants weighing less than 2.5 kg as low birthweight babies, because in many cases accurate records of gestational age cannot be obtained. Nevertheless, where accurate records do exist it has been possible to estimate the incidence of intrauterine growth failure (IUGR). The incidence depends on the definition of IUGR. A common definition is 'Birthweight for gestational age below the tenth percentile' (Metcoff 1977), although this cut-off certainly includes some normally-grown small babies.

The tenth centile cut-off itself varies from one set of standards to another, due to population differences and in the case of Lubchenko's birthweight standards (Lubchenko et al. 1963) lower standards may be due to the high altitude at which the data was collected. Reduced oxygen tension at high altitude is believed to slightly retard fetal growth by decreasing the oxygen available to the fetus for anabolic processes.

IUGR is recognised to be multifactorial in origin. Maternal cigarette smoking is undoubtedly one causative agent (Abernathy et al. 1966). Nicotine reduces the blood supply to the fetus by causing vasoconstriction, and carbon monoxide reduces the oxygen carrying capacity of hemoglobin. Cigarette smoking thus interfers with the transfer of oxygen and nutrients to the fetus.

Other factors associated with reduced fetal growth are a low pre-pregnancy maternal weight, maternal age, alcoholism and low socio-economic status; whether these factors are causative, and how they operate is unknown.

While multiple factors are associated with IUGR, in many cases no cause can be identified. However in the final analysis of the role of zinc in fetal development these factors will have to be considered. In this study the relevant data has been recorded, but not included in this thesis.

SIGNIFICANCE

Intrauterine growth failure can have permanent consequences. Although catch-up growth occurs in some children, some remain stunted (Walther and Raemaeker 1982, Harvey et al. 1982, Fledelius 1982). It appears that there are critical periods for the growth

of some cell types. Dobbing and Sands (1970) showed that by twenty weeks of gestation the human brain has developed its adult quota of neurones. Impairment of growth during this period may be irreversible. For example, microcephaly and mental retardation among survivors of the atomic bomb attacks on Japan, occurred mainly in those infants who were irradiated early in the second trimester (Miller and Blot 1973, Otake and Schull 1980).

Thus the sequelae of intrauterine growth failure can be severe, but without clearly defined causal factors such as an atomic explosion diagnosis is imprecise and difficult.

Ultrasound identifies some growth retarded fetuses in utero, so that early delivery and appropriate feeding can be instituted in an attempt to minimise the damage caused by pre-natal starvation (Cambell 1974). However post-natal identification of these babies is often haphazard (Brook 1983). Because of the lack of proper routine post-natal anthropometric measurements, the true extent of growth retardation is not known and consequently rational investigative treatment of the sequelae has hardly begun. Effective preventive measures will also require an understanding of the pathophysiology.

IDENTIFICATION OF IUGR INFANTS

Accurate measurements of length as an index of stunting are rarely made in neonatal intensive care nurseries, because neonatometers and the personnel trained to use them are not generally available (Brook 1983). Skinfold thickness measurements (an index of fat stores) and estimates of lean tissue deposition by calculation of arm muscle circumference remain in the realms of research.

Clinicians therefore rely on clinical assessment to identify the stunted baby, because accurate anthropometric measurements are not made. In practice, full-term (37 weeks) babies weighing more than 2.5kg are presumed to be normally grown in the absence of gross congenital abnormalities. Thus birthweight becomes the sole index of growth.

Accurate standardized methods for the anthropometry of infants exist and have been reviewed by Cameron (1977). Intrauterine growth curves have been developed (for review see Brandt 1977) and are usually available in the clinical setting, for assessing anthropometric measurements. However they are only as useful, as the measurements are accurate.

Many studies that have attempted to correlate maternal factors with IUGR have used birthweight as the only index of fetal growth. This greatly reduces the value of otherwise careful and extensive studies. The inadequacy of birthweight alone as an index of normal fetal growth in full term infants was emphasised by Miller and Hassanein (1973).

Critical assessment of fetal growth depends on accurate knowledge of gestational age. Gestational age is calculated from the first day of the last menstrual period (LMP). Even when this is known with certainty, the true conceptual age will vary, depending on the date of fertilization. When errors in reporting LMP are suspected or LMP is unknown, gestational age must be assessed by other means. Physical characteristics (other than growth) and neurological development of the neonate afford an independent estimate of maturity, (Dubowitz et al. 1970).

In looking at patterns of IUGR Daikou et al. (1979) found that birthweight of full term infants, even when gestational age was taken into account, failed to identify 16 out of 30 infants who displayed clinical features of malnutrition.

The establishment of standards for intrauterine growth has been reviewed by Metcoff (1977). While there must be reservations about constructing longitudinal growth curves from cross sectional data, the numbers are large enough to allow considerable confidence. The assumption is being made however, that the growth of babies born before 37 weeks was previously normal (Campbell 1974).

Usher and McLean (1969) published Canadian Standards for measurements in 7 dimensions of Caucasian infants born between 25 and 44 weeks of gestation. These measurements are detailed in the methodology section. More recently Brenner et al. (1976) in the U.S.A. have developed birthweight curves that can be adjusted for parity, sex and race. It is interesting to note that the 10th centile at 40 weeks gestation is 2760g, not 2500g which is commonly used (Metcoff 1977). New Canadian standards have been published recently (Blinder et al. 1984). The data base is much larger than that of Usher and McLean and sex differences have been analysed.

Tools for identifying babies who have experienced intrauterine deprivation are available. All that remains is to standardize measuring techniques at the clinical level, and to decide which growth standards to apply. After that is

accomplished the difficult problem of classifying these babies can be better approached.

CLINICAL CLASSIFICATION OF PRE-NATALLY DEPRIVED INFANTS

Intrauterine growth retardation (IUGR) encompasses variable patterns of growth failure the etiologies of which are often obscure. Both the type, (Rosso & Winick 1974) and the timing (Villar & Belizan 1979) of the prenatal insult appear to be important. Chromosome abnormalities, malformations and genetic causes have been described as 'intrinsic' to the feto-placental unit, while maternal malnutrition has been identified as one of several extrinsic causes (Rosso and Winick 1974). However this distinction may be artificial in that Hurley (1981) has clearly demonstrated that a deleterious genetic defect in the mutant pallid strain of mouse can be prevented by addition of large amounts of manganese to the diet, and conversely feeding a manganese deficient diet produces a 'phenotype' pallid mouse. It therefore seems unwise to label any form of IUGR as intrinsic since it suggests intervention is not possible.

Current systems of classification of IUGR should be considered as only interim approaches until a truly etiological description can be given. Metcoff's view (1977) that present

classifications should be seen as representatives of a family of possible impaired growth patterns seems to do justice to current evidence.

CRITERIA FOR CLASSIFICATION

Three measurements are commonly used to classify IUGR babies: Head circumference, crown-heel length, and birth weight. When the deficit in growth is represented by a proportional decrease in these three measurements, the infant is said to be symmetrically growth retarded, or a Type I IUGR (Rosso and Winick 1974). A 15-20% reduction in cell number in the fetal brain and other organs has been demonstrated in this type (Winick 1969), and such infants could be described as stunted.

Asymmetric (Type II or brain-sparing) IUGR is defined as a head circumference and length that are normal or near normal, while the weight/length ratio is reduced, so that these infants appear wasted. Considerable overlap between the two types occurs in practice, and the logic of defining two groups on the basis of three variables is questionable, if the three variables are to some extent dependent upon each other, as seems to be case (Daikoku et al. 1979).

Type I IUGR has been observed in the babies of chronically malnourished Indian (Ghosh et al. 1971) and Mexican (Urrusti et

al. 1972) women. About 30% of small-for-dates fetuses referred for ultrasonography at Queen Charlotte's Hospital London U.K. conformed to this pattern (Campbell 1974), and no obvious maternal factor (such as chronic malnutrition) was noted. Meadows et al. (1981) recorded low leucocyte zinc levels presumptive of zinc deficiency in 22 women who gave birth to such babies.

Follow-up studies on Type I babies in whom the rate of growth of the fetal head slowed before the 26th week of gestation have demonstrated reduced cognitive skills and motor ability at 5 years of age (Harvey et al. 1982).

Villar and Belizan (1982) have related the growth velocity curves for fetal length and weight to the timing of the insult that results in growth retardation. Since the growth velocity curve for length peaks at mid-pregnancy, they consider that stunting or Type I IUGR results from fetal deprivation occurring in the late 1st or early 2nd trimester. Chronic maternal malnutrition would thus be expected to result in Type I IUGR, and does occur where chronic malnutrition is endemic (Ghosh et al. 1971). A fetal deficit of any essential nutrient occurring at this time would theoretically have an effect on overal growth as well as some more specific effects.

Type II IUGR, which is characterised primarily by a weight deficit is attributed to a process starting between the 27th and 30th week of gestation, when the velocity of fetal weight gain begins to increase.

Earlier theories suggested that Type II growth retardation resulted from hypoxia, and that faced with a lack of oxygen, the fetus could preferentially shunt oxygen and nutrients to the brain (Gruenwald 1963), resulting in brainsparing growth retardation. It is difficult to see why length should also be relatively spared, if this were indeed the case.

Type III growth retardation is generally agreed to result from a failure of placental support in the last month of pregnancy. These wasted infants have loose skin, and reduced skinfold thicknesses. Severe late onset toxemia of pregnancy is often associated with this type of fetal malnutrition, as is post-maturity (Metcoff 1977).

CAUSATION

Although current evidence suggests that the timing of the insult is an important determinant of the type of IUGR that develops, little progress has been made in identifying specific causative factors.

Metcoff (1980) has extensively examined the relationships between anthropometric measurements of fetal growth and numerous maternal factors. When known non-nutritional factors were controlled, he demonstrated that fetal growth could be predicted from a set of 13 nutrition-related maternal variables measured at mid-pregnancy. The concept of the importance of a pattern of nutrient levels (in particular of amino acids) emerged. Large babies were associated with lower than average plasma zinc levels, and lower plasma levels of several amino acids, suggesting a different equilibrium was reached between maternal and fetal demands, in mothers who produced unexpectedly large babies. In a previous study from the same centre, poor fetal growth was associated with low plasma zinc levels in the mother (Crosby et al. 1977) but these results are not mutually exclusive, since large babies were not considered separately from those of normal weight in this study, and the proportion of large babies, in relation to the total number, would be small.

These two studies are very valuable in that the authors recognised the complexity of the factors affecting fetal growth and used multivariate analysis to dissect out those of the most importance. The correlations found involved extensive biochemical assays at mid-pregnancy which may or may not be an appropriate time to make measurements. Longitudinal studies are required to choose the most appropriate time to assess maternal nutritional status.

6. MECHANISM OF ACTION OF ZINC DEFICIENCY ON FETAL DEVELOPMENT

INTRODUCTION

Three broad areas of zinc activity have been identified.

(i) Zinc containing and zinc activated enzymes, of which there are at least seventy.

(ii) Cell membrane integrity.

(iii) T - Cell mediated immunity.

In attempting to define the role of zinc in fetal development, research has focussed primarily on the first of these areas, though the effects of zinc on membrane lipids are receiving increasing attention (for review see Bettger and O'Dell 1981).

ZINC AND NUCLEIC ACID SYNTHESIS

Failure of development of the fetus in animals fed marginally zinc deficient diets is well documented (Hurley 1977; Miller et al. 1968), as are the teratogenic effects of serious zinc deficiency (for review see Hurley 1981). The disturbances of morphogenesis that occur in the fetuses of animals fed zinc deficient diets and the fact that zinc deficiency exerts its

effect primarily on rapidly proliferating tissues such as the skin and gonads, has been linked to its role in nucleic acid synthesis (for review see Hurley 1980). For example in embryonic tissue the purine and pyrimidine salvage pathways which recycle nucleotides are normally very active. 'De novo' synthesis alone is insufficient to provide for the rapid accumulation of nucleic acids which occurs during development. Zinc deficient rat embryonic tissue exhibits depressed levels of activity of thymidine kinase, one of the enzymes that functions in the pyrimidine salvage pathway (Duncan and Hurley 1980). DNA polymerase activity was likewise reduced, and zinc deficient rat embryos incorporated less tritiated thymidine into the head region, than did normal controls (Eckert and Hurley 1977).

Another aspect of zinc deficiency which may be related to its role in nucleic acid synthesis is its effect on chromosomes. In pregnant rats given a zinc deficient diet, chromosomal abberations were detected both in maternal bone marrow and in fetal liver cells. The most common abberations were gaps and terminal deletions (Bell et al. 1975).

CELL DIVISION

In the micro-organism Euglena Gracilis cell division is

blocked by zinc deficiency even when the DNA content of the cells has doubled. Earlier work had shown zinc localizing on the spindle and nucleolus, during mitosis (Fujii 1954). This suggests a possible role for zinc in the formation of the mitotic apparatus (Falchuk et al. 1975a).

Further work with synchronously dividing cells showed that zinc deficiency could block the cell in any stage of its life-cycle, and this might be related to a requirement for zinc during the initiation and continuation of DNA synthesis, and for RNA and protein synthesis (Falchuk et al. 1975b).

BONE DEPOSITION

The activity of bone alkaline phosphatase is acutely sensitive to dietary zinc levels in animals (Prasad 1981), as is serum alkaline phosphatase in pregnant women (Hambidge et al. 1983). Thus bone deposition is a zinc dependent process and to confirm this, skeletal abnormalities have been demonstrated in zinc deprived (1.3ppm Zn in the maternal diet) rat fetuses (Hickory et al. 1979).

PROTEIN METABOLISM

The suppressed activity of RNA polymerase (Terhune and Sanstead 1972), in addition to the deranged nucleic acid synthesis described in section 1, will clearly have serious repercussions on protein synthesis, but zinc appears to be involved in other aspects of protein metabolism. A marginally zinc deficient diet (6ppm) fed throughout pregnancy to rats, has been demonstrated to result in anorexia, greatly reduced nitrogen retention and low birthweight off-spring (Greeley et al. 1980). The anorexia may be associated with the protein level in the diet, since rats on a short term zinc deficient diet (1ppm) select low protein but iso-caloric diets as compared to controls on a zinc-adequate diet (Reeves and O'Dell 1981). Energy and protein utilization were inefficient in the zinc deficient group and plasma amino acid levels were imbalanced. This imbalance was reflected in reductions in the plasma levels of leucine, iso-leucine and valine.

Metcoff (1980) implicated altered maternal plasma amino acid patterns in human fetal growth failure, and actually induced IUGR in rats by feeding an excess of the small non-essential neutral amino acids (serine, glycine and alanine) when threonine was the limiting amino acid (Metcoff et al. 1981).

Zinc deficiency has also been shown to reduce the incorporation of histidine into the proteins of rat skin, muscle and kidney (Hsu and Rubenstein 1982).

Thus, while the exact relationships between zinc, protein metabolism and fetal development are far from clear, an effect of zinc deficiency on maternal and/or fetal protein metabolism may well be implicated in fetal growth failure.

ESSENTIAL FATTY ACID METABOLISM

A connection between zinc deficiency and essential fatty acid (EFA) metabolism was postulated by Bettger et al. (1979, 1980). A deficiency of zinc, or of EFA, produces similar symptoms of growth failure, skin lesions and reproductive abnormalities, but the effects of the single deficiencies added together were not as severe as the effects of a double deficiency (Bettger et al. 1980).

Linoleic, gamma-linolenic and di-hommo-gamma linolenic acid increase the absorption of zinc from the gut in young rats (Cunnane S.C. 1982b); the mechanism by which this occurs is not known. It might be the result of altered membrane permeability due to changes in the lipid composition of the plasma membrane

of the intestinal brush-border. On the other hand, the effect might be mediated by prostaglandins (PG) synthesised from EFA, causing increased blood flow and hence better absorption of zinc, since Prostaglandin E1 also increased zinc absorption in the rat's gut (Cunnane 1982b).

A number of zinc dependent enzymes have been identified in the desaturation and elongation of membrane fatty acids, so that zinc deficiency could exacerbate poly-unsaturated fatty acid (PUFA) deficiency (Clejan et al. 1982). Since PUFAs are the precursors of prostaglandins, zinc deficiency may affect PG synthesis. In two brief reports, Cunnane presents some evidence that PG synthesis in rat uterus and placenta are abnormal in zinc deficient rats (Cunnane 1981a, 1981b). Further work appears to be necessary to characterize the effect of zinc deficiency on the synthesis of the various prostaglandins. Tissue specific differences also require further investigation. However if the prime effect of zinc deficiency in the placenta is shown to be an increase in PGF2 and 6-keto PGF1, as Cunnane suggests, then the vaso-constricting effect of these substances would lead to decreased placental perfusion. Decreased blood flow through the placenta has been shown to cause IUGR in rats (Wigglesworth 1964) and in monkeys (Hill et al. 1971).

In Summary: Biochemical theories abound as to how zinc deficiency might affect human fetal growth. The question remains: Can it be shown that it is sub-clinical zinc deficiency that causes some of the previously inexplicible fetal growth failure? The first step in this process is to identify sub-clinical zinc deficiency and link it to abnormal fetal growth.

THE THESIS

While animal experiments have shown that zinc is an important element for normal fetal growth and development, the role of zinc deficiency in the abnormal development of the human fetus requires further investigation. The major difficulty is the detection of sub-optimal zinc nutriture in humans. The measurement of plasma zinc so frequently utilized to assess zinc status is now recognized to be an inadequate test under many circumstances (Solomons 1979). Thus the decline in plasma zinc throughout pregnancy, detected by several researchers (Table 1), may or may not, reflect a declining zinc status.

It has been suggested that leucocyte zinc may be an alternative and practical indicator of total body zinc status (Keeling et al. 1980, Meadows et al. 1981, Prasad 1983).

The demonstration that leucocyte and muscle zinc correlate (Jones et al. 1981, Meadows et al. 1981) is important because it indicates that leucocyte zinc, unlike plasma zinc, does reflect a major zinc pool. Thus a decline in leucocyte zinc during pregnancy could reflect a fall in muscle zinc, which represents the largest rapidly turning over pool of zinc in the body (Babcock et al. 1982).

Whether maternal tissue zinc depletion is ever severe enough to interfer with the normal growth and development of the fetus, will be determined by comparing zinc levels in pregnancy for babies born with congenital abnormalities, or intrauterine growth retardation (IUGR), with those of normal babies born an appropriate size for gestational age (AGA). Although Meadows et al. (1981) found that mothers who produced SGA babies had significantly lower leucocyte zinc levels at term, than those who produced AGA babies, no longitudinal study of leucocyte zinc for SGA babies was done. A longitudinal study might make it possible to identify mothers at risk of producing a SGA baby because of their low zinc status, early enough in pregnancy for intervention to be possible.

Since birthweight alone fails to identify 30% of babies with some degree of growth failure (Miller and Hassanain 1973), anthropometric measurements as well as birthweight will be correlated with maternal zinc levels.

It is recognized that intrauterine growth retardation (IUGR) is multifactorial in origin, and that zinc may, or may not, be one of many independent variables that are associated with fetal growth. However the purpose of this study is to first establish whether leucocyte zinc is a practical and reliable indicator of zinc status, then to describe the behaviour of leucocyte zinc in

pregnancy and finally to correlate longitudinal maternal leucocyte zinc measurements in pregnancy with fetal growth.

Following the preliminary work reported in this thesis, multivariate analysis may be an appropriate tool for identifying the fraction of the variance in fetal growth, that is attributable to maternal zinc status.

MATERIALS AND METHODS

1. MATERIALS

Heparin (1000 units/ml.) Harris Laboratories.

30 ml disposable plastic syringes. Becton-Dickinson.

Butterfly 21 venipuncture sets. Abbott Ireland Ltd.

Sodium diatrizoate 50%. Winthrop.

Ficoll 400. Pharmacia.

Blood dilution vials. Sarsted.

Lay-flat polyethylene tubing.

Zinc standard solution (1 000 ug/L). Canlab.

Analytical grade chemicals. J.T. Baker.

Sartorius Electronic Balance 2004p.

Atomic Absorption Spectrophotometer Instrumentation Laboratories IL551.

Plastic, rather than glass containers were used for samples and solutions. All containers and solutions were checked weekly for contamination with zinc.

2. SUBJECTS AND CONTROLS

New patients, attending the obstetrics clinic at the Ottawa General Hospital, who knew the date of their last menstrual period (LMP), were asked to participate in the study. Multiple pregnancies were subsequently excluded.

Non-pregnant female volunteers of child-bearing age acted as controls.

The study was approved by the ethics committees of the University of Ottawa Medical School, and the Ottawa General Hospital, and written informed consent was obtained from all subjects.

3. MATERNAL BLOOD COLLECTION

A 30ml sample of venous blood was collected three times during pregnancy and once at delivery. The times chosen were 9 $\pm$ 3, 20 $\pm$ 3, and 32 $\pm$ 3 weeks of gestation. It was not possible to schedule blood collection always at the same time of day, so diurnal variation in plasma zinc was not controlled. Most samples were drawn between noon and 5 pm and were not fasting samples, and this may account for our values for plasma zinc being slightly

lower than other reported values in pregnancy.

Blood samples were collected in heparinized plastic syringes, (approximately 7 units of heparin per millilitre of blood), no more than the dead space of the syringe being filled with heparin. Zinc contamination due to heparin was negligible at this concentration.

Each new lot number of syringes was checked for zinc contamination and none was ever found. In contrast, when blood was collected in trace-metal free vacutainers (Becton Dickinson) variable and significant zinc contamination was found, so this method of blood collection was not used.

4. MUSCLE BIOPSY

Those women who required a Caesarian section were asked to given written informed consent for a muscle biopsy to be taken at surgery.

Samples were handled only with stainless steel surgical instruments, rubber gloves and talc being found to cause high levels of zinc contamination.

The same muscle, rectus muscle, was biopsied each time, because of reports that zinc content varies between different muscle groups (O'Leary and Hegarty 1981). The zinc content of red muscle has been reported to be three times that of white muscle (Cassens et al. 1967).

A piece of muscle measuring approximately 0.5 x 1.5 cm was sufficient to allow triplicate analyses of each sample. Samples were stored at -20° until analysis.

5. CORD BLOOD COLLECTION

Cord blood was drawn into a heparinized 30 ml plastic syringe as soon as the placenta was delivered. If the placenta was delivered more than five minutes after the baby, the blood did not yield a satisfactory sample of leucocytes. Since the blood clotting mechanism is initiated as soon as the placenta separates, it is likely that delay in delivery of the placenta, resulted in the aggregation of leucocytes, and hence difficulty in separation.

6. DIETARY DATA

Sixteen three-day diet records were kept at the time of blood sampling, so that dietary zinc intake could be correlated with

blood zinc levels.

Subjects were instructed how to keep a diet record and given diet record forms and stamped addressed envelopes in which to return them.

The 'NUTS' nutrient data base (Quilchena Consulting Ltd.) was used for computerized dietary analysis.

7. ANTHROPOMETRIC MEASUREMENTS OF NEONATES

Within forty-eight hours of birth the following measurements were made: Crown-to-heel and crown-to-rump length, head, chest, mid-upper-arm, and mid-thigh circumference, triceps and sub-scapular skinfold thickness and weight.

The methods of Usher and McLean (1969) were used and the techniques for measurements were those reviewed by Cameron (1978). Reproducibility was within 0.5 cm except for skinfolds which was 0.5 mm, and crown to heel length which was within 1.0 cm. It is not easy to completely extend the knee joint of a new-born in order to make the latter measurement, and two people are necessary, one to hold the baby's head, and the other to straighten the knees.

8. SEPARATION OF PLASMA

5 ml of the heparinized blood sample was centrifuged at 350 g for 5 minutes in a capped zinc-free plastic centrifuge tube. The supernatant plasma was removed with a plastic transfer pipette and stored in a plastic vial at -20° C until analysis. Thawed samples were diluted 1:10 with 0.5 M HCL for zinc analysis. Reproducibility of results on 10 samples of pooled plasma was 1.13%.

9. PREPARATION OF LEUCOCYTES

(i) Density Gradient Layering.

20-25 ml of heparinized blood was layered on to 10 ml of Ficoll-Hypaque solution of density 1.07-1.08 g/L, in each of two blood separation vials. The vials were covered and allowed to stand for 20-30 minutes. Erythrocyte aggregation occurred at the interface, thus removing the red cells from the upper layer and leaving a leucocyte rich plasma. This was transferred to a plastic centrifuge tube and spun at 160-200 g for 5 minutes. The density gradient layering technique has been reviewed by Boyum (1968).

(ii) Lysis of the Remaining Red Cells.

After pouring off the supernatant, the cell button was re-suspended by tapping the bottom of the tube. The red cells were lysed by adding 3 ml of double de-ionized water and shaking for exactly 13 seconds. 1 ml of quadruple stength Hartman's solution was added to restore isotonicity, and the sample was centrifuged immediately at 160-200 g for 3 minutes. The supernatant containing red cell ghosts and hemoglobin was discarded and the inside of the tube was wiped with zinc free paper tissue.

(iii) Removal of Extra-Cellular Fluid.

2 ml of isotonic magnesium chloride solution were added, the cells were re-suspended and poured into a specially formed lay-flat polyethylene tube which had previously been weighed. Details of how to make these tubes can be found in the paper of Baron and Ahmed (1969).

The lay-flat tube containing the leucocytes was centrifuged at 120 g for 3 minutes. The supernatant was removed; the inside of the tube was wiped as previously; the tube was sealed and stored at -20° C before drying.

(iv) Drying of Leucocytes.

The leucocytes were freeze dried or oven dried at 80° C overnight, to constant weight. The tubes were then re-sealed and stored in a dessicator until weighed.

(v) Wet Ashing of Leucocytes.

Zinc was extracted from the weighed specimen of dried leucocytes by wet-ashing in 5 ml of 1 M analytical hydrochloric acid in the lay-flat tube. The tube was re-sealed and the specimen was ashed overnight on a rotary shaker.

(vi) Discussion of the Methodology for the Isolation of Leucocytes.

Leucocytes collected by the above method were morphologically normal on microscopic examination, and viable as judged by the Trypan Blue exclusion test, and the observation of amoeboid movement of granulocytes.

The ratio of platelets to leucocytes was one to one in the suspension of cells in isotonic magnesium choride (mean of six different specimens). The final centrifugation at low speed can be presumed to have further decreased the platelet contamination.

The zinc content of platelets has been estimated to be 0.2 - 0.45 ug/10^9 cells (Foley et al. 1968) so that zinc contamination from platelets in the specimen of leucocytes would be negligible since reported values for leucocyte zinc on a cell count basis range from 9.5 to 12.9 ug/10^9 cells.

Red cell contamination due to incomplete lysing of erythocytes was eliminated by thorough resuspension of the cells, lysing and by adjusting the time allowed for the initial aggregation of the red cells on the Ficoll-Hypaque solution. Considerable individual variation occurred in the time taken for the supernatant to be sufficiently free of red cells to allow the pipetting-off of the white cells. Cord blood was extremely slow to separate (45-60 minutes) while blood taken in late pregnancy separated in as little as ten minutes. No explanation for these variations has been found.

It is recognised that like muscle taken for biopsy, leucocytes are a mixed tissue, but the difference in zinc content between neutrophils and lymphocytes is reported to be small (Whitehouse et al. 1982). Variation in the proportion of the different type of cells would therefore not be likely to affect the zinc content of leucocytes as a whole to a significant degree.

The most persistent difficulty that arose was the clumping of cells, which sometimes occurred after centrifugation at any stage of the isolation procedure. Clumping was more likely to occur if

the blood was layered on to cold Ficoll-Hypaque solution, and if the cells were exposed to g forces greater than 250 g. Clumping makes the use of cell number as the reference measurement for zinc content impossible, since the cells cannot be counted. Visible clumps of cells should be removed and where severe clumping occurs the specimen should be discarded, since the micro-environment of clumped cells is rapidly depleted of oxygen and nutrients and the viability of the cells is doubtful. Slight clumping frequently occurred at the magnesium chloride wash stage, right at the end of the preparation. This was tolerated since the reference measurement used in this study was dry weight rather than cell number.

Yield of leucocytes was very variable and appeared to be related to the time taken for the blood to separate. Yields of 10-15 mg dry weight were usual from 25 ml of blood in pregnancy. Smaller yields from controls and cord blood, that weighed more than 2 mg but less than 5 mg were analyzed by ashing in 2-3 ml of hydrochloric acid instead of in 5 ml.

(vii) Methods of Expression of Results.

Other researchers have expressed leucocyte zinc in terms of cell number, wet weight and dry weight (see table 2). Total protein and DNA are other possible reference measurements. Abnormalities of cell volume occur in some diseases; variations in

TABLE 2

REPORTED VALUES FOR LEUCOCYTE ZINC IN MAN MEASURED BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

Reference	Amount (ug)	Units	SD
Milunsky et al. 1970	95	/10^{10} cells[a]	44
Prasad et al. 1978	112	/10^{10} cells	19
Nishi, Y. 1980	129	/10^{10} cells[a]	33
Jones et al. 1980	70	/g dry weight	8[b]
Meadows et al. 1981	58	/g dry weight	8[b]

[a] Value adjusted to 10^{10} cells.

[b] S.E.M.

cell volume and hence in wet weight, render the latter a useless reference measurement in these circumstances.

Cell counts have been used more frequently but clumping, as previously described, can result in the loss of irreplacable specimens.

Preliminary experiments using the Lowrie and Coomassie Blue protein determination on leucocytes indicated that the latter did not measure total protein in leucocytes. The method used to determine total protein, if this were chosen as the reference measurement, would thus be of considerable significance.

In malnutrition, the choice of reference measurement is particularly difficult, because when total cell protein is reduced, the zinc concentration on a dry weight basis may be normal, while on a cell count basis, it may be low. Gross malnutrition is not common in Canadian pregnant women, therefore the choice of dry weight as the reference measurement seems reasonable.

10. PREPARATION OF MUSCLE SAMPLES FOR ZINC ANALYSIS

Muscle samples were macerated with stainless steel scalpel blades, on an acid-washed petri-dish or watch glass. Samples were dried at 80°C to constant weight overnight in an oven, then transferred to a dessicator. Triplicate samples weighing

approximately 0.02 g were accurately weighed (to 0.01 mg) and transferred to a teflon decomposition vessel (Gaffin 1979) containing 2.5 ml of concentrated nitric acid (Ultrex grade). The sample was digested at 135°C for 90 minutes. After cooling, the sample was made up to 50 ml volume with double-deionized water in an acid-washed volumetric flask, and aspirated directly into the flame of an Atomic Absorption Spectrophotometer.

(ii) Discussion of the Methodology for Analysis of Zinc in Muscle.

The importance of avoiding contamination of the specimen during handling procedures cannot be over-emphasized and the avoidance of contact with rubber, talc and glass which has not been extensively acid-washed, has already been mentioned.

The mean recovery of zinc after the decomposition procedure was 102.6% in a series of three standard additions experiments.

The mean variation between samples from each muscle specimen was 3.97%. Some variation is to be expected, apart from experimental error, because muscle is a mixed tissue, and the samples were not rendered fat-free, although visible fat was removed.

11. ZINC ANALYSIS

All samples were read in the flame mode of an Atomic Absorption Spectrophotometer (1L 551 Instrumentation Laboratories) using background correction at 213.9 nm. Working standards of concentration 0.05, 0.1, 0.2, 0.3 mg/ml zinc were made up from stock standard 1 mg/l zinc (Canlab). These were acidified and stored in plastic containers. Dilution errors due to faulty pipette calibration or technique were ruled out. Pipetting accuracy was $\pm$ 0.48% and matrix interference due to protein and sodium in plasma was negligible, being less than the coefficient of variation for within-run sampling.

Within run coefficients of variation were:

plasma 2.1%
leucocytes 1.5% n=10
muscle 2.0%

Day to Day coefficients of variation were:

plasma 6.0%
leucocytes 2.9% n=6
muscle 3.8%

RESULTS

Results are presented in 7 numbered sections, and discussed in corresponding sections in the Discussion.

Where necessary, brief explanatory notes have been included with each table or figure. Further details can be found in the Methodology.

1. ESTABLISHING THE LABORATORY METHODS

TABLE 3

13 samples of leucocytes were prepared from one large blood sample obtained from the Red Cross. The coefficient of variation was calculated, both with and without two results that were clearly erroneous because they were approaching three standard deviations from the mean.

These results are discussed in Section 1 of The Discussion.

TABLE 3

REPEAT ANALYSES OF LEUCOCYTE ZINC

ON THE SAME BLOOD SAMPLE

Sample Number	Leucocyte Zinc (uM/kg)	Dry Weight (mg)
1	839.7	4.89
2	865.5	5.89
3	823.6	8.51
*4	997.1	6.37
5	792.8	9.38
6	857.8	7.37
7	902.3	7.12
8	842.5	11.80
9	844.8	17.11
10	829.0	15.49
*11	672.4	13.42
12	785.3	10.42
13	846.1	17.05
	n = 13	n = 11
	$\bar{x}$ = 838.4	$\bar{x}$ = 839.0
	SD = 72.61	SD = 32.43
	CV = 8.7%	CV = 3.9%

* Excluded from statistics on the right, because values approach 3SD from the mean.

2. CONTROLS

TABLE 4

NORMAL VALUES FOR LEUCOCYTE AND PLASMA ZINC

IN 31 HEALTHY WOMEN OF CHILD-BEARING AGE

		Leucocyte Zinc (uM/kg)	Plasma Zinc (uM/L)
Results	mean *	823.2 ± 104.9	12.11 ± 1.802
	normal range ($\bar{x}$ ± 2SD)	613.4 to 1033	8.506 to 15.71
Meadows et al. (1981)	mean *	887 ± 125	14.2 ± 1.6

* Results are mean ± standard deviation (SD).

N.B. Meadows' results have been changed to S.I. units and SD has been calculated from given n and SEM.

Peripheral blood samples were collected from the controls during the course of the study and analysed alongside the experimental group in both studies.

TABLE 5

CHANGES IN LEUCOCYTE AND PLASMA ZINC OVER THREE MONTHS IN A GROUP OF ELEVEN CONTROLS

Month	Mean leucocyte zinc (uM/kg d.w.)	Mean plasma zinc (uM/L)
first	802 ± 23	11.32 ± 0.52
second	784 ± 30	11.55 ± 0.56
third	789 ± 27	11.88 ± 0.45

Results are mean ± SEM.

Controls were non-pregnant women of child-bearing age.

The changes in the group means from month to month are not significant.

In the course of the study it became clear that in pregnancy significant changes in leucocyte zinc might be occurring. Therefore eleven non-pregnant women were followed over approximately the same time interval and concurrently with the pregnant women. There were no significant alterations in the group means, but see Table 6.

TABLE 6

RELATIVE STANDARD DEVIATIONS OF LEUCOCYTE AND PLASMA ZINC, OVER 3 TO 7 MONTHS IN 11 HEALTHY FEMALE VOLUNTEERS

	$\bar{x}$	S.D.	C.V.
Leucocyte zinc (uM/kg)	798.4	71.89	9.00%
Plasma zinc (uM/kg)	11.61	1.211	10.43%

This data shows the mean coefficient of variation for 11 individuals' leucocyte and plasma zincs over the time period.

TABLE 7

LINEAR REGRESSION OF LEUCOCYTE ZINC AND PLASMA ZINC FOR 44 PAIRS OF CONTROL VALUES

	leucocyte zinc uM/kg	plasma zinc uM/L		leucocyte zinc uM/kg	plasma zinc uM/L
1	795	10.71	23	780	12.24
2	841	9.33	24	795	11.32
3	979	8.72	25	933	11.17
4	780	12.24	26	780	11.93
5	902	11.62	27	719	10.86
6	826	12.08	28	688	13.00
7	765	11.78	29	749	13.92
8	795	12.08	30	887	12.85
9	811	12.39	31	811	14.84
10	811	12.08	32	704	12.39
11	902	13.46	33	918	13.00
12	749	8.72	34	979	11.62
13	826	12.24	35	749	7.65
14	795	12.24	36	658	10.09
15	719	11.01	37	688	10.40
16	658	11.62	38	795	9.18
17	811	11.62	39	826	10.25
18	841	9.79	40	749	12.85
19	780	12.85	41	826	12.70
20	872	10.55	42	719	11.93
21	857	11.62	43	704	12.39
22	811	13.61	44	719	12.39

n = 44 r = 0.010

'r' is not significant

3. CORRELATION OF LEUCOCYTE ZINC AND PLASMA ZINC WITH MUSCLE ZINC

FIGURE 1

LINEAR REGRESSION OF LEUCOCYTE ZINC WITH MUSCLE ZINC

Maternal rectus muscle zinc is plotted against maternal peripheral venous blood leucocyte zinc. Rectus muscle and blood were obtained at Caesarian section.

Methods of collection, storage and analysis are described in the Methodology.

The correlation is significant.

Note:

(The equation for a straight line is: $y = ax + b$ where a = slope and b = intercept on the y axis.)

FIGURE 1

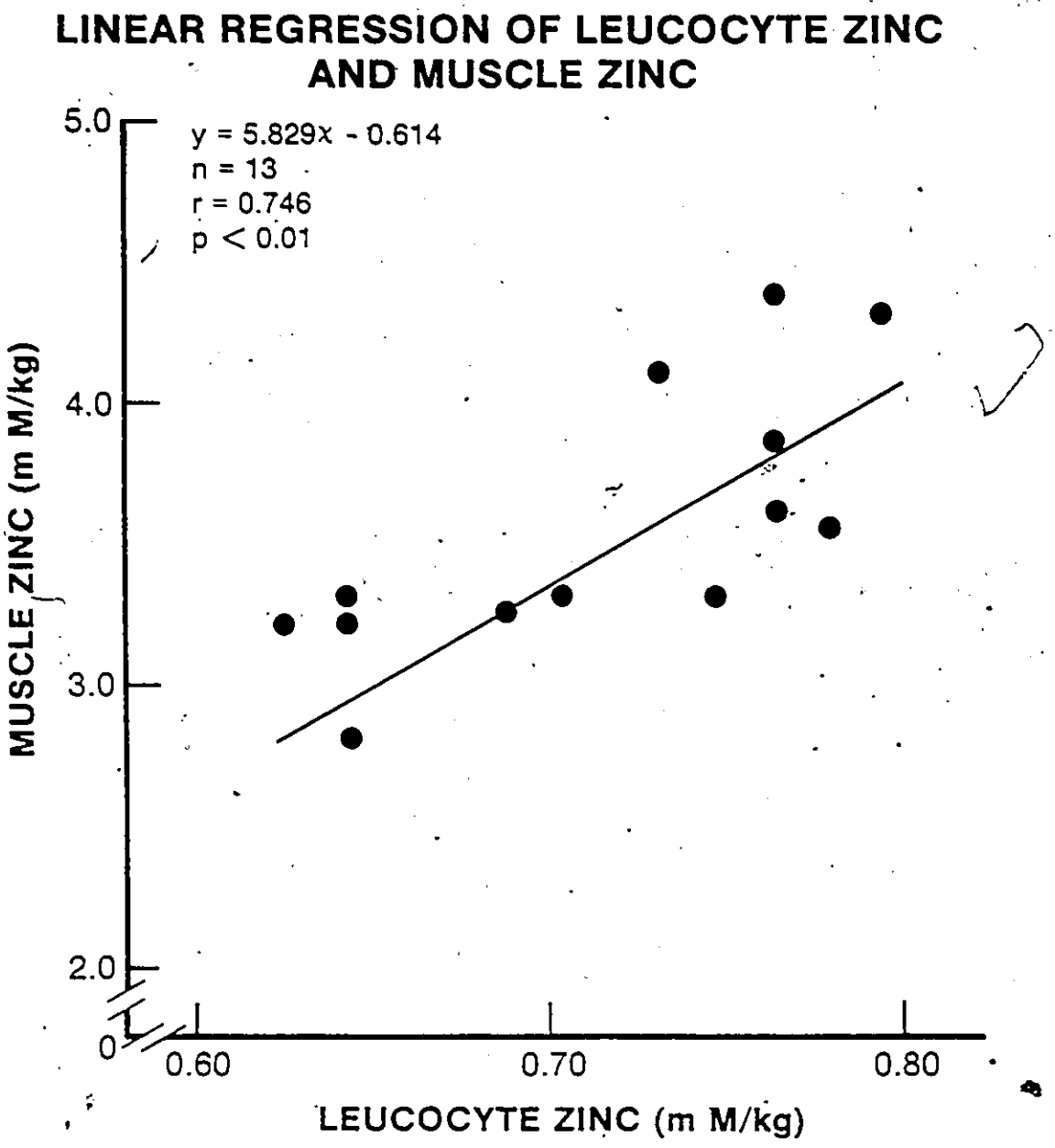

LINEAR REGRESSION OF LEUCOCYTE ZINC AND MUSCLE ZINC
y = 5.829x - 0.614
n = 13
r = 0.746
p < 0.01
MUSCLE ZINC (m M/kg)
5.0
4.0
3.0
2.0
0
0.60
0.70
0.80
LEUCOCYTE ZINC (m M/kg)

TABLE 8

LINEAR REGRESSION OF PLASMA AND MUSCLE ZINC

Plasma zinc (uM/L)	Muscle zinc (mM/kg)
12.24	2.79
9.18	3.30
9.33	3.23
9.02	4.10
8.41	3.30
7.80	4.31
10.25	3.25
7.65	3.56
8.72	3.85
8.72	3.61
6.27	3.30
9.33	3.24

r = -0.491

'r' is not significant

4. LEUCOCYTE AND PLASMA ZINC IN PREGANCY

TABLE 9

LONGITUDINAL DATA FOR LEUCOCYTE AND PLASMA ZINC IN PREGNANCY

42 women provided blood samples suitable for leucocyte zinc determination, at the times indicated. A further 6 who were missing a leucocyte zinc value (poor yield of leucocytes or insufficient blood sample) supplied samples suitable for plasma zinc analysis.

Zinc values at 9 ± 3 weeks were compared with controls using the two-tailed Student's t-test 'A priori' planned comparisons were made between zinc values at 9 ± 3 weeks and at delivery and between leucocyte zinc values at 9 ± 3 weeks and at 20 ± 3 weeks, and between 32 ± 3 weeks and delivery. Plasma zinc at term was compared to plasma zinc at 9 ± 3 weeks.

TABLE 9

LONGITUDINAL DATA FOR LEUCOCYTE AND PLASMA ZINC IN PREGNANCY

Weeks of Gestation	Leucocyte Zinc (uM/kg d.w.)	Plasma Zinc (uM/L)
	n = 42	n = 48
9 ± 3	769 ± 15 ***	11.31 ± 0.25
20 ± 3	733 ± 10 **	9.23 ± 0.18
32 ± 3	748 ± 11	8.54 ± 0.16
At term	729 ± 11 **	8.24 ± 0.28 *
non-pregnant controls (n = 31)	823 ± 19	12.11 ± 0.32

Note: Multiple births, premature births and gross congenital abnormalities excluded.

Controls were non-pregnant women of child-bearing age.

Results are mean ± SEM.

** significantly less than leucocyte zinc at 9 ± 3 weeks $p < 0.05$
* significantly less than plasma zinc at 9 ± 3 weeks $p < 0.001$
*** significantly less than control leucocyte zinc $p < 0.05$

TABLE 10

LONGITUDINAL DATA FOR LEUCOCYTE AND PLASMA ZINC, IN THE FIRST, SECOND AND THIRD TRIMESTER OF PREGNANCY

The time required to collect and separate blood obtained at deliveries was 3-6 hours per sample. Once a sufficient number (42, see Table 9) of term leucocyte zinc values had been obtained to describe the pattern of leucocyte zinc up to delivery, attendance at deliveries was curtailed. Obviously these term values could be of no use in predicting fetal outcome and the time spent in obtaining further results could not be justified.

Thus, 81 women who carried to term, provided blood samples suitable for leucocyte zinc determination at the times shown. A further 8 provided samples suitable for longitudinal analysis of plasma zinc.

Zinc values at 9 $\pm$ 3 weeks were compared with controls using the two-tailed student's t-test. 'A priori' planned comparisons were made between zinc values at 9 $\pm$ 3 weeks and at 20 $\pm$ 3 weeks or at 32 $\pm$ 3 weeks.

TABLE 10

LONGITUDINAL DATA FOR LEUCOCYTE AND PLASMA ZINC IN THE FIRST, SECOND AND THIRD TRIMESTER OF PREGNANCY

Weeks of Gestation	Leucocyte Zinc (uM/kg d.w.) n = 81	Plasma Zinc (uM/L) n = 89
9 ± 3	760 ± 9.3 ***	10.80 ± 0.19
20 ± 3	734 ± 6.9 **	8.96 ± 0.14
32 ± 3	743 ± 7.7	8.50 ± 0.14 *
non-pregnant controls (n = 31)	823 ± 19	12.11 ± 0.32

Note: Multiple births, premature births and gross congenital abnormalities excluded.

Controls were women of child-bearing age.

Results are mean ± SEM.

*** significantly less than control value $p < 0.05$
* significantly less than plasma zinc at 9 ± 3 weeks $p < 0.001$
** significantly less than leucocyte zinc at 9 ± 3 weeks $p < 0.05$

TABLE 11

COMBINED LONGITUDINAL AND CROSS-SECTIONAL DATA FOR LEUCOCYTE AND PLASMA ZINC IN PREGNANCY

133 women provided blood samples at three or less of the planned times in pregnancy. This combined longitudinal and cross-sectional data provides a very similar profile of leucocyte and plasma zinc in pregnancy to the profile provided by the longitudinal data in Tables 9 and 10.

Note:

In addition to the significant differences noted on Tables 9, 10, and 11, significant correlations between mean leucocyte and mean plasma zinc across pregnancy can be demonstrated.

	a	b	r	p	) when
Table 9	0.040	-21	0.89	.05	)y=leucocyte
Table 10	0.037	-19	0.91	.01	) zinc
Table 11	0.039	-20	0.91	.05	)x=plasma
					) zinc

The interpretation of these correlations is discussed on page 111 where they are also compared to the correlation or lack of correlation exhibited in Table 7 (page 78) and Table 12 (page 88).

TABLE 11

COMBINED LONGITUDINAL AND CROSS-SECTIONAL DATA

FOR LEUCOCYTE AND PLASMA ZINC IN PREGNANCY

Weeks of Gestation	Leucocyte Zinc (uM/kg d.w.)	Plasma Zinc (uM/L)
	n = 81	n = 89
9 ± 3	754 ± 9.0 ***	10.67 ± 0.18
	(93)	(101)
20 ± 3	735 ± 6.0 **	8.92 ± 0.13
	(109)	(110)
32 ± 3	743 ± 6.0	8.53 ± 0.12
	(126)	(127)
At term	731 ± 9.0 **	8.32 ± 0.21 *
	(71)	(72)
non-pregnant controls (n = 31)	823 ± 19	12.11 ± 0.32
	(31)	(31)

Note: Multiple or premature births and gross congenital abnormality excluded.

Results are mean ± SEM.

Numbers in parentheses denote sample size.

*** significantly less than controls $p < 0.01$
* significantly less than plasma zinc at 9 ± 3 weeks $p < 0.001$
** significantly less than leucocyte zinc at 9 ± 3 weeks $p < 0.05$

[see table 9 notes for methodological and statistical details]

TABLE 12

CORRELATION OF LEUCOCYTE AND PLASMA ZINC

IN PREGNANCY, FOR INDIVIDUALS

Weeks of Gestation	Correlation Coefficient 'r'	Sample Size 'n'
9 ± 3	0.200 *	93
20 ± 3	-0.005	109
32 ± 3	0.015	126
At term	0.006	71

* $p < 0.05$

This table should be compared to Table 7 (page 78). Only in pregnancy, and then only in the first trimester, is there any significant correlation between an individual's leucocyte and plasma zinc at a given point in time.

TABLE 13

A COMPARISON OF LEUCOCYTE ZINC IN PREGNANCY BETWEEN MOTHERS OF LGA, AGA, AND SGA BABIES

Mothers were grouped according to whether their babies weighed $<$ 10th centile, *(SGA) $>$ 10th but $<$ 90th centile (AGA) or $>$ 90th centile (LGA) for gestational age.

Mean leucocyte zinc at 32 $\pm$ 3 weeks gestation was significantly lower in the group of mothers who produced large infants, than in the other two groups ($p < 0.05$) using the Kruskall-Wallis non-parametric test for differences between samples having different variance.

At 20 $\pm$ 3 weeks gestation the mean leucocyte zinc levels of mothers of both large and small infants were lower than those of mothers of average size infants and these differences approached significance using the non-parametric Kruskall-Wallis test ($p = 0.06$).

The distribution of values at 32 $\pm$ 3 weeks gestation is depicted by the histogram that follows this table.

* Centiles were calculated from the Canadian anthropometric standards established by Usher and MacLean (1969)

TABLE 13

A COMPARISON OF LEUCOCYTE ZINC IN PREGNANCY BETWEEN MOTHERS OF LGA, AGA AND SGA BABIES

	Leucocyte Zinc (uM/kg d.w.)			
	First Trimester	Second Trimester	Third Trimester	At Term
SGA	737 ± 22 (n=10)	707 ± 17** (n=13)	751 ± 19 (n=17)	742 ± 27 (n=10)
AGA	751 ± 10 (n=76)	742 ± 7 (n=87)	747 ± 7 (n=99)	730 ± 9 (n=54)
LGA	777 ± 44 (n=8)	708 ± 12** (n=10)	692 ± 11* (n=11)	723 ± 34 (n=7)

* Significantly different from the other two centile groups at this point in pregnancy $p < 0.05$

** Approaching significant difference from the other group at this point in pregnancy $p = 0.06$

Results are mean ± SEM.

FIGURE 2

LEUCOCYTE ZINC OF MOTHERS OF SGA, AGA AND LGA INFANTS AT 32 WEEKS GESTATION

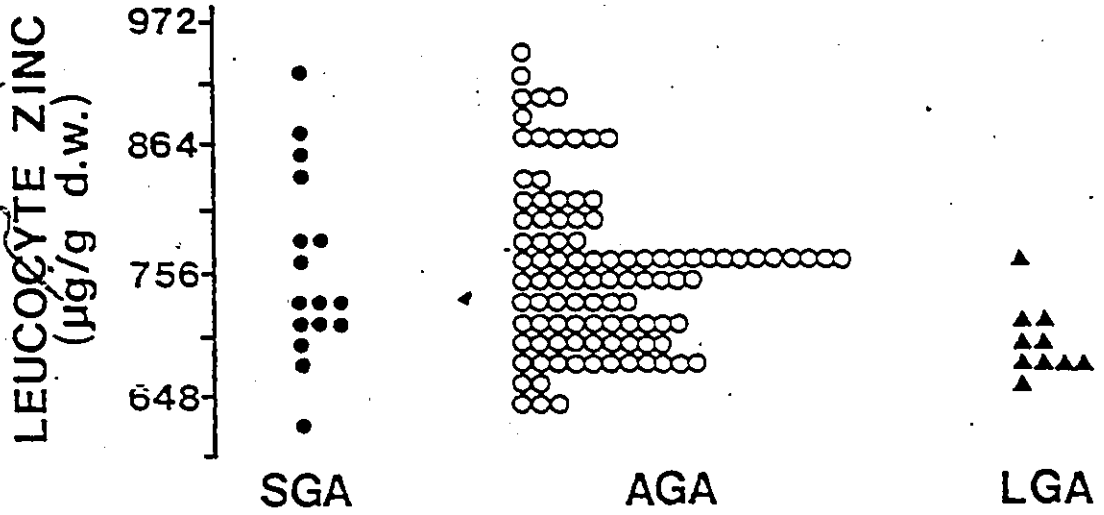


TABLE 14

LONGITUDINAL DATA FOR MATERNAL LEUCOCYTE AND PLASMA ZINC IN PREGNANCY FOR AGA INFANTS

33 women produced normal full-term infants who weighed between the 10th and the 90th centile birthweight for gestational age (AGA), and provided samples of leucocytes and plasma at the appropriate times.

TABLE 14

LONGITUDINAL DATA FOR MATERNAL LEUCOCYTE AND PLASMA ZINC IN PREGNANCY FOR AGA INFANTS

Weeks of Gestation	Leucocyte Zinc (uM/kg d.w.)	Plasma Zinc (uM/L)
	n = 33	n = 38
9 ± 3	767 ± 17	11.14 ± 0.29
20 ± 3	745 ± 12	9.15 ± 0.21
32 ± 3	763 ± 12	8.50 ± 0.19
At term	732 ± 13 **	8.22 ± 0.27 *

Results are mean ± SEM.

Numbers in parenthese are numbers of subjects.

* significantly lower than plasma zinc at 9 ± 3 weeks gestation $p < 0.001$

** significantly lower than leucocyte zinc at 9 ± 3 weeks gestation $p < 0.05$

TABLE 15

A COMPARISON OF PLASMA ZINC IN PREGNANCY BETWEEN MOTHERS OF LGA, AGA AND SGA BABIES

Plasma Zinc (uM/L)

	First Trimester	Second Trimester	Third Trimester	At Term
SGA	11.3 ± 0.2 (11)	9.3 ± 0.5 (13)	8.8 ± 0.4 (15)	8.4 ± 0.5 (10)
AGA	10.6 ± 0.2 (83)	8.8 ± 0.1 (88)	8.5 ± 0.1 (102)	8.2 ± 0.2 (54)
LGA	11.3 ± 0.5 (8)	9.3 ± 0.3 (10)	8.9 ± 0.3 (11)	8.8 ± 1.1 (8)

There are no significant differences between the three groups at any point in time.

See notes for Table 13 for interpretation of this table.

5. ANTHROPOMETRY OF NEONATES

TABLE 16

ANTHROPOMETRY OF NEONATES; DESCRIPTIVE STATISTICS

Neonates were measured within forty-eight hours of birth using standard measuring techniques reviewed by Cameron (1978). Birthweight was recorded on reception of the baby to the nursery by the nurse on duty. This weight was checked against weight at the time other anthropometric measurements were made. Agreement was excellent except in a few cases where obvious errors had been made in which case the second weight was used. A loss in weight of 2-3% per day is usual in the early neonatal period, and short of weighing every baby at birth, which was not feasible in this study, this was the most reliable means of recording birthweight.

TABLE 16

ANTHROPOMETRY OF NEONATES: DESCRIPTIVE STATISTICS

Variable	Mean	SD
Birthweight (g)	3319	530
Head circumference (cm)	34.5	1.5
Mid-upper arm circumference (cm)	10.6	1.1
Mid-thigh circumference (cm)	15.4	1.6
Chest circumference (cm)	32.5	2.0
Triceps skinfold thickness (mm)	3.7	0.1
Subscapular skinfold thickness (mm)	3.8	0.1
Crown to rump length (cm)	33.3	1.9
Crown to heel length (cm)	50.0	2.5

Full-term singleton births only, included.

Full-term defined as gestational age $> 37 < 42$ weeks.

Minimum number of subjects = 119

TABLE 17

LINEAR REGRESSION ANALYSIS OF "a priori" SELECTED NEONATAL ANTHROPOMETRIC MEASUREMENTS AND MATERNAL LEUCOCYTE ZINC IN PREGNANCY

An "a priori" selection of birthweight, crown-heel length and thigh circumference was made, as these were believed to be the best anthropometric indicators of overall, skeletal and soft tissue growth, respectively. This selection was done to reduce the likelihood of finding correlations by chance if all anthropometric measurements were correlated.

TABLE 17

LINEAR REGRESSION ANALYSIS OF 'A PRIORI' SELECTED NEONATAL ANTRHOPOMETRIC MEASUREMENTS AND MATERNAL LEUCOCYTE ZINC IN PREGNANCY

		Leucocyte zinc at:		
		9 weeks gestation	20 weeks gestation	32 weeks gestation
Birthweight	r	0.143	-0.032	-0.123
	n	94	110	127
	p	0.09	0.37	0.09
Crown-heel length	r	0.131	-0.074	-0.123
	n	90	105	121
	p	0.11	0.23	0.09
Mid-thigh circumference	r	0.15	0.02	-0.03
	n	86	100	113
	p	0.08	0.42	0.37

Full-term (> 37 < 42 weeks gestation) single live births only.

No significant correlations were found.

TABLE 18

LINEAR REGRESSION ANALYSIS OF 'A PRIORI' SELECTED NEONATAL ANTRHOPOMETRIC MEASUREMENTS AND MATERNAL PLASMA ZINC IN PREGNANCY

		9 ± 3 weeks gestation	20 ± 3 weeks gestation	32 ± 3 weeks gestation
Birthweight	r	-0.219	-0.045	-0.068
	n	102	111	128
	p	0.01*	0.32	0.22
Crown-heel length	r	-0.108	-0.034	0.080
	n	97	106	121
	p	0.15	0.37	0.19
Mid-thigh circumference	r	-0.112	-0.003	-0.059
	n	94	101	113
	p	0.14	0.49	0.27

Full-term (>37 < 42 weeks gestation) single live births only.

* p = 0.01

The "a priori" selection of neonatal anthropometric measurements described in Table 17 were next correlated with maternal plasma zinc at 9 ± 3, 20 ± 3 and 32 ± 3 weeks gestation. There is a negative correlation between birthweight and plasma zinc at 9 ± 3 weeks gestation, which reaches significance at the 99% level.

6. FETAL (CORD) LEUCOCYTE AND PLASMA ZINC

TABLE 19

ZINC IN MATERNAL AND FETAL LEUCOCYTES

AND PLASMA AT DELIVERY

	leucocyte zinc (uM/kg)	plasma zinc (uM/L)
maternal	723 ± 13 (26)	8.32 ± 0.23 (32)
fetal	877 ± 26* (26)	12.98 ± 0.51* (34)

* significantly higher than maternal values $p < 0.001$

Results are mean ± SEM

Numbers in parentheses indicate number of subjects

Fetal blood was obtained from the umbilical vein after delivery of the infant, once the cord stopped pulsating. If the placenta was delivered rapidly, blood was drawn from a vein on the fetal side of the delivered placenta. Cord blood was drawn in one or two heparinized plastic 30 ml syringes with an 18 gauge stainless steel needle. Samples of leucocytes from cord blood tended to be small, so the largest possible sample of blood was drawn (30-60 ml).

TABLE 20

LINEAR REGRESSION OF FETAL ZINC LEVELS WITH MATERNAL ZINC LEVELS AT DELIVERY

Equations:

Fetal leucocyte zinc, y = a x maternal leucocyte zinc, x + b
Fetal plasma zinc, y = a x maternal plasma zinc, x + b

A. LEUCOCYTE ZINC

r = 0.306 — 'r' is not significant at this n, but approaches significance.

n = 26

a = 0.199

b = 0.727 (micromoles/kg)

B. PLASMA ZINC

r = -0.003 — 'r' is not significant.

n = 33

a = -0.004

b = 13.38 (micromoles/litre)

7. DIETARY DATA

TABLE 21

ZINC INTAKES FROM THREE DAY FOOD RECORDS

IN THE THIRD TRIMESTER OF PREGNANCY

	Mean Intake (mg/day)	% RDNI Canada	% RDA U.S.A.
Overall	17.7 ± 8.9 (16)	171	88.5
From Food Only	13.0 ± 4.2 (11)	130	65.0
From Food and 15mg Zn in a Prenatal Supplement	29.6 ± 5.8 (5)	296	148

Numbers in parentheses indicate sample size.

Results are mean ± SD.

Instruction in the keeping of three-day food record was given to pregnant subjects who volunteered for this additional aspect of the study. Intakes have been calculated using the NUTS data base (Quilchena Consulting Ltd.) Mean zinc intakes have then been calculated as percentages of both the Canadian Daily Recommended Nutrient Intake (RDNI) (Health and Welfare Canada 1983) and the American Recommended Daily Allowance (RDA).

TABLE 22

CORRELATION OF ZINC INTAKE WITH LEUCOCYTE ZINC AND PLASMA ZINC

Zinc intake (mg/day)	Leucocyte zinc (uM/kg)	Plasma zinc (uM/L)
9.9	719	11.01
11.8	719	8.11
11.9	780	8.87
23.7	749	8.72
28.6	780	12.08
30.9	688	7.65
12.8	857	7.80
10.1	826	7.49
16.9	749	5.97
19.2	719	8.87
25.9	688	8.41
11.9	857	7.80
15.0	811	7.49
6.5	780	9.64
8.9	704	11.01
38.8	933	9.02
n = 16	r = 0.149 N.S.	r = 0.056 N.S.

Blood was sampled within 48 hours of the food record being kept.

DISCUSSION

1. LABORATORY METHODS

Table 3 shows the repeatability for measurements of leucocyte zinc. There is a problem with occasional contamination with exogeneous sources of zinc (which is a ubiquitous element), and/or weighing errors. For analysis of values from large groups of subjects occassional weighing errors tend to cancel each other out and infrequent contamination has little effect on mean values. However they are not acceptable if a single result is to be interpreted as an indicator of zinc status. (A weighing error occurs when a container becomes damp, or picks up static electricity which disturbs the electronics of the sensitive balance used to weigh to $\pm$ 0.01 mg). Gross errors were assumed to have occurred when the result in question approached $\pm$ 3SD from the mean, but on single samples smaller errors cannot be detected.

In longitudinal clinical studies the investigator can rarely repeat an analysis that goes wrong. The timing of collection of the sampler in this study was crucial, and the turn-around time from sample collection to zinc analysis was often one month and sometimes more, due to technical problems with equipment. Thus by the time a questionable result was obtained it was too late to repeat it.

If this method is to be adapted for clinical determination of patients' zinc status it may be necessary to use the furnace mode of the Atomic Absorption Spectrophometer (AAS). This permits analysis of a sample ten times smaller than does the flame mode. Triplicate or more samples could be analysed from one patient, and the zinc content could be analysed on a cell count basis to overcome the problem of weighing to $\pm$ 0.001 mg. Whitehouse et al. (1982) reported zinc analysis of granulocytes using this method. The 'noise level' and sensitivity for zinc of the AAS used in this study, and the limitations of calculating a weight of 3-30 mg. from the difference between the empty and full weight of the lightest possible containers (Lay-flat tubing) which weighed about 350 mg. made it imperative to get the largest possible sample of leucocytes from the 25 ml blood sample. Fortunately in pregnancy yields were good, and most samples weighed about 15 mg. d.w. If the cells are wet-ashed in 5 ml of HCL the concentration of the sample is in the mid-range of the standard curve, and background 'noise' does not raise the coefficient of variation to unacceptable levels.

When a very small sample was obtained ($<$5 mg. d.w.) it was ashed in 2 or 3 ml. of HCL. This improved the precision but left no sample for repeat readings in the event of problems with aspiration into the flame or with the stability of the standard curve.

The methodology for the analysis of plasma zinc is in widespread use and the coefficient of variation for ten samples of pooled plasma was 1.1% which compares well with other reports (Breskin et al. 1983).

To summarize: The laboratory methods were sufficiently precise to distinguish differences between groups. For a clinically applicable determination of zinc depletion in an individual, a larger blood sample (60-75 ml.) would yield enough cells for triplicate analysis using the present method alternatively the furnace mode of the AAS would allow triplicate measurements on 10 ml. of blood.

2. CONTROLS

Table 4 establishes a normal range for leucocyte and plasma zinc in women of usual child-bearing age. These figures are 7.2% and 15% respectively lower than those reported by Meadows et al. (1981). Possible reasons for this include the different populations we were sampling, timing of sampling and contamination. My control values for plasma zinc are much closer to those of Hambidge et al. (1983). [13.12 $\pm$ 1.499 uM/L] than to Meadow's. There is a diurnal variation in plasma zinc, the highest levels occurring in the morning, before eating (Metland

and Brubakk 1973). Thus the lower values in this study may have been due to diurnal variation, since most samples were collected in the afternoon.

The longitudinal study of leucocyte and plasma zinc in controls showed no significant trend (Table 5). Seasonal variations have been reported for hair zinc (Hambidge et al. 1976). Individuals' variation with time for leucocyte zinc, was not much less than that of plasma zinc (Table 6). Due to the limitations of the precision of the method already discussed, these results must be interpreted with caution. However the CV in Table 6 is 9%, whereas in Table 3 it is $< 4\%$, and it therefore seems likely that leucocyte zinc is indeed relatively labile, but is is reasonable to interprete changes of 10% or more as potentially important.

Evidence presented in section 3 of the results and discussed in the following section suggests that leucocyte zinc is a useful indicator of current zinc status, and thus we should accept that rapid and important changes in zinc status may occur without clinical signs of deficiency.

As reported by Jones et al. (1981) there is no correlation between leucocyte and plasma zinc (Table 7).

To Summarize: The normal range for leucocyte zinc in non-pregnant women is 613-1030 uM/kg dry weight. The mean is close to but lower than the mean of the normal group in the series of Meadows et al. (1981). For plasma zinc the normal non-fasting P.M. plasma zinc is 8.51 to 15.7 uM/L.

3. LINEAR CORRELATION OF LEUCOCYTE AND PLASMA ZINC WITH MUSCLE ZINC

Figure 1 (page 80) shows the linear relationship of muscle and leucocyte zinc at delivery and Figure 3 (page 110) shows the close agreement of my data with that of Meadows et al. (1981). Clearly, however, Meadow's group found a wider range of zinc concentrations in both muscle and leucocytes. It is not possible to explain this, except that we were sampling different populations.

The results of the current study confirm that leucocyte zinc depletion reflects muscle zinc depletion and is hence a useful clinical indication of a deficit in the largest body pool of available zinc.

Plasma zinc on the other hand does not correlate with muscle zinc (Table 8) and therefore is not a useful indicator of overall zinc status.

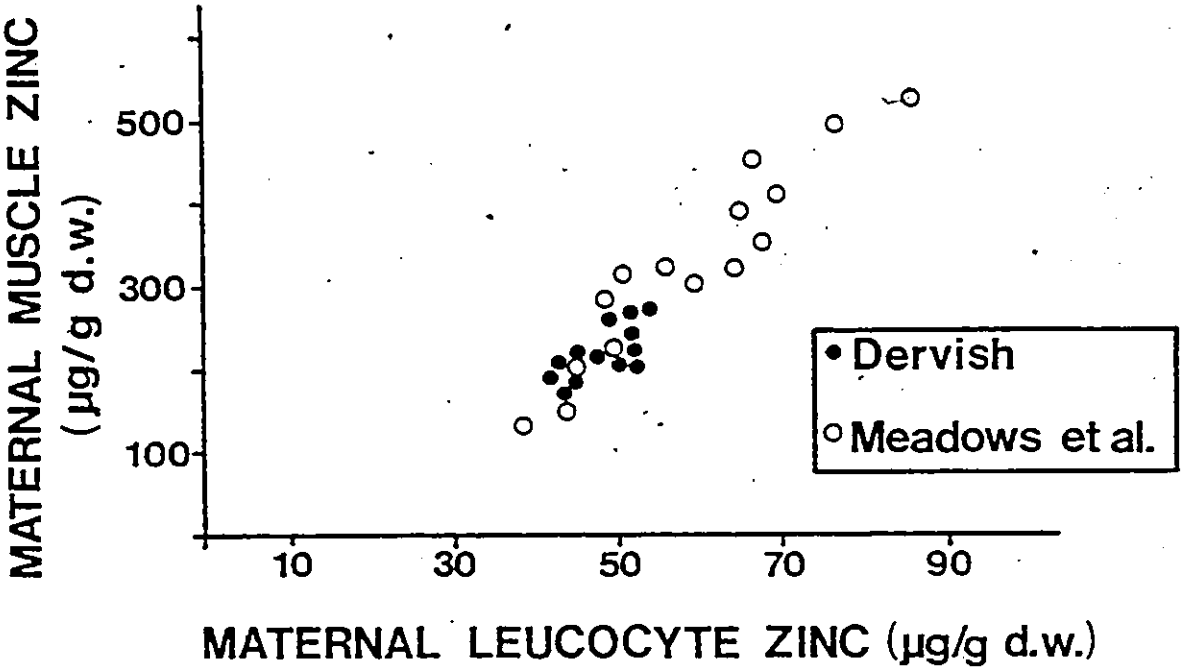
The variations in leucocyte zinc that occur in apparently healthy individuals (Table 6) are thus an indication that there are labile reserves of zinc in both leucocytes and muscle, and that at least a part of intracellular zinc in these two tissues behaves in the same way. Glucocorticoids and LEM (page 23) have been shown to mediate zinc movement between plasma and liver, but what factors control the zinc content of other tissues remain largely unknown.

It is possible that there are mechanisms for moving not only plasma zinc to the liver for redistribution to tissues with a high demand for zinc, but also for the transfer of intracellular zinc from one tissue to another. If this is the case, then it may be necessary to measure the zinc content of a number of tissues under a particular set of circumstances in order to distinguish overall deficiency from a benign redistribution of tissue zinc. From the results obtained in this study it is clear that variations of 10-12% in tissue zinc can be considered normal (Table 6).

Table 8 confirms the findings of Jones et al. (1981) and Meadow's group. There is no significant correlation between plasma zinc and muscle zinc.

FIGURE 3

LEUCOCYTE ZINC AS AN INDICATOR OF ZINC STATUS IN PREGNANCY



4. ZINC STATUS IN PREGNANCY

The data in Tables 9, 10 and 11 which shows the early difference between pregnant and non-pregnant leucocyte zinc values suggest that there is a change in zinc metabolism early in pregnancy. Neither balance studies nor zinc levels of other tissues have been reported this early in pregnancy, but Hambidge et al. (1983) and Breskin et al. (1983) noted a significant fall in plasma zinc in the first trimester, which was not related to haematocrit changes, serum copper increases, or dietary intake.

This early change in zinc metabolism in the first trimester of pregnancy requires further investigation. It is important to obtain muscle biopsies at this stage to determine whether the relationship between leucocyte and muscle zinc holds in early pregnancy, and to measure the zinc content of maternal liver, embryos and their supporting tissues to see if redistribution can explain the early fall in leucocyte zinc.

Of great interest in this study was the only case of multiple congenital malformations. This infant was growth retarded and the malformations exhibited similarity to some of those seen in zinc deficient rat fetuses, namely short mandible, clubbed feet, urogenital anomalies and skeletal malformation. The mother of this child had the lowest recorded first trimester leucocyte zinc (566 millimoles per kilogram), but a normal plasma zinc (13.46

micromoles/litre). Her leucocyte zinc remained low throughout pregnancy and she was noted to have a perineal rash by the obstetrician. Rashes around body orifices are recognized symptoms of zinc deficiency. It is clearly extremely important to document normal changes in leucocyte and plasma zinc in the first trimester so that truly deficient states can be recognized.

The further significant fall in leucocyte zinc by mid-pregnancy confirms the findings of Meadow's group (1981). If the relationship between muscle and leucocyte zinc holds in mid-pregnancy it would be valid to conclude that at this point the zinc reserves of healthy women who produce normal babies become somewhat depleted. One can speculate that zinc is being transferred from one maternal pool to another, or to a fetal pool: i.e. leucocyte zinc is not reflecting the zinc status of the maternal-fetal unit. (Fetal zinc demands will be considered in section 5).

The slight and insignificant rise in leucocyte zinc by the 32nd week (Tables 9, 10 and 11) would be unremarkable except that it is present in the data of Meadows et al. (1981) too. Tighter scheduling and more frequent blood sampling would be necessary to clearly define what is happening at this time of rapid fetal growth.

By delivery, (Table 9) there is no doubt that women who produce normal babies have a degree of tissue zinc depletion, since the previous section of this discussion clearly established a positive correlation between leucocyte and muscle zinc at this time. It is important to determine the time course of return to normal tissue zinc levels in the immediate post-natal period.

Unlike the data of Meadows et al. (1981), Tables 9 and 10 show longitudinal changes in zinc status, (except for the comparison with the controls). This is important because the standard deviation for group mean leucocyte and plasma zinc is quite high (Table 4) [sampling error could occur especially with small samples and cross-sectional data].

Table 11 however shows that with larger samples, partly cross-sectional data gave the same profile of zinc status in pregnancy as longitudinal data. The significant fall in plasma zinc across pregnancy has been reported previously (Table 1) and was discussed in the literature review (page 25). Table 12 shows that although there are cross-pregnancy changes in both leucocyte and plasma zinc there is a weak correlation between leucocyte and plasma zinc in the first trimester only. In controls (Table 7) there was no correlation. The appearance of this weak correlation in early pregnancy is difficult to explain.

The correlations displayed by mean leucocyte and mean plasma zinc in Tables 9, 10, and 11 (see page 86) are statistically, but not biologically, significant. The fall in plasma zinc is largely explained by hemodilution. (For review see Solomons (1979). This could not account for a similar decline in leucocyte zinc. Furthermore, if the fall in plasma zinc caused a fall in leucocyte zinc, or vice versa, then a correlation would have been found at each time interval in individuals. It was not (Table 12) . This is an elegant example of how a correlation can exist without causation, and of why it is important to form a hypothesis first, then seek a correlation, and finally try to prove causation.

5. MATERNAL ZINC STATUS AND INFANT ANTHROPOMETRY

At no point in pregnancy did mothers of SGA infants as a group have signficantly lower zinc status than mothers of AGA or LGA infants. At 20 weeks gestation both SGA and LGA groups had lower mean leucocyte zincs than did the AGA group, and this difference approached significance ($p = 0.06$). With larger numbers a significant difference may have emerged (Table 13, Figure 2). Plasma zinc did not differ significantly between the 3 groups of mothers at any time (Table 15). This agrees with the work of Meadows et al. (1981).

There was overlap at the lower end of the range of leucocyte zinc in all three groups at all points in pregnancy. This can be seen at 32 weeks gestation in Figure 2. It is not likely therefore that leucocyte zinc will predict individual women who will have small or large babies. Nevertheless as mentioned previously (page 111) one subject with the lowest leucocyte zinc value in the first trimester and consistently low values thereafter had a SGA baby with congenital abnormalities.

The significantly lower leucocyte zinc at 32 weeks gestation in mothers of LGA babies is a surprising finding. It suggests a new hypothesis: Fetal growth may determine maternal zinc status in later pregnancy. The original hypothesis and the new one are not mutually exclusive. Although this study does not support the first hypothesis, it should be remembered that neither zinc deficiency nor SGA babies appear to be very common in the population examined. Meadow's study may well have drawn on a more nutritionally diverse population in the Greater London area of the U.K. Since the possibility exists that there are two valid hypotheses, analysis of this data by multiple regression analysis to determine the degree of variance in birthweight and other anthropometric measurements that is attributable to maternal zinc status may be inappropriate, except possibly in the first trimester when the demands of fetal growth are negligible.

Metcoff (1980) suggested that ~~low~~ maternal levels of some nutrients (including zinc) reflect a different maternal-fetal equilibrium when the fetus is large, than that found when the fetus is of average size. Certainly the fall in leucocyte zinc in the LGA group at 20 and 32 weeks gestation occurs at a time when fetal growth velocity is high.

The average fetus weighs 3.4 kg and contains 17.3 mg/kg of zinc, or about 60 mg. (Shaw 1979). This represents close to 4% of average total maternal zinc stores. The 2.8% fall in leucocyte zinc seen across pregnancy in mothers of AGA babies (Table 14) is thus not an unreasonable fall to attribute to fetal growth, particularly in view of the concerns about dietary zinc intake in pregnancy expressed by a number of authors (Jameson 1982, Sanstead et al. 1983, Hunt et al. 1979). The question remains, is a fall in maternal tissue zinc desirable and what implications does it have for pregnancy outcome in women whose pregnancies are numerous and frequent, or who habitually produce LGA babies?

Table 16 describes the anthropometric measurements made at birth. They are in good agreement with those of Usher and McLean (1969). Subsequent multivariate analysis will indicate whether maternal zinc status in the first trimester is an independent determinant of any of these measurements.

The data in Tables 17 and 18 show only one significant correlation: birthweight is inversely correlated with plasma zinc in the first trimester. It will be important to see whether after controlling for the confounding factors (smoking, gestational age, maternal age, weight and parity, pregnancy weight gain and infant's sex) this correlation holds. McMichael et al. (1982) found a similar correlation at mid-pregnancy but plasma zinc did not account for any of the variance in birthweight when multivariate analysis was done.

6. FETAL ZINC STATUS

Table 19 indicates that cord blood leucocyte and plasma zinc values are significantly higher than maternal values ($p < 0.001$). The latter was noted by Meadows et al. (1983); the former should be viewed with caution because the methodology for the separation of cord blood needs improving. In this study a lengthy separation time (page 66 and 67) resulted in small samples of leucocytes (2-5 mg. d.w.); this decreased the accuracy of these analyses compared to those of maternal leucocytes. Meadows et al. (1983) describe using the same methodology for the separation of cord and maternal blood, with results being higher (not significantly) for cord blood.

In their 1983 paper, Meadows' group also report a significant linear correlation between maternal and fetal leucocyte zinc

($r = 0.3$, $n = 89$, $p < 0.001$). Comparison of my regression equation (Table 20) with theirs is not possible since theirs is not included in the paper; neither does their plot of fetal leucocyte zinc against maternal leucocyte zinc include a 'best fit' straight line. It is clear however that with the same correlation coefficient (0.3) but larger sample size, they were able to demonstrate a significant relationship, while I could not. From their results, they suggest that a low maternal tissue zinc status causes low tissue zinc in the fetus, which may account for the intrauterine growth retardation exhibited by the babies with the lowest leucocyte zinc levels.

Too few cord blood samples were obtained from IUGR babies in my study, to make any comments on this. The relationship between maternal leucocyte zinc, cord leucocyte zinc and IUGR has not been determined by the results collected. However I am in agreement with Meadows; that there is no correlation between maternal plasma zinc and cord plasma zinc at delivery (Table 20).

7. DIETARY DATA (Tables 21 and 22)

The mean daily zinc intake from food was 13.0 ± 4.2 mg/day which is slightly higher than the most recently reported North American zinc intake in pregnancy (11.3 ± 4.1 mg/day) from Hambidge et al. (1983). This intake has been expressed as a percentage of the Canadian RDNI and American RDA to emphasize the difficulty of interpreting the dietary intake in terms of current recommendations.

The rationale for the Canadian RDNI covers zinc acquisition of the fetus in the last two trimesters, but makes no allowance for zinc in increased placental, uterine and mammary tissues, or in increased maternal red blood cells, extracellular fluid and plasma volume. Neither does it allow for the high loss of zinc in colostrum and breast milk post-partum. It is possible that increased maternal absorption of zinc in late pregnancy as demonstrated in rats (Davies and Williams 1977) takes care of this. The average full-term infant contains 60 mg of zinc (Shaw 1979), the majority of fetal growth occurring in the second and third trimester. Thus the average daily accrual of zinc by the fetus in the last two trimesters is 0.3 mg/day which allowing for a bio-availability of about 30% (WHO 1973, and Recommended Nutrient Intakes for Canadians 1982) is supplied by 1 mg/day extra dietary zinc. The 1.5 mg/day extra RDNI thus only covers this and the usual "safety" allowance for individuals with greater than average requirements. Where is the allowance for extra maternal tissue?

By comparison the American RDA of 5 mg/day extra zinc throughout pregnancy is both generous and difficult to supply from the normal North American diet which supplies 5 mg/1000kcals.

Jameson's recommendation seems to be based on the assumption that the average weight gain in pregnancy (12 kg) contains the same amount of zinc as 12 kg of lean tissue i.e. (30 mg/kg). (He

recommended 350-400 mg extra per pregnancy, see literature review page 14). The mean daily intake of the group of women in the present study did not prevent apparent tissue zinc depletion, and when Hambidge's group (1983) provided a zinc supplement (15 mg/day) serum alkaline phosphatase activity increased, but plasma zinc did not. It would be interesting to see if tissue zinc depletion could be prevented by a modest zinc supplement.

Dieticians and health educators use Canada's Food Guide to plan diets for pregnancy and to advise pregnant clients with regard to their food habits. Good sources of dietary zinc are not at present given any special attention; the extra food recommendations for the second and third trimester of pregnancy include 250-500 ml. of milk which supplies 0.77 mg. of zinc and 80-160 cals per 250 ml (Murphy et al. 1975). This does not meet the zinc requirement of the third trimester, even if the most conservative estimate (The Canada RDNI) is used. Only red meat, eggs, liver, or oysters could supply more zinc per calorie than milk. It is true that in addition to the extra recommended milk other food up to a total daily extra energy intake of 300 kcals is recommended, and perhaps it is here that emphasis could be placed on foods that are excellent sources of zinc.

In conclusion: To judge the adequacy of zinc intake by the present RDNI or RDA is difficult. Available data suggests the Canadian RDNI may be too conservative while the American RDA

appears generous (see Sandstead 1981 for review of methods used to establish dietary recommendations in pregnancy).

With an average daily intake from food only of 130% of the RDNI, women in this study exhibited tissue zinc depletion. Whether this represents dietary deficiency or a physiological response to pregnancy remains to be established. It is however certain, that such reductions in tissue zinc do not indicate a level of deficiency that adversely affects fetal development in most mothers.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AGA	Appropriate for gestational age
CV	Coefficient of variation
d.w.	Dry weight
IUGR	Intrauterine growth retardation
LEM	Leucocyte endogenous mediator
LGA	Large for gestational age
LMP	Last menstrual period
mM/kg	Millimoles per kilogram
MT	Metallothionein
ppm	Parts per million
r	Correlation coefficient
RDA	Recommended daily allowance
RDNI	Recommended daily nutrient intake
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
uM/kg	Micromoles per kilogram
uM/L	Micromoles per litre
x	Sample mean
WHO	World Health Organization
Zn-MT	Zinc metallothionein

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