ZINC DEFICIENCY AND THE DEVELOPING EMBRYO

A Thesis Submitted for the

Degree of Doctor of Philosophy

by

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any institution, and to the best of the candidate's knowledge contains no material previously published or written by any other person, except where due reference is given.

I. R. Record

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Abstract

Despite efforts by several groups of workers over the last several years, the deleterious effects of a nutritional zinc deficiency on the development of rat embryos and fetuses is yet to be completely understood. This thesis details the results of *in vitro* and *in vivo* studies by the author which provide new insights into the mechanisms whereby a maternal zinc deficiency evokes a teratogenic response in developing rat embryos.

Initial studies *in vitro* using normal 9.5 d rat embryos and zinc-deficient rat serum indicated that it was not possible to directly induce a teratogenic zinc deficiency using embryo culture. Subsequent investigations into the development of zinc-deficient rat embryos *in vitro* revealed that approximately half the embryos explanted appeared abnormal at day 9.5, and continued to develop abnormally *in vitro* regardless of the zinc status of the medium. The remaining embryos were morphologically normal, and continued to develop normally in culture.

In order to understand the appearance of two populations of zinc-deficient 9.5 d rat embryos, further studies were carried out *in vivo*. These experiments confirmed observations of others that zinc-deficient rats consumed their diet cyclically over a 4-day period, during which time the circulating zinc levels fluctuated according to the anabolic or catabolic state of the dam. Morphological studies by the author, using a variety of techniques, established for the first time that during the critical periods of organogenesis (day 9.5 to 11.5), low maternal serum zinc levels were associated with the appearance of dysmorphologies and also with unscheduled cell death in the embryonic tissue, particularly in the neural tube, limb-buds and somites.

In further studies it was found that, although morphological anomalies could be induced as early as day 3 of gestation, these appeared to be reversible, with no lasting effect being observed until the embryos were exposed to zinc deficiency during the post-implantation period.

Further experiments into the development of zinc-deficient fetuses showed that the capacity for repair was limited, and litter loss was a common occurrence. Examination of zinc-deficient fetal rat brains has since revealed areas of abnormal cellular necrosis in regions of extensive mitotic activity.

From these studies it can be concluded that, in rats, zinc deficiency induces a cyclical feeding pattern accompanied by fluctuations in the circulating zinc levels. During periods of low zinc availability to the embryo, abnormal cell necrosis occurs within the embryonic or fetal tissue leading to the observed terata. The precise timing of the feeding cycle with respect to the gestational age of the litter dictates the organs affected, and the degree to which they are malformed.

CONTENTS

CHAPTER 1

ZINC AS AN ESSENTIAL TRACE ELEMENT

1.1 INTRODUCTION

Since 1500 BC, zinc (in the form of calomine) has been used as a topical aid to wound healing. It was not until 1869 that RAULIN recognised that zinc was essential for the growth of *Aspergillus niger* and ascribed some physiological importance to the metal. Sixty-five years later, two independent groups of workers (BERTRAND & BHATTACHERJEE 1934; TODD *et al* 1934) demonstrated that the metal was essential for the growth of rats. At the time this was regarded as an interesting biological quirk, but was thought not to be of human (or animal) significance due to the high level of zinc in soils and foodstuffs. However in the early 1960's, a clinician working in Iran treated a number of hypogonadal dwarfs with zinc, and attributed their growth failure and lack of sexual competence to a dietary deficiency of the metal (PRASAD *et al* 1961). This largely serendipitous finding demonstrated that zinc deficiency was not only a theoretical possibility, and was directly responsible for focussing attention on the role of zinc in the growth, development and well-being of both humans and animals. As a result of intensive investigation by many groups of workers, it is now apparent that the metal plays a major role in every aspect of cellular metabolism.

It is the purpose of this chapter to review the existing literature on the physiological, biochemical and nutritional roles and requirements of zinc with special reference to embryonic and fetal growth and cell division. At this point it should be noted that the literature regarding zinc deficiency is large (more than 1350 articles in the years 1976 - 1985). The author has made every effort to include reference to all

relevant publications by others in the body of the thesis, however space precludes mention of all such articles in the introduction, thus preference has been given to recent, key or historic studies relevant to the present investigation.

1.2 CLINICAL MANIFESTATIONS OF ZINC DEFICIENCY

The absence of sufficient dietary zinc can produce a wide range of symptoms in humans and animals and this fact has been reviewed thoroughly by UNDERWOOD (1977), PRASAD (1979) and BERGLUND (1984). Features of this deficiency include anorexia, growth retardation (e.g. TODD *et al* 1934; MILLS *et al* 1969), parakeratosis (e.g. TUCKER & SALMON 1955; BARNEY *et al* 1967,1969; DIAMOND *et al* 1971; ALVAREZ & MEYER 1973), alopecia (DAY & McCOLLUM 1940), delayed wound healing (PORIES *et al* 1967; PRASAD & OBERLEAS 1974), impaired spermatogenesis (FOLLIS *et al* 1941; UNDERWOOD & SOMERS 1969; DIAMOND *et al* 1971), failure to maintain pregnancy and induction of fetal malformations (BLAMBERG *et al* 1960; HURLEY & SWENERTON 1966; HICKORY *et al* 1969; MILLS *et al* 1969; APGAR 1970; HURLEY & SHRADER 1972; WARKANY & PETERING 1972; APGAR 1973; DREOSTI *et al* 1985a), increased sensitivity to both teratogens (JACKSON & SCHUMACHER 1979; HACKMAN & HURLEY 1981a,b; RECORD *et al* 1982a,b; MILLER *et al* 1983; SATO *et al* 1985) and also carcinogens (FONG *et al* 1978), depressed immunocompetance (GROSS *et al* 1979) and various behavioural abnormalities (PRASAD *et al* 1961; CATALANOTTO 1978; WALRAVENS *et al* 1978a; BURNET 1981;HLSSON 1981).

Probably one of the first noticeable effects of zinc deficiency is a reduction in food intake, closely followed by a cessation of growth (TODD *et al* 1934; MILLS *et al* 1969; MILLER 1971). Force feeding of zinc-deficient animals has shown that the growth retardation is not solely due to inanition (MILLS *et al* 1969; CHESTERS & QUARTERMAN 1970; MILLER 1971; MASTERS *et al* 1983) but appears to be a specific metabolic response to a lack of zinc, possibly associated with a reduced rate of food

utilisation (CHESTERS & QUARTERMAN 1970; MILLER 1971) leading to the observed defects in the synthesis of DNA, RNA and protein. There is also mounting experimental evidence to suggest that the inanition and subsequent maternal catabolism caused by zinc deficiency might have a protective role during fetal development (MASTERS *et al* 1983; HURLEY 1985; RECORD *et al* 1985b,c,d,f,1986,this thesis).

Skin lesions are amongst the most prominent signs of a severe zinc deficiency. Parakeratosis in swine was recognised as a symptom of zinc deficiency as early as 1955 (TUCKER & SALMON 1955) and has been characterised as a thickening of the skin with excessive keratinisation and the retention of epithelial cell nuclei (MILLER & MILLER 1962; UNDERWOOD 1977) which has been detected in the oesophagus and skin of rats, mice, swine and monkeys (FOLLIS *et al* 1941; TUCKER & SALMON 1955; BARNEY *et al* 1967,1969; DIAMOND *et al* 1971; ALVAREZ & MEYER 1973). This condition has also been found in humans suffering from *acrodermatitis enteropathica*, a genetic disease characterised by the lack of an intestinal zinc-binding protein (FREIER *et al* 1973; THYRESSON 1974; WALRAVENS *et al* 1978).

Impaired wound healing in humans is another important aspect of zinc impoverishment. PORIES *et al* (1967) were the first to demonstrate a significant increase in the rate of wound healing compared with untreated controls, when subjects were given 150mg zinc daily following surgery for pilinodal sinuses. Similar results were obtained in studies involving severe burns and major surgery (CALHOUN & SMITH 1968; HENZEL *et al* 1970), and ulcers of the leg (HALLBOOK & LANNER 1972) and stomach (FROMMER 1975). In animal studies the evidence for a theraputic effect of zinc is equivocal. NORMAN *et al* (1975) and SANDSTEAD *et al* (1970) were unable to demonstrate any beneficial effect of zinc on experimental wound healing in normally nourished animals, however LAVY (1972) reported a significant increase in the rate of wound healing in rats. PRASAD & OBERLEAS (1974) have demonstrated that in zinc-deficient animals wound healing is

significantly retarded, and attributed this to a reduction in thymidine kinase activity, DNA synthesis and cell division.

In animal models, spermatogenesis and the development of primary and secondary sexual characteristics in the male (FOLLIS *et al* 1941; UNDERWOOD & SOMERS 1969; BARNEY *et al* 1969; DIAMOND *et al* 1971; NEATHERY *et al* 1973) and all phases of reproduction in the female (HURLEY & SHRADER 1972 ; APGAR 1975; WATANABE 1983) can be affected as a result of zinc impoverishment.

Aside from the more morphological and physiological effects of zinc deficiency other, perhaps more subtle but nevertheless important defects in both behaviour and learning have been reported. Disturbances of taste and smell acuity are consequences of zinc deficiency in both humans and animals (CATALANOTTO 1978). Mental lethargy has been reported to occur in zinc-deficient human subjects in Egypt and Iran (PRASAD *et al* 1961) as well as in cases of *acrodermatitis enteropathica* (WALRAVENS *et al* 1978a; OHLSSON 1981). Other reported neurological features include jitteriness, impaired concentration, depression and mood lability in both children and adults (HENKIN *et al* 1975; SIVASUBRAMANIAN & HENKIN 1978). Table 1.1 details some of the reported effects of zinc impoverishment.

Table 1.1 Clinical manifestations of human zinc deficiency.

congenital abnormalities
growth retardation
hypogonadism/delayed sexual maturation
hypospermia
alopecia
skin lesions
diarrhoea
immune deficiencies
phagocyte chemotactic defects
cellular immune defects
behavioural disturbances
night blindness
impaired taste acuity (hypogeusia)
impaired collagen synthesis and wound healing

It has been suggested that zinc is associated with brain function at a neurophysiological level, principally in relation to the hippocampus which is involved in memory, cognitive function and the integration of emotion (SAHGAL 1980). This evidence is largely circumstantial, and it is thought that the metal plays an important role in the metabolism of glutamic acid in the mossy fibre layer, or it may be involved in a stable zinc-glutamate storage complex in the giant terminal boutons (DREOSTI & RECORD 1984).

1.3 AETIOLOGY OF ZINC DEFICIENCY

SANDSTEAD *et al* (1976) suggested that the majority of human zinc deficiencies are conditioned rather than frank, and proposed that many of the more common conditioning factors influence the zinc status by chelating dietary zinc and rendering it less available. The possible pathogenic factors have been thoroughly listed by BERGLUND (1984) and are reproduced in Table 1.2. Whilst it is true that many of these factors will influence the zinc status, epidemiological studies suggest that many individuals, even in the world's more affluent areas regularly ingest amounts of zinc close to or below the estimated minimum requirement (WHITE 1979; LYON *et al* 1979; HOLDEN *et al* 1979; HUNT *et al* 1979; RECORD *et al* 1985a). It is possible therefore that a true estimate of the zinc status of an individual cannot be made unless all such factors are considered.

Table 1.2 Aetiology of human zinc deficiency.[a]

DECREASED INTAKE
anorexia nervosa
chronic uraemia
abnormal food pattern selection
experimental depletion
total parenteral nutrition without added zinc
DECREASED ABSORBTION
high-phytate diet
high-fibre diet
high dietary iron:zinc ratio
geophagia
acrodermatitis enteropathica
coeliac disease
short-bowel syndrome
jejeuno-ileal bypass
alcoholic cirrhosis
pancreatic insufficiency
steatorhoea
DECREASED UTILISATION
protein deficiency (?)
alcoholic cirrhosis
acute infection/inflammation (?)
INCREASED LOSS
diarrhoeal fluid loss
ileostomy fluid loss
coeliac disease
inflammatory bowel disease
intestinal parasitosis
pancreatic-cutaneous fistula
pancreato-coeliac fistula
nephrotic syndrome
thiazide diuretics
parenteral EDTA administration
oral D-penicillamine therapy
viral hepatitis
alcoholism
acute alcoholic pancreatitis
alcoholic cirrhosis
haemolytic anaemias
post-surgery/post-trauma
extra corporeal dialysis (occasional)
thermal burn exudation
exfoliative dermatitis
INCREASED REQUIREMENT
neoplastic diseases
post-burn re-epithelialisation
growth spurts
pregnancy
lactation
psoriasis (?)

(a) After Berglund 1984.

1.4 ZINC REQUIREMENTS AND INTAKES

Establishment of a reliable figure for the human zinc requirement has been based largely on isotopic balance studies on different populations which indicated an approximate metabolic requirement in excess of 6 mg/day (SANDSTEAD 1982). Estimates of zinc balance in patients undergoing total parenteral nutrition suggested that the requirement was between 2 and 6 mg/day, or around 75 ug Zn/Kg. (LOWRY *et al* 1979; PHILLIPS 1982). These values have been accepted by various bodies as an indication of the metabolic requirement of normal adults, although requirements for zinc depend greatly upon the dietary availability of the metal and have been the subject of several *in vivo* and *in vitro* studies (PECAUD *et al* 1975; REINHOLD *et al* 1976).

Estimates of the overall bioavailability of zinc are varied, however SANDSTROM & CEDERBLAD (1980) suggest that the availability of zinc is seldom above 30%, and may even be less. Other physiological parameters such as pregnancy might increase zinc absorbtion (SWANSON & KING 1982), and there is evidence to show that absorbtion is also increased during zinc deficiency (WILKINS *et al* 1972).

The significance and relationship of various dietary parameters which might facilitate or hinder zinc absorbtion and utilisation in man are unclear, as are changing requirements during growth, pregnancy and lactation. The National Health and Medical Research Council in Australia has recently accepted figures for the Recommended Dietary Allowance of zinc for various populations, in which they have taken into account the estimated metabolic requirements and bioavailability together with a 50% safety margin (DREOSTI 1982). This author has used these published figures to estimate the metabolic requirements for individuals of different ages (Table 1.3).

Table 1.3 Apparent metabolic and dietary allowances of zinc for different ages.[a]

Age	Estimated Metabolic Requirement (mg)	Dietary allowance for bioavailability of		
		10%	20%	40%
0-4 mo.	1	12	6	3
5-12 mo.	2	20	10	5
1-10 yr.	4	40	20	10
11-17 yr.	6	60	30	15
18+ yr male	6	60	30	15
18+ yr female	6	60	30	15
pregnancy	8	80	40	20
lactation	10	100	40	20

(a) modified from Dreosti 1982.

These levels of zinc are not readily met by the average western diet (Table 1.4) and it is possible that subclinical or frank zinc deficiencies do occur with much greater frequency than is currently thought (PRASAD 1979). In a recent study, the zinc intakes of some premenopausal women in South Australia were found to be significantly lower than the estimated daily requirement (RECORD *et al* 1985a)

Table 1.4 Zinc levels (mg/100g edible portion) in some common foodstuffs.

Oysters	up to 100	Breakfast cereal	1 - 3
Beef	4	Bread	1 - 2
Lamb, bacon	3 - 4	Sausages	1 - 2
Shellfish	3 - 4	Pies, pasties	1 - 2
Cheese	3 - 4	Eggs, fish	1 - 2
Nuts, beans	2 - 4	Biscuits, sweets	< 1
Kidney, Liver	4	Fruit	< 1
Heart	2	Beverages	< 1
Pork	2		

SOURCE: McCance and Widdowson's "The Composition of Foods"; ed. Paul, A.A. & Southgate, D.A.T., HMSO London 1977.

1.5 INTERACTIONS OF ZINC WITH OTHER SUBSTANCES

Interactions between zinc and other essential and toxic trace elements, hormones, vitamins and other substances such as alcohol, fibre and drugs abound in the literature. The location of interactions can be at one or more separate or related sites within the living animal (Table 1.5).

Table 1.5 Possible sites of interaction between zinc and other substances.

LOCATION	EXAMPLE	EFFECT
Foodstuffs	Phytate	Influences bioavailability
Intestinal Mucosa	Cu,Ca	Competition for absorbtive sites or effects on absorbtive capacity of mucosa
Transport	Cu	Competition for sites on transport proteins, e.g. albumin
Intracellular	Cd,alcohol	Antagonism or synergism of metabolic processes or interference with integrity of structural proteins

Dietary calcium and copper both interfere with zinc absorbtion (VAN CAMPEN 1969; BECKER & HOEKSTRA 1971) when present at excessive levels. Copper acts by direct competition for intestinal binding and transport sites (DAVIES 1980) whereas calcium as well as phytate form insoluble complexes in the alkaline intestine (OBERLEAS *et al* 1966). Dietary fibre has also been shown to decrease the availability of zinc (REINHOLD *et al* 1976). It is possible that both dietary fibre and phytate may contribute to the occurrence of zinc deficiency in populations subsisting largely on bread and the products rich in fibre and phytate (REINHOLD 1971). It is of interest in this context to note that the Iranian dwarves subsisting on similar diets consumed levels of zinc above the current RDA (15 mg/day)

even though clinical studies and trials showed them to be zinc-deficient (PRASAD *et al* 1963). CASEY and HAMBIDGE (1980) provided an interesting contrast as these workers found the Tokelau islanders had an extremely low zinc intake (4.5 mg/day) with no apparent adverse effects.

Some workers have found pronounced changes in the zinc content of tissues and the metabolism of zinc associated with alcohol ingestion, whilst others have found lesser effects (DREOSTI 1984 (review)). Chronic effects of alcohol on zinc metabolism are however more likely to be associated with severe liver damage.

Additional factors, perhaps not apparently concerned with such mechanisms can have profound influences on the interactions (Table 1.6).

Table 1.6 Factors which might influence interactions between zinc and other substances.

Species
Individual (?genetic)
Chemical form of the substance
Environmental conditions
Stage of maturity
Sex
Physiological state (pregnancy, lactation, catabolism, anabolism)
Subclinical disease
Route of exposure
Recycling of substances within the body

Substances which have been consistently reported to interact with zinc are presented in Table 1.7. Most of these interactions fall outside the direct scope of this thesis, and have been reviewed or discussed thoroughly in the indicated references as well as in other places, e.g. UNDERWOOD (1977), PRASAD (1979). LEVANDER & CHENG (1980), BERGLUND (1984).

Table 1.7 Interaction of other substances with zinc.

Substance	Possible interaction	Reference
VITAMINS		
A	Alcohol (retinol) reductase	Smith 1980
B1	Decreased B1 status increases tissue zinc levels	Gershoff 1968
B6	B6 may affect tissue zinc status	Gershoff 1968; Ikeda *et al* 1979
E	Both protect against peroxidative damage	Bettger *et al* 1980
ESSENTIAL TRACE ELEMENTS		
Manganese	Direct competition or substitution	Wallwork *et al* 1983;
Copper	Competition for binding and transport sites	Kirchgessner *et al* 1981 Wallwork *et al* 1983
Iron	Competetion for binding and transport sites	Rama & Planes 1981; Fairweather-Tate *et al* 1984
Calcium	Competition for binding sites	Record *et al* 1982c; Prasad 1979
Selenium	Involvement with selenomethionine and SH groups	Whanger *et al* 1980
TOXIC METALS		
Cadmium	Competition for enzyme and binding sites	Samarawickrama & Webb 1979; Frazier 1981 Record *et al* 1982a,b
Lead	Competition for binding sites	Cerclewski & Forbes 1976
OTHER NUTRIENTS		
Phytate	Chelation of zinc	Reinhold *et al* 1976
Fibre	Chelation of zinc	Reinhold *et al* 1976
Essential fatty acids	Possible antioxidants	Bettger & O'Dell 1981
Citrate	Zinc chelation	Lonnerdal & Hurley 1984
Protein	Reduced zinc availability	Van Campen & House 1974

DRUGS		
Acetazolamide	? Carbonic anhydrase inhibition	Hackman & Hurley 1981a
Alcohol	Membrane lipid peroxidation Increased excretion	Dreosti 1984 Lindeman *et al* 1978
Corticosteroids	? Increased excretion	Flynn *et al* 1971
Penicillamine	Chelation and excretion	Klingberg *et al* 1976
Salicylate	Mediated through prostaglandins Chelation of zinc	Hackman & Hurley 1981b Vormann *et al* 1986
Thalidomide	? Chelation of zinc	Jackson & Schumacher 1979
Valproic acid	? Chelation of zinc	Hurd *et al* 1983

1.6 ABSORBTION AND INTERMEDIARY METABOLISM OF ZINC

Absorbtion of zinc in rats occurs mainly from the duodenum, jejeunum and ileum , with very little being absorbed from the stomach or colon (SAHAGIAN *et al* 1966; METHFESSEL & SPENCER 1973) although PEARSON *et al* (1966) provided evidence that zinc was actively transported against a concentration gradient in distal gut segments. BECKER & HOEKSTRA (1971) were much more equivocal and stated that "zinc absorbtion is variable in extent and highly dependent upon a variety of other factors". These other factors included body size, level of dietary zinc and the presence of other potentially interfering substances. Zinc absorbtion is also under homeostatic control. SCHWARZ & KIRCHGESSNER (1974) found the absorbtion of ^{65}Zn by zinc-deficient rats to be double that of control animals. WILKINS *et al* (1972) also reported similar effects.

Serum zinc is available to and incorporated by different tissues at different rates. Uptake by bone and brain is relatively slow, as is its subsequent release, thus those pools are not readily available for other tissues (MACINTOSH & LUTWAK-MANN 1972). Liver, kidney, spleen and

pancreatic cells have relatively high rates of zinc turnover, while the pools in muscle and erythrocytes have a lower exchange rate. Radioactive zinc is also rapidly transported through the placenta to the developing fetus (BERGMAN & SOREMARK 1968).

Excretion of zinc occurs primarily via the faeces, with lesser proportions being lost in urine, perspiration and menstrual fluid. Much of the faecal zinc is exogenous, that is unabsorbed in the gut, and therefore varies greatly within and between individuals. The remainder occurs through the loss of pancreatic enzymes and the sloughing off of intestinal cells. In healthy humans, urinary loss is less than 1 mg/day, compared with the dietary intake of 10-15 mg (ROBINSON *et al* 1973; SPENCER *et al* 1978). Patients with kidney damage, or recovering from major surgery or burns can have increased rates of urinary loss (SULLIVAN & LANKFORD 1962; PRASAD *et al* 1965).

Significant losses of zinc can occur in perspiration, especially in hot weather. A normal individual in the tropics excreting 4 L/day of sweat could lose about 4mg zinc/day, although zinc-deficient subjects can exert homeostatic control over this loss (MILNE *et al* 1983), and reduce the amount to about 2 mg/day (PRASAD *et al* 1963). Menstrual losses are small, but in the short term might be of significance in a zinc-impoverished individual. UNDERWOOD (1977) estimates the total zinc loss during the menstrual period to be about 450 ug Zn, which averages out at about 16 ug Zn/day over the cycle, or approximately 65 ug/day during menstruation.

1.7 BIOCHEMICAL ROLES OF ZINC

The first demonstration of a zinc metalloenzyme was reported by KEILIN & MANN in 1940 when they showed that carbonic anhydrase contained zinc. Since then this list has grown substantially and currently over 100 zinc metalloenzymes from different species are recorded. Each of the six categories of enzymes nominated by the International Union of Biochemistry

(IUB) Commission on enzyme nomenclature contain examples of zinc metalloenzymes (Table 1.8).

Table 1.8 Zinc metalloenzymes.[a]

Name	source
Class I - Oxidoreductases	
alcohol dehydrogenase	yeast, liver
D-lactate dehydrogenase	barnacles, bacteria
D-lactate cytochrome reductase	yeast
superoxide dismutase	vertebrates, plants, fungi, bacteria
Class II - Transferases	
transcarboxylase	*P. shermanii*
aspartate transcarbamylase	*E. coli*
phosphoglucomutase	yeast
RNA polymerase	wheatgerm, bacteria, viruses
DNA poymerase	sea urchin, *E. coli*, T_4 phage
reverse transcriptase	oncogenic viruses
nuclear poly(A)polymerase	rat liver, viruses
terminal dNT transferase	calf thymus
mercaptopyruvate sulphur transferase	*E. coli*
thymidine kinase	rat liver, fetuses
Class III - Hydrolases	
alkaline phosphatase	mammals, bacteria
5' nucleotidase	bacteria, lymphoblast, plasma
fructose 1,6-biphosphatase	mammals
phosphodiesterase (exonuclease)	snake venom
phospholipase C	*B. cereus*
cyclic nucleotide phosphodiesterase	yeast
nuclease	microbes
x-amylase	*B. subtilis*
x-D-mannosidase	mammals, plants
aminopeptidase	mammals, fungi, bacteria
aminotripeptidase	rabbit intestine
dipeptidase	mammals, bacteria
angiotensin-converting enzyme	mammals
procarboxypeptidase A	pancreas
procarboxypeptidase B	pancreas
carboxypeptidase A	vertebrates, crustacea
carboxypeptidase B	mammals, crustacea
carboxypeptidase (other)	mammals, crustacea, plants, bacteria
DD-carboxypeptidase	*S. albus*
Elastase	*P. aeruginosa*
neutral protease	vertebrates, fungi, bacteria
collagenase	mammals, bacteria
aminocyclase	pig kidney, microbes

dihydropyrimidine aminohydrolase	bovine liver
dihydroorotase	*Clostridium oroticum*
p-lactamase II	*B. cereus*
creatininase	*P. putida*
AMP deaminase	rabbit muscle
inorganic pyrophosphatase	yeast
nucleotide pyrophosphatase	yeast
Class IV - Lyases	
fructose 1,6-diphosphate aldolase	yeast, bacteria
L-rhamulose-1-phosphate aldolase	*E. coli*
carbonic anhydrase	animals, plants
o-aminolaevulinic acid dehydratase	mammalian liver, erythrocytes
glyoxalase I	mammals, yeast
Class V - Isomerases	
phosphomannose isomerase	yeast
Class VI - Ligases	
t-RNA synthetase	*E. coli*, *B. stearothermophilus*
pyruvate carboxylase	yeast, bacteria

(a) After Vallee, 1983

Zinc enzymes have been subdivided on the basis of their affinity for the metal. Those with high affinity where the zinc is tightly bound in a definite stoichiometry, with a degree of specificity are termed zinc metalloenzymes, whereas those proteins which have a low binding affinity with a low specificity are termed zinc-enzyme complexes. Zinc plays an important role in both the structure and function of enzyme complexes. Both attributes can be found in the enzyme alcohol dehydrogenase (EC 1.1.1.1) which has 4 zinc ions per molecule. Two of these ions are essential for catalytic activity and are bound to the enzyme via an imidazole and two cystinyl groups and are much less susceptible to exchange or extraction with ligands (DRUM *et al* 1967; ECKLUND *et al* 1974).

Extensive investigations into the reponses of enzyme activities in zinc-deficient tissues have shown responses ranging from none to rapid reductions in activity (Table 1.9). Most enzymes are not rate limiting in their metabolic pathways, thus a reduction in activity has little effect on

cell metabolism. As the fractional rate of degradation of different enzymes varies greatly, as does their affinity for zinc, it is hardly surprising that the responses of individual enzymes to zinc deficiency have been so varied.

Table 1.9 Enzymes having decreased activity due to zinc deficiency.

Enzyme	Reference
Alcohol dehydrogenase	Prasad & Oberleas 1971
RNA polymerase	Terhune & Sandstead 1972
DNA polymerase	Duncan & Hurley 1978a
Alkaline phosphatase	Iqbal 1971, Dreosti *et al* 1980a
Leucine aminopeptidase	Park *et al* 1985
Carboxypeptidase A,B	Hsu *et al* 1966
Maltase	Park *et al* 1985
Lactase	Park *et al* 1985
Sucrase	Park *et al* 1985
Ornithine carbamoyl transferase	Rabbani & Prasad 1978
Carbonic anhydrase	Iqbal 1971
2'3'cyclic nucleotidase	Prohaska *et al* 1974
Glutamate dehydrogenase	Dreosti *et al* 1981a
Dopamine beta hydroxylase	Wenk & Stemmer 1982
Phenylethanolamine N-methyl transferase	Wenk & Stemmer 1982
Thymidine kinase	Record & Dreosti 1979
3'phosphohydrolase	Dreosti *et al* 1981a

1.8 ZINC IN CELL REPLICATION AND DIFFERENTIATION

Although most studies into the teratology of zinc deficiency have been carried out using rats, other species such as mice (DREOSTI *et al* 1986), monkeys (SWENERTON & HURLEY 1980) and chickens (BLAMBERG *et al* 1960) show similar effects. The first report of a teratological maternal zinc deficiency in mammals appears in 1966 when HURLEY & SWENERTON maintained rats from weaning to maturity on diets containing 9 ug Zn/g. After mating the females were placed on diets completely deficient in zinc for the duration of pregnancy. In this study 98% of the zinc-deficient fetuses were grossly malformed and growth retarded. In subsequent studies this group demonstrated not only that the period of zinc deprivation could be

commenced at the time of conception (HURLEY *et al* 1971), but that relatively short periods of zinc deficiency (4 days) around the time of organogenesis could induce lasting malformations (HURLEY & SHRADER 1972). Studies by other workers (e.g. MILLS *et al* 1969; WARKANY & PETERING 1972; VOJNICK & HURLEY 1977; HICKORY *et al* 1979; RUTH & GOLDSMITH 1981; ROBINSON & HURLEY 1981(a,b); RECORD *et al* 1985b,c,d,1986,this thesis) have confirmed and extended these observations to show that all organ systems can be affected both biochemically and morphologically, even to the extent of demonstrating that 3 days of maternal dietary deficiency affected pre-implantation embryos (HURLEY & SHRADER, 1975).

Histological examination of malformed fetuses (DIAMOND & HURLEY 1970; HURLEY & SHRADER 1972; WARKANY & PETERING 1972) and autoradiographic studies (SWENERTON *et al* 1969; ECKHERT & HURLEY 1977) have not reported any cellular or sub-cellular changes due to zinc deficiency, however it was reported (SWENERTON *et al* 1969; HURLEY & SHRADER 1972; ECKHERT & HURLEY 1977) that there was an increased number of mitotic figures in certain tissues suggesting that cells were being blocked in mitosis and failing to complete cell division. Whilst confirming these earlier observations, studies conducted as part of this thesis suggest there is not a specific inhibition of mitosis.

There have been numerous attempts to distinguish between reduced rates of increase of cell number and cell size (MACAPINLAC *et al* 1968; WILLIAMS & CHESTERS 1970; HSU *et al* 1969). Most studies of this type have involved labelling newly synthesised DNA, RNA or protein with a suitable radioactive precursor, followed by subsequent determination of the specific activity of the product (HSU *et al* 1969; SWENERTON & HURLEY 1969; GREY & DREOSTI 1972; FERNANDEZ-MADRID *et al* 1973; DUNCAN & DREOSTI 1973,1974; ECKHERT & HURLEY 1977; DREOSTI *et al* 1980a; RECORD 1980).

In general, studies on the concentration of DNA in organs of zinc-deficient animals have failed to find any real differences (WILLIAMS &

CHESTERS 1970; McKENZIE *et al* 1975; RECORD 1980; RECORD *et al* 1980a) however this is not unequivocal as other groups (e.g. HIRSCH & HURLEY 1978) have found reduced concentrations as well as total levels of DNA in zinc-deficient rat embryos. Several groups of workers (PRASAD & OBERLEAS 1974; DREOSTI & HURLEY 1975; DUNCAN & HURLEY 1978a; RECORD & DREOSTI 1979; RECORD 1980) have reported decreased activities of thymidine kinase and DNA polymerase in zinc-deficient fetal tissues. Whilst the latter enzyme is essential for DNA synthesis, the role of thymidine kinase is not well understood. Although the enzyme is synthesised in response to a mitogenic stimulus, the product, TMP, can be synthesised either *de novo* from dUMP or by the salvage pathway from endogenous thymidine. Studies from this laboratory (RECORD 1980; RECORD *et al* 1980b; DREOSTI *et al* 1985a) have indicated that in zinc-deficient fetal rat liver, the contribution of the salvage pathway to DNA synthesis declined by the same amount as the *de novo* path (70%), whereas in the brain, the total DNA synthesis decreased by 40%, and the contributions of the salvage and *de novo* paths declined by 70% and 16% respectively. It would appear therefore that DNA synthesis is spared in the fetal brain relative to the liver and that the contribution of the salvage pathway reflects more reliance upon the activity of thymidine kinase in the central nervous system. It was however observed that the flux of ^{3}H-thymidine increased following methotrexate treatment in both replete and zinc-deficient animals, indicating that the activity of thymidine kinase was not the rate limiting step in the synthesis of DNA and suggesting that other enzymes such as DNA polymerase might be involved.

CHESTERS (1978,1982) pointed out that both thymidine kinase and DNA polymerase activities are reduced by similar extents in zinc-deficient tissues. Since thymidine monophosphate can be derived from *de novo* synthesis, it is not obvious why a reduced thymidine kinase activity should be linked to DNA polymerase activity. In addition, the inhibition of ^{3}H-thymidine incorporation by lack of zinc is paralleled by a reduction in

the number of cells whose DNA became labelled (FUJIOKA & LIEBERMAN 1964; RUBIN 1972; SARYAN *et al* 1979). This suggests that such zinc-deficient cells either synthesised DNA at a normal rate or not at all. It has been suggested (CHESTERS 1982) that lack of zinc abolishes the establishment of the metabolic pathways for DNA synthesis within a proportion of the cells while the remainder develop normally.

The patterns of r-RNA synthesised after addition of EDTA to cultured cells by incorporation of ^{3}H-uridine have been studied (CHESTERS 1975). The results suggested that synthesis of r-RNA and processing of 32-S to 28-S r-RNA was reduced, as was the survival of 28-S r-RNA. SOMERS & UNDERWOOD (1969) reported that the activity of RNAase was increased as a result of zinc deficiency, which could account for the subsequent observations.

From the evidence presented above it can be argued that reduced cellular proliferation cannot be explained on the basis of a general reduction of DNA or RNA polymerase activities, but occurs at the level of the individual cell. Studies by FALCHUK *et al* (1975a,b) demonstrated that, in *Euglena gracilis*, depletion of the zinc content of the medium resulted in arrest of the cell in G2. This group subsequently showed that zinc was also required for transition from G1 to S, S to G2, and G2 to M (FALCHUK *et al* 1977). More recently it has been proposed that zinc ions act as a second messenger in the process of mitogenic induction (GRUMMT *et al* 1986).

It would therefore appear that zinc may play a role in the processes altering the genetic potential of the cell and permitting the synthesis of new enzymes and proteins. DNA synthesis is particularly sensitive, because the enzymes required for it are not constitutive, but are induced at the appropriate stage of the cell cycle. These enzymes may however be constitutive in permanently dividing cells, such as malignant cells and those of the basal layers of the oesophagus.

1.9 HUMAN BIRTH DEFECTS AND MATERNAL ZINC STATUS

Despite the great emotional, intellectual and economic cost to society, little is known about the cause of human congenital abnormalities and spontaneous abortions.

Many groups of workers (Table 1.10) have implicated low maternal serum zinc levels with spontaneous abortion, congenital malformations, atonic bleeding and excessive duration of parturition. Whilst some of these studies were carried out in the less well developed areas of the world, it is of relevance to note that studies in the U.S.A., Sweden, Britain and West Germany have shown similar results.

Table 1.10 Adverse pregnancy outcomes and low zinc status in humans.

Condition	Reference
Anencephalus	Damyanov & Dutz 1971 Sever & Emanuel 1973 Cavdar *et al* 1980 Stewart *et al* 1981 Soltan & Jenkins 1982
Myelomeningeocele	Jameson 1976
Spina Bifida	Bergmann *et al* 1980
Anencephalus	Hambidge *et al* 1975
Exencephalus, microcephalus	Hurd *et al* 1983
Chronic bleeding	Jameson 1976
Small-for-age	Meadows *et al* 1981 Crosby *et al* 1977 Simmer & Thompson 1985 Patrick *et al* 1982
Eclampsia	Bassiouni *et al* 1979 Cherry *et al* 1981

To illustrate the magnitude of the problem of fetal and neonatal loss, the author has extracted information regarding the causes of death in Australia for the year 1983 (Australian Bureau of Statistics 1985a). Whilst it was reported that cardiovascular problems and malignant disease were the causes of 73% of the 110,000 deaths, about 1% (1000) were due to congenital anomalies and a further 0.7% to other perinatal conditions (Table 1.11). In the same year, approximately 1600 still-births (post-22 weeks of gestation) were reported. Whilst not all of the deaths were attributable to congenital defects or nutritional problems, it is of interest to note that the incidence of perinatal deaths is greater than that due to motor vehicle accidents (i.e. 2.6%).

Although available data on the incidence of birth defects is scant, data compiled since 1980 suggest that the rates of such defects in Australia are similar to those reported throughout the world. By collating such data from various sources, it can be estimated that of every 1,000 human conceptions, only about 340 give rise to "normal" children (Table 1.11). Indeed if minor or major anomalies are not detected until later in life were to be included, this figure would be substantially larger.

Table 1.11 Fate of 1,000 human conceptions.

Fate	Number lost	Number remaining	Reference
Presumptive unrecognisable loss	500	500	Craft 1982 Hertig 1967
Spontaneous abortion (3-28 weeks)	75	425	W.H.O. 1970
Stillbirth (post 28 weeks)	9	416	A.B.S. 1985b
Early neonatal death	4	412	Machin 1975 Bauld *et al* 1974
Major malformations detected at birth	6	406	Perinatal Statistics Unit 1985
Major malformations detected by 12 months	14	392	Lamy & Frezal 1961
Minor malformations detected at birth	54	338	Marden *et al* 1964

Due to difficulties in obtaining information from single groups of workers regarding each stage of development, and also possible differences in definition, locality and time of the study, the data in Table 1.11 can only be regarded as approximate.

The aetiology of birth defects is largely unknown, however BECKMAN & BRENT (1984) have estimated the relative contributions due to various teratogenic stimuli (Table 1.12).

Relatively few malformations can be attributed directly to some cause e.g. vitamin A excess, alcohol, specific drugs or hyperthermia etc. The majority of cases (65%) have an unknown aetiology, and even though infants with autosomal genetic disease e.g. Down's, Turner's syndrome or chromosomal abnormalities (e.g. Fragile-X) can be identified, there are few clues as to why such defects should occur in these infants, but not siblings. It is exceedingly unlikely that a single nutritional deficit such as zinc deficiency could account for even the majority of these unexplained events, but it is possible that a deficit of this metal

contributes to the total number of defects, and may act synergystically with other teratogenic stimuli.

Table 1.12 Aetiology of human malformations[a].

Suspected cause	%
Autosomal genetic disease	15-20
Chromosomal abnormalities	5
Maternal conditions	
diabetes	4
endocrinopathies	
nutrition deficiencies	
drug addictions	
Maternal infections	3
Mechanical deformities	1-2
Chemicals, drugs, radiation, hyperthermia	<1
Unknown	
? polygenic	65
? Multifactorial	
(gene-environment interactions)	
? spontaneous	
? synergystic action of teratogens	

(a) After Beckman & Brent 1984

1.10 AIMS OF THIS THESIS

Despite the substantial efforts expended in the study of the teratogenic response to zinc deficiency, many questions remain unanswered. Although evidence has been presented in the past to implicate zinc deficiency in all aspects of cellular replication, the development of such abnormalities during the critical periods of organogenesis has received little attention. The aims of the present study were two-fold. In the first instance it was intended to examine the *in vivo* and *in vitro* development of zinc-deficient rat embryos during the early critical period

of organogenesis (from day 9.5 to 11.5 of gestation) in order to gain further insights into the morphological aberrations and associated physiological and biochemical changes. The second aim was to examine the influence of maternal dietary zinc deficiency prior to day 9 and between days 11 and 20 to further elucidate the role of zinc in the development of the embryos and fetuses over those periods.

Early studies described in this thesis were performed using embryos growing both *in vivo* and *in vitro* and a variety of biochemical and morphological techniques were applied. As a result of the observations obtained in these studies, further experiments were carried out to examine the effects of maternal dietary zinc deficiency on the subsequent development of the fetus until day 20 and also early development prior to day 9. In addition, the opportunity was taken to study the mouse embryo to examine some of the effects of zinc deprivation on the development of this species and to compare these observations with those made on the rat.

In order to make this thesis more readable, the author has elected to preceed each series of chapters with a description of the relevant developmental events occurring over that period. Each chapter ends with a discussion of the aspects covered. Chapter 10 presents an overall discussion of the studies, together with the conclusions which can be drawn.

Full papers, and abstracts of communications containing work from this thesis which have been presented at conferences constitute Appendix I.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

The rats used in these studies were of the Sprague-Dawley strain which was originally obtained from the Animal Resource Centre, Perth, Western Australia. Animals from the original stock were randomly mated and subsequent generations maintained in the facilities of CSIRO Division of Human Nutrition, Adelaide, South Australia.

When required for experimental purposes, females of the desired age were caged overnight with males of the same strain. The following morning, successful matings were determined by vaginal lavage. Copulation was presumed to have taken place at the mid-point of the dark cycle (approximately midnight). Thus the day of detection of sperm was taken to be day 0.5 of gestation. Immediately thereafter the pregnant animals were allotted randomly to the appropriate experimental and control groups.

Whilst on the dietary regimes, animals were housed individually in plastic boxes equipped with stainless steel mesh floors to prevent coprophagy. Glass-distilled water was allowed *ad libitum*, and food was provided according to the requirements of the individual study.

All animal experiments were carried out within the specification of the CSIRO/NH&MRC guidelines on the use of animals in scientific experiments, and received prior approval by the appropriate ethics committee.

2.2 DIETS

Prior to the experimental feeding period, animals were maintained on a

commercially available pelleted diet (PRO-RAT, Milling Industries, Adelaide, South Australia). Briefly, the semi-synthetic zinc-deficient and replete diets were prepared in the laboratory as described elsewhere (RECORD 1980;RECORD *et al* 1986). Soy flour (SOYBAKE, H.J.Langdon & Co, Melbourne, Victoria) was heated in an oven at 105^{o}C for 1hr to inactivate the trypsin inhibitory factor. Suitable amounts (typically 1.5Kg) were then extracted for 1hr with hot 95% alcohol prior to filtration in a Buchner funnel. The material was then suspended in approximately 8*l* of distilled water, 15g of EDTA added and the pH adjusted to 4.5. The mixture was then stirred for 2hr and filtered. This process was repeated twice more, and then the flour was washed three times with metal-free distilled water to remove traces of EDTA. After a further extraction with re-distilled ethanol, the resultant metal-free flour was air-dried and subsequently stored at -20^{o}C until required.

The extraction procedure reduced the zinc content of the flour from an initial 40-50 ug/g to less than 0.5 ug/g. The final protein content was about 46%, the remainder being mainly insoluble carbohydrate. The flour was then used as the basis for the diet which contained, on analysis, less than 0.5 ug Zn/g. When required, zinc (in the form of zinc sulphate) was finely ground with sucrose and incorporated into the diet to a final level of 100ug Zn/g prior to addition of the oil (RECORD 1980;RECORD *et al* 1986). All batches of the semi-synthetic diet were assayed for zinc and copper prior to use. The composition of the diet is detailed in Table 2.1.

For comparative purposes, the formulation of the commercial and semi-synmthetic diets are compared with the recommendation of the American Institute of Nutrition (National Academy of Sciences 1978).

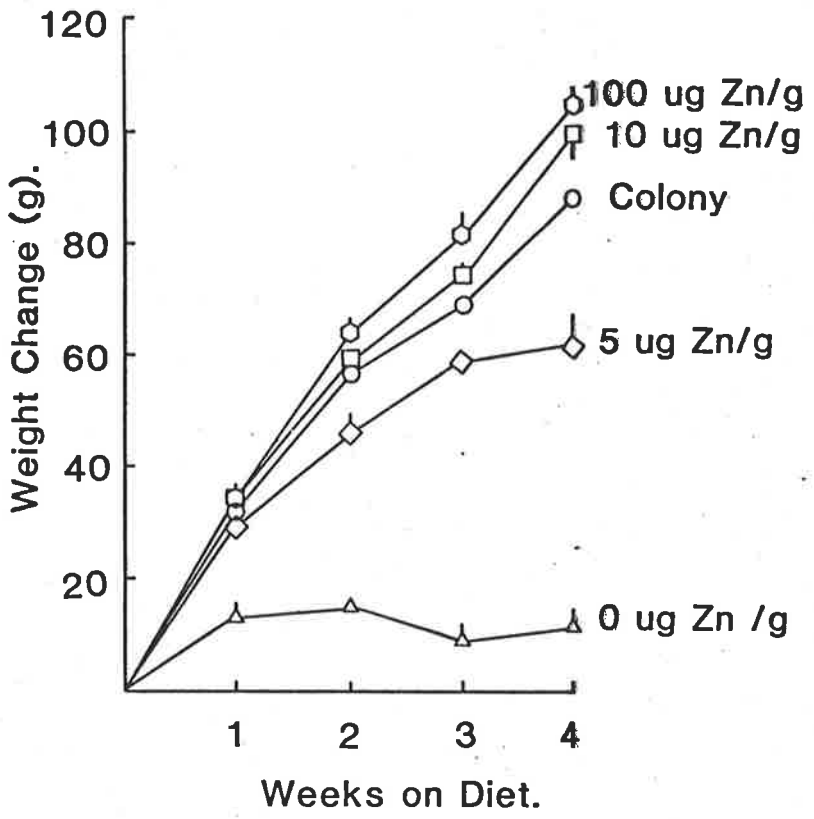
Table 2.1 Comparison of commercial and semi-synthetic diets with recommendations of the National Academy of Science.

Constituent	Commercial	Semi-synthetic	N.A.S
Protein	23%(mixed protein)	19%(Soy)	12%(ideal)
Crude fat	3.1%	8%[a]	5%
Digestible energy	12800 KJ/Kg	16000 KJ/Kg	15900 KJ/Kg
Crude fibre	4.8%	N.D.	N.S.
Sucrose	N.S.	44%	N.S.
Calcium	1.1%	0.5%	0.5%
Phosphorus	0.96%	1.0%	0.4%
Zinc	61 mg	100 mg[b]	12 mg
Copper	5 Mg	12 mg	5 mg
Iron	66 mg	190 mg	35 mg
Manganese	102 mg	28 mg	50 mg
Molybdenum	1 mg	N.D.	N.S.
Iodine	2 mg	7 mg	0.15 mg
Sodium chloride	0.86%	0.6%	0.05%
Magnesium	421 mg	470 mg	400 mg
Choline chloride	700 mg	500 mg	1000 mg
Methionine	4.2 g	3.5 g	6 g
Vitamin A	22500 IU	7500 IU	4000 IU
Thiamine	58 mg	15 mg	4 mg
Riboflavin	5 mg	5 mg	3 mg
Niacin	18 mg	15 mg	20 mg
Calcium Pantothenate	20 mg	25 mg	8 mg
Pyridoxine	5 mg	15 mg	6 mg
Vitamin B12	120 ug	15 ug	50 ug
Ascorbic Acid	N.D.	50 mg	N.S.
Vitamin D3	4000 IU	750 IU	1000 IU
Vitamin E	36 IU	60 IU	30 IU
Vitamin K	8.3 mg	12.5 mg	50 ug
Folic acid	2 mg	0.3 mg	1 mg
Biotin	110 ug	125 ug	N.S.
Inositol	N.D.	250 mg	N.S.
Para-amino benzoic acid	N.D.	5 mg	N.S.

N.D.: not determined
N.S.: not specified
a : sunflower oil
b : zinc was omitted from the zinc-deficient diets

In order to ensure that no essential components were omitted from the soy-flour based diet, growth curves were obtained for weanling rats allowed free access to diets containing different levels of zinc for 4 weeks and compared with the growth rates obtained from similar animals fed the commercial diet *ad libitum* (Figure 2.1). The results indicated that once supplemented with zinc, the semi-synthetic diet was adequate in this respect, and that omission of zinc did cause a rapid cessation in growth.

.Fig. 2.1 Growth of weanling rats fed diets containing varying levels of zinc.



2.3 REMOVAL OF TISSUES

At the end of the experimental period, animals were anaesthetised with ether and blood samples collected by cardiac puncture and allowed to clot

prior to recovery of the serum. The uteri were then exposed and removed. In the case of post-implantation rat and mouse embryos, the uterus was cut lengthwise with fine scissors and the decidual tissues containing the embryos progressively removed to a petri dish containing either Hank's balanced salt solution (HBSS) or phosphate-buffered saline (PBS) adjusted to pH 7.3. The ovaries were also removed to count the number of corpora lutea.

Once removed from the uteri, the decidual tissue was divided into halves using fine stainless steel forceps under a dissecting microscope. The embryo was teased away from the remainder of the decidual tissue, and the parietal yolk sac, trophoblast and Reichert's membranes removed. In the case of 9.5 day rat embryos which were to be cultured, examination and staging was carried out as described in Chapter 3.

Older (11.5d) rat and 9.5d mouse embryos were examined for the presence of blood islets and vessels in the visceral yolk sac, and the degree of rotation of the embryo was noted. The visceral yolk sac was then removed, and, if required, retained for protein determination. The degree of fusion of the allantois with the chorion (to form the chorio-allantoic placenta) was examined then this and the amnion were removed. The crown-rump length of the embryo was determined using an eye-piece graticule in the microscope, the number of somite pairs counted and the embryo examined for the presence of morphological anomalies and photographed if required. In order to preserve uniformity, standard scoring sheets were used for each litter. The procedures to this point followed similar lines to those suggested by BROWN & FABRO (1981).

Embryos thus recovered were either stored individually at -20^{o}C for subsequent protein analysis, or fixed for histological (LM, TEM or SEM) examination.

In the case of the 20d fetuses, the uteri and ovaries were removed, the number of corpora lutea determined and the number and position of both

fetuses and resorption sites noted. After removal of the fetal membranes, fetuses and placentae were weighed individually and the fetuses examined for the presence of external malformations. Some fetuses and their placentae were retained for trace element analysis whilst the remainder were decapitated and the body cavity opened prior to immersion in Bouin's fixative for at least 48h. After this period, the fixed carcasses were examined for the presence of internal malformations and the heads sectioned as suggested by WILSON (1965). Representative examples of defects were photographed when they were observed.

2.4 BIOCHEMICAL ANALYSIS

2.4.1 Protein.

Embryonic and yolk-sac proteins were determined by a modification of the method of LOWRY *et al* (1951). Once removed from the uterus, tissues were stored frozen until an experiment had been completed. The tissues were then digested in a solution containing 0.05% Triton X-100 in 0.1M NaOH. After digestion, the protein content of equal aliquots was determined exactly as described by LOWRY *et al* 1951), except that the standard (crystalline bovine serum albumin, Sigma, St. Louis) was dissolved in the above solution.

2.4.2 Trace Elements.

All trace element analyses were performed on a Perkin-Elmer HG-500 Atomic Absorbtion Spectrophotometer (Perkin-Elmer, Norwalk, N.J.). Sera and other fluids were diluted with glass-distilled water and the zinc and copper levels determined by the direct aspiration technique (WILKINS *et al* 1972). Tissues, diet samples and faeces were wet-ashed in a mixture of nitric and perchloric acids. Residues were then diluted appropriately with glass-distilled water prior to trace element determination (RECORD 1980; RECORD *et al* 1982a).

2.5 LIGHT MICROSCOPY.

2.5.1 Paraffin Sections.

The fixative and staining method described by FRASER (1982) was used throughout. Briefly, embryos were fixed for 48h in a modified Bouin's fixative, dehydrated and embedded in paraffin wax. Serial sections (6uM) were then cut and mounted on slides (12 sections/slide). Alternate slides were then stained with either cresyl violet for observation and counting of mitotic figures, (FRASER 1982) or with Groat's haematoxylin and eosin for general histological examination. The results of this technique are desribed in Chapter 5.

2.5.2 Light and Transmission Electron Microscopy.

Embryos were fixed overnight in cold (4°C) 0.1M sodium cacodylate buffer containing 2% paraformaldehyde and 3% glutaraldehyde adjusted to pH 7.3. The embryos were then washed once in 0.1M cacodylate buffer, post-fixed for 1h in 1% aqueous osmium tetroxide, dehydrated through a graded series of alcohols (50% - 100%) and embedded in Spurr's resin (TAAB, Berks, U.K.). Semi-thin (0.5 - 1.0uM) sections (Porter-Blum MT2-B) were stained with hot toluidine blue (0.025%) - borax (0.5%). Thin sections were stained with uranyl acetate and lead citrate prior to examination with a JEOL 100S electron microscope at 60 kV (TULSI 1983).

2.5.3 Scanning Electron Microscopy.

Embryos were fixed, post fixed and dehydrated in the same manner as those destined for TEM studies, but were then critical-point dried from iso-amylacetate using carbon dioxide as the transition fluid BALZERS-UNION, Liechtenstein). Specimens were then mounted on aluminium stubs using either silver dag or double-sided tape, sputter coated with gold and palladium to thickness of either 50 or 100 nM, and examined with an ETEC scanning electron microscope at 5 or 20 KeV.

2.6 STATISTICS.

Experimental results were evaluated by either analysis of variance (ANOVA) or Pearson's correlation techniques and were carried out using either the Statistical Package for the Social Sciences (SPSS) Version 6.0 (Nie *et al* 1975) and the facilities available through the CSIRONET computer network (CSIRONET, Canberra, A.C.T.) or a smaller, purpose-written package (STATSPACK) running on an APPLE-compatible micro-computer (LINGO PC-128).

2.7 MISCELLANEOUS.

Techniques pertaining to only one study or chapter are described in the body of the thesis.

CHAPTER 3

GROWTH AND DEVELOPMENT OF ZINC-DEFICIENT RAT EMBRYOS *IN VITRO*

3.1 INTRODUCTION

Investigations into the adverse effects of zinc deficiency using pregnant animals are complicated by the possible mobilisation of maternal zinc stores, and the influence of hormonal changes. Because of these factors many efforts have been made in recent years to use cell or tissue preparations to study the effects of zinc deficiency *in vitro*. The advantage of such a technique would be to render it possible and practicable to control precisely the availability of zinc, or of other interacting substances to the cells in question.

Probably the first efforts in this direction were by FUJIOKA & LIEBERMAN (1964) who demonstrated a reduced incorporation of [^{3}H]-thymidine into the DNA of rat liver after perfusion with EDTA. FALCHUK *et al* (1975, 1977) have used cultures of an eukariotic organism *Euglena gracilis* to study the role of zinc in cell division with some success. This particular organism was studied because it was capable of growing well in a chemically defined medium and also could be synchronised in a specific phase of the cell cycle by varying the lighting conditions. Although yielding valuable insights into some of the actions of zinc during the cell cycle, extrapolation from this model to either the animal or human setting is uncertain.

Several groups (CHESTERS 1972; WILLIAMS & LOEB 1973; DUNCAN & DREOSTI 1975) have studied the transformation and replication of mitogen stimulated lymphocytes as well as other cell lines (LIEBERMAN & OVE 1962; RUBIN 1972; GRUMMT *et al* 1986) in zinc-deficient media. Whilst the model also provided a relatively easy method of obtaining growing or synchronised cells with an

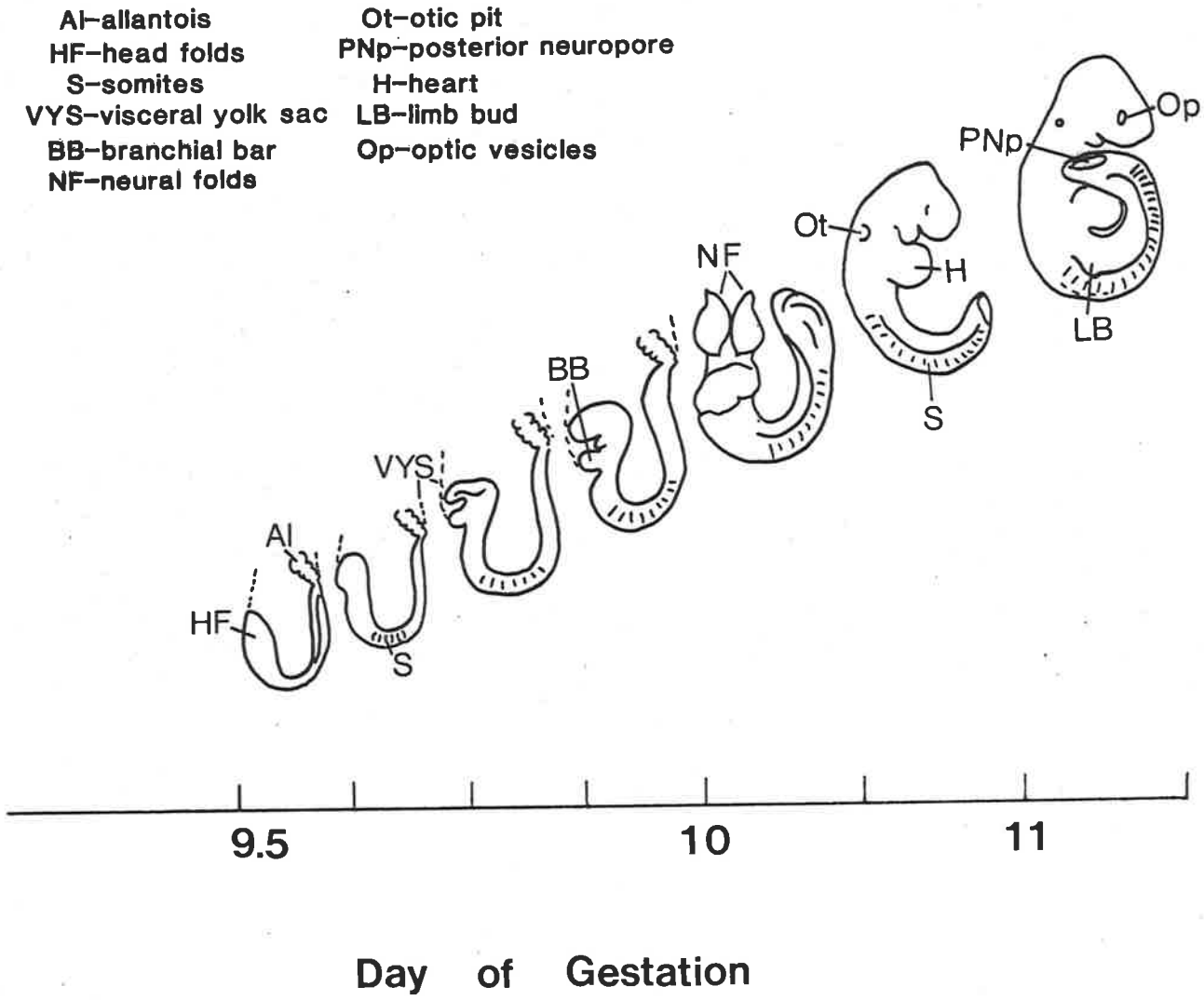
appropriate decrease in the incorporation of [^{3}H]-thymidine into DNA, these studies did not have the capacity to identify the effects on the developing embryo or fetus. Possibly the only satisfactory attempt to culture embryos in a zinc-deficient environment was reported by INIGUEZ *et al* (1978) who cultured chick embryos in media from which zinc was extracted by dithizone. Although limited in extent, this study did demonstrate that growth and development *in vitro* could be adversely affected by zinc deficiency, and that these effects could be reversed by addition of near-physiological levels of zinc.

For the purposes of the current study it was decided not to use this model, but to use the whole rat embryo culture procedure developed by NEW *et al* (1966,1978), and successfully used by many other groups of workers to study various other teratogens. This technique was felt to be of particular relevance due to the high incidence of neural tube defects observed in zinc deficient fetuses (HURLEY & SHRADER 1972) which arise during the times covered by the incubation period.

These methods which have been developed over the last few years allow early head-fold stage rat embryos (9.5 days) to be removed from the uteri and cultured in a medium composed of 90% rat serum with added antibiotics. During the following 48h of culture, the embryo undergoes development comparable with that *in vitro* (NEW *et al* 1976). Briefly, the developmental stages are as follows and are depicted diagrammatically in Figure 3.1.

After 9.5 days of gestation the embryo is contained within a spongy mass of decidual tissue. Surrounding the embryo proper are the trophoblast, parietal yolk sac, Reichert's membrane and the visceral yolk sac. Within the visceral yolk sac the embryo is visible as a translucent thickening of the yolk sac with a thinner neural groove. Above the embryo is the amnion dividing the egg cylinder with the allantois visible as a small bud. Over the next 24 hours (*in vivo* or *in vitro*), the allantois extends to fuse with the chorion to form the chorio-allantoic placenta.

Fig 3.1 Development of the rat embryo from day 9.5 to day 11.5.



The embryo itself begins to take form as the neural folds increase in size and separate from the visceral yolk sac. The eyes and heart begin to develop and the somites start to form. At this stage the embryo is in a dorsally concave position, but begins to rotate about its long axis to a dorsally convex position as the neural tube begins to close. In the period from day 10.5 to 11.5 rotation is completed, vitelline vessels connect the embryo to the visceral yolk sac circulation and umbilical vessels connect the embryo to the chorio-allantoic placenta. The neural tube has closed, except for a small opening at the posterior end, the fore-limb buds are visible at about the level of the tenth somite, the primitive heart is functioning, the optic and otic vesicles are clearly defined and the branchial bars are clearly visible.

Techniques of this type have proven valuable in studying the effects of certain teratogens such as alcohol (WYNTER *et al* 1983), acetaldehyde (POPOV 1981), cadmium (Klein *et al* 1980; RECORD *et al* 1982a,b), metabolic inhibitors (WALSH & CHRISTIAN 1984; SCHMID 1985) and other fetotoxins.

The majority of such *in vitro* studies have involved the addition of potential teratogens to the incubation media, however relatively few have attempted to use serum deprived of a growth factor. COCKROFT (1979) successfully removed a range of small growth factors (inositol, pantothenic acid, folic acid) by dialysis of normal serum, and in an earlier study STEELE *et al* (1974) grew embryos in serum obtained from vitamin E-deficient rats and restored growth and development by the addition of the vitamin *in vitro*.

In this, the first study presented in this thesis, the rat embryo culture technique as described by NEW *et al* (1978) was used to remove embryos from the influence of maternal zinc stores. The aims were to establish whether a teratogenic zinc deficiency could be established *in vitro* and by culturing normal rat embryos in zinc-deficient serum for 48h to determine whether any developmental anomalies observed under such

conditions reflected those observed *in vivo*. This chapter presents in greater detail the results of work already presented elsewhere (RECORD *et al* 1984a,1985e).

3.2 MATERIALS AND METHODS

Virgin female Sprague-Dawley rats (200-230g) were placed overnight with males of the same strain. Mating was determined by vaginal smear and was designated day 0.5 of gestation.

Animals used as embryo or serum donors were fed either a stock colony diet (PRO-RAT diet, Milling Industries, Adelaide, S. Australia) or a zinc-deficient or replete diet prepared from EDTA-extracted soya-bean flour (Chapter 2) from the day of mating. Animals were allowed free access to the zinc-deficient diet, which contained less than 0.5ug Zn/g. The zinc-replete diet was prepared in the same manner, except that zinc sulphate was added to a final level of 100ug Zn/g diet. The zinc-replete animals received 12g food daily, an amount based on the average daily food consumption of the zinc-deficient group. All animals receiving the soya-flour-based diets were housed individually in stainless steel and plastic cages throughout the experiment.

On day 9.5 of gestation the dams were anaesthetised with ether and serum samples collected for zinc determinations by flame atomic absorption spectroscopy (WILKINS *et al* 1972). The embryos were removed from the uterus and the decidual tissue, trophoblast, parietal yolk sac and Reichert's membrane were removed with fine forceps (NEW 1966). Undamaged, early head-fold embryos were placed in 60ml screw-capped bottles containing 3.0ml of prewarmed medium which had been equilibrated with 5% O_2, 5% CO_2 and 90% N_2. After 20h the medium was re-gassed with 20% O_2, 5% CO_2 and 75% N_2 and at 40h with 40% O_2, 5% CO_2 and 55% N_2. At the end of the 48h incubation period, the embryos were examined for the presence of heart-beat, yolk-sac circulation and for morphological development (BROWN &

FABRO 1981). Individual yolk sacs and embryos were stored frozen for subsequent protein determination by the method of LOWRY *et al* (1951).

The media used in these studies consisted of 90% immediately centrifuged, heat-inactivated pooled rat serum (STEELE & NEW 1974) supplemented with streptomycin sulphate (66ug/ml) and penicillin-G-sulphate (6ug/ml), which was then made to volume with water (KLEIN *et al* 1980). Where appropriate, carrier-free ^{65}Zinc (378mCi/mg Zn, Radiochemical Centre, Amersham, UK) was added to the medium to a final specific activity of about 0.4uCi/ug. The radioactivity of the media and tissue samples was determined using a Nuclear-Chicago model 1185 gamma counter. Results were evaluated by analysis of variance.

3.3 RESULTS

The first phase of the study was aimed at discovering whether normal embryos (i.e. obtained from dams fed the colony diet) would grow in serum obtained from zinc-deficient animals. The concentration of zinc in the zinc-deficient media, obtained as a pool from approximately 25 non-pregnant animals fed the zinc-deficient diet for 20 days, was 0.56ug Zn/ml, less than half that of control cultures (Table 3.1).

Embryos from zinc-replete dams cultured in zinc-deficient media grew and developed to the same extent as those cultured in sera from zinc-replete animals. The only major difference was a small decrease in the protein content of the yolk sac. It therefore appeared that either the 9.5 day egg cylinders could maintain their growth on the stores of zinc already present within the tissues, or that they were able to accumulate sufficient zinc from the medium to continue their growth. These aspects were explored in the next phase of the study.

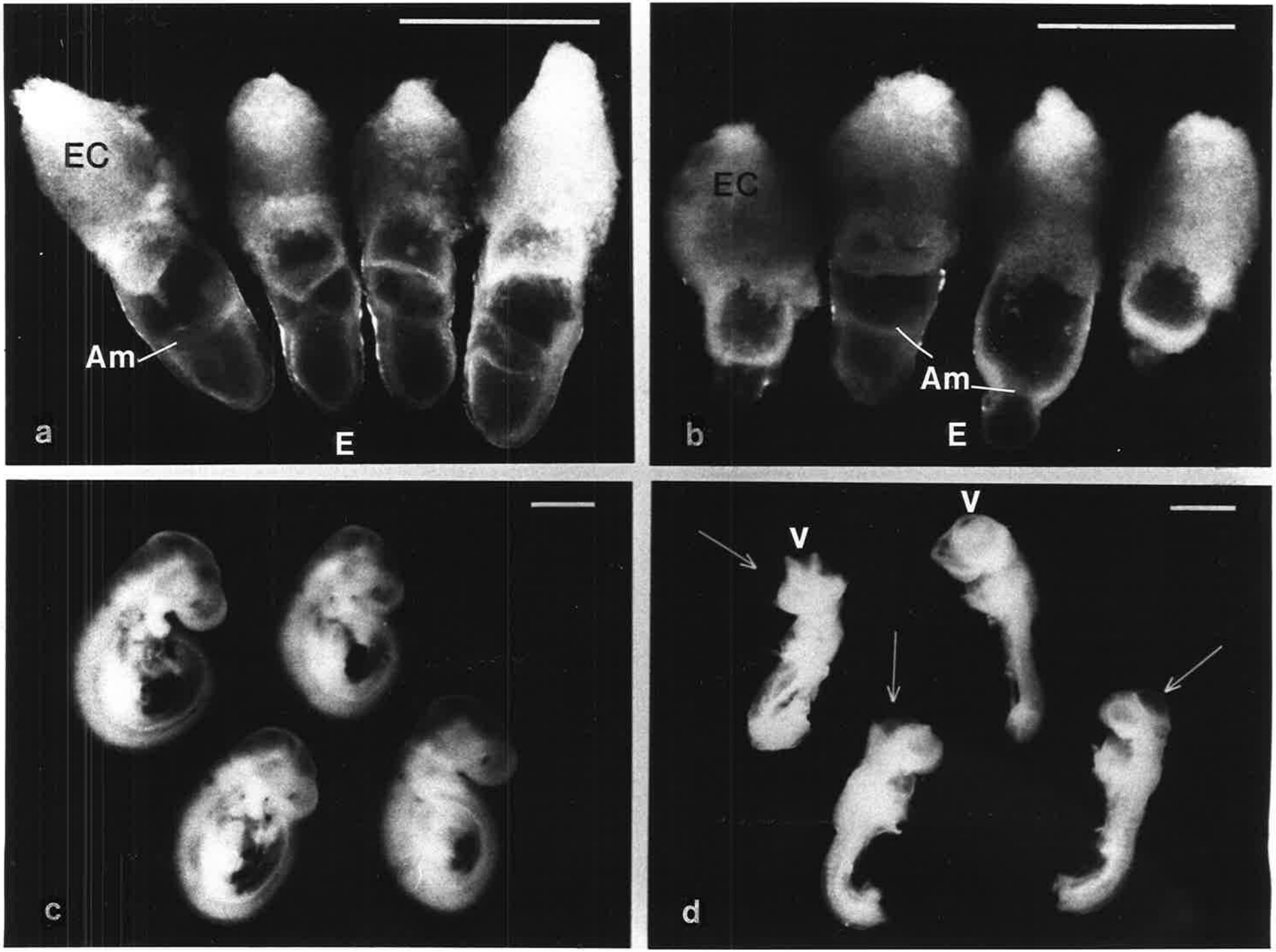
Table 3.1 Growth and development of normal embryos in zinc-deficient and replete media[a].

	Medium	
	Replete	Deficient
Zinc concentration (ug Zn/ml)	1.29	0.56
No. embryos	43	12
Embryo protein (ug)	114.6 ± 5.6	114.8 ± 7.9
Somite number	22.5 ± 0.4	22.8 ± 0.4
Yolk sac protein (ug)	103.0 ± 3.5	89.5 ± 5.6[b]
Abnormal yolk sac circulation	4	2
Abnormal rotation	6	1
Abnormal allantoic fusion	5	0
Open neural tubes	2	0

a: Means ± SEM of the number of embryos indicated.
b: Significantly less than control value $p < 0.05$

In the second experiment embryos obtained from either zinc-deficient or replete dams were cultured in zinc-replete or deficient media. In several incubations, extra zinc was added to the media in an attempt to overcome any potential growth retardation. During dissection of the 9.5 day zinc-deficient embryos it was noted that they fell into two broad morphological categories. The first group appeared normal when viewed under the dissecting microscope (Fig. 3.2a). The second group were generally smaller, and the embryonic pole of the egg cylinders were developmentally abnormal or retarded (Fig. 3.2b). Embryos from any one dam had similar characteristics. Both normal and abnormal embryos were cultured but in order to reduce the chances of selecting immature embryos only the larger and more normal of the latter group were chosen for culture.

Figure 3.2. a, normal 9.5 day egg cylinders removed from zinc-deficient rats. b, grossly abnormal egg cylinders removed from other zinc-deficient dams at the same gestational age. c, embryos grown from egg cylinders similar to those in 3.2 (a). The embryos have developed normally. d, embryos grown from egg cylinders similar to those in 3.2 (b). The embryos are malformed in that they have failed to rotate, they are anophthalmic, the neural tubes are open (arrowheads) and there are large fluid-filled vesicles on the heads (arrows). EC, ectoplacental cone; Am, amnion; E, embryonic pole. Bar= 1mm.


EC
Am
a
E
EC
Am
b
E
c
V
V
d

As with the previous studies, zinc-replete embryos grew well in both zinc-deficient and zinc-replete media. The apparently normal zinc-deficient embryos also grew well in both media (Fig. 3.2c). Those embryos classed as abnormal at the start of the incubation were still retarded in growth and development after 48h in culture, even in the presence of physiological levels of zinc (Table 3.2, Fig 3.2d).

Table 3.2 Growth of zinc-deficient or replete rat embryos in zinc-deficient or replete media[a].

Embryo Status	Medium	Zinc concentration (ug Zn/ml)	No. embryos	Embryo protein (ug)	Yolk Sac protein (ug)	Somites
Replete	Replete	1.07	28	102.1±4.4	94.9±2.4	21.4±0.5
Replete	Deficient	0.56	20	123.2±8.3[b]	104.3±5.9	20.6±1.5
Deficient (normal)	Deficient	0.56	50	111.9±4.5	102.5±4.1	21.1±0.3
	Replete	1.07	16	122.7±5.8b	97.3±6.7	21.6±0.6
Deficient (abnormal)	Deficient	0.56	54	65.9±3.8[c]	76.7±3.2[c]	17.7±0.5[c]
	Replete	1.07	8	51.5±9.0[c]	50.4±5.0[c]	17.4±1.4[c]

a: values are means±SEM of the number of embryos indicated
b: significantly greater than replete embryos in replete medium $p < 0.05$
c: significantly less than deficient (normal) or replete embryos $p < 0.05$

Morphological development of the embryos is presented in Table 3.3. Of the abnormal 9.5 day embryos cultured for 48h, 57% had defective yolk sac circulation, 24% had failed to complete rotation, the allantois had not fused with the chorion in 52% and 57% had open neural tubes. On the heads of several embryos there were large fluid-filled vesicles apparently separating the endoderm and mesoderm (Figure 3.2d).

The incidence of such defects in the zinc-replete embryos was minimal, however there was a moderate increase in the number of deformities in the more normal zinc-deficient embryos cultured in zinc-replete medium. Due to

the relatively small numbers involved the importance of this cannot be assessed.

Table 3.3 Morphological development of zinc-deficient or replete embryos in zinc-deficient or replete media[a].

Embryo Status	Medium	No. Embryos	Normal Yolk sac Circulation	Normal Rotation	Normal Allantoic Fusion	Open Neural Tubes
Replete	Replete	28	27 (96)	21 (75)	27 (96)	1 (4)
Replete	Deficient	20	16 (80)	17 (85)	18 (90)	2 (10)
Deficient (normal)	Deficient	50	50 (100)	45 (90)	46 (92)	3 (6)
	Replete	16	16 (100)	14 (88)	14 (88)	4 (25)
Deficient (abnormal)	Deficient	54	23 (43)	13 (24)	28 (52)	31 (57)
	Replete	8	0 (0)	2 (25)	5 (63)	4 (50)

a : percentages of embryos in parentheses.

Radioactive ^{65}Zinc was added to several of the latter incubations in order to provide an estimate of the ability of the conceptus to acquire zinc from the medium (Table 3.4). On the basis of the specific activity of ^{65}Zinc in the medium, the amount of zinc accumulated by the yolk-sacs and embryos was calculated. Aside from a small reduction in the ability of the yolk sac of the abnormal zinc-deficient embryos to accumulate zinc, the level of radioactivity in the zinc-deficient tissues was no different to that of the controls. It is, however of interest to note that the yolk sacs accumulated approximately twice as much zinc as the embryos (Table 3.4), even though the total protein content of the two tissues was similar (Table 3.3).

Table 3.4 Accumulation of ^{65}Zinc by embryos and yolk sacs cultured *in vitro*.

Embryo Status	Medium	Zinc Concentration	No. Embryos	ng Zn/mg protein: Embryo	ng Zn/mg protein: Yolk sac
Replete	Replete	1.07	26	35.1 ± 1.0	80.2 ± 1.5
Deficient (normal)	Deficient	0.56	24	38.2 ± 1.9	80.1 ± 3.9
	Replete	1.07	8	41.0 ± 0.94	84.8 ± 2.9
Deficient (abnormal)	Deficient	0.56	16	34.5 ± 2.1	70.5 ± 3.7

3.4 DISCUSSION

The reasons as to why a dietary zinc deficiency can induce teratogenic effects *in vivo* but not *in vitro* are not entirely clear, but there would appear to be some maternal effect which renders circulating zinc unavailable to the embryo. It is apparent from these studies that the ability of zinc-deficient or replete embryos to acquire sufficient zinc from the medium is unaffected. From the results in Table 3.4 it can be calculated that each conceptus accumulates approximately 70-80ng of zinc during this period. Considering 4 embryos per incubation, this means that up to 240ng of zinc are required over the 48h. Each incubation contains 3ml of medium at a level of (in the zinc-deficient medium) 0.56ug/ml, thus there is 1.6ug zinc present. The growth over the 48h period therefore requires only 240/1600 i.e. about 15% of the total zinc present, or less than 5% per embryo. Although the bioavailability of the zinc present in the serum is unknown, it can be suggested that effects on growth are unlikely until the zinc concentration falls below about 0.2ug/ml. In addition it has since been shown (WYNTER *et al* 1983) that in the case of alcohol, the presence of the teratogen during the first 12h of culture is a necessary prerequisite for a teratogenic response. Thus, in retrospect, it is extremely unlikely that a teratogenic zinc deficiency affecting organs

developing during this period could be induced within this time *in vitro* unless the embryo already had a marginal zinc status at the time of explantation.

It is possible however to suggest explanations for some of these observations, some of which will be examined in Chapters 4 and 5 of this thesis.

With the progression of zinc deficiency, zinc is lost first from the circulating low molecular weight serum fractions such as albumin and amino acids, but is more tightly bound by the much larger alpha-2-macroglobulin. (PARISI & VALLEE 1970; PRASAD & OBERLEAS 1970; PARRY 1977). Which accounts for approximately 20% of the circulating zinc. It is possible that Reichert's membrane which is removed prior to culture *in vitro* might act as an ultra filter *in vivo*, and exclude such large molecules from the embryonic environment, thus preventing their uptake and digestion by the visceral yolk sac. The data presented in Table 3.4 also suggest that the yolk sac, which has first contact with zinc in the medium has a significantly higher affinity for the metal than the embryo, and that under conditions of zinc stress *in vivo*might not be able to meet both its own requirements as well as those of the embryo. It is also possible that the heat-treatment of serum which is required for satisfactory embryonic development might partially denature zinc-binding proteins rendering them more available for pinocytosis and digestion by the visceral yolk sac (MOORE *et al* 1977) and allowing an uptake of zinc not normally encountered *in vivo*.

It should also be noted that not only is Reichert's membrane removed prior to culture, but also the parietal yolk sac, trophoblast and the decidual tissue are all present *in vivo*. As each of these tissues, as well as the uterus is growing, there is a positive requirement for zinc. Although zinc requirements of the uterus are met by the dam's circulation, passage of the maternal blood through the peri-embryonic sinuses is slow,

and the uterine fluid which is the sole source of nutrients for the developing embryo must pass through these tissues (BECK 1981). It is possible that during an *in vivo* zinc deficiency, the flux of zinc into the region might not be sufficient to meet the demands of both embryonic and extra-embryonic tissues. By contrast, the embryo in culture is continually exposed to a large volume of medium with relatively few barriers between it and the zinc present in the medium.

3.5 CONCLUSIONS

Despite the lack of success in inducing a teratogenic zinc deficiency *in vitro*, these studies have shown that some of the effects occur prior to day 9.5 of gestation, and have provided the basis to facilitate the investigations of abnormalities due to zinc deficiency which are discussed in subsequent chapters of this thesis.

CHAPTER 4

BIOLOGICAL AND MORPHOLOGICAL EFFECTS OF ZINC DEFICIENCY DURING PREGNANCY

4.1 INTRODUCTION

The purpose of the experiments reported in Chapter 3 was to remove the maternal influence and grow rat embryos in a zinc-deficient environment where the onset of teratogenesis could be studied. Due to the lack of success in this respect, and the observation that some zinc-deficient embryos were already malformed at day 9.5 of gestation, attention was turned to the development of zinc-deficient embryos *in vivo*.

In the consideration of the teratogenic potential of any treatment, whether it be a deprivation or an excess of a substance, the effect of the treatment upon the mother must be considered. Maternal metabolism is of particular importance when a dietary deficiency is being considered, as the ability of the dam to mobilise tissue reserves of the nutrient in question will influence the development of the embryo. Indeed, recent studies using rats (MASTERS *et al* 1983) and monkeys (GOLUB *et al* 1982; HURLEY 1985) have provided substantial evidence to indicate that maternal catabolism increases the availability of maternal zinc to the developing fetus, thus compensating to some extent for a dietary deficit of the metal.

Except for serum and other body fluids, tissue zinc levels of adult animals fed zinc-deficient diets generally do not appear to decrease unless the deficiency extends for considerable periods of time (DREOSTI *et al* 1980b). This is probably due to the presence of the metal in stable structural proteins or other macromolecules with slow turnover rates (Chapter 1). Thus it would appear that, whilst the zinc-deficient animal has large stores of zinc in such organs as liver, bone and muscle, these reserves are largely unavailable for reutilisation by other tissues or the

developing embryo (HURLEY & SWENERTON 1971).

This chapter details studies performed on pregnant Sprague-Dawley rats to discover any explanation for the difference in the appearance of zinc-deficient embryos discussed in the previous chapter.

4.2 MATERIALS AND METHODS

All rats used in this study were of the Sprague-Dawley strain, bred in the colony as described earlier. Until the commencement of the experiment they were maintained on the colony diet (Chapter 2) and allowed free access to de-ionised water.

Whilst on the experimental diets (Chapter 2), zinc-deficient rats were housed in either stainless steel and plastic cages or in metabolism cages of similar construction (TECHNIPLAST, Italy). Where appropriate urinary, faecal and tissue samples were collected and zinc determinations performed as described earlier (Chapter 2). At the end of the study period the uteri of pregnant rats were removed and the embryos recovered and assessed as described in Chapter 2.

The uptake of [^{125}I]-Polyvinylpyrollidone (PVP) was estimated as described earlier (RECORD *et al* 1982b). Briefly the procedure was as follows: Visceral yolk sacs, still containing the embryos, were incubated in 90% serum (3ml) equilibrated with 40% O_2, 5% CO_2, 55% N_2 for 30 min. [^{125}I]-PVP (44.6uCi/mg, M.W. 30 000-40 000 daltons, Radiochemical Centre, Amersham, U.K.) was added to the medium to a final concentration of 9ug/ml. After 60 min incubation, the bottles were cooled rapidly in ice and the yolk sacs washed 4 times with 5ml portions of 0.9% NaCl. The yolk sacs were then dissected free from other embryonic tissues and counted separately in a gamma counter (Nuclear Chicago, model 1185, Des Plains, Ill). The protein content of each yolk sac was determined as described in Chapter 2. As previously reported (RECORD *et al* 1982b) the uptake of [^{125}I]-PVP over this time is linear, and non-specific accumulation of the

substrate is minimal at 4°C. As pinocytosis involves the engulfment of extracellular fluid, and any macromolecules contained within the medium, the rate of this process was expressed as microlitres of medium accumulated per mg yolk-sac protein per hr (WILLIAMS *et al* 1975).

4.3 RESULTS

The first experiment was designed as a preliminary study to assess the *in vivo* development of zinc-deficient and replete rats. All embryos were assessed for morphological development, some were selected for TEM and LM studies, but the majority were retained for protein estimation. Results for the groups are presented in Table 4.1.

Table 4.1 Reproductive performance of zinc-deficient and replete dams[a].

	Zinc-deficient (ad lib fed)	Zinc-replete (restricted fed)	Zinc-replete (ad lib fed)
n	7	5	5
Maternal weight change	-2.0±6.4[c]	+21.4±4.2[d]	N.D.
Maternal serum zinc	0.47±0.08[c]	1.07±0.04[d]	1.16±0.06[d]
Corpora lutea	14.3±0.5	15.8±0.7	N.D.
Live embryos (dead[b])	14.9±0.8(4)	12.2±1.1(2)	10.2±1.8(4)
Yolk sac diameter (mm)	4.26±0.11[c]	4.46±0.06[d]	4.89±0.05[e]
Yolk sac protein (ug)	168±13	173±5	168±9
Embryo length (mm)	2.59±0.22[c]	3.56±0.07[d]	4.02±0.07[e]
Somites	21.3±0.9[c]	24.3±0.3[d]	24.3±0.2[d]
Embryo protein (ug)	141±16[c]	262±25[d]	239±14[d]

a: values are means ± SEM of the number of dams indicated.
b: total number of dead embryos
c-e: values in any one row with different superscripts are signiicantly different $p < 0.05$
N.D. not determined

In the zinc-deficient group there were large variations in maternal weight change, maternal zinc levels and embryonic size. Yolk sac diameter was significantly reduced as a result of a maternal zinc deprivation, however the protein content was not affected. Embryonic growth was however significantly reduced in terms of crown-rump length, somite number and protein content (all $p < 0.001$). Further examination of parameters affected by zinc deficiency showed some unexpected correlations (Table 4.2).

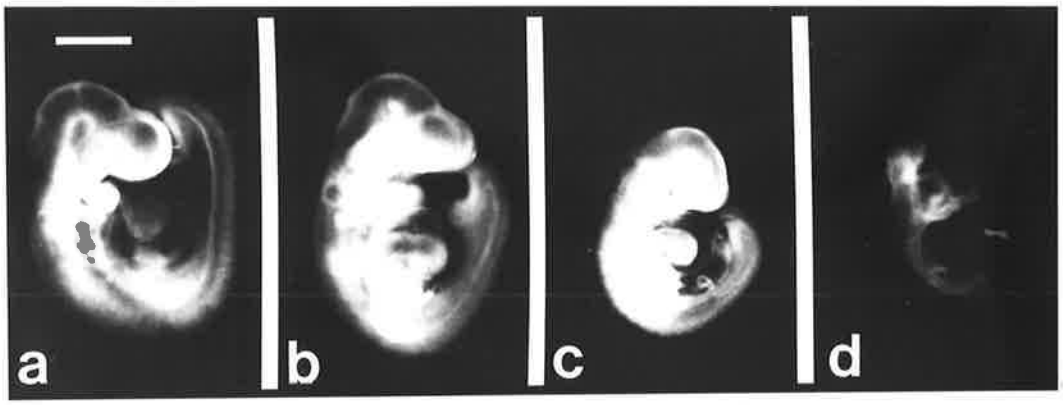


Figure 4.1. One zinc-replete (a) and three zinc-deficient (b-d) 11.5-day embryos taken from different dams. Embryo b appears morphologically normal; embryo c has craniofacial abnormalities and an enlarged pericardium, while embryo d has failed to rotate, has an enlarged pericardium, open anterior neural tube and anophthalmia. Bar=1mm.

In this same series of animals there were strong negative correlations between the changes in maternal weight and the maternal serum zinc levels, and between maternal zinc levels and both embryonic protein and length. This was supported by a similar but non-significant relationship between maternal serum zinc and somite number. In addition, maternal weight changes were positively correlated with all parameters of embryonic growth in the zinc-deficient group, but negatively correlated with the same parameters in the zinc-replete group (although only the relationship with somites was significant) (Table 4.2).

Table 4.2 Correlation matrix for maternal and embryonic variables of zinc-deficient, zinc-replete dams taken separately and together.

		Maternal serum zinc	Live embryos	Yolk sac diam.	Yolk sac protein	Embryo length	Somites	Embryo protei
Maternal	-Zn	-.85[b]	.49	.44	.08	.87[b]	.68[a]	.85[b]
Weight	+Zn	.64	.10	-.33	-.52	-.19	-.80[a]	-.37
Change	All	.46	-.08	.41	-.03	.75[b]	.53[a]	.61[a]
Maternal	-Zn	-	-.19	-.21	-.03	-.68[a]	-.67	-.72[a]
Serum	+Zn	-	.62[a]	.20	-.24	-.04	-.37	-.43
Zinc	All	-	-.43	.65[b]	.04	.63[b]	.57[a]	.56
Live	-Zn	-	-	.22	.04	.38	-.44	.37
Embryos	+Zn	-	-	.38	.38	.44	.36	.20
	All	-	-	-.35	-.06	-.29	-.43	-.28
Yolk	-Zn	-	-	-	.75[a]	.61	.84[b]	.65[a]
Sac	+Zn	-	-	-	.35	.82[b]	.29	.22
Diam.	All	-	-	-	.47	.83[c]	.76[c]	.70[c]
Yolk	-Zn	-	-	-	-	.42	.50	.50
Sac	+Zn	-	-	-	-	.46	.70[a]	.94[c]
Protein	All	-	-	-	-	.31	.36	.49
Embroyo	-Zn	-	-	-	-	-	.82[a]	.98[c]
Length	+Zn	-	-	-	-	-	.37	.46
	All	-	-	-	-	-	.86[c]	.90[c]
Somites	-Zn	-	-	-	-	-	-	.88[b]
	+Zn	-	-	-	-	-	-	.66[a]
	All	-	-	-	-	-	-	.84[c]

a: $p < 0.05$
b: $p < 0.01$
c: $p < 0.001$

The inverse relationship between maternal weight change and the serum zinc levels suggested the possibility that the zinc-deficient dams might have been utilising circulating zinc to maintain their own growth, leaving less zinc for the developing litters. On the other hand, low serum zinc levels were associated with larger embryos, which initially suggested that the litters might have been draining the available pool of maternal zinc. Evidence from the zinc-replete groups indicated less of an effect of circulating zinc, but did suggest that embryonic growth was, to some extent

compromised by maternal growth.

It was also noted that there were large differences between individual litters, although the differences between embryos of any one litter were smaller. This can be seen in Table 4.3 where the coefficients of variation of embryonic protein between and within litters are compared.

Table 4.3 Co-efficient of variation between and within litters of zinc-deficient and replete embryos.

	Within litters	Between litters
Zinc-deficient	22.5	29.0
Zinc-replete (restricted fed)	13.4	21.0
Zinc-replete (ad lib fed)	14.9	13.1

Individual morphological abnormalities are presented in Table 4.4. Several litters were free from malformations, i.e. defects which would have caused intra-uterine death or substantial malformations at birth. A further 16% had malformations which were considered minor at this stage of development, but might have either developed into major defects later, or been eliminated during a catch-up phase of growth.

Table 4.4. Defects in organ systems of individual zinc-deficient litters.a

Rat	1	2	3	4	5	6	7	TOTAL	%
Total embryos	13	19	14	15	14	14	15	104	100
Yolk-sac circulation	2	8	9	5	0	3	1	28	27
Allantoic function	1	15	11	6	0	2	1	36	35
Flexion	2	17	12	5	0	2	1	39	38
Heart	1	2	0	1	0	1	0	5	5
Caudal neural tube	0	1	6	1	0	0	0	8	8
Hind-brain	0	1	3	4	0	4	15	27	26
Mid-brain	0	2	7	4	0	4	14	31	30
Fore-brain	0	6	12	2	0	4	15	39	38
Optic vesicle	0	11	12	2	0	3	0	28	27
Otic vesicle	0	3	12	2	0	3	0	20	19
Fore-limbs	0	2	2	1	0	1	0	6	6
Dead	0	2	2	0	0	1	0	5	5

(a) Of the 104 embryos 12% (i.e. 12) had open cranial neural tubes

The results from this phase of the study suggested that the yolk sac was largely unaffected by zinc deficiency. As the yolk sac accumulates nutrients by pinocytosis, for subsequent digestion and transport of the materials to the developing embryo it was thought appropriate at this stage to eliminate the possibility that the function of the yolk-sac was affected by zinc deficiency. Yolk sacs were obtained from separate groups of rats (both zinc-deficient and replete) and incubated in either zinc-deficient, zinc-replete or zinc-deficient serum + 2ppm zinc and the uptake of [^{125}I]-PVP determined. The results are presented in Table 4.5.

Table 4.5 Uptake of [^{125}I]-PVP by zinc-deficient and replete yolk-sacs *in vitro*.

	ul medium accumulated/h/mg protein[a]		
	Zinc-deficient	Zinc-replete	Zinc-deficient + 2ug Zn/ml
Zinc-deficient yolk-sacs	1.42±0.07	1.33±0.09	1.35±0.09
Zinc-replete yolk-sacs	1.68±0.09[b]	1.47±0.10	1.43±0.09

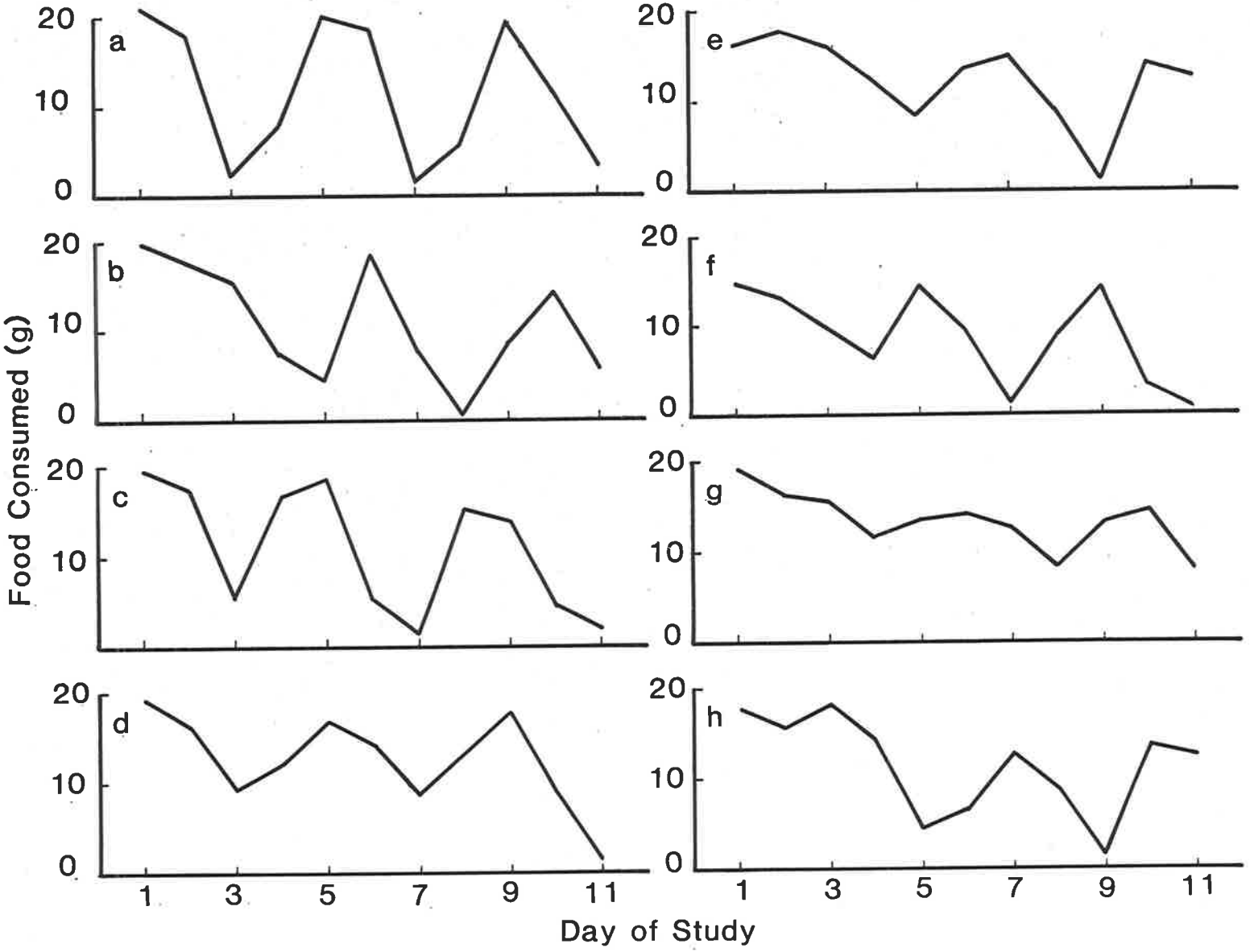
(a) Values are means ± SEM of 3-5 determinations per rat.
(b) Significantly greater than any other mean ($p < 0.001$)

There was no significant effect of maternal zinc deficiency on the ability of the yolk sac to accumulate nutrients by pinocytosis, which is thought to be the rate-limiting step of embryonic nutrition (WILLIAMS *et al* 1976; MOORE *et al* 1978). The increased rate of pinocytosis of the zinc-replete yolk sacs in the zinc-deficient medium was not expected and cannot be explained, save to note that 9.5d zinc-replete embryos cultured in zinc-deficient media also grew better than those cultured in zinc-replete media (Chapter 3).

In order to discover the reasons for the variability between the zinc-deficient litters, several other studies were carried out.

In the first of these, pregnant rats were confined to metabolism cages for the first 11 days of gestation, and fed either the zinc-deficient diet ad lib, or the zinc-replete diet at the average daily quantity consumed by the zinc-deficient group. Food intakes, maternal weight, urinary and faecal quantities as well as zinc balance were monitored for the whole period. At sacrifice, after 11.5 days gestation, embryos were removed from the uteri for morphological and biochemical examination and serum trace element levels determined. The average food intake was 11.6g/day, although this fluctuated extensively (Fig 4.2).

Fig 4.2 Voluntary food intakes of individual pregnant rats fed zinc-deficient diets.



Maternal weight also fluctuated on a daily basis, in line with the alterations in both dietary intake and faecal and urinary output. The average weight gain in the zinc-replete group (allowed a constant 12g food/day) was 19g, wherease the average weight change in the zinc-deficient group was only +5g, although this varied between -14g and +20g.

Faecal zinc concentration declined throughout the experimental period in the zinc-deficient animals, but the urinary zinc excretion remained relatively constant. In contrast, possibly due to altered bioavailability, the faecal zinc concentration in the control animals increased once they were placed on the soya-bean based diet. Calculation of the total zinc output showed that the zinc-deficient group were in a negative zinc balance, whereas the zinc-replete group were in positive balance throughout the study period (Table 4.6).

Table 4.6 11-Day zinc balance for zinc-deficient (ad lib fed) and replete (restricted fed) pregnant rats.[a]

Dietary zinc (ug/g)	Weight change (g)	Total food consumption	Faecal zinc output (ug)	Urinary zinc output (ug)	Balance[a] (ug)
< 0.5	1.8±5.6	129±6.4	1177±199	53.8±3	-1167±200
100	18.7±3.0	132	10720±514	66.1±4.4	2414±514

a: Based on dietary zinc contents of 0.5ug Zn/g and 100ug Zn/g respectively.

As with the previous study, there was a profound effect of maternal zinc deficiency on both embryonic size and development (Table 4.7).

Table 4.7 Reproductive performance of zinc-deficient and zinc-replete (restricted-fed) dams confined to metabolic cages[a].

	Zinc-deficient	Zinc-replete (restricted-fed)
n	8	6
Maternal serum zinc (ug/ml)	0.63±0.11[c]	1.07±0.03
Live embryos (total dead)	13.4±0.7 (5)[b]	10.8±1.1 (0)[b]
Yolk sac diameter (mm)	4.33±0.09[c]	4.64±0.03
Yolk sac protein(ug)	173±6[c]	204±9
Embryo Length (mm)	3.13±0.25[c]	3.82±0.08
Somites	21.1±1.1[c]	24.4±0.2
Embryo protein (ug)	205±21[c]	345±18

a: Values are means±SEM of the number of dams indicated.
b: Total number of dead embryos.
c: Significantly less than zinc-replete values $p < 0.05$

In this study there was also a small decrease in yolk sac size (both diameter and protein content), although the effect on the embryo was not as pronounced as that observed earlier. The type and distribution of the malformations noted in these embryos was also similar to the previous study (Table 4.8). Of the 112 zinc-deficient embryos examined, 36 (34%) were either dead or malformed. Only two of the zinc-replete embryos (3%) showed any sign of dismorphogenesis.

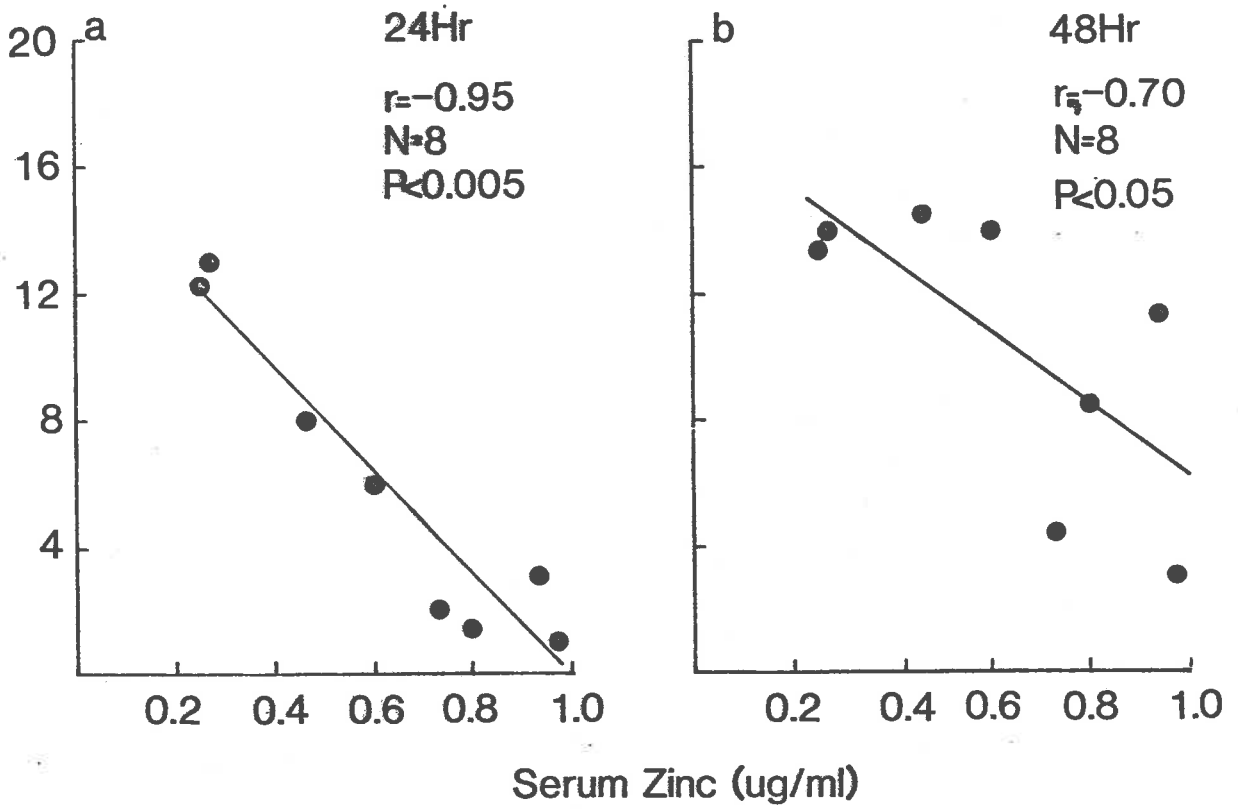
Table 4.8. Defects in organ systems of zinc-deficient embryos (experiment 2).

	No. with defect								total	%
Rat no.	1	2	3	4	5	6	7	8		
Yolk sac circulation	0	0	12	0	0	1	0	1	14	13
Allantoic fusion	0	2	3	0	1	1	0	0	7	7
Flexion	0	0	11	1	1	1	0	0	14	13
Heart	0	2	1	1	1	1	0	0	6	6
Cranial neural tube	0	0	9	0	2	0	0	0	11	10
Caudal neural tube	0	0	0	0	1	0	0	0	1	1
Optic system	0	0	5	0	1	6	0	2	14	13
Fore-limb	0	0	9	0	1	0	0	0	10	9
Total Malformed	0	3	14	1	3	7	0	3	31	29
Total live	14	11	14	12	16	11	14	15	107	(100)

There was a strong ($r = 0.91$; $n=8$; $p < 0.0001$) negative correlation between the serum zinc levels on the day the zinc-deficient dams were killed with the food intake over the previous night (Fig 4.3). There was little correlation between the serum zinc levels and food intake for the period 72-48h before killing, but the earlier 24h food consumption was directly correlated (Fig. 4.3). It was also apparent that there was an inverse relationship between serum zinc levels and the size of the embryos when they were removed from the uterus after 11.5 days of gestation (Fig. 4.4).

In order to ascertain that the correlation between serum zinc levels and dietary intake was not artefactual, the study was repeated using non-pregnant animals. As with the first group, the relationship was highly significant ($r=-0.97$; $n=16$; $p < 0.0001$) (Fig 4.5). The serum copper concentrations showed no relationship to the dietary intake or the zinc levels.

Fig. 4.3 Correlation of serum zinc levels with food consumption on various nights prior to bleeding.



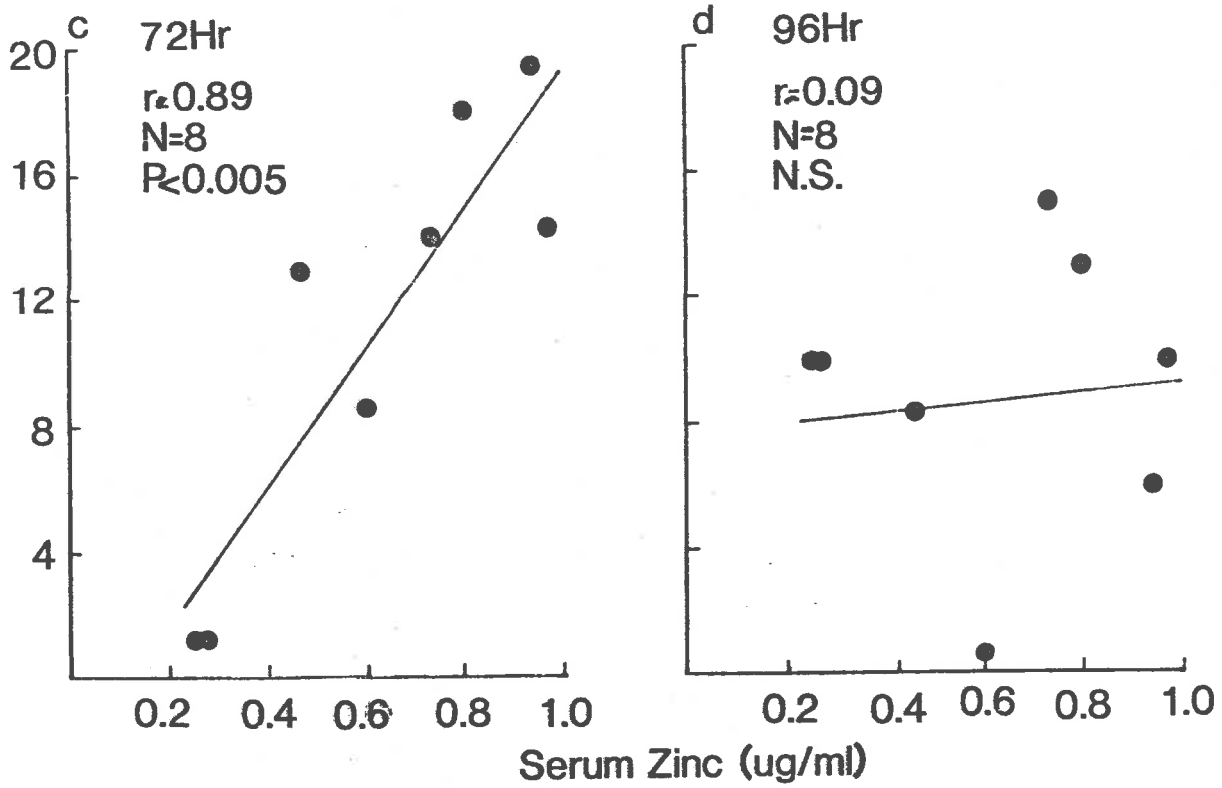
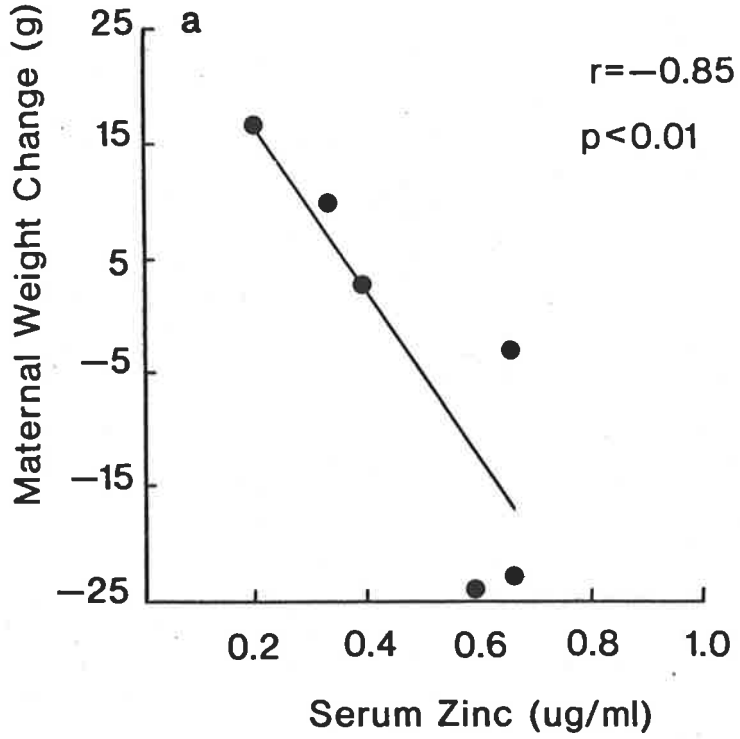


Fig. 4.4 Relationships between maternal serum zinc levels of ad-lid fed zinc-deficient dams and maternal weight change and embryonic protein content



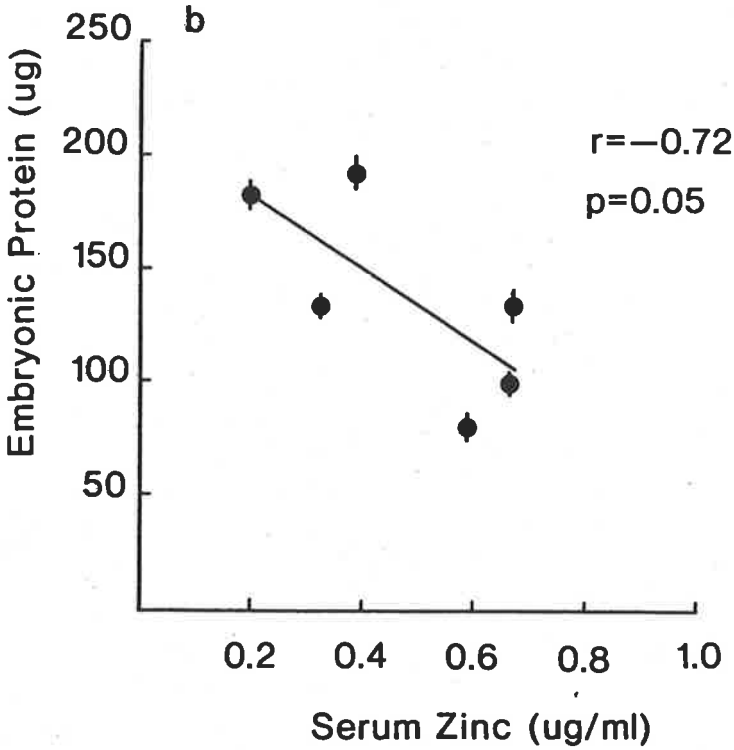
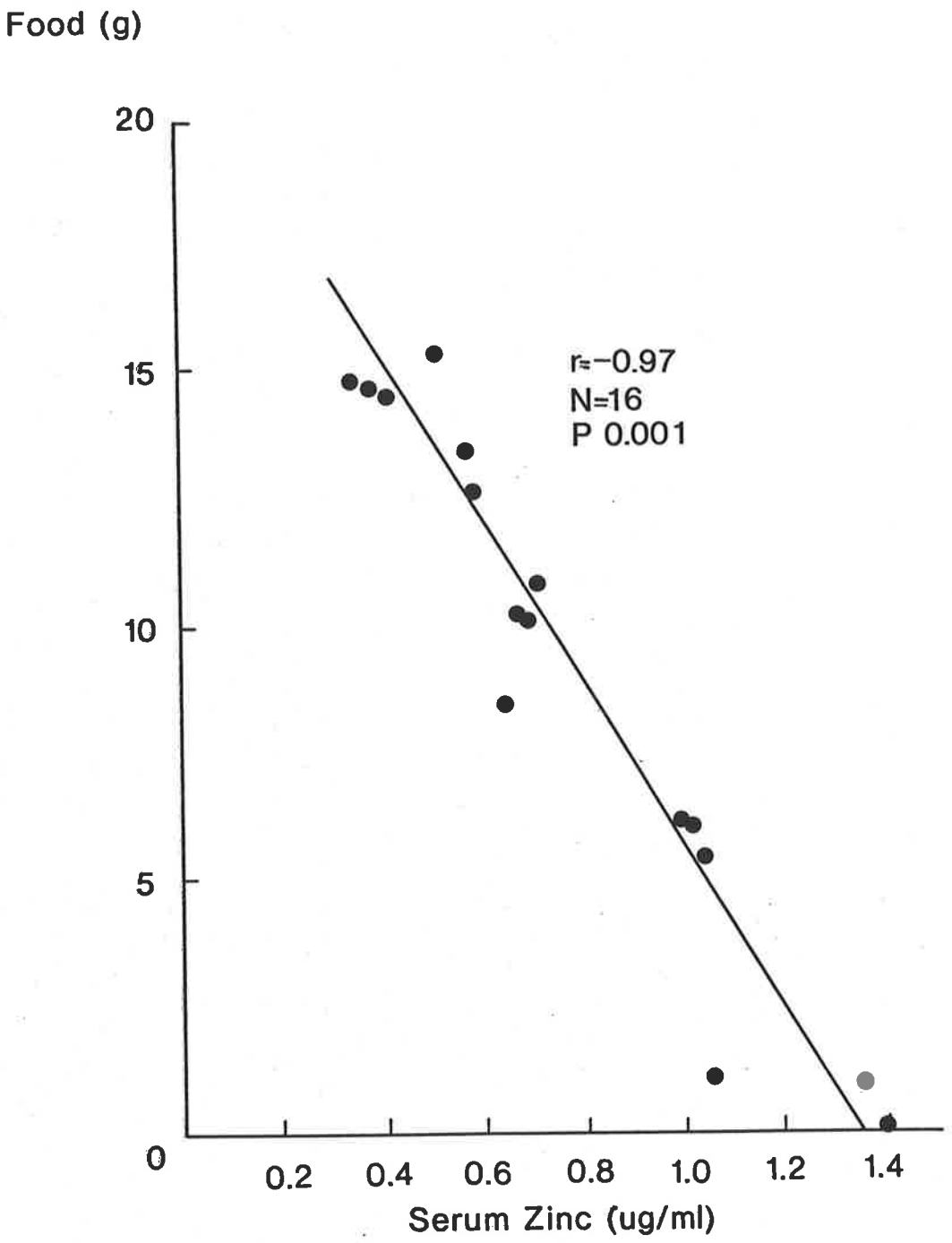


Fig. 4.5 Correlation between intakes of zinc-deficient diet and serum zinc levels.



The next study was aimed at determining whether the serum zinc level of zinc-deficient animals could be manipulated by dietary control after 11 days on the diet. In this experiment animals were divided into three groups. One was fed the zinc-replete diet on an ad lib basis for 11 nights. The second group (of zinc-deficient animals) was fasted on the 10th night and allowed food ad lib on the 11th, but the last group (also zinc-deficient) was fasted on the 11th night (prior to bleeding). Zinc and copper levels were determined on serum samples obtained the next morning (Table 4.9).

Table 4.9 Serum zinc and copper levels of animals after 11 days of dietary treatment.[a]

Group	n	Serum zinc (ug/ml)	Serum copper (ug/ml)
Zinc-replete (ad lib)	6	1.60±0.08	1.69±0.06[b]
Zinc-deficient (fasted night 10, fed night 11)	6	0.66±0.06[b]	2.17±0.14
Zinc-deficient (fed night 10, fasted night 11)	6	1.38±0.06	1.97±0.10

(a) values are means±SEM of the number of animals indicated.
(b) significantly less than other groups. $p<0.005$

The results suggested that serum zinc levels could be controlled (to a certain extent) by dietary manipulation, and that it would be practicable to regulate the serum zinc levels by dietary means alone.

Further studies were undertaken to confirm the report by WILKINS *et al* (1972) as to the rapidity with which low serum zinc could be induced. Animals were allowed 20g of either the zinc-deficient or replete diet on the first night. Half of each group was fasted on the next night whilst

the other half received 20g of the appropriate diet. Animals fed the replete diet, as well as the zinc-deficient animals which were fasted had normal serum zinc levels on the next day. Those which received 20g of zinc-deficient diet had, however, significantly reduced levels of serum zinc (Table 4.10).

Table 4.10 Response of serum zinc to short-term feeding and fasting[a].

Diet 1st night	Diet 2nd night	Serum zinc (ug/ml)	Serum copper (ug/ml)
+Zn	+Zn	1.45±0.06	1.70±0.02
+Zn	0	1.45±0.06	1.71±0.03
-Zn	-Zn	0.88±0.07	1.44±0.07
-Zn	0	1.50±0.05	1.82±0.10

(a) Values are means±SEM of 4 animals per group.
(b) Significantly less than other groups $p<0.005$.

In this experiment alone the serum copper levels also declined along with the zinc. The explanation of this phenomena is uncertain. The results suggested that serum zinc levels could be controlled (to a certain extent) by dietary manipulation, and that it would be practicable to regulate the serum zinc levels by controlling the food intakes. Accordingly two other studies on pregnant animals were carried out. In the first, zinc-deficient (and replete) animals were placed on a dietary regime which was identical to that followed by the dam showing the greatest degree of teratology in the previous study (Fig. 4.2c). Cresyl red (a faecal marker) was added to the first two days' food to allow faeces generated from the colony diet to be distinguished from those derived from the semi-synthetic diet. Animals were housed in metabolism cages and faecal samples collected daily, the

"colony-diet faeces" being collected separately from those from the soy diet.

All animals were in a negative balance throughout the experiment. Over the 11 days the average loss was 800±50ug of zinc. Faecal zinc concentrations dropped rapidly from a mean of about 275ug Zn/g wet weight to a minimum of 19ug Zn/g. As with the first study, the faecal zinc concentration paralleled the food intake to a certain extent (Table 4.11).

The maternal serum zinc levels at the end of the experiment were normal(1.30±±0.03ug Zn/ml) yet embryonic development was poor and the incidence of teratology high (data not shown).

Table 4.11 Food intake, faecal output and zinc excretion of cyclically fed zinc-deficient dams.[a]

Day of study	Food intake (g)		Faecal weight (g)	Zinc excretion (ug)	Zinc concentration (ug/g)
1	16.8±0.59	Colony (8)	1.29±0.41	269.4±46.5	271.2±36
		Deficient (5)	1.28±0.25	125.4±16.4	106.5±12.3
2	16.5±0.36	Colony (3)	0.63±0.26	178.7±69.0	287.2±9.2
		Deficient (8)	1.54±0.20	104.5±21.5	91.3±29.6
3	5.0±0.03		1.43±0.32	51.1±18.5	35.7±6.7
4	16.4±0.07		2.00±0.23	59.4±11.1	29.1±3.3
5	17.6±0.58		1.6±0.26	29.6±3.2	20.2±1.8
6	5.1±0.02		0.96±0.19	19.5±3.7	20.9±1.1
7	1.4±0.03		0.43±0.11	11.5±2.8	18.6±4.2
8	15.0±0.04		2.04±0.18	52.6±6.9	31.5±2.6
9	13.6±0.15		1.71±0.30	30.9±2.2	18.9±2.3
10	4.4±0.07		1.01±0.22	22.5±5.9	19.3±2.1
11	1.8±0.17		1.23±0.09	5.6±2.8	30.1±7.0

(a) 8 dams confined to metabolism cages for 11 days each.

The studies conducted thus far suggested that the teratogenic effect of zinc deficiency was not simply due to a dietary deficit of the metal, but could be modified by the food intake. it seemed likely that the specific feeding cycle of an individual rat could induce low circulating maternal zinc levels at critical periods of organogenesis, thus producing a preponderance of specific malformations.

In the next study it was decided to examine more thoroughly the effect of cyclical feeding on pregnant dams. Accordingly a total of 25 dams were allotted to one of 4 groups at random. Group 1 (8 dams) was fed the zinc-deficient diet in a similar pattern to that which was followed by the dam which had the most severely affected litter (Fig 4.6a). Group 2 dams (4) were fed to the same schedule but allowed zinc-replete diet. Group 3 animals (9) were fed to a schedule designed to be the inverse of the first cycle and were paired with Group 4 (4 dams) fed the zinc-replete diet (Fig 4.6b). After 11.5 days gestation the dams were killed and the embryos removed for examination as described earlier. The results are presented in Tables 4.12 and 4.13.

Fig. 4.6 Feeding regime of zinc-deficient and replete dams.

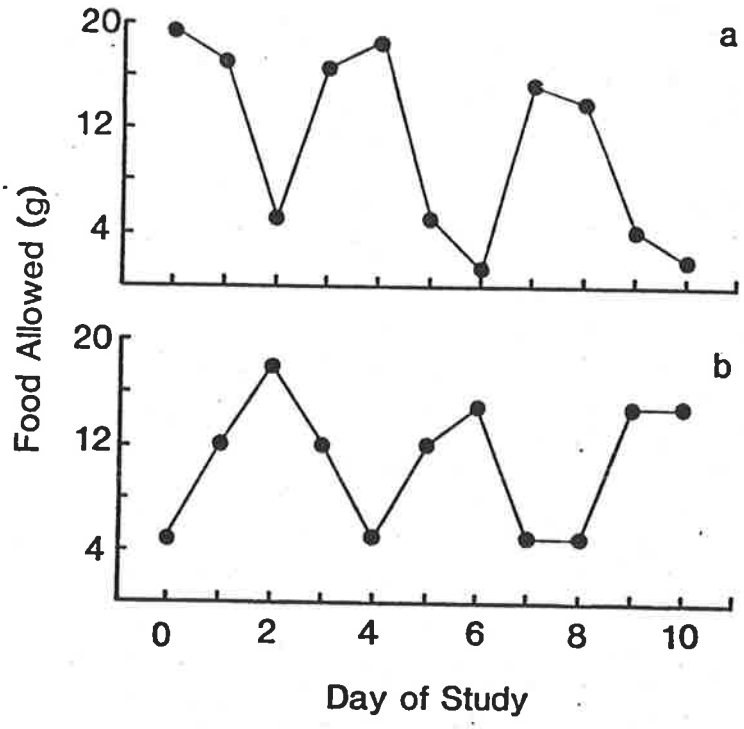


Table 4.12 Growth and development of zinc-deficient and replete rat embryos from cyclically fed dams.

Zinc status	Group 1 deficient	Group 2 replete	Group 3 deficient	Group 4 replete
No. dams with total resorptions	0/8	0/4	4/9	0/4
Maternal serum zinc (ug Zn/ml)	1.13±0.04	1.04±0.05	0.50±0.06[a]	1.06±0.03
Total no. embryos	105	42	76	52
Dead embryos (%)	6	0	6	4
Live embryos/dam	13.1±0.7	10.5±1.4	8.3±2.9[a]	12.3±0.7
Malformed embryos	67	2	2	2
Yolk sac protein (ug)	161±12	145±13	144±6	172±13
Crown-rump length (mm)	1.56±0.06[a]	3.67±0.08	2.53±0.10[a]	3.62±0.10
Somite no.	17.4±0.5[a]	23.7±0.2	21.8±0.3[a]	23.4±0.4
Embryo protein	128±29[a]	216±7	175±13[a]	251±6

a: Significantly less than respective control value $p < 0.05$

Table 4.13 Defects in organ systems of 11.5 day embryos obtained from cyclically fed zinc-deficient or replete dams.[a]

Organ system affected	Group 1	Group 2	Group 3	Group 4
Total live embryos	99	42	70	48
Yolk sac circulation	29	2.3	1.3	1.9
Allantoic fusion	27	2.3	1.3	1.9
Rotation	51	4.6	1.3	1.9
Open cranial neural tube	32	0	1.3	0
Anophthalmia	21	0	1.3	0
Heart	10	0	1.3	0
Fore-limbs	26	0	1.3	3.8

a: percentage of embryos affected.

These results showed that high intakes of zinc-deficient diets on days 8 and 9 of gestation (Group 1) increased the degree of uniformity between the litters, and also increased the rate of malformations. Specifically,

the total malformation rate (including embryonic death) was about 70%, with 32% of the embryos having open neural tubes. Embryonic growth, as judged by crown-rump length, somite number and protein content was greatly reduced in this group as compared to the pair-fed controls (Group 2). Even though the embryos were grossly undersized and malformed, the maternal serum zinc level was normal.

There was also a high incidence of total embryonic loss in the zinc-deficient group fed higher amounts of the diet on days 6 and 7 of gestation. The remaining litters were morphologically normal, although slightly growth retarded.

A further short study was undertaken to discover whether the zinc levels in the uteri, embryos and other tissues of cyclically fed zinc-deficient dams were substantially altered after 9.5 days gestation. These results are presented in Table 4.14.

The zinc-deficient dams failed to gain weight and the concentrations of zinc in serum, kidney, and the products of conception (embryos plus decidual tissue) were significantly lower than either the pair-fed or ad lib fed control group. Uterine zinc content was lower, but not significantly so, due to the large variation in one of the control groups (Table 4.14).

Table 4.14 Zinc concentration in tissues of pregnant rats fed zinc-deficient diets in a cyclical manner for 9.5 days.

	Group		
	Deficient	Replete (cycled)	Replete (ad lib)
Weight change (g)	-0.5±6.8[a]	9.3±1.7	34.0±2.0
Serum zinc (ug/ml)	0.45±0.06[a]	1.17±0.03	1.07±0.05
Conceptus weight (mg)	35±2	40±3	40±4
Conceptus zinc (ug Zn/g)	12.9±0.3[a]	16.2±0.3	16.3±0.4
ug Zn/conceptus	0.457±0.045[a]	0.645±0.061	0.627±0.051
Uterus (ug Zn/g)	12.1±0.3	14.9±1.8	14.1±0.5
Liver (ug Zn/g)	24.6±1.1	27.5±1.2	28.2±0.5
Kidney	19.1±0.3[a]	24.2±0.7	24.7±0.8
Bone	162±13	160±6	170±9
Muscle	12.2±2.0	13.1±3.0	10.6±1.0

a: Significantly less than either control $p < 0.05$

4.4 DISCUSSION

The *in vivo* studies reported in this chapter not only provide explanations for and confirmation of some of the observations reported in Chapter 3, but are also pertinent to findings by other workers.

The fact that a dietary zinc deficiency can be established rapidly is well documented (DREOSTI *et al* 1968; WILKINS *et al* 1972; HURLEY *et al* 1982). It is not therefore surprising that after only a few days of dietary zinc deprivation, there will be little circulating zinc available for the developing embryo. Indeed it has been demonstrated (HURLEY & SHRADER 1975) that a maternal zinc deficiency can have serious consequences after only three or four days.

These facts are complicated by the observation that zinc-deficient rats have a cyclical feeding pattern with a period of about 4 days

(CHESTERS & QUARTERMAN 1970; WILLIAMS & MILLS 1970; GRIFFITH & ALEXANDER 1972; CHESTERS & WILL 1973; WALLWORK *et al* 1981,1982;WALLWORK & SANDSTEAD 1983). Reports relating the cyclical feeding pattern to changes in serum zinc concentrations are, however, infrequent. CHESTERS & WILL (1973) reported a high correlation between the dietary intake of a zinc-deficient animal and the serum zinc concentrations on the following day. WALLWORK *et al* (1981) also noted that animals at the high point of the feeding cycle had significantly lower serum zinc levels than those in the anorexia phase. This aspect has been expanded upon in this thesis and in associated publications (RECORD *et al* 1985b,c,d,e,1986; DREOSTI *et al* 1985a).

A study by MASTERS *et al* (1983) produced the first indication that the feeding/fasting cycle might be important in the fetal response to zinc deficiency when they noted that the incidence and severity of teratogenic malformations in zinc-deficient rats was enhanced when the dams were force-fed zinc-deficient diet to maintain their food intake during the latter part of pregnancy. Conversely zinc-deficient rats which were fasted tended to have more normal fetuses. These results were attributed to an increase in maternal catabolism during the fasting state, leading to a release of zinc from tissue stores (HURLEY 1985). In the force-fed animals maternal catabolism was increased, leading to a depletion of the serum zinc levels.

From the studies reported in this chapter it is apparent that zinc-deficient dams regulate their food intake for some as yet undefined reason. During this time large fluctuations occur in the the serum zinc levels, probably as a result of changes in the maternal anabolism/catabolism (GIUGLIANO & MILLWARD, 1983,1984). Thus it can be suggested that while the dam restricts her food intake, zinc is released from maternal stores, and is available to the embryo or fetus. When the dam returns to an anabolic state and increases her food intake, the serum zinc levels fall resulting in a reduced availability of zinc to the fetus.

In this way, production of specific malformations are linked intimately to the feeding and fasting stages of the cycle.

It would seem that animals with a high food intake on days 6 and 7 of gestation (and hence low serum zinc levels) had a significant loss of embryos without evidence of implantation having occurred. If the feeding phase was delayed until days 8 and 9 (that is, the beginning of the organogenesis phase) when the embryo is most susceptible to teratogenic stimuli (BEAUDOIN 1979) then multiple gross abnormalities were produced which probably would have resulted in intra-uterine death. This could be of major significance when the defects involved included one or more defects such as open neural tubes, failure to rotate or failure of the allantois to fuse with the chorion to form the chorio-allantoic placenta.

It was also notable that, as was the case in the studies reported in Chapter 3, the yolk sac size was relatively unaffected by maternal zinc deficiency. Indeed the smallest group of zinc deficient yolk sacs contained only 16% less protein than the equivalent controls (Tables 4.7 and 4.13), whereas the embryonic protein content was reduced by 40%. Yolk sac function (as measured by [^{125}I]-PVP uptake was also unaffected. These observations lend further support to the hypothesis that the yolk sac removes zinc from the uterine fluid, but retains the metal at the expense of the embryo, and furthermore provides evidence to refute any notion that the yolk sac is involved in this teratological response.

4.5 CONCLUSIONS.

In this study it is probable that, during the feeding cycle, zinc-deficient animals alternate between a fasting, catabolic state when maternal tissues release zinc into the serum, and a feeding, anabolic state accompanied by a nett uptake of zinc from the serum to the maternal tissues.

If allowed to continue their pregnancy, each dam would undergo 4 or 5

waves of low serum zinc, each exerting its influence on the developmental events occurring at that time, and so contribute to the wide range of deformities observed near term. These aspects will be discussed in Chapters 6 and 7.

CHAPTER 5

HISTOLOGICAL APPEARANCE OF ZINC-DEFICIENT EMBRYOS

5.1 INTRODUCTION

Despite the large number of studies conducted into the teratogenic effects of a maternal dietary zinc deficiency, there there have been few reports of any histopathological changes in embryonic or fetal tissues. In the majority of studies (e.g. DIAMOND & HURLEY 1970; WARKANY & PETERING 1972; HURLEY & SHRADER 1972; ECKHERT & HURLEY 1977) examination of zinc-deficient embryos and fetuses from day 12 to day 20 of gestation showed only reflections of the gross morphological abnormalities. It was reported, however, that despite the reduced labelling of cells by [^{3}H]-thymidine, there was an apparent increase in the mitotic index of zinc-deficient embryonic tissue (HURLEY & SHRADER 1972; ECKHERT & HURLEY 1977), leading to the proposal that the primary effect of zinc deprivation was to cause a blockage in mitosis, and a disruption of the order of cell division resulting in the observed terata.

Other studies have however shown cellular and subcellular abnormalities in zinc-deficient three and four day old embryos (HURLEY & SHRADER 1975) and a reduction of cell density in regions of the hippocampi of 20-day fetal rats (DREOSTI & FRASER 1984). In studies on other tissues and species, subcellular changes due to zinc deficiency have been observed repeatedly. These include chromosomal abnormalities in sperm and ova (WATANABE *et al* 1983), testicular atrophy (DIAMOND & HURLEY 1970), apoptosis in the intestine (ELMES 1977; KOO & TURK 1977; ELMES & JONES 1980) and pancreas(FELL *et al* 1973; KOO & TURK 1977).

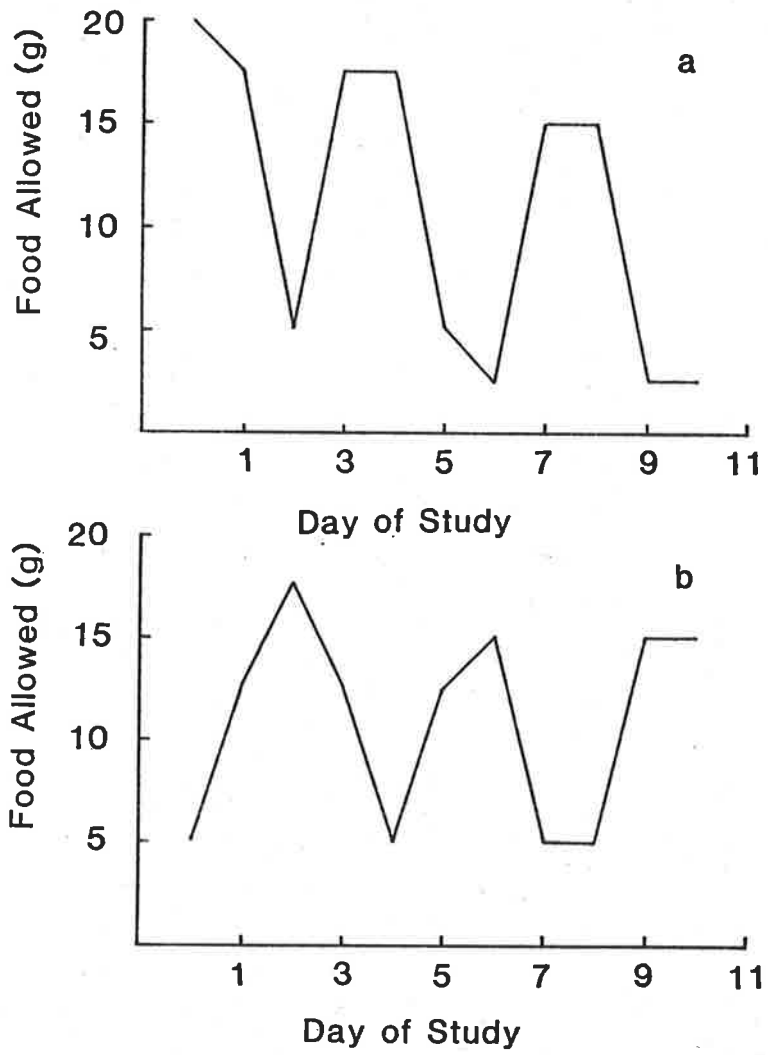
In accordance with the aims of this thesis, embryos collected from studies reported earlier, as well as those from specifically designed

histopathological studies have been examined at the level of the light microscope (LM) and also using transmission (TEM) and scanning electron microscope (SEM) techniques.

5.2 MATERIALS AND METHODS.

All methods of animal husbandry, diet preparation and tissue recovery and preparation have been described earlier. Where required, animals were cyclically fed to the schedules depicted in Figures 5.1a,b, and embryos were removed at 9.5, 10.5 or 11.5 days of gestation. Embryos taken for LM studies were fixed and stained according to the method of FRASER (1982) as detailed in Chapter 2. Sections were stained with both cresyl violet to enhance the mitotic figures and haemotoxylin and eosin to aid cytological assessment. Semithin sections (1um) were also taken prior to TEM studies and also used for LM observations.

Fig. 5.1 Feeding regime of zinc-deficient and replete dams.



5.3 RESULTS.

In the first study, embryos were removed from zinc-deficient(ad lib)and zinc-replete (restricted-fed) dams after 11.5 days of gestation. Representative embryos from each litter were processed for TEM, SEM and LM (plastic and paraffin) observation after examination and photography under the dissecting microscope.

Scanning electron micrography of abnormal zinc-deficient embryos failed to reveal any major pathologies or defects not demonstrable using light microscopy. Figure 5.2a shows a typical zinc-replete 11.5 day embryo which has completed rotation. The neural tube has fused, the fore-limb buds are clearly visible and the heart and brachial bars are well formed. Figures 5.2b and c show earlier stages of development. The first of these (Fig. 5.2b) shows a late 10-day embryo which was at the start of rotation, and the neural tube had fused, except for small regions at the cranial and caudal extremities. The other embryo (Fig. 5.2c) was removed from the uterus earlier on day 10, before rotation had commenced and while the neural folds were still elevated. Figure 5.2d is is a higher power micrograph of the cranial neuropore in Figure 5.2b, showing the junction between the neuroderm and endoderm as well as the point of closure of the neural tube.

Figure 5.3a shows a typical grossly malformed zinc-deficient 11.5-day embryo which has not rotated, the allantois had not fused with the chorion to form the chorio-allantoic placenta and is clearly visible. At the time of recovery the embryo had an enlarged pericardium (since collapsed) and a poorly developed head, principally in the mesencephalic region. There were no apparent otic pits or optic placodes and only one brachial bar had formed. Comparison of Figure 5.3a with figures 5.2a,b or c shows that the abnormalities are not merely reflections of a reduced rate of growth of the embryos due to zinc deprivation.

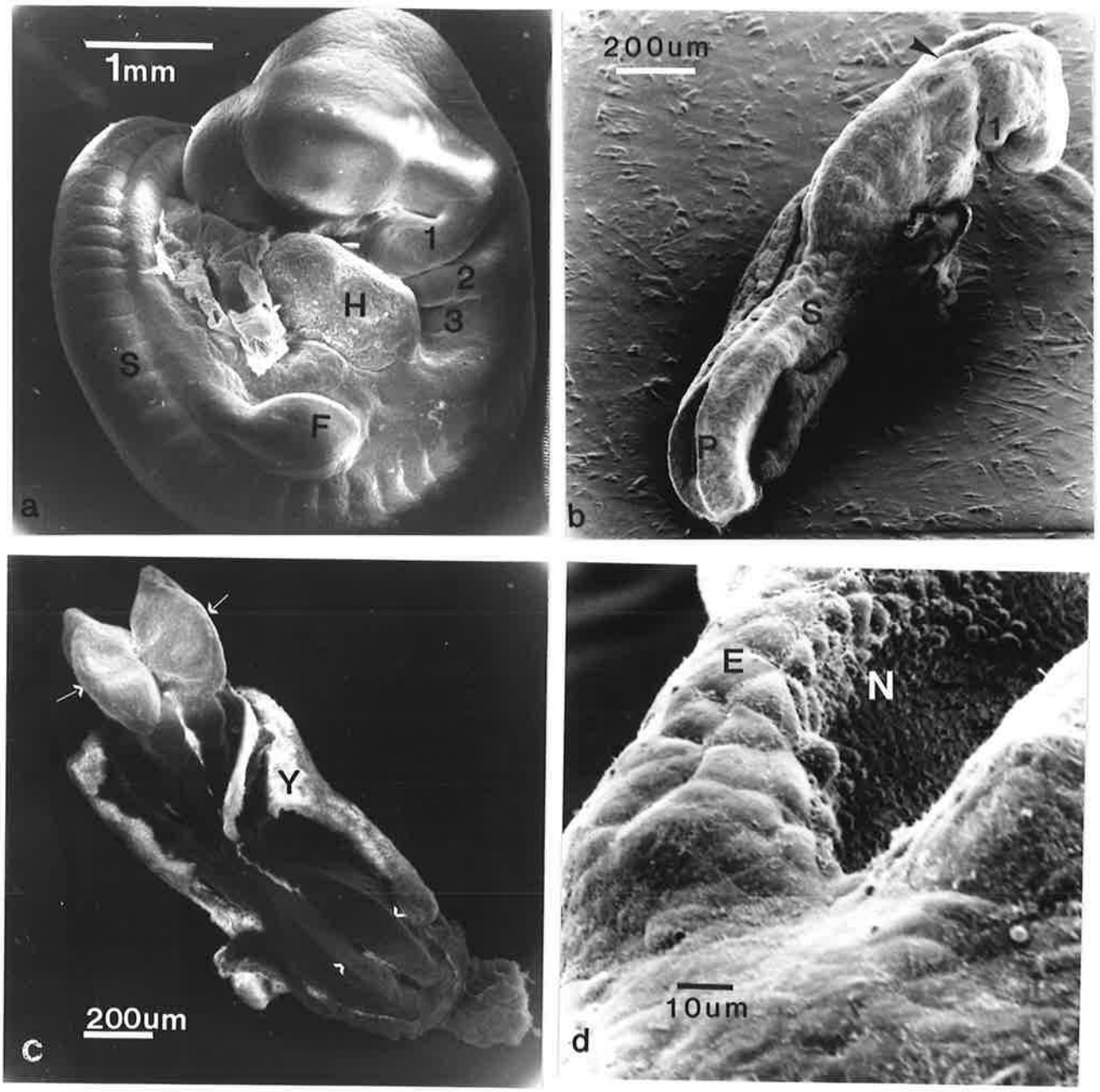
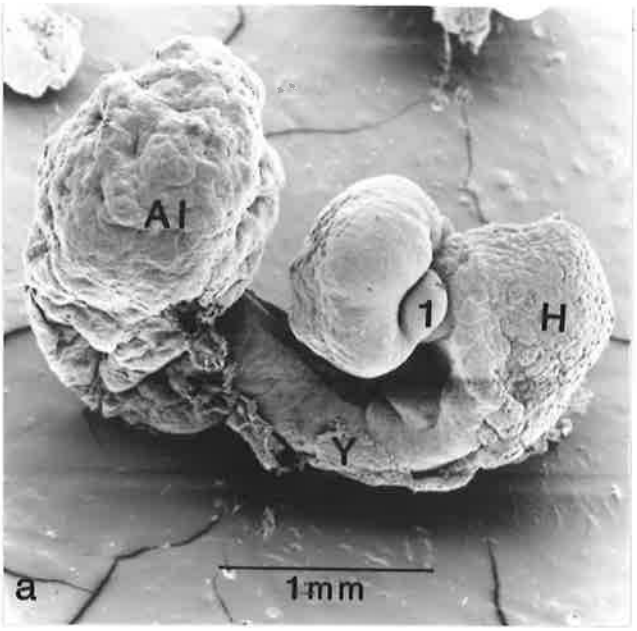


Figure 5.2. a. Zinc-replete 11.5 day embryo. S, somite; F, fore-limb bud; H, heart; 1,2,3, brachial bars. b. Late 10.5 day zinc-replete embryo with an open anterior neuropore (arrow). The embryo had not completed rotation and the fore-limb buds are not visible.P, posterior neuropore; Y, visceral yolk sac. c. Zinc-replete early 10.5 day embryo showing the cranial neural folds (arrows) and caudal neural folds (arrowheads). The surface of the neural epithelium is clearly visible (N). d. Higher magnification of the region indicated by the arrow in (b) showing the point of closure of the neural tube. E, endoderm; N, neuroderm. Magnifications as indicated.



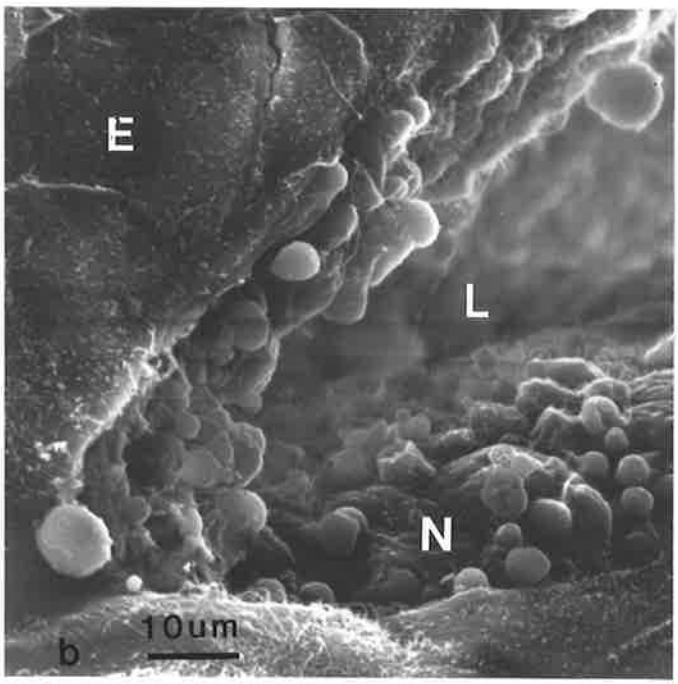


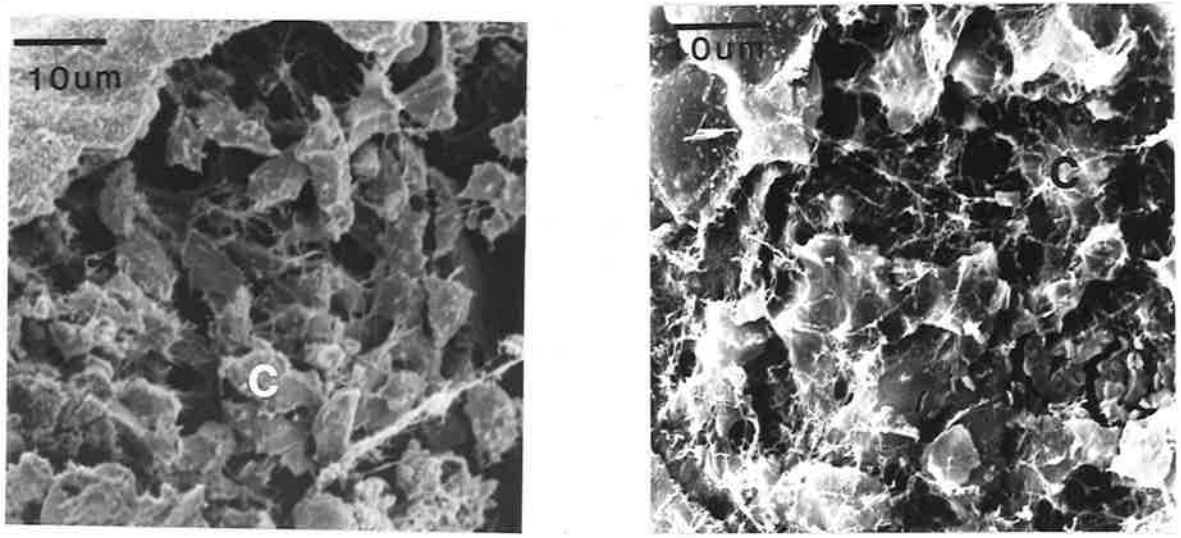
Figure 5.3. a. Grossly malformed 11.5 day zinc-deficient embryo showing lack of rotation, unfused allantois (A), emlarged pericardium (H) and only one brachial bar (1). No optic or otic vesicles were seen and the neural tube was open (not visible). b. Point of closure of the neural tube of a similar embryo, showing the endoderm (E) and cells budding and blebbing into the lumen (L) from the neuroderm (N). Magnifications as indicated.

The region at the neuroepithelial/endodermal junction at the open neural tube of a similar embryo was photographed at a higher magnification (Fig. 5.3b). Comparison of the micrographs in Figures 5.2d and 5.3b show that the lumenal surface of neural epithelium of the zinc-deficient animal was much rougher than that of the control embryo, and instead of a smooth border between the neuroepithelium and the endoderm , there was a ruffled edge with cell blebbings and budding into the lumen.

In an exploratory procedure a portion of the endoderm caudal to the position of the optic vesicle was lifted off several embryos (using adhesive tape) to allow examination of the mesoderm. As can be seen in Figure 5.4a, the mesoderm of the control embryo appears more closely packed with cells and there are more intracellular projections and connections than in the zinc-deficient embryo (Fig. 5.4b).

Light microscopy (paraffin sections) of grossly malformed zinc-deficient embryos again reflected the abnormal morphology seen under the dissecting microscope and the scanning electron microscope. Sections through the cranial region of a zinc-replete 11.5d embryo (Figs. 5.5a,b) show an essentially smooth lumenal surface with the neuroepithelial cells arranged in a regular pattern with mitotic cells visible at the lumenal border. Outside the neuroepithelium the irregular-shaped mesodermal cells are relatively close-packed with a dense extra-cellular fibrillar network. Early blood vessels are also present. The deformed zinc-deficient embryos (Figs 5.5c,d), sectioned at approximately the same region, showed a highly convoluted lumenal surface with evidence of cells blebbing and budding into the neural tube lumen. The basal margin of the neural epithelium was also convoluted in the same manner. Mitotic figures were visible in the appropriate position along the lumenal border.

Figure 5.4. Scanning electron micrographs of (a) cranial mesoderm of a zinc-replete 11.5 day embryo and (b) a similar region of a zinc-deficent embryo of the same age showing a reduction in the density and extent of the extra-cellular fibrillar network. c, cell bodies. Magnifications as indicated.



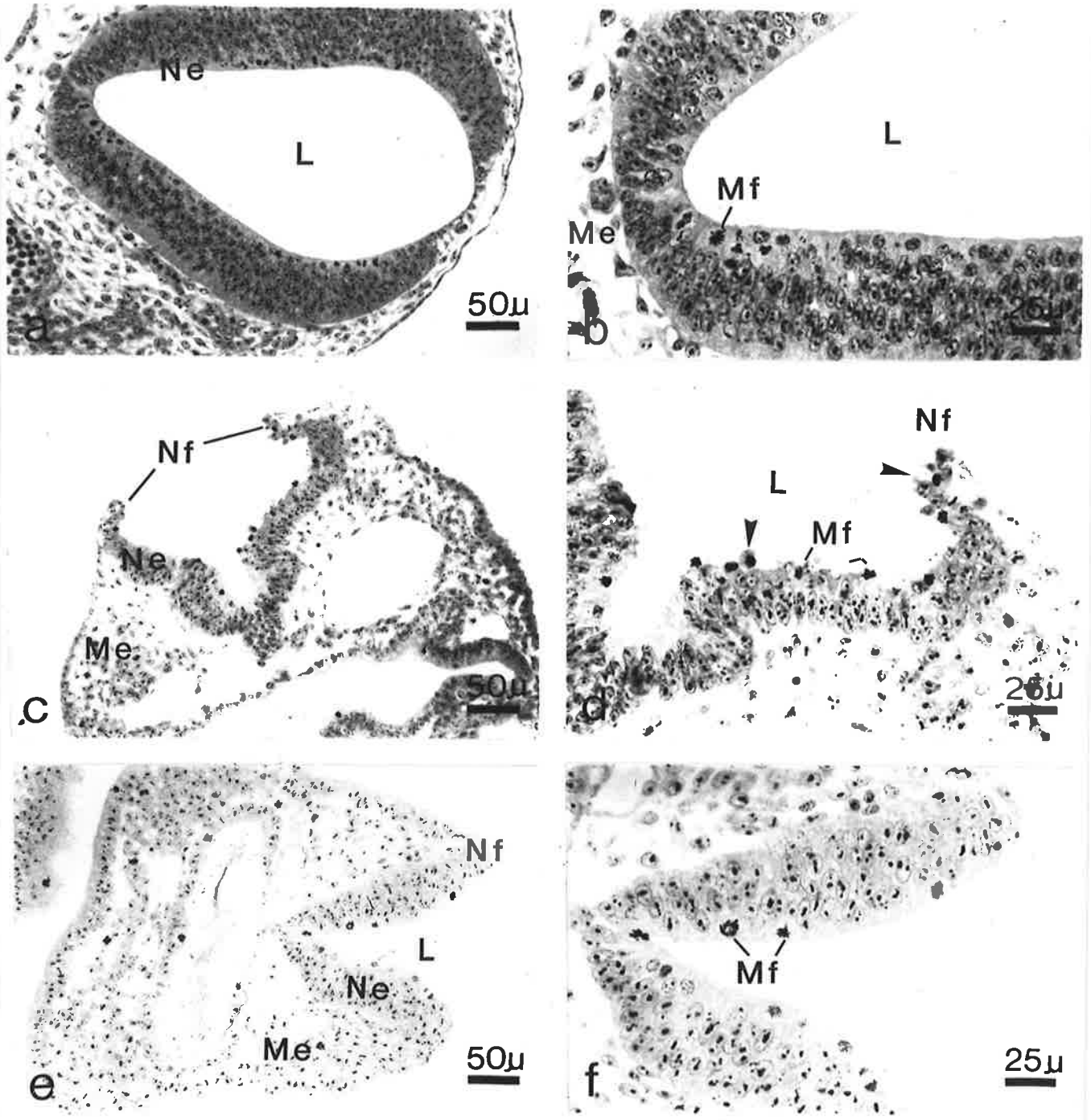
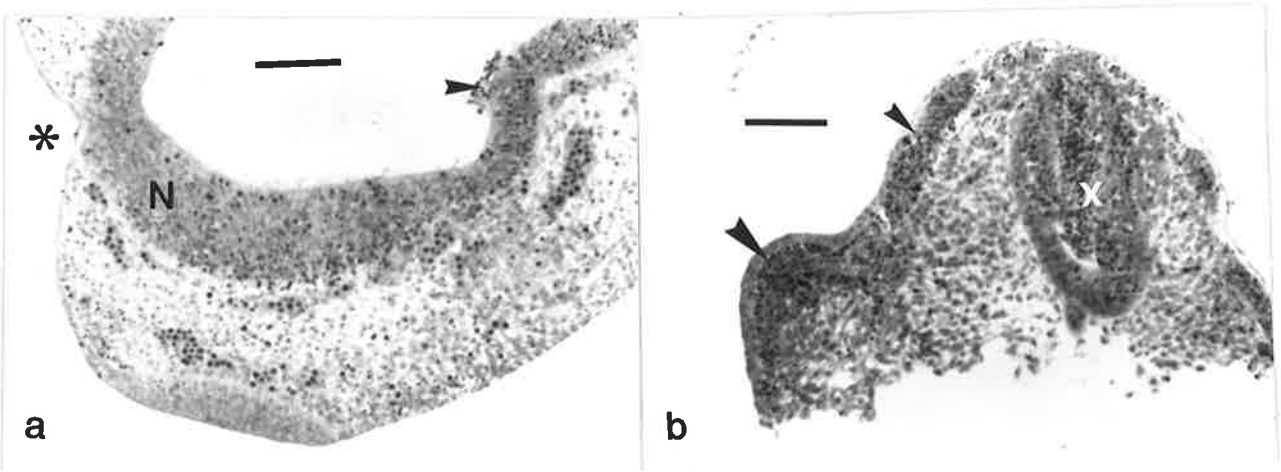


Figure 5.5. Light micrographs of the cranial regions of zinc-replete and deficient (abnormal) embryos. a,b. Zinc-replete embryo showing the neuroepithelium (Ne) and a portion of the mesoderm (Me). Mitotic figures are clearly visible at the lumen (L) of the neuroderm (MF). c,d. Section through the open cranial neural tube of an 11.5 day zinc-reficient embryo showing the distorton of the neural tube with cells entering the lumen (arrows). Mitotic figures are still present (MF). e,f. Comparable section through a 10.5 day zinc-replete embryo showing a much more organised neuroepithelium. Fraser's cresyl violet stain, magnifications as indicated.

The mesoderm was more sparsely populated with cells than the control embryos, and the cells were rounded rather than irregular and with fewer inter-cellular projections. Again, to demonstrate that the abnormal zinc-deficient embryos were not merely growth-retarded, normal zinc-replete 10.5-day embryos were sectioned and photographed at the same level (Fig. 5.5e and f). These sections showed a highly organised cellular arrangement, completely unlike that presented in Figures 5.5c and d.

In this phase of the study, apparently normal zinc-deficient embryos were also sectioned (Fig. 5.6). Unlike the abnormal embryos, these showed evidence of severe cellular changes. Dark staining inclusions were visible in the neural epithelium in both cranial and caudal regions with major tissue disruption producing occlusion of the caudal neural tube in some cases. The somites, brachial bars and limb-buds were also severely affected, although other areas of the mesoderm appeared normal.

Figure 5.6. Sections through the cranial (a) and caudal (b) regions of an apparently normal zinc-deficient embryo showing darkly staining cells in the neural epithelium (arrow, a), limb bud (large arrow, b) and somite (small arrow, b). The caudal lumen of the neural tube is completely occluded (X). The asterisk indicates the rostral closure of the neural tube where natural cell death is sometimes observed. Bar= 25um.



Transmission electron microscopy of these embryos showed a large variety of cells, extrusions, inclusions and extracellular debris, some aspects of which are illustrated in Figures 5.7 and 5.8. The first three of these micrographs (Figs. 5.7a,b,c) show regions of the neural epithelium with large extracellular spaces. The extracellular material appeared to consist of cells and cell particles of varying size and staining intensity. Cellular inclusions also ranged from light, frothy material to dense bodies containing degenerating intracellular organelles (Fig. 5.7d),and under higher magnification other cells showed evidence of swollen rough endoplasmic reticulum and occasionally distended mitochondria. Many apparently normal cells, including those undergoing mitosis contained such debris and inclusions, thus it is probable that many of the particles were the remains of fragmented dead cells which had been phagocytosed by healthy cells. Similar effects were also seen in the mesoderm (Fig. 5.8). In contrast, the only region of control (zinc-replete) embryos to show any similar signs of cell death was the area in the mid-line of the rostral part of the telencephalon consistent with that occurring after the fusion of the neural tube.

Mitotic counts were performed on sections of the cranial and caudal neural tubes as well as the limb-bud region of zinc-replete and zinc-deficient rat embryos. Because the morphologically normal embryos contained large areas of necrotic cells which on occasion appeared similar to mitotic figures, or obscure the mitoses, only the morphologically abnormal zinc-deficient embryos were included (Table 5.1).

Figure 5.7. Transmission electron micrographs of the cranial neural ectoderm of an animal similar to that in Figure 5.6, showing what appear to be fragments of dead cells engulfed by apparently normal neighbours (a,b). Large extra-cellular spaces are visible (*), and fragments of cells also appear in the lumen. Figure 5.7c shows a fragment of a dead or dying cell containing a wide variety of inclusions such as degenerating organelles (large arrow) and swollen rough endoplasmic reticulum (small arrow). Figure 5.7d is a higher magnification of another cell containing a wide variety of inclusions. L,lumen; M, mitotic cell; N, nucleus. Magnifications as indicated.

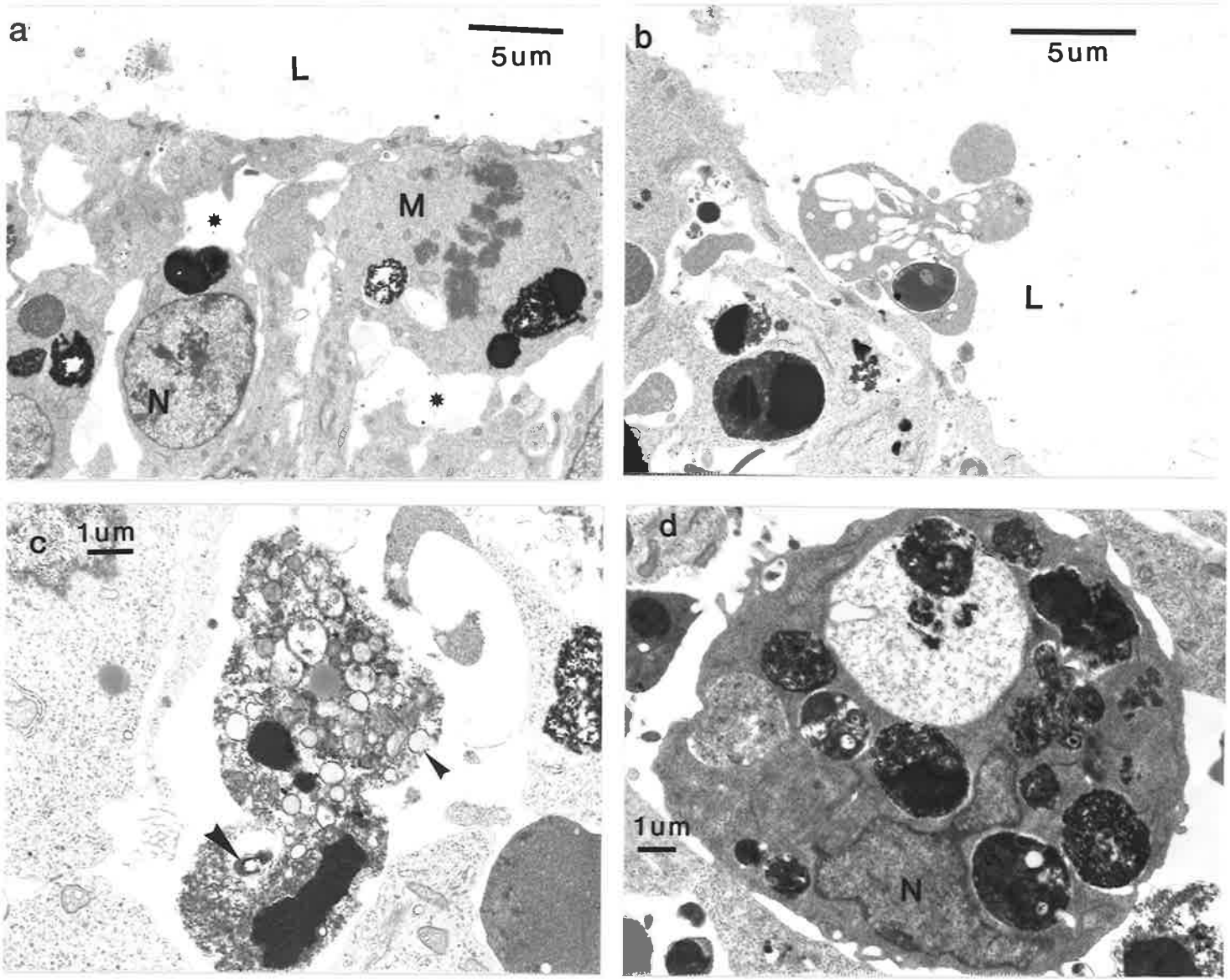

a
L
5um
M
N
b
5um
L
c
1um
d
1um
N

Figure 5.8. Transmission electron micrographs of mesodermal cells from the cranial region of an apparently normal 11.5 day zinc-deficient embryo showing (a) cells containing a large number of vesicles and (b) a cell containing a large amount of flocculent material. Except for the floc, which is similar to that shown in Figure 5.7, the cytoplasm of this cell appears normal, unlike that in (a).

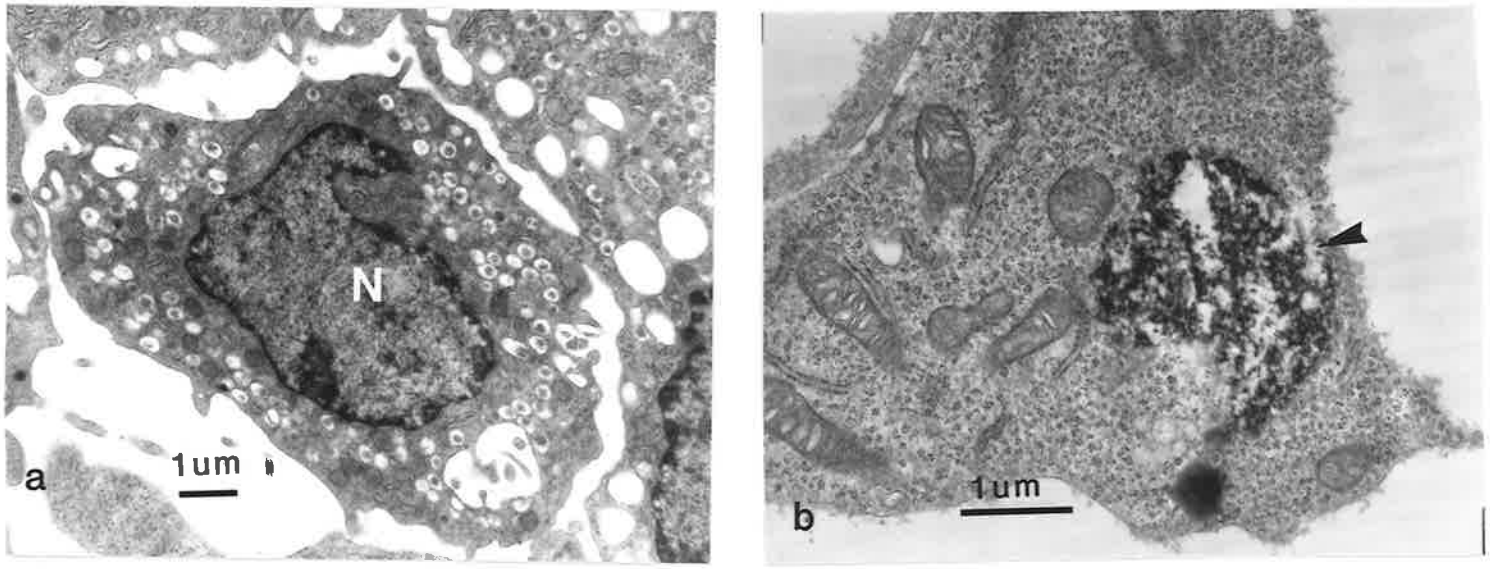


Table 5.1. Mitotic indices of zinc-deficient and replete 11.5d rat embryos.[a]

	Zinc-deficient	Zinc-replete
Maternal serum zinc (ug/ml)	0.72±0.06[b]	1.08±0.03
Cranial neural tube (total cells counted)	6.8±0.4[b](5842)	5.0±0.2 (6838)
Caudal neural tube (total cells counted)	8.1±0.6[c](1579)	6.5±0.4 (2954)
Limb-buds (total cells counted)	6.5±0.3[c](3790)	5.6±0.3 (4486)

a : Means±SEM of 10 zinc-deficient and 7 zinc-replete embryos
b : $p < 0.005$
c : $p < 0.05$

In addition, the length of the lumenal border in both cranial and caudal sections, and the areas of the limb-buds were determined. From these measurements, the estimates of cell density in the neuroepithelium (in terms of cells/1000μm) were calculated, as were cell densities in the limb-buds (cells/1000μm^2) (Table 5.2).

Table 5.2. Estimates of cell densities in the cranial and caudal neuroepithelium and in the limb-buds of zinc-deficient and replete embryos.[a]

	Zinc-deficient	Zinc-replete
Cranial neural tube (cells/1000μm)	648±67	867±25
Caudal neural tube (cells/1000μm)	666±58[b]	829±28
Limb-buds (cells/1000μm^2)	7.68±0.42[b]	7.69±0.20

a : Means±SEM of 10,zinc-deficient and 7 zinc-replete embryos.
b : $p < 0.05$

In order to determine whether the increased mitotic index associated with zinc deficiency arose as a result of a blockage at some specific stage of mitosis, differential counts were performed on the mitotic figures. These results (Table 5.3) suggest that the ratio of prophase nuclei might

have been reversed as a result of zinc deficiency, however this difference was not statistically significant.

Table 5.3. Differential mitotic counts of zinc-deficient and replete rat embryos.[a]

	Prophase	Metaphase	Anaphase	Telophase
Cranial				
Replete (7)	41.6±2.3	43.0±2.8	8.6±0.5	6.8±1.3
Deficient (10)	43.3±1.5	40.8±1.3	8.8±0.5	7.1±0.5
Caudal				
Replete (7)	41.0±1.7	42.0±3.2	10.5±1.7	6.5±1.3
Deficient (10)	43.7±2.6	40.3±3.2	9.0±0.6	6.0±0.8
Limb-buds				
Replete (7)	41.2±3.6	42.6±2.4	4.7±0.9	11.6±2.6
Deficient (10)	44.9±2.3	36.0±2.3[b]	6.1±0.8	13.0±1.8

a : Mean±SEM of the number of embryos indicated
b : Significantly less than control $p < 0.05$

Consideration of these results, together with the other observations on the induction of malformations by the imposition of specific feeding cycles led to the hypothesis that periods of low circulating maternal zinc levels induced by high intakes of zinc-deficient diet were capable of inducing abnormal cellular necrosis during the organogenetic period. Once the food intake of the dam declined and levels increased due to release of zinc from the maternal stores, cellular growth and differentiation proceeded normally. This hypothesis was tested by feeding pregnant animals the zinc-deficient or replete diet to the cycles depicted in Figure 5.1 to induce minimal serum zinc levels on days 8 and 9 (Group A) or days 10 and 11 (Group B). Embryos were removed at 9.5, 10.5 or 11.5 days gestations for examination by light and transmission electron microscopy to detect cellular and subcellular anomalies. Table 5.4 lists the food intakes of the dams on the night before they were killed and the maternal serum zinc concentration at the time of death.

Table 5.4 Maternal serum zinc concentrations and food intakes of cyclically fed dams.[a]

	Day of gestation					
	9.5		10.5		11.5	
Group	Food intake (gm)	Serum zinc (ug/ml)	Food intake (gm)	Serum zinc (ug/ml)	Food intake (gm)	Serum zinc (ug/ml)
-Zn A	14 (4)	0.37±0.2	4 (5)	0.90±0.07	2 (5)	1.09±0.04
+Zn A	14 (2)	1.27±0.2	4 (5)	1.00±0.03	2 (6)	1.16±0.04
-Zn B	5 (4)	1.28±0.1	15 (3)	0.44±0.08	15 (3)	0.36±0.04
+Zn B	5 (3)	1.31±0.6	15 (3)	1.27±0.01	15 (3)	1.17±0.05
+Zn Ad lib[b]	- (3)	1.05±0.1	- (3)	1.19±0.11	- (3)	1.16±0.06

a : Values are means±SEM of the number of dams in parentheses
b : Food intake not determined

Under the dissecting microscope, Group B zinc-deficient embryos at 9.5 days were morphologically identical to zinc-replete embryos, whereas those from Group A had underdeveloped embryonic poles similar to those described in Chapter 3. At 10.5 days Group A zinc-deficient embryos were smaller than their controls and appeared developmentally retarded. Group B zinc-deficient embryos on the other hand were normal although small. After 11.5 days gestation the embryos from Group A dams were still abnormal, whereas those from Group B still appeared morphologically normal.

Microscopic examination of the 9.5 day embryos from group A zinc-deficient dams showed substantial areas of necrosis within the embryo, but primarily in the neural folds (Fig. 5.9a). Cellular debris apparently extruded from the neural epithelium was visible within the lumen of the egg cylinder. Other cell types such as the mesoderm and yolk-sac endoderm appeared relatively unaffected. Group b embryos were totally unaffected at this stage (Fig. 5.9b).

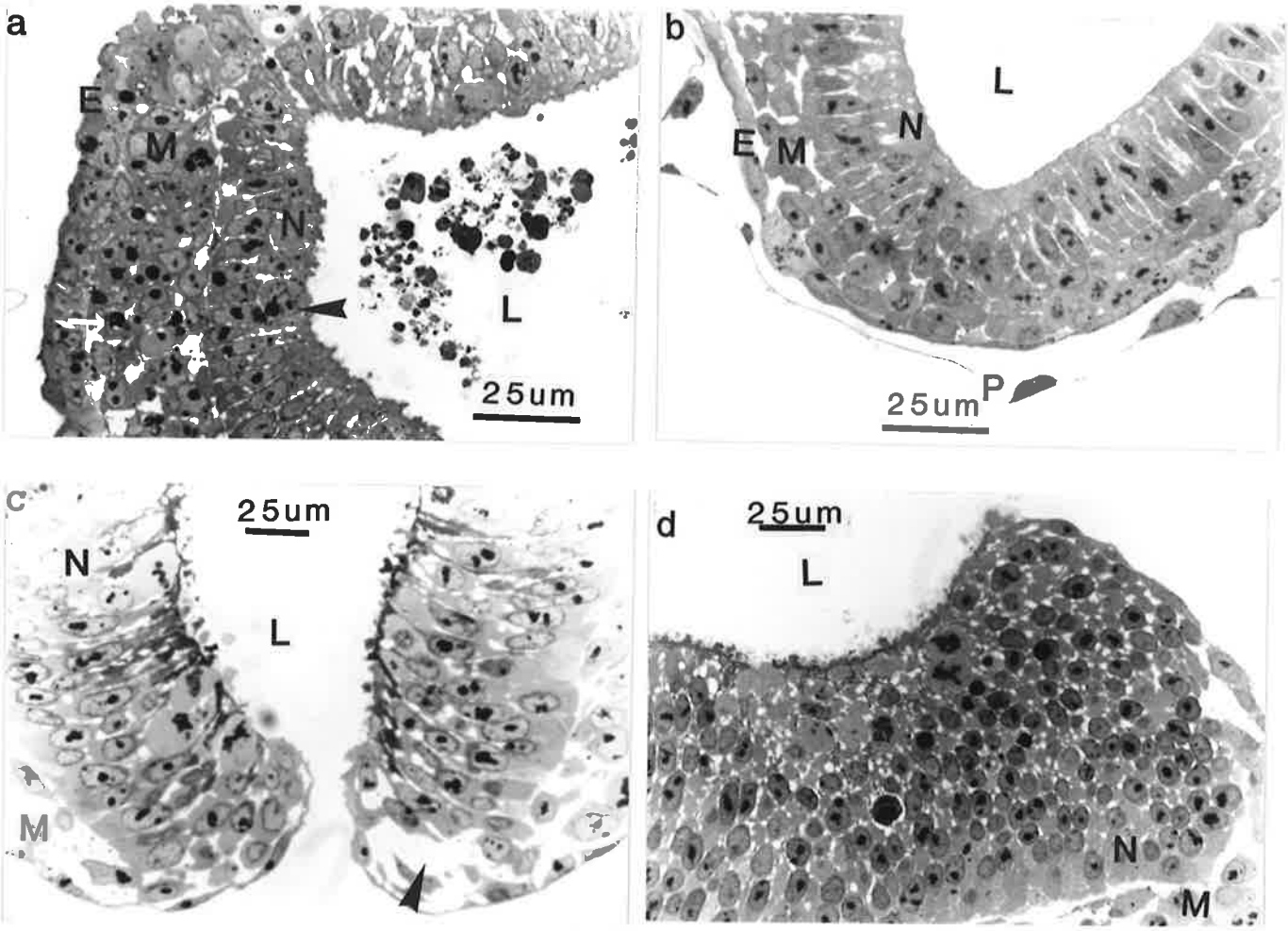


Figure 5.9. (a), transverse section through a Group A zinc-deficient 9.5 day egg cylinder showing cell necrosis in the folds (arrow) and extensive cell debris in the lumen . (b), a similar zinc-deficent Group B embryo apparently unaffected by zinc-deficiency. (c), 10.5 day Group A zinc-deficient embryo prior to closure of the neural tube. Large extra-cellular spaces are visible (arrow) and some discharge of material into the lumen is evident. (d), a similar section through a Group B zinc-deficient embryo showing some apparently necrotic cells (arrow). E, yolk sac endoderm; M, mesoderm; N, neural ectoderm; L, lumen. Magnifications as indicated.

Isolated foci of necrotic cells were occasionally observed in the neural epithelium of zinc-deficient Group A embryos at 10.5 days of gestation as well as in the mesoderm. Extracellular spaces were more apparent in this region and cellular extrusions could be seen in the lumen of the neural tube. Group B embryos showed limited evidence of necrosis in the neural epithelium, although not in the mesoderm (Figures 5.9c,d).

The histological appearance of 11.5-day embryos taken from Group A zinc-deficient dams was identical with the grossly malformed zinc-deficient embryos described earlier, whilst the Group B zinc-deficient dams showed large areas of cellular necrosis, as described earlier.

5.4 DISCUSSION

The cellular necrosis described in these zinc-deficient embryos was more pronounced and extensive than the programmed cell death which is a normal feature of embryonic development. At this stage (11.5d) in the normal rat embryo, cell death is limited to a small line of cells in the region where the neural tube most recently closed (SCHLUTER 1973; GEELAN & LANGMAN 1977). The necrosis induced by zinc deficiency was widespread throughout the neuroepithelium, somites and limb-buds as well as some other mesodermal areas. Histologically the dead, dying cells and inclusions appeared similar to those produced as a result of teratogens such as cadmium (WEBSTER & MESSERLE 1980), ethanol (BANNIGAN & BURKE 1982), ethylnitrosourea (FUJIWARA 1980) and arsenic (MORRISSY & MOTTET 1983). To the author's knowledge the only other reported instances of embryonic cell necrosis due to a dietary insufficiency are limited to deficits of riboflavin (SHEPARD 1968), vitamin A (PALLUDAN 1966), pantothenic acid (GIROUD *et al* 1955) and folate (JOHNSON 1964).

Necrosis caused by a dietary zinc deficiency has previously been described in the small intestine (ELMES 1977; ELMES & JONES 1980), the pancreas (FELL *et al* 1973; KOO & TURK 1977) and testes (UNDERWOOD & SOMERS

1969), but not in embryonic or fetal tissue. Examination of the published electron micrographs of these other workers suggested that the events leading to cell death might be similar to those observed in this study.

Although it is premature to speculate on the reasons for the cytotoxicity of zinc deficiency in these tissues, the histological appearance suggests some metabolic derangement. Whether this is due to an inability of the cells to synthesise DNA (e.g.ECKHERT & HURLEY 1977), or to effects on the synthesis or degradation of RNA (SOMERS & UNDERWOOD 1969), or the induction of membrane lipid peroxidation (CHVAPIL 1976) causing membrane damage remains unclear. Some of these possibilities will be discussed in Chapter 10.

Zinc deficiency seemed to affect particular cells or groups of cells in regions of the embryo undergoing especially rapid cell division, such as the lateral portion of the rostral neural tube, and the caudal region (neural tube, somites, limb-buds) in the 11.5d embryos and analagous regions in earlier embryos. Even in severely necrotic regions of the developing embryo occasional apparently normal cells and mitotic figures were observed, suggesting either that the deficit of zinc acts at a specific time during the cell cycle, or that some cells are able to accumulate and utilise zinc released as a result of the death of a nearby cell. Except for the increased mitotic index of the zinc-deficient tissues which confirmed previous observations (HURLEY & SHRADER 1972; FELL *et al* 1973; ECKHERT & HURLEY 1977), there was no evidence of a significant increase in the proportion of cells in any stage of mitosis. Indeed it can be suggested that the increased rate of proliferation (at the time of high maternal serum zinc levels) is due to an increased availability of zinc to the embryonic tissue and an attempt to re-establish the population of cells. The reduced cell density in the neural epithelium is probably as a result of the cell necrosis in the 9.5 or 10.5 day embryos' neural folds. The absence of any similar reduction in cell density in the limb-buds is

probably due to the fact that the limb-bud primordia do not start to differentiate until day 10, when the maternal serum zinc levels are increasing.

Cell death has not been reported in even the most extensive studies of other zinc-deficient embryos and fetuses (DIAMOND & HURLEY 1970; HURLEY & SHRADER 1972; ECKHERT & HURLEY 1977). It is possible that in these earlier studies, only grossly malformed embryos were selected for histological study or that after day 11, when the placenta has become fully functional, the embryo or fetus has easier access to the relatively large pool of circulating maternal zinc. In addition, after day 11 the decidual tissue, which has a large mass compared with the embryo regresses and possibly contributes to the nutrient requirement of the embryo. Furthermore the growth rate of the embryo or fetus decreases with age in mid-gestation (KOHLER *et al* 1972), thus the proportion of cells for which extra zinc is required declines. Although larger cell numbers are involved it is possible that these dividing cells acquire sufficient zinc from other quiescent cells within the embryo to maintain their integrity. It does appear however that even in older (17-day) fetuses, limited cell death can be observed in the ependymal region of zinc-deficient fetal rat brains (DREOSTI *et al*, unpublished observations).

5.5 CONCLUSIONS

From the observations in this and preceding chapters, the following sequence of events leading to the production of congenital anomalies produced by zinc deficiency can be suggested. Once fed a zinc-deficient diet, dams enter a cyclical feeding pattern, the exact timing of which is influenced by poorly defined metabolic parameters. This cycle, which has a period of about 4 days, is accompanied by inverse fluctuations in the maternal serum zinc levels (CHESTERS & WILL 1973; RECORD *et al* 1985b,c,d,f,1986; Dreosti *et al* 1985). Prior to the formation of the

chorio-allantoic placenta, at about day 11 in the rat, the embryo derives its nutrients, including zinc, from the uterine fluid which is a plasma exudate sensitive to changes in the maternal serum zinc concentration (GALLAGHER & HURLEY 1980). During periods in which the maternal serum zinc level is low, foci of rapidly proliferating cells undergo necrosis, possibly as a result of damage to lysosomal or other intracellular membranes (CHVAPIL 1976) or because of an inability to replicate or differentiate. As a result of this necrosis, neighbouring cells engulf the debris thus producing large extracellular spaces affecting the final structure of the organ. The location of the necrosis, and hence the structure affected depends on the gestational time at which the maternal zinc level is minimal. When the dam enters a fasting, catabolic state, zinc is released from maternal tissue stores and becomes available to the embryo (MASTERS *et al* 1983; RECORD *et al* 1985b,d,1986) allowing the embryo to resume both cell division and differentiation at an increased rate producing an elevation in the mitotic index. Material from the fragmented cells would also become available through phagocytosis for re-utilisation in other non-necrotic cells within the embryo, thus allowing such cells to continue growth and differentiation.

Although cell death is not a prerequisite for a teratogenic response such events have been reported to occur as a result of a wide range of treatments (SCOTT 1977). It is possible that, in the case of maternal zinc deficiency, necrosis is responsible for early effects on the embryo, but that in later development inhibition of growth or cell division is responsible for some of the more subtle defects.

CHAPTER 6

EFFECTS OF CYCLICAL FEEDING OF ZINC-DEFICIENT DIETS FOR THE FIRST HALF OF GESTATION ON FETAL OUTCOME IN RATS

6.1 INTRODUCTION

Previous chapters of this thesis have described the effects of cyclical feeding of zinc-deficient diets on the development of the rat embryo prior to day 11.5 of gestation. Embryos exposed to low maternal serum zinc levels on gestational days 8 and 9 were grossly malformed on day 11.5. Typical deformities included failure of the allantois and chorion to fuse to form the chorio-allantoic placenta, open neural tubes as well as other defects which could prove fatal to the developing embryo. Embryos exposed to normal maternal zinc levels on gestational days 8 and 9, but subjected to low levels of zinc availability on days 10 and 11 were morphologically normal but contained large areas of necrotic cells (*vide supra*) in the lateral region of the cranial neural tube, as well as in the fore-limb buds, somites and neural tube throughout the caudal region.

The purposes of this study were firstly to assess the subsequent development of zinc-deficient rat embryos exposed to specific feeding cycles during the first half of gestation and allowed a commercial zinc-replete ration until just prior to delivery. Particular attention was paid to the presence of resorption sites in an attempt to correlate the embryonic survival at day 11 with that of day 20 fetuses. A second purpose was to confirm, if possible, previous epidemiological (JAMESON 1976; METCOFF 1980; McMICHAEL *et al* 1982; FEHILY *et al* 1986) and experimental (DREOSTI *et al* 1985a) associations of small-for-age babies or fetuses with higher maternal serum zinc levels at the end of gestation.

During the period from day 11 to day 20 the embryo continues its rapid

growth and development. Over this time the wet weight increases from less than 5mg to in excess of 3g at day 20. Many of the external features which have already appeared continue to develop and the internal organs differentiate and become functional.

The posterior neuropore closes at about 25 somites (11 days), the lens placodes of the eye form, Rathke's pouch contacts the infundibulum and the diencephalon and the hypothalamus can be distinguished. The heart continues its development from an s-shaped tubule, and the intra-ventricular septum forms. In the developing kidney, the mesonephron and gonadal ridges appear and lung buds and the liver anlage are present.

At about 13 days, or shortly after, the nasal processes fuse ventral to the nasal pits, the medial processes fuse with the maxillary processes to form the upper lip, and the mandibular arches have fused to form the lower jaw. Palatine processes appear on the maxillary arches and the primary palate forms on the frontonasal process. The mandibular processes fuse to form the floor of the mouth and the tongue bud appears. The pleuroperitoneal fold which later encloses the viscera appears, and the cloaca has begun to form and partition. In the nervous system the cerebral hemispheres are visible, the auditory portion of the ear develops, the metencephalon thickens in a region which will become the cerebellum.

Individual digits start to form in first the front, then the hind limbs and skeletal cartilage appears in the ribs at about day 15. The mandible starts the process of ossification and the mandibular and hyoid cartilages merge. Externally the eyelids begin to form and hair papillae form on the maxillary processes and trunk. Chloroid plexuses appear in the ventricles and the lens vesicles are completed and detach from the epidermis. In the circulatory system the aorta separates from the pulmonary artery, the aortic arches adopt the adult configuration and the heart is completely partitioned except for the foramen ovale. At this stage the gonads become identifiable as ovaries or testes, and the cloaca becomes

divided into the urogenital sinus and rectum.

On day 16 the digits in the fore-paws separate, with the hind paws following a day later. Pinnae cover the ears, the eyelids fuse, papillae appear on the tongue, the palatal shelves elevate and fuse, and the umbilical hernia is withdrawn. In the nervous system the choroidal fissure forms, the hippocampal formation becomes distinguishable and the corpora quadrigemina are recognizable in the mesencephalon. The renal glomeruli and tubules are distinguished and fluid begins to accumulate in the kidney. The paramesonephric ducts unite to form the utero-vaginal canal. In the male, the testes lie behind the bladder, the seminal vesicles appear on the walls of the deferens and the prostatic buds appear in the urethra.

6.2 MATERIALS AND METHODS

Preparation of the diets and animal husbandry techniques were as described in Chapter 2. In this study pregnant Sprague-Dawley rats (200-220g) were randomly allotted to one or other of the treatment groups as follows:

Group A: Zinc-replete, semi-synthetic diet ad lib throughout gestation.

Group B: Zinc-replete, semi-synthetic diet in restricted amounts (12g/d) throughout gestation.

Group C: Zinc-deficient, semi-synthetic diet ad lib throughout gestation.

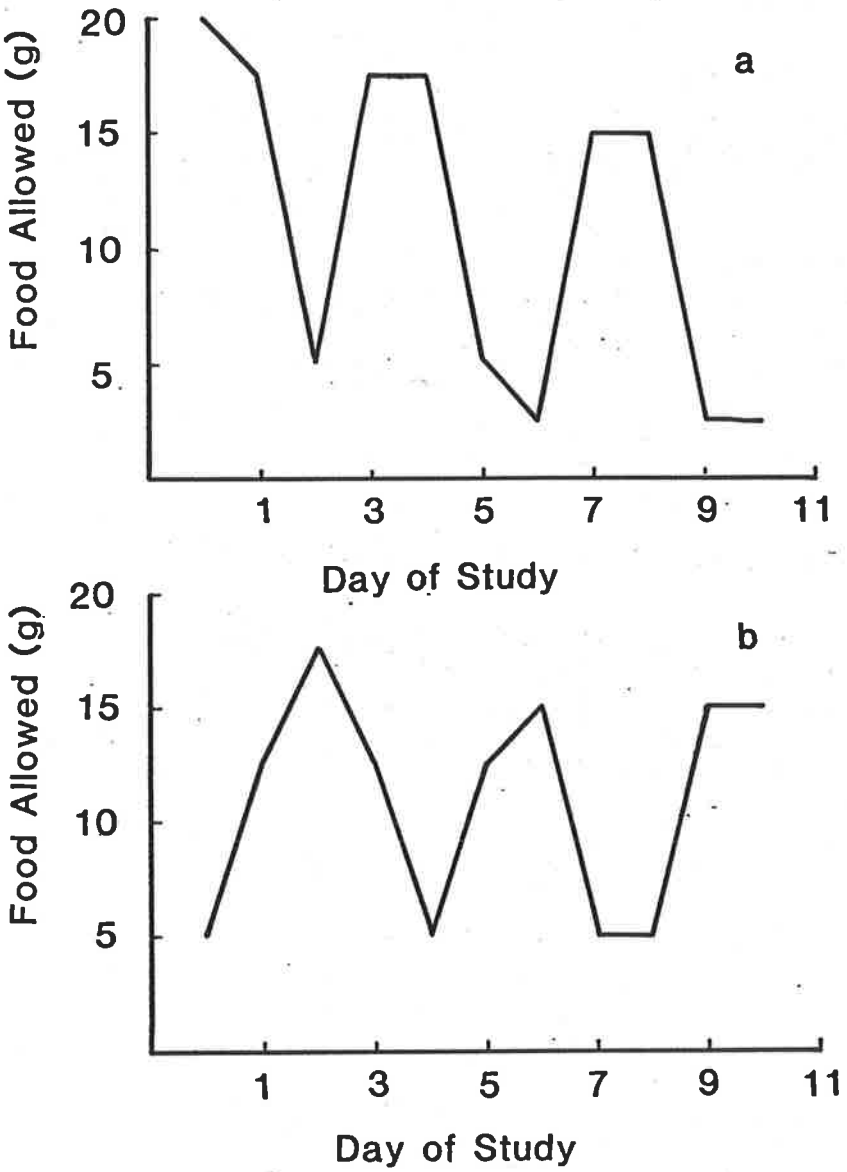
Group D: Zinc-replete, semi-synthetic diet as in Figure 6.1a from detection of sperm to day 11, then commercial diet ad lib.

Group E: As per Group D, but were supplied the zinc-deficient diet.

Group F: Zinc-replete, semi-synthetic diet as in Figure 6.1b from detection of sperm to day 11, then commercial diet ad lib.

Group G: As per Group F, but were supplied the zinc-deficient diet.

Fig. 6.1 Feeding regime of zinc-deficient and replete dams.



The commercial diet contained 62ug Zn/g and the zinc-replete and deficient diets 100 and <0.5ug Zn/g respectively (Chapter 2).

On the 20th day of gestation dams were anaesthetised with ether, blood samples collected by cardiac puncture for serum zinc estimation and the uteri and ovaries removed. Each uterus was examined for the presence of

fetuses and resorption sites. Where possible these were counted. If no such sites were visible, the ovaries were examined for the presence of degenerating corpora lutea to confirm that the animal had been pregnant. Surviving fetuses were weighed and examined for the presence of internal and external malformations by the method of WILSON (1965).

For calculation of the results, those animals which had lost the entire litter were excluded, except where specified. Statistics were evaluated by analysis of variance, and where appropriate the standard Pearson correlation was used.

6.3 RESULTS

Of the dams which carried fetuses to day 20, those fed the zinc-deficient diet throughout gestation had significantly lower serum zinc levels than similar animals fed the zinc-replete diet (in either ad lib or restricted amounts) (Table 6.1). Dietary zinc deficiency during the first half of pregnancy (Groups E and G) was associated with a higher maternal serum zinc level on day 20 than similar animals fed zinc-replete diets throughout gestation (Groups D and F).

Group C animals were the only group to fail to gain weight, even when the total mass of the litter was included. Indeed when the litter mass was discounted there was a large (approximately 25%) weight loss. The only other group to show a reduced weight gain was that fed the zinc-replete diet in restricted amounts throughout pregnancy (Group B).

Table 6.1 Reproduction in zinc-deficient and replete pregnant rats.

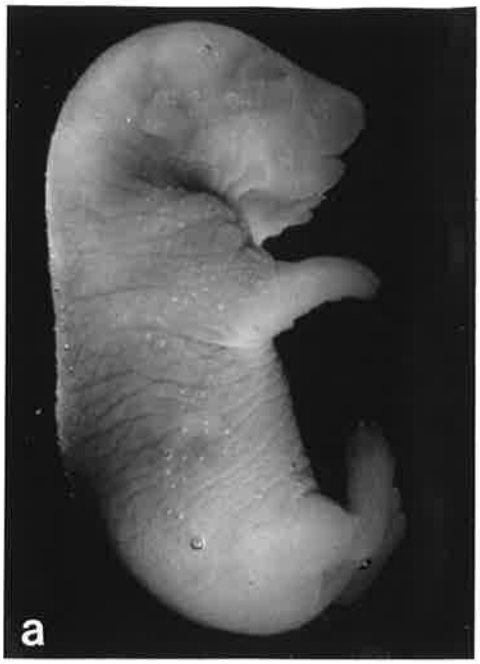
Treatment group	No. of rats mated	No. of rats with live young	Maternal weight change (g) +litter	Maternal weight change (g) -litter	Maternal serum zinc (ug/ml)
A	4	4	108±4	63±2	0.86±0.14
B	6	6	55±7[a]	12±3[a]	0.56±0.05[a]
C	8	7	-6±7[a]	-26±5[a]	0.36±0.05[a]
D	5	5	94±15	47±11	0.85±0.05
E	9	5	102±14	59±10	0.92±0.09
F	3	3	80±34	29±24	0.80±0.01
G	5	4	83±13	55± 4	0.95±0.01

a: Significantly less than zinc-replete, ad lib fed group, $p<0.05$.

There was little difference in the number of implantation sites (including animals with and without surviving fetuses) due to any dietary treatment. The average number of surviving fetuses was significantly less in the zinc-deficient Groups C and G, but not Group E. Fetal and placental weights were however significantly reduced only in Group C, as was the total mass of the conceptuses (fetal and placental tissue).

The incidence of gross fetal malformations ranged from 84% in Group C (fed exclusively the zinc-deficient diet) to 45% and 37% respectively in Groups E and G (fed zinc-deficient diet in the first half of gestation) to an average of 3.7% in the combined zinc-replete groups. When the number of resorption sites in all dams was included, 91% of the implants in Group C were affected, 73% and 61% in Groups E and G, but only 10.6% in the control animals. Only single defects were found in the zinc-replete groups, whereas multiple defects were common in those fetuses exposed to some period of maternal zinc deficiency (Fig. 6.2).

Figure 6.2. Representative defects observed in rat fetuses exposed to maternal zinc deprivation for 20 days. Fetus (a) is a normal zinc-replete fetus; Fetus (b) has ectrodactyly, clubbed feet and a missing tail; Fetus (c) has ectrodactyly and bilateral cheiloschisis; (d) shows an umbilical hernia, clubbed feet and short tail; (e) shows cleft palate and (f) shows hydrocephalus.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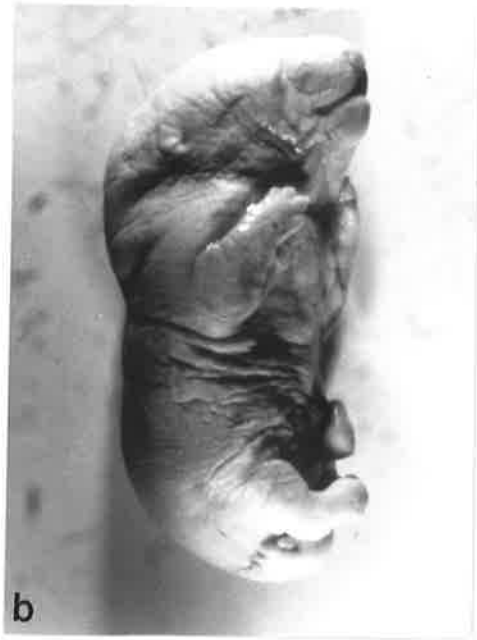
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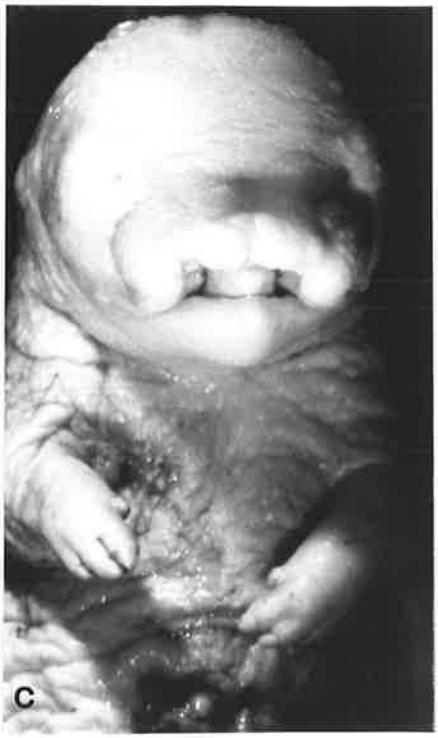
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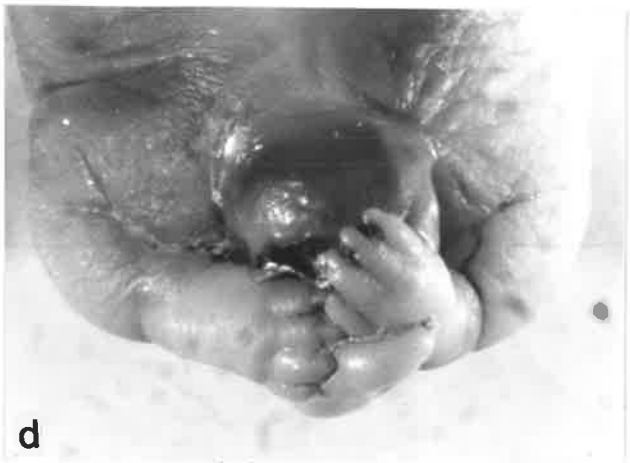
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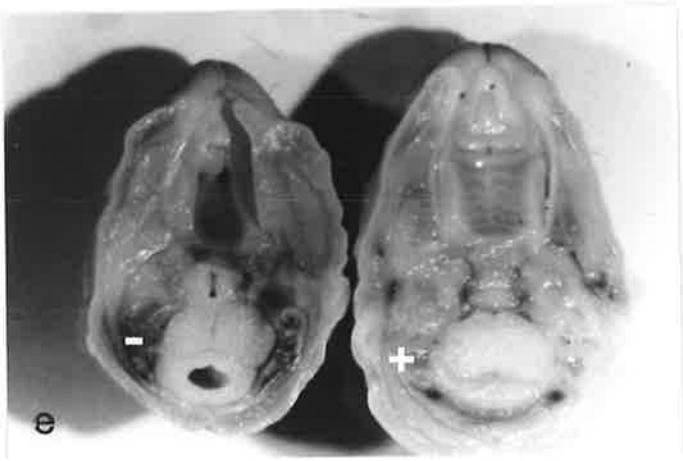
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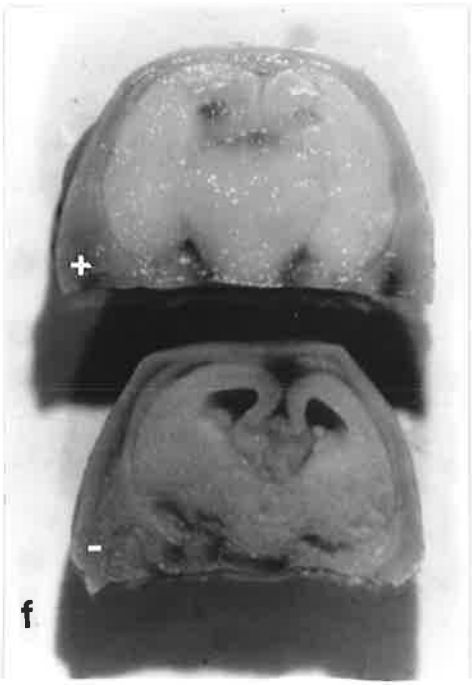
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Table 6.2 Growth and development of zinc-deficient and replete rat fetuses[a].

Treatment group	Implant sites (dams)	Live fetuses per dam	Fetal weight(g)	Placental weight(g)	Malformations No. (% live)	Total affected sites
A	12.3 (4) ±1.0	11.3 (4) ±0.9	3.56 ±0.07	0.52 ±0.03	3 (6.7)	7 (14.3)
B	13.2 (6) ±1.5	12.5 (6) ±1.5	3.07 ±0.08[b]	0.44 ±0.02[b]	1 (1.3)	5 (6.3)
C	12.1 (8) ±0.8	7.9 (7) ±1.7[b]	2.14 ±0.15[b]	0.36 ±0.02[b]	46 (84)	88 (90.7)
D	12.6 (5) ±1.2	12.0 (5) ±1.5	3.38 ±0.10	0.55 ±0.03	2 (3.3)	5 (7.9)
E	12.6 (9) ±0.8	11.2 (5) ±2.5	3.36 ±0.10	0.53 ±0.03	25 (45)	82 (72.6)
F	14.0 (3) ±0.7	12.7 (3) ±1.8	3.47 ±0.40	0.57 ±0.06	2 (5.3)	6 (14.3)
G	12.2 (5) ±2.4	7.6 (5) ±3.0	3.09 ±0.12[b]	0.45 ±0.05	14 (37)	37 (60.7)

a : values are means±SEM of the number of dams with live young
b : significantly less than zinc-replete, ad lib fed group, $p<0.05$.

Comparisons were also made between the types of malformations observed in the three groups of litters exposed to the zinc-deficient diets (Table 6.3). Defects of all organ systems examined were found in Group C litters(Fig. 6.2). Palatal and mandibular defects as well as herniations were absent in the fetuses from Groups E and G. The incidence of tail and urogenital defects were similar in Groups E and G, but none of the Group E fetuses had a- or syndactyly whereas 24% of the Group G fetuses were affected. The incidence of brain defects was higher in Group E, although this was largely due to the presence of mild to moderate hydrocephalus in all 15 fetuses of one dam. The incidence of any defect in Groups E and G was substantially less than that of Group C. These results compare well with those reported by HURLEY & SHRADER (1972) for fetuses delivered from dams fed the zinc-deficient diet for 21 days or for the first 10 and 12 days of gestation.

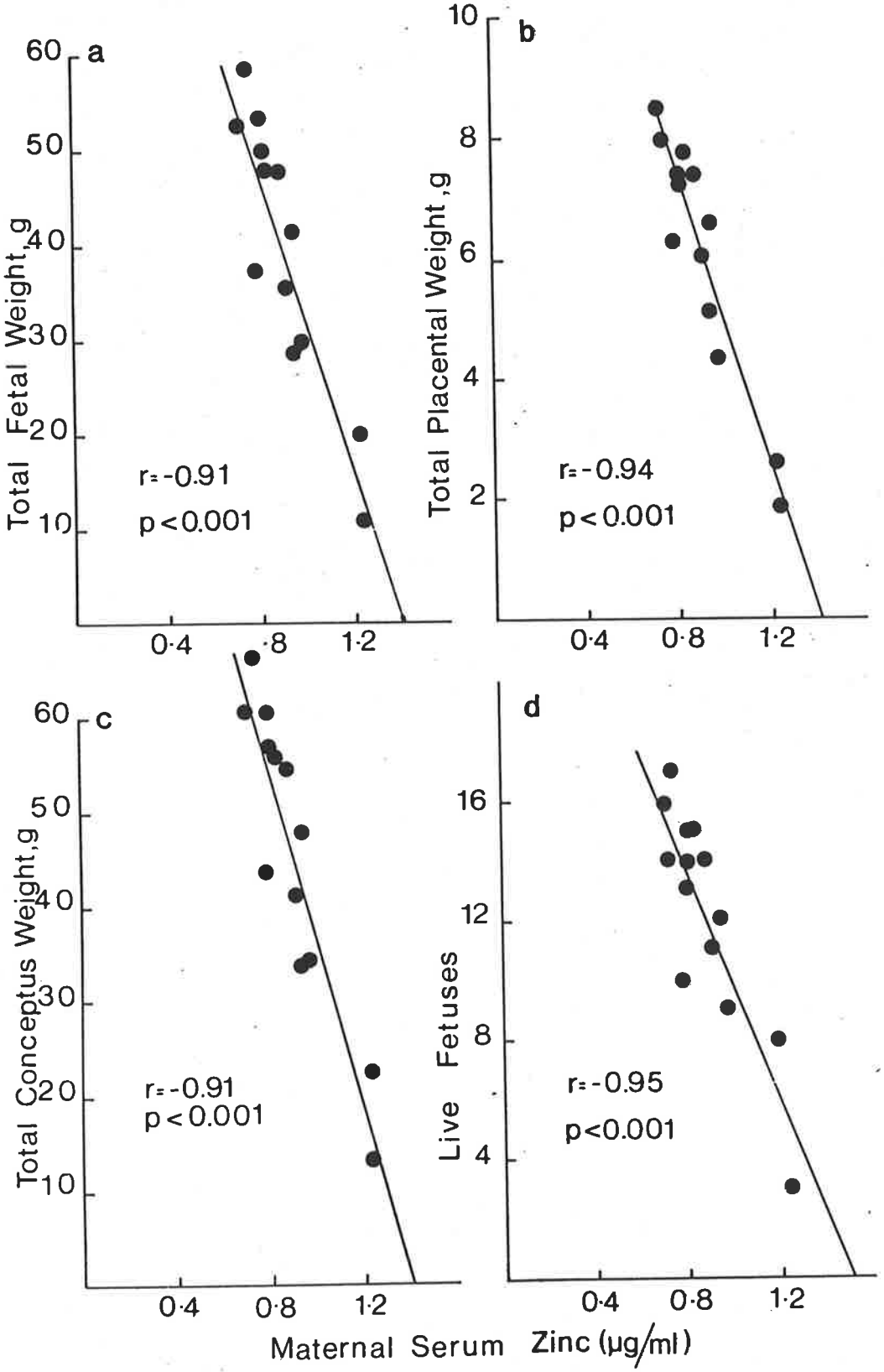
Table 6.3 Incidence of major malformations in fetuses from dams fed zinc-deficient or replete diets.

Group	+Zn	C	E	G
Live fetuses examined	218	55	56	38
Incidence	%	%	%	%
Micro- or anophthalmia	0.	12.7	1.8	2.7
Brain	0.	70.9	28.6*	15.8
Palate	0.5	47.3	0.	0.
Micro- or agnathia	0.	14.5	0.	0.
A- or syndactyly	0.	20.0	0.	23.7
Club Foot	0.5	27.3	1.8	0.
Tail	1.0	47.3	14.3	18.4
Umbilical Hernia	0.	12.7	0.	0.
Urogenital	2.0	25.5(21)	3.6(4)	10.5

*: Includes 15 mild-moderate hydrocephalies from 1 dam.

Correlations were also observed between the maternal serum zinc levels of dams and various growth parameters of their litters. In Group A, the ad lib fed zinc-replete control animals, there was no apparent relationship between maternal serum zinc levels and maternal body weight changes, litter weight or fetal number. There were strong correlations between the litter parameters and and maternal serum zinc in all other groups (Fig. 6.3).

Fig. 6.3 Relationship between maternal serum zinc levels at day 20 and litter growth in dams fed the zinc deficient diet for the first half of pregnancy.



In Group B, the zinc-replete restricted-fed animals, there also appeared to be a significant correlation between maternal weight change and the serum zinc level, however, this was not apparent in the other groups .

6.4 DISCUSSION

The teratological features of zinc deficiency observed in this study agree well with those described by HURLEY & SHRADER (1972) and subsequently by ROGERS *et al* (1985) and DREOSTI *et al* (1985). When zinc-deficient diets were fed to rats according to different cycles for only the first 11 days of pregnancy, marked differences were observed between the two sets of litters. Group E dams, which were fed the zinc-deficient diet to induce low maternal serum zinc levels on days 8 and 9 of gestation had a higher incidence of total litter resorption than either of the other two zinc-deficient groups. This was probably due to the induction of gross, fatal malformations, including absence of neural tube closure and failure of the allantois to fuse with the chorion to form the chorio-allantoic placenta (Chapter 4; RECORD *et al* 1985b,c,f;1986). The principal defect in the live fetuses of this group involved the tail which is most sensitive to teratogenic insult around 10.5 of gestation (BARR 1973).

The other cycled group fed the zinc-deficient diet to induce low serum zinc levels on days 10 and 11 not only had an appreciable level of tail abnormalities, but a high incidence of fore-limb defects. In contrast to the other studies reported in this thesis, there did not appear to be any loss of litters due to the period of low maternal zinc status around the time of implantation. As expected, fetuses from dams fed the zinc-deficient diet ad lib throughout gestation showed a wide range of malformations which were induced at varying developmental stages.

The results from the section of the study on the animals fed the colony diet (containing 62ug Zn/g), as well as those from the animals fed the zinc-replete diet in restricted amounts (Group B) provide strong

experimental evidence linking the number of fetuses (and the total mass of the conceptuses) with the level of circulating maternal serum zinc.

At day 20, the normal litter of 12 fetuses contains approximately 800ug of zinc (this thesis, Chapter 7), most of which is accumulated during the last 48h. Assuming the dam consumes 40g of food over this period, the colony diet (62ug Zn/g) would supply approximately 2500ug of zinc. Obligatory urinary and faecal losses over this period appear to be approximately 2000ug (DREOSTI *et al* 1980b; Chapter 4), thus it can be seen that this level of dietary zinc is marginal. Animals fed the zinc-replete diet in restricted amounts (12g/day of 100ug Zn/g) would acquire about 2400ug zinc from dietary sources. It can be suggested therefore that the requirements of the litter are a major determinant in the level of circulating zinc as evidenced by the strong relationships between litter mass and maternal serum zinc levels. This aspect will be explored in greater detail in Chapter 7.

6.5 CONCLUSIONS.

Even though dietary zinc deficiency was used as the teratogenic agent in the early stages of gestation, such treatment was unlikely to have had any prolonged effect on the zinc status of the dams, as evidenced by the higher serum zinc levels in these dams when compared with the pair-fed controls at the end of pregnancy. Indeed it would seem likely that most agents which reduce either final litter weight or litter number during gestation might have a similar effect on the maternal serum zinc status.

Although extrapolation from animal to human studies is difficult, previous clinical studies (JAMESON 1976; McMICHAEL *et al* 1982; METCOFF 1983; FEHILY *et al* 1986) have suggested that in the case of small-for-age babies, there is also an inverse relationship between the weight of the newborn and the maternal serum zinc concentration. It can therefore be suggested that events in early gestation which retard embryonic or fetal growth might be

reflected at the end of pregnancy in the smaller baby exerting less demand on the circulating zinc pools of the mother. This effect could be of considerable clinical significance if the mother's zinc intake was marginal.

CHAPTER 7

EFFECT OF MATERNAL AGE ON THE TERATOGENICITY OF ZINC DEFICIENCY

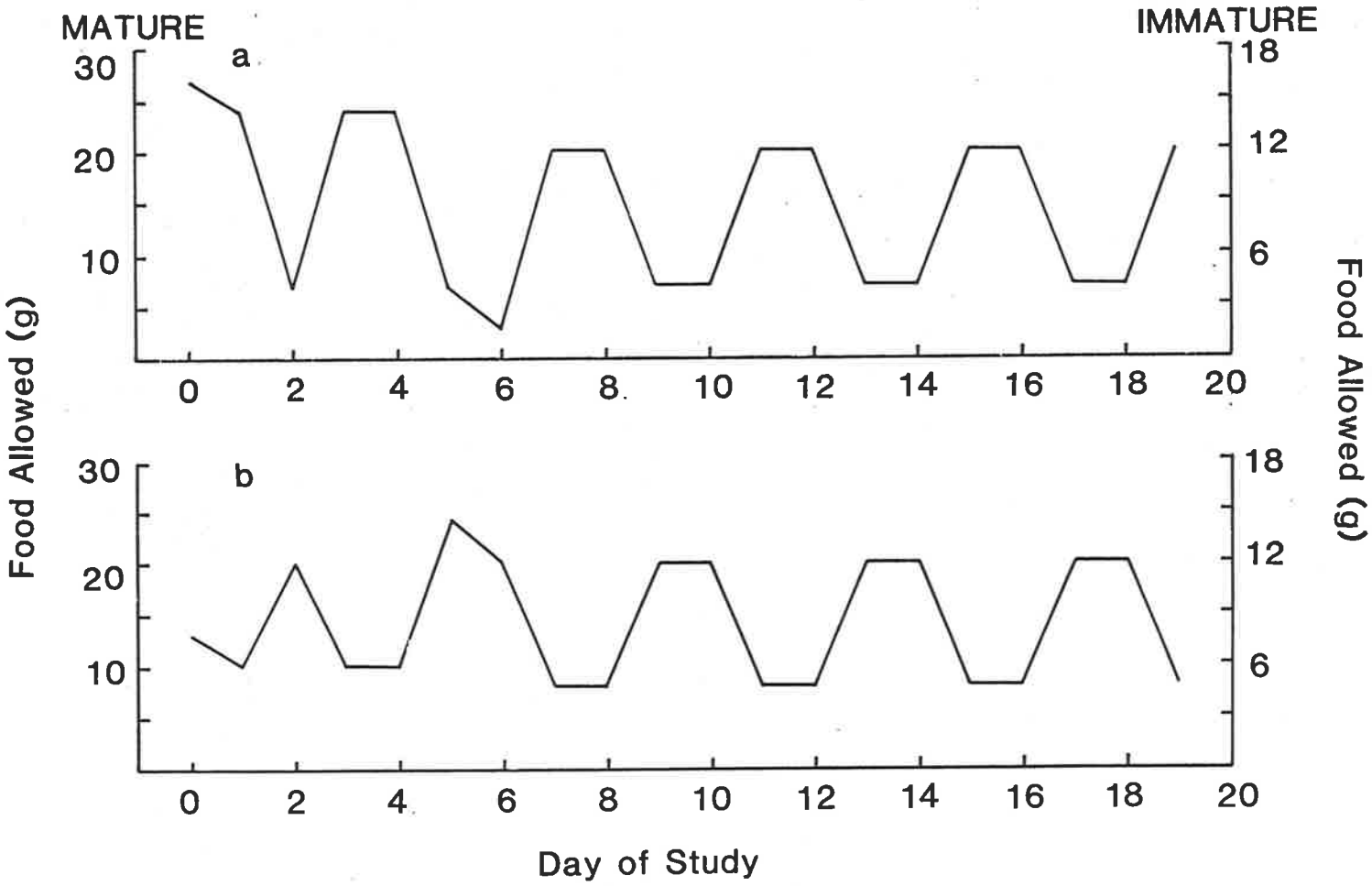
7.1 INTRODUCTION

In the previous chapter attention was drawn to the effect of litter size on maternal serum zinc concentration at the end of gestation. These observations suggested that, in the case of zinc, the metabolic stress produced by the litter during the latter part of pregnancy could only just be met by the dam. Other chapters of this thesis and earlier studies (MASTERS *et al* 1983; HURLEY 1985) have provided evidence to show that maternal catabolism and anabolism have profound effects on the supply of zinc to the litter. This second study of fetal development was designed to investigate other parameters such as maternal weight and the levels of trace element stores which it was felt might influence embryonic and fetal development. On the basis of previous evidence it was thought that older, heavier rats growing at a slower rate would firstly require less zinc for growth, and secondly be able to mobilise more zinc from their own dietary supply and tissue reserves. On the other hand, young dams undergoing a relatively rapid increase in body weight would have both smaller available zinc pools and a greater zinc requirement for growth. It was therefore suggested that the litters of young zinc-deficient dams would be affected earlier, and to a greater extent than those of older animals.

7.2 MATERIALS AND METHODS.

Animal husbandry and analytical techniques have been described in Chapter 2. For the purpose of this study, mature (av. wt. 320g) and juvenile (av. wt. 180g) dams were placed on a strict diet regime from the time of detection of pregnancy (Fig. 7.1a,b).

Fig 7.1 Feeding regime of zinc-deficient and replete mature and immature dams.



To compensate for differences in body weight, the quantities of diet (either zinc-deficent, containing less than 0.5ug Zn/g or zinc-replete, containing 100ug Zn/g) allowed to animals of each group were adjusted on a $(\text{body-weight})^{0.75}$ basis as suggested by MILLWARD *et al* (1981). The feeding patterns chosen were designed to elicit the maximal teratological response on day 11 (Cycle A) or the minimum response (Cycle B). Dams were killed on either day 11 of gestation to allow examination of the embryos, or on day 20 to examine the fetuses and to collect fetal and maternal tissues for trace element analysis.

7.3 RESULTS.

7.3.1 Embryonic development.

Of the 25 animals in this section of the study, two (both juvenile, one from each zinc-deficient group) had observable degenerating corpora lutea, but no implantation sites and were excluded from the study. Only minor, non-significant maternal weight changes were detected. Maternal serum zinc levels were reduced marginally as a result of dietary zinc deficiency, but the only major decline was in the Group B animals which had been fed the night before. There were no significant differences in the number of live embryos per dam, although the zinc-deficient Group A embryos from young dams were smaller in terms of length, somite number and protein than their respective pair-fed controls. Embryos from the mature dams, whether zinc-defiicient or replete were significantly larger than embryos from comparably cycled juvenile mothers (Table 7.1).

As can be seen from Table 7.2, embryos from Group A zinc-deficient dams were morphologically normal. Similar embryos from the juvenile group were however grossly malformed with rotational defects predominating, followed by anophthalmia, open neural tubes and placental defects.

Table 7.1 Growth and development of zinc-deficient and replete rat embryos at day 11.5.

Group	n	Maternal weight change	Live embryos per dam	Maternal serum zinc	Crown-rump length	Somite No.	Protein (ug)
MATURE							
A -Zn	3	+1.7±2.5	12.0±1.41	1.00±0.06	3.42±0.30	23.9±1.3	166±36[a]
A +Zn	3	+5.7±5.1	12.0±1.9	1.31±0.08	3.61±0.17	24.3±1.5	248±55
JUVENILE							
A -Zn	5	-11.4±5.7	10.0±2.8	1.01±0.10	2.01±0.6[a]	15.4±5.4[a]	83±40[a]
A +Zn	3	-0.7±4.7	12.3±1.1	1.19±0.11	3.29±0.3	21.4±2.5	189±17
B -Zn	5	-0.2±1.6	13.6±0.8	0.43±0.03[a]	3.28±0.2	23.1±1.2	210±39
B +Zn	4	+1.0±2.4	12.5±2.1	1.19±0.03	3.17±0.3	22.0±1.7	199±48

a: Significantly less than the respective control $p<0.05$.

Table 7.2 Malformations observed in zinc-deficient rat embryos at day 11.5.

	MATURE		JUVENILE			
	A -Zn	A +Zn	A -Zn	A +Zn	B -Zn	B +Zn
No. Dams	3	3	5	3	5	4
No. Embryos	37	44	61	39	71	50
Dead (%)	1 (3)	8 (18)	11 (18)	2 (5)	3 (4)	0
Allantoic fusion %	0	1	36	0	2	0
Flexion %	0	1	68	7	3	0
Neural tube closure %	0	1	41	3	3	0
Optic %	0	0	47	0	2	0

7.3.2 Fetal development.

Reproductive performances of the rats allowed to continue their pregnancy until the 20th day of gestation are presented in Table 7.3. All animals had either normal or degenerating corpora lutea in their ovaries, however there was a substantial loss of litters in both juvenile zinc-deficient groups. Only the results from animals with surviving fetuses have been included.

There was no alteration in the gross body weight of the juvenile groups, however the mature zinc-deficient dams lost weight. The pair-fed zinc-replete animals gained weight, and both ad lib fed zinc-replete groups gained significantly more than any other group. When the mass of the litter (total weight of fetuses and litters) was included, only the ad lib fed groups increased their body mass. The number of live fetuses was significantly reduced in both juvenile zinc-deficient groups, but not in the mature dams. Maternal serum zinc levels were significantly reduced in all zinc-deficient groups, although due to the dietary cycling, those of Group B (i.e. animals fasted on the previous night) were slightly higher than those of Group A (animals fed on the previous night).

As with the embryos, fetuses and placentae from the juvenile ad lib fed group were smaller than those of the older dams. Dietary restrictions also reduced fetal weights, and a superimposed zinc deficiency caused even greater reductions. Placental weights were not significantly affected, except by food restriction.

The incidence of fetal malformations was negligible in the zinc-replete litters, however 89% of the fetuses from the mature zinc-deficient dams and 65% from the juvenile groups were severely malformed (Table 7.4). Brain defects (principally hydrocephalus) were common on both juvenile zinc-deficient groups, with the only other significant cluster being a high incidence of tail defects in Group A. Fetuses from Group A mature dams had a high incidence of caudal defects (tail and clubbed feet), and 46% had brain

defects. Those fetuses from Group B also had a large number of tail defects, but the incidence of clubbed feet was less whilst that of a- or syndactyly was greater.

Table 7.3 Growth of zinc-deficient and replete rat fetuses at day 20.

Group	n	Maternal weight change (g)	Carcass weight change (g)	Maternal serum zinc (ug/ml)	No. live fetuses	Ave. fetal weight (g)	Ave. placental weight (g)	Total litter weight (g)
MATURE								
A -Zn	4	-18.0[b] ±7.1	-37.3 ±12.9	0.39[b] ±0.05	8.8[c] ±3.1	2.0[b] ±0.22	0.35 ±0.04	19.4[b] ±5.8
A +Zn	3	39.7 ±31.1	-19.3 ±17.3	0.82 ±0.13	16.3 ±2.7	3.11 ±0.39	0.42 ±0.01	59.7 ±14.9
B -Zn	3	-33.3[b] ±12.3	-49.0[b] ±15.0	0.60[b] ±0.07	6.7[b] ±3.2	2.24[b] ±0.34	0.38 ±0.06	15.9[b] ±6.3
B +Zn	4	75.5 ±35.5	16.8 ±37.5	0.86 ±0.09	163.8 ±0.9	3.04 ±0.10	0.44 ±0.01	58.6 ±4.1
Ad lib +Zn	4	140.8[a] ±16.1	88.5[a] ±16.3	0.97 ±0.06	12.5 ±2.5	3.56 ±0.15	0.57[a] ±0.05	52.2 ±10.7
JUVENILE								
A -Zn	5	-3.4 ±5.4	-32.2 ±5.8	0.30[b] ±0.10	11.4 ±1.5	2.27 ±0.17	0.34 ±0.03	28.9 ±3.0
A +Zn	5	24.0 ±5.7	-15.6 ±3.8	0.62[c] ±0.07	15.6 ±0.4	2.23 ±0.14	0.30 ±0.02	39.6 ±2.8
B -Zn	4	-1.3 ±7.6	-31.3 ±7.0	0.34[b] ±0.30	13.8 ±0.3	1.88 ±0.04	0.30 ±0.02	30.0 ±1.0
B +Zn	5	4.8 ±4.5	-24.8 ±5.3	0.87 ±0.12	11.6 ±2.0	2.32 ±0.16	0.34 ±0.03	29.7 ±4.3
Ad lib +Zn	5	107.8[a] ±13.0	60.4[a] ±12.5	0.89 ±0.09	13.0 ±0.8	3.19[a] ±0.13	0.47[a] ±0.02	47.2[a] ±2.4

a: Significantly greater than any other group $P < 0.05$
b: Significantly less than pair-fed control $p < 0.05$
c: Significantly less than ad lib control $P < 0.05$

Table 7.4 Incidence of Malformations in fetuses of rats fed zinc-deficient diets cyclically throughout gestation.

	JUVENILE			MATURE		
	Zinc-replete	Zinc-deficient A	Zinc-deficient B	Zinc-replete	Zinc-deficient A	Zinc-deficient B
No. of dams with live fetuses	15/15	5/8	4/8	11/15	4/5	3/4
No. of dams with abnormal fetuses	1/15	4/5	3/4	0/11	4/4	3/3
No. of fetuses	201	57	55	177	35	20
	No. %	No. %	No. %	No. %	No. %	No. %
No of fetuses	1 0	36 63	37 67	0 0	32 91	17 85
Eye	1 0.5	0 0	1 2	0 0	14 40	0 0
Brain	0 0	35 61	37 67	0 0	16 46	0 0
Agnathia	0 0	0 0	1 2	0 0	1 3	0 0
A- or syndactyly	0 0	0 0	0 0	0 0	2 6	3 15
Club feet	0 0	0 0	0 0	0 0	32 91	5 25
Tail	0 0	8 14	0 0	0 0	32 91	17 85
Umbilical hernia	0 0	0 0	0 0	0 0	1 3	0 0
Urogenital	0 0	0 0	1 2	0 0	3 9	0 0

Despite the small sample size in some of the groups, the zinc concentrations in maternal and fetal tissues revealed some interesting trends. There were no significant differences in maternal muscle and bone zinc concentrations, however in both mature and juvenile animals, maternal liver zinc concentrations were reduced in Group A dams when compared with Group B. Fetal brain and placental zinc concentrations were unaffected by the experimental treatments. Liver zinc concentrations were marginally reduced by dietary restriction and significantly reduced by maternal zinc deficiency. In this latter respect, fetuses from the younger dams were much more affected than those of the older animals. Carcass (body-liver-brain) zinc concentrations

were also affected in the juvenile groups but not in the mature groups. Total fetal zinc (fetus + placenta) was significantly reduced in both groups by dietary limitation and even more by zinc deficiency.

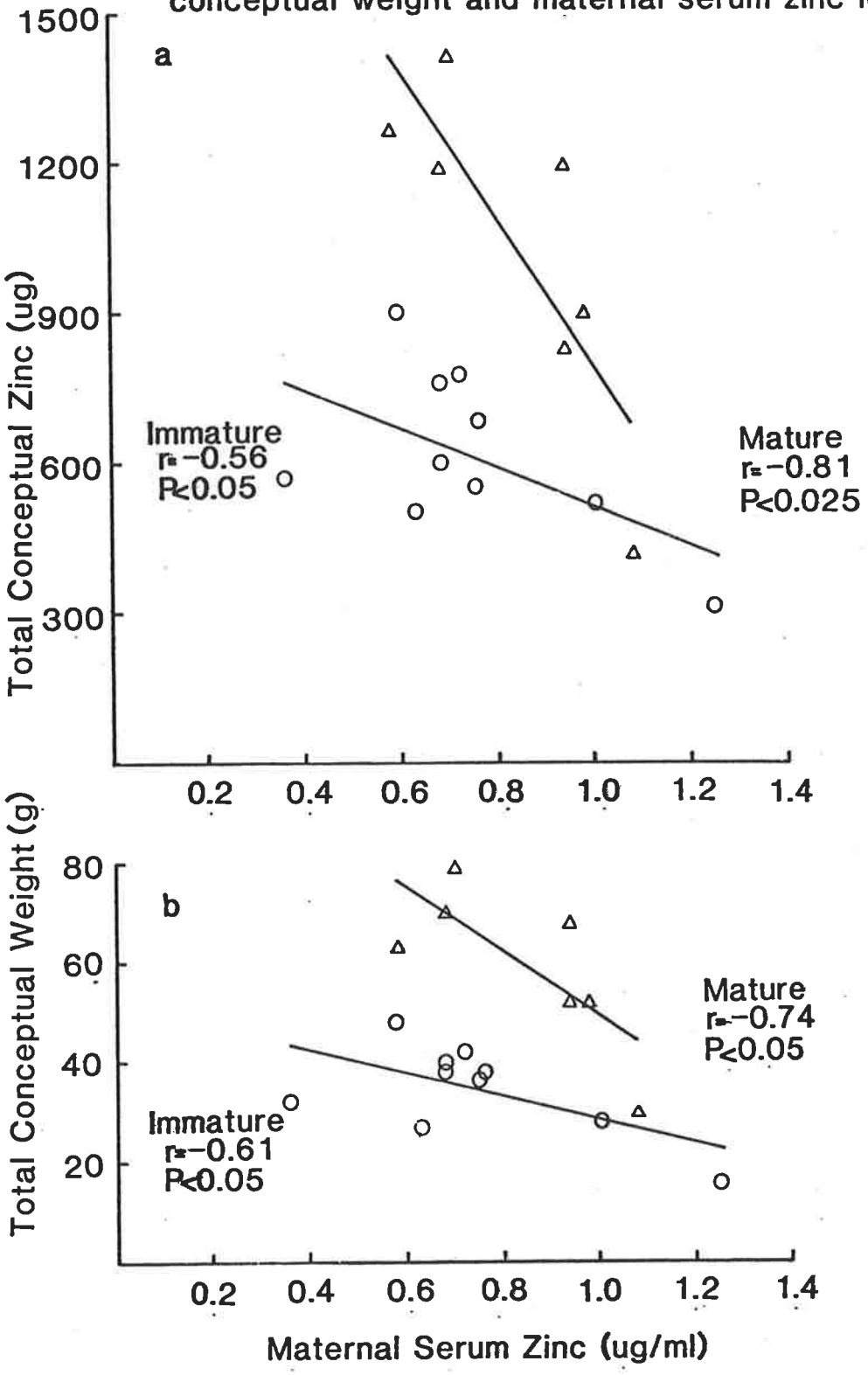
Table 7.5 Maternal and fetal tissue zinc concentrations.

	Maternal			Fetal				
Group	Liver ug/g	Muscle ug/g	Bone ug/g	Brain ug/g	Liver ug/g	Carcass ug/g	Placenta ug/g	Total (ug)
MATURE								
A -Zn	24.4 ±2.5	17.1 ±1.7	177 ±10	9.2 ±0.2	43.6 ±4.6	14.0 ±0.2	9.7 ±0.7	39.4 ±5.3
A +Zn	23.8 ±2.1	17.6 ±0.8	199 ±9	9.2 ±0.3	50.1 ±8.6	13.3 ±0.4	10.4 ±0.3	58.4 ±11.3
B -Zn	31.5 ±2.5	15.0 ±1.0	178 ±11	8.5 ±0.7	43.5 ±6.3	13.9 ±0.5	9.9 ±0.5	41.6 ±7.5
B +Zn	30.1 ±1.1	17.5 ±0.1	174 ±7	8.5 ±0.4	65.8 ±5.2	14.3 ±0.3	11.5 ±0.6	62.0 ±5.1
+Zn ad lib	28.2 ±0.5	17.2 ±0.8	146 ±26	8.4 ±0.2	73.3 ±5.9	15.4 ±0.4	11.4 ±0.3	84.3 ±8.7
JUVENILE								
A -Zn	22.5 ±3.7	16.7 ±1.7	119 ±18	9.2 ±0.5	20.3 ±0.2	11.1 ±0.2	11.8 ±1.6	30.4 ±5.7
A +Zn	23.9 ±1.2	18.4 ±0.8	160 ±18	9.4 ±0.2	56.9 ±2.8	14.4 ±0.4	12.0 ±0.2	45.4 ±4.2
B -Zn	28.6 ±1.1	20.0 ±0.6	174 ±53	8.7 ±0.1	14.9 ±1.1	9.8 ±0.2	11.5 ±1.6	24.7 ±1.5
B +Zn	29.0 ±1.4	18.6 ±0.8	156 ±3	9.2 ±0.1	61.6 ±3.7	15.0 ±0.7	12.0 ±0.4	48.2 ±5.0
+Zn ad lib	25.4 ±2.3	16.7 ±0.5	163 ±4	9.2 ±0.2	73.8 ±4.3	15.6 ±1.4	12.4 ±0.3	74.6 ±6.5

As with the previous study (Chapter 6) correlation between various fetal and maternal parameters were examined. Maternal serum zinc levels in the zinc-replete pair-fed groups were inversely related to both total conceptual mass and total conceptual zinc although there was no apparent

relationship between these parameters in the other groups (Figure 7.2). Maternal serum zinc was also the only parameter observed to be correlated (directly or inversely) with litter size or zinc content.

Fig 7.2 Relationship between total conceptual zinc, total conceptual weight and maternal serum zinc levels.



7.4 DISCUSSION.

Results of this study demonstrate clearly the differences in sensitivity to the teratogenic effects of zinc deficiency between older (heavier) and younger (lighter) animals, and enlarge on the observations of others (MASTERS *et al* 1983; HURLEY 1985). In the first part of this study, apart from confirming the observations in Chapter 4 and subsequent publications (RECORD *et al* 1985b,c,d,1986; DREOSTI *et al* 1985), it was apparent that even under the most teratogenic conditions (food cycle A), embryos from older zinc-deficient dams were morphologically normal, although retarded in growth. Embryos from the younger dams subjected to the same feeding cycle were, on the other hand, grossly malformed and stunted. Also in accord with previous studies, young animals subjected to the reverse cycle (B) were slightly smaller than their controls, but otherwise unaffected.

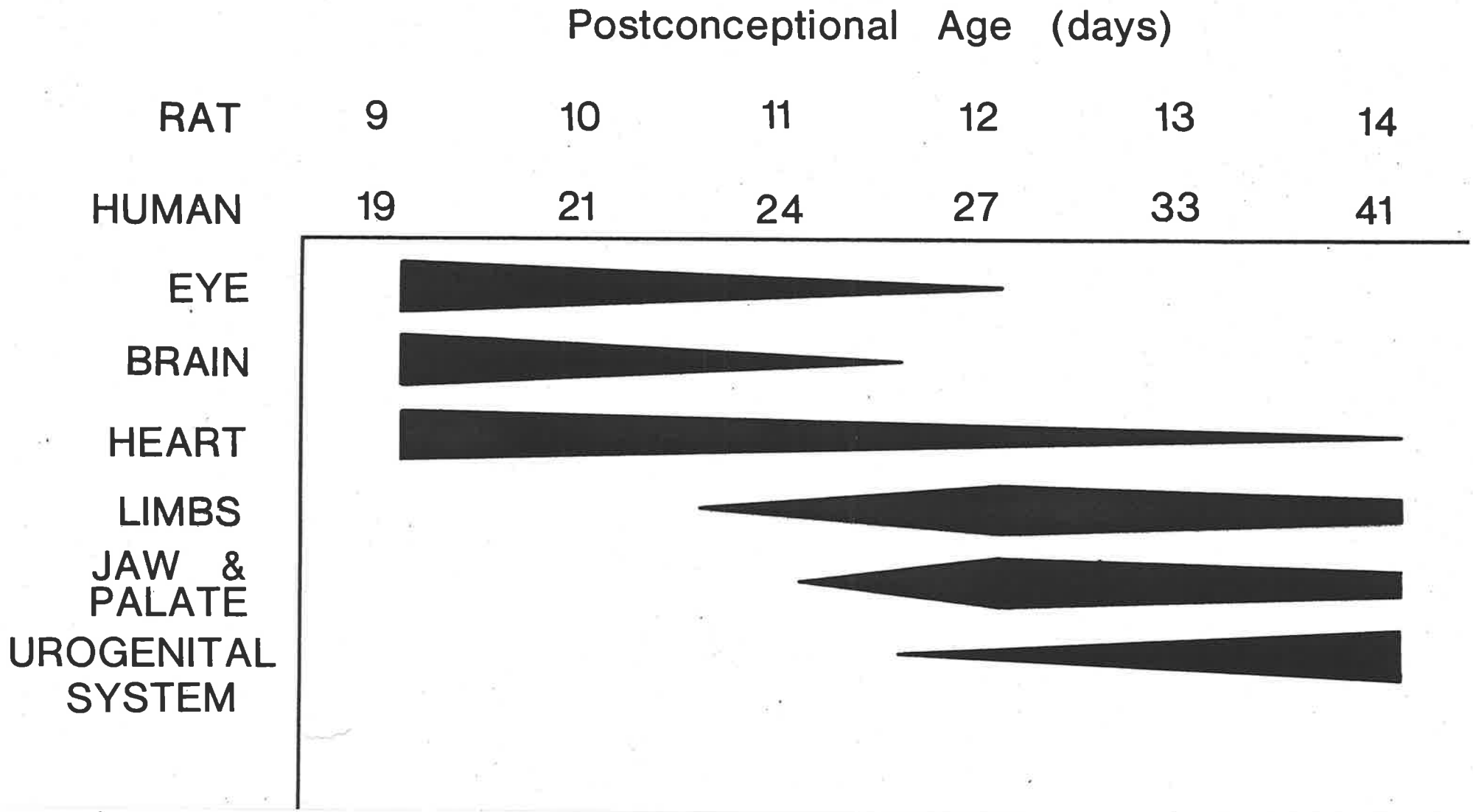
From the large number of juvenile animals which had lost their litters by day 20 of gestation, it can be deduced that the major deformities observed on day 11 proved lethal to the developing fetuses. This was understandable in the case of Group A dams whose embryos had a high incidence of rotational, placental and neural tube defects. Less expected was the high rate of embryonic or fetal loss in the Group B zinc-deficient juvenile group. Previous observations (Chapter 5; RECORD *et al* 1985b,c,d) have shown that such embryos contain large areas of necrotic cells in the neural epithelium, somites and limb buds. It would seem therefore that this degree of damage cannot be accomodated by the embryo which subsequently perishes. Many embryos from the mature dams managed to survive the critical periods of organogenesis, and developed less severe, but still major, defects. By comparing the periods of minimal maternal serum zinc levels with the known times of maximum sensitivity of organ systems (Fig. 7.3), it can be seen that most of the observed defects occurred during the period of 11 to 14 days. Due to the

juxtaposition of the feeding cycles (as one group was entering a fast and the other leaving), as well as the extended period over which the organs develop, it is not surprising that both groups displayed some similarities in the types of malformation.

As was observed by other workers using either marginally zinc-deficient diets (HALAS *et al* 1982; GREELEY 1984; HERZFELD 1985; FAIRWEATHER-TATE 1985b) or essentially zinc-free diets (HURLEY & SWENERTON 1966; HURLEY & SWENERTON 1971; APGAR 1975; MASTERS *et al* 1983; REINSTEIN *et al* 1984; ROGERS *et al* 1985) concentrations of zinc in the livers and carcasses of all zinc-deficient fetuses were reduced when compared with the pair-fed controls. In the present experiment there were also large differences between fetuses from young and old zinc-deficient dams fed to the same schedules. The extra zinc available to the fetuses probably arises as a result of tissue catabolism resulting in a nett weight loss of the older dams. Younger dams are probably more under the influence of hormones such as growth hormones thus do not catabolise tissue as readily (MILLWARD *et al*, 1981). This provides further evidence to support the suggestion that maternal-fetal zinc transfer is limited in the case of the juvenile dam.

There is no clear explanation for the reductions in maternal liver zinc concentrations in all Group A animals. As these dams were allowed the maximal amount of diet on the night before they were killed, it is possible that all were forced into an anabolic state, with zinc either being redistributed to other organs and used for tissue growth, or passed to the intestinal tract and subsequently excreted. Indeed balance studies (Chapter 4) showed that faecal zinc excretion increased during the feeding phase of the cycle.

Fig 7.3 MAJOR ORGAN SYSTEMS INFLUENCED BY TERATOGENS



Maternal muscle and bone zinc concentrations did not alter significantly as a result of dietary zinc deficiency, an observation which agrees with other reports (HURLEY & SWENERTON 1966; APGAR 1975; HERZFELD *et al* 1985). However it must be considered that the "average" 250g rat contains approximately 5800ug of zinc (WILLIAMS *et al*, 1977), and the "average" zinc-deficient litter in this study contained only 300ug zinc, i.e. about 5% of the total, it is unlikely changes would be detected unless zinc were lost preferentially from a particular tissue.

The lack of correlation between maternal serum zinc levels and either mass or zinc content of the conceptuses in the ad lib and zinc-deficient groups suggests that, in the first instance sufficient zinc was ingested to meet the requirements of the litter, and in the second that the maternal serum zinc levels had decreased to a functional minimal value. The fact that a negative correlation exists between these parameters in the restricted fed animals suggests that the dietary intake of these animals is marginal for fetal development. Calculations performed in Chapter 6, and evidence from both HURLEY & SWENERTON (1971) and WILLIAMS *et al* (1977) show that during the last few days of gestation, approximately 200-250ug zinc is transferred to the litter each day. Data from Chapter 4 showed that, over the first half of pregnancy, rats consuming 12g diet/day (i.e. 1200ug Zn/day) excreted approximately 1000ug/day, leaving only 200ug/day for tissue growth. Over the 24hr before death, young rats in this study were allowed either 400 or 1200ug zinc and the older animals either 750 or 2000ug zinc. Even if all the dietary zinc could be absorbed, rather than the more usual estimates of 10-40%, it can be seen that a major proportion of the zinc would be required for fetal development. This suggests that even with an appropriate dietary zinc concentration, the absolute amount of zinc may limit fetal growth.

7.5 CONCLUSIONS.

From these studies it can be seen clearly that it is not only the dietary zinc concentration which has a profound effect on embryonic and fetal development, but that the metabolic state of the dam, and her requirements for, or ability to relase zinc also play a significant role in the development of the litter. It is possible that these observations may have some relevance in the human situation when the dietary zinc intake is marginal, and may help to provide an indication of the type of woman most likely to be at risk of complications during pregnancy.

CHAPTER 8

EFFECT OF ZINC DEFICIENCY ON THE RAT EMBRYO PRIOR TO DAY 9.5

8.1 INTRODUCTION

The studies reported in this chapter were carred out in order to expand some of the observations reported in the earlier chapters. In the initial studies on day 11.5 embryos it was noted that, on some occasions, zinc-deficient dams allowed access to relatively large amounts of diet around day 6 (the time of implantations in the rat) had no implantation sites in the uteri, although corpora lutea were present (e.g. Table 4.13). As will be discussed in Chapter 9, there is also a substantial embryonic loss in zinc-deficient mice, with no evidence of implantation having occurred.

It was felt that there were three major events which could be affected by maternal zinc deficiency and lead to the loss of embryos. The first was fertilisation, which has been shown to be sensitive to zinc deficiency (HURLEY & SWENERTON 1966). In order to avoid complications of this type, it has been found necessary to refrain from instituting the zinc-deficient regime until after mating. It is therefore unlikely that this process was affected in any of the studies reported in this thesis. It was also thought possible that early embryonic development might be compromised with the embryo perishing prior to implantation. Indeed it has already been reported (HURLEY & SHRADER 1975) that three and four-day-old rat embryos show abnormalities due to a maternal zinc deficiency. The third possibility was that the process of implantation was inhibited by zinc deficiency, either by affecting the priming of the uterus by progesterone and oestrogen (NALBANDOV 1971) or by inhibiting trophoblastic outgrowth or uterine stromal proliferation.

It was therefore decided to place pregnant rats on cycled zinc-deficient or replete diets for the first 3, 5, 7 or 9 days of gestation, then allowing them to return to a zinc-replete diet until day 11.5 when the embryos would be removed and assessed for any effects on growth and morphology. In addition, embryos were also flushed from the fallopian tubes of some dams after 3 days of gestation in order to establish firstly that fertilisation and ovulation had taken place, and secondly to confirm the findings of HURLEY & SHRADER (1975) that such embryos were affected by zinc deficiency and could resume normal development. In this way it was hoped to ascertain whether the time of implantation was particularly sensitive to a reduction in circulating zinc.

The developmental changes which are encompassed in this study are as follows.

The first cleavage of the fertilised ovum usually occurs about 24h after copulation, although some eggs do not divide until 48h after ovulation (NICHOLAS 1962). As the zygotes progress towards the uterus, they undergo more or less regular cell divisions, so that at the end of 4 days the embryo is composed of about 16 cells arranged around a blastocyst cavity. This stage probably represents the first morphogenetic change in the development of the embryo (DUCIBELLA *et al* 1975). Until the formation of the blastocyst cavity, the cells of the morula are totipotent, but once the cavity has formed, the position of the cell largely determines its fate.

The blastocyst "hatches" from the zona pellucida on the fifth day of pregnancy, an event which may be initiated by endogenous hormone secretions of the embryo (DIKMANN & DEY 1974).

Implantation of the embryo into the uterine wall at about day 6 requires a complex set of interactions between the trophectoderm and the uterine tissue. Implantation appears to rely heavily upon hormonal

conditions of the uterus which must occur during a 12h period soon after the zona pellucida have been shed. The rat appears to be unique in that the uterus must have been primed with progesterone for at least 48h, followed by a burst of oestrogen (NALBANDOV 1971), prior to implantation which occurs progressively during the next 24-48h.

8.2 MATERIALS AND METHODS

In order to elicit the greatest response, dams weighing between 170-190g were used as they appeared to have a greater sensitivity to low zinc status (Chapter 7). Animals were mated as described in Chapter 2 and fed either the zinc-deficient or replete diet in a cyclical manner similar to that described earlier, except that the dams were allowed alternate periods of unrestricted access to food or were completely fasted (Table 8.1). In this manner it was hoped to elicit maximal fluctuations in serum zinc levels. Dams were allowed unrestricted access zinc-replete diet from days 3, 5, 7 or 9 in order to ascertain the subsequent effects of a transitory zinc deficiency.

Table 8.1 Feeding regime of zinc-deficient and replete rats.

Day	Cycle	
	A	B
0	Ad Lib	Ad Lib
1	Ad Lib	Fast
2	Ad Lib	Fast
3	Fast	Ad Lib
4	Fast	Ad Lib
5	Ad Lib	Fast
6	Ad Lib	Fast
7	Fast	Ad Lib
8	Fast	Ad Lib
9	Ad Lib	Fast
10	Ad Lib	Fast
11	Sacrifice	

In addition several animals (zinc-deficient and replete) were killed on day three and their fallopian tubes and uteri flushed with Hanks Balanced Salt Solution from the fimbriated ostium to recover and examine

the early embryos (HURLEY & SHRADER 1975). The remainder of the animals were killed on day 11 and the embryos removed for examination as described earlier (Chapter 2).

8.3 RESULTS

8.3.1 3 Day rat embryos.

As can be seen from Table 8.2 there were a large number of abnormal 3-day embryos in the zinc-deficient group compared with those from the zinc-replete dams.

Table 8.2 Effects of maternal zinc deficiency on rat embryos after 3 days gestation.

	Group	
	+Zn	-Zn
No. dams with corpora lutea	10	9
Corpora lutea/dam	14.7±0.7	14.0±0.9
Embryos recovered/dam	8.6±0.7	7.3±1.0
% recovery	59	52
Malformed embryos/dam	1.1±0.6	5.7±1.3[a]
% Malformed embryos	13	77
Maternal serum zinc	1.26±0.03	0.78±0.08[a]

a : Significantly different to control value $p < 0.001$

The major abnormalities in the zinc-deficient embryos included slow development (approximately 20% of the recovered embryos were of only 1 or 2 cells) whilst the remainder of the abnormal embryos showed gross fragmentation of most blastomeres as reported by HURLEY & SHRADER (1975), however there was usually at least one normal blastomere.

8.3.2. 11.5 Day rat embryos

As can be seen from Table 8.3, the feeding of a zinc-deficient diet (irrespective of the cycle) did not cause a significant reduction in either the number of dams continuing their pregnancy (as judged by the number of dams with corpora lutea but no embryos) or the number of embryos implanting. The food intake did however seem to affect the implantation rate. Those dams fasted on day 7 (both zinc-replete and deficient) had a lower implantation rate, although this was not consistent as the animals fed the zinc-deficient diet (Cycle A) until day 9, then allowed zinc-replete diet suffered no apparent implantation loss.

Table 8.3 Reproductive performance of zinc-deficient and replete rats.

Days of cycled Feeding		Group A+Zn	A-Zn	B+Zn	B-Zn
0-3	Dams with corpora lutea	5	6	-	-
	Dams with embryos	5	6	-	-
	Implantation rate (%)	91	84	-	-
0-5	Dams with corpora lutea	6	6	5	7
	Dams with embryos	6	4	5	6
	Implantation rate (%)	83	51	88	84
0-7	Dams with corpora lutea	13	14	10	12
	Dams with embryos	11	9	10	10
	Implantation rate (%)	65	63	91	86
0-9	Dams with corpora lutea	4	6	5	4
	Dams with embryos	3	6	4	3
	Implantation rate (%)	67	100	81	81

Growth of the embryos decreased marginally with the length of time the dams were fed the zinc-deficient diets (Table 8.4). The incidence of malformations observed in both groups of zinc-deficient embryos at day 11.5 was greater than the pair-fed animals in all cases, although the incidence increased sharply when the animals continued their zinc-deficient regime

until day 9. There was however no apparent increase in the mortality rate. It was also of interest to note that the growth of the zinc-replete group A embryos maintained on the regime until day 9 was also slightly retarded when compared to the other zinc-replete group.

Table 8.4 Growth and development of zinc-deficient and replete rat embryos.

Days of cycled Feeding		Group A+Zn	Group A-Zn	Group B+Zn	Group B-Zn
0-3	Embryos	69	65	-	-
	Length (mm)	3.45±0.10	3.34±0.32	-	-
	Somites	24.7±0.3	23.6±1.8	-	-
	Dead	0	5	-	-
	Malformed	1 (1%)	7 (11%)	-	-
0-5	Embryos	59	59	63	81
	Length (mm)	3.40±0.10	3.12±0.28[a]	3.67±0.10	3.25±0.19[a]
	Somites	23.7±0.4	22.6±1.5	24.4±0.5	22.5±1.1[a]
	Dead	2	9	5	4
	Malformed	1 (2%)	6 (10%)	0	5 (6%)
0-7	Embryos	110	118	118	139
	Length (mm)	3.32±0.09	3.12±0.10[a]	3.50±0.07	2.77±0.14[a]
	Somites	24.3±0.5	22.9±0.5[a]	24.3±0.4	20.0±1.1[a]
	Dead	5	2	9	10
	Malformed	5 (5%)	9 (8%)	1 (1%)	16 (12%)
0-9	Embryos	33	63	48	39
	Length (mm)	2.54±0.23	2.85±0.3	3.70±0.3	3.17±0.6[a]
	Somites	19.4±1.9	21.4±1.06	25.4±1.1	21.4±3.4[a]
	Dead	5	1	2	4
	Malformed	4 (12%)	29 (46%)	0	7 (18%)

a : significantly less than the respective control group $p<0.05$.

8.4 DISCUSSION

Although the results of this study did not provide experimental evidence to support the suggestion that the process of implantation could be adversely affected by zinc deficiency, even though the dams were under considerable metabolic stress, several important effects have been highlighted.

In the first instance, the effect of a maternal zinc deficiency on very early (3.5 day) embryos has been confirmed. This was first reported

by HURLEY & SHRADER (1975) but to the author's knowledge has not been repeated. The reasons for the apparent fragmentation or death of the blastomeres is not known, however it might be attributable to the same effect that was observed in the older rat and mouse embryos (Chapters 5 and 9).

In other studies (HURLEY & SHRADER 1972) dams fed a zinc-deficient diet for only the first 4 days of pregnancy had normal fetuses at day 21, suggesting that the pluripotent nature of the pre-implantations blastomers allowed complete recovery. In this study it was apparent that there were significantly more malformed embryos at day 11.5 in the group exposed to zinc deficiency for the first 3 days of gestation than in the control group. The possibility exists that, in the current study, the dams, due to their age and metabolic requirement for zinc, were unable to supply sufficient metal to the embryos to continue normal development.

Until after day 7 the incidence of malformations remained relatively constent, despite the cyclical feeding. This is in accord with previously established principles that the effect of a teratogenic stimulus applied before about day 8 of gestation is either embryotoxic or has little effect (BEAUDOIN 1978). There was however a dramatic increase in the number of abnormal embryos in the rats fed the zinc-deficient diet until day 9, confirming the observations discussed in Chapters 3 and 5.

8.5 CONCLUSIONS

It was apparent from this study that a zinc deficiency during the period 7-9 days was sufficient to cause a large increase in the incidence of abnormalities observed at day 11, thus confirming findings reported earlier in this thesis. It was also confirmed that pre-implantation embryos could be affected by maternal zinc deprivation, but the majority had sufficient regenerative capacity to develop normally. No specific effect on the process of implantation was detected.

CHAPTER 9

GROWTH AND DEVELOPMENT OF THE ZINC-DEFICIENT MOUSE EMBRYO

9.1 INTRODUCTION

Despite the extensive investigations into the effects of a maternal dietary zinc deficiency on the *in utero* development of the rat, surprisingly little is known about a deficit of this metal on the mouse. The teratogenicity of zinc deficiency in this species has been described in conjuction with acetazolamide (HACKMAN & HURLEY 1981a) and as a co-teratogen with alcohol (MILLER *et al* 1983; KEPPEN *et al* 1985) and alone (SATO *et al* 1985; DREOSTI *et al* 1986).

The latter report was based upon the results of studies reported in this thesis (Chapters 4 and 5) and elsewhere (RECORD *et al* 1985b,c,d,f,1986; DREOSTI *et al* 1985) and established that the strain of mice used (C57B2/6J) were highly sensitive to zinc deficiency and required at least 5 ugZn/g diet to retain any fetuses and more than 10ug Zn/g diet to avoid the occurrence of malformations. These observations are in agreement with those of previous studies (HACKMAN & HURLEY 1981a; MILLER *et al* 1983; KEPPEN *et al* 1985; SATO *et al* 1985). Of particular importance was the previous study from this laboratory (DREOSTI *et al* 1986) which showed clearly that, unlike rats, mice consumed their diet on a constant basis, with no cyclical feeding pattern. The suggestion was made that due to the absence of food cycling, and the transient rises in maternal serum zinc levels associated with a maternal fast, the mouse embryos were subjected to extended periods of zinc impoverishment.

The studies described in this chapter were conducted both to confirm these earlier findings (DREOSTI *et al* 1986) and to compare the effects of maternal zinc deprivation with the effects reported for the rat in Chapters 4 and 5. Accordingly pregnant mice were allowed free access to diets of

varying zinc contents (<0.5ug Zn/g, 5.0ug Zn/g or 100ug Zn/g) and the embryos removed for examination after 9.25d gestation (equivalent to 11.5d in the Sprague-Dawley rat).

9.2 MATERIALS AND METHODS

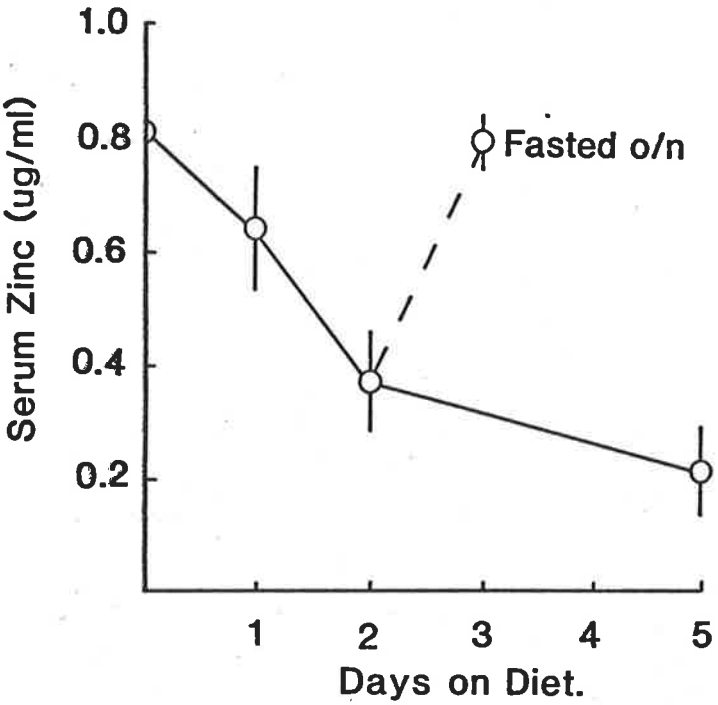
Mice of the C57Bl/6J strain were obtained at 8-10 weeks of age from the Gilles Plains Animal Resource Centre, Adelaide, South Australia, and allowed free access to food and water as described in Chapter 2. Female mice were placed in a darkened cage with males (4 females/2 males) for 2h from 08.30 to 10.30h each morning and examined hourly for the presence of a vaginal plug. Thereafter they were housed individually in stainless steel and plastic cages and allowed free access to various diets containing either <0.5, 5 or 100ug Zn/g diet. On the 9th day of gestation (after 220hr gestation) dams were anaesthetised with ether, maternal blood samples collected by cardiac puncture and the ovaries and uteri excised. The number of corpora luteae were counted, and embryos removed from the uterus and dissected away fro the extra-embryonic tissues for morphological assessment and protein determination. Representative embryos from each dietary group were also fixed in Karnovsky's fixative for light microscopy as described earlier (Chapter 2).

9.3 RESULTS

In common with rats, the serum zinc level of mice decreased rapidly following institution of a zinc-deficient regime, and rose over an overnight fast (Fig. 9.1). In the mice fed the two lower levels of zinc there was a substantial pre-implantation embryonic loss and also a small decrease in the number of implanted embryos. There was a surprisingly high proportion of dead embryos in the control (100 ppm zinc) group, however the incidence of malformations was considerably higher in the groups fed low levels of zinc and ranged from 100% in the zinc-deficient group to 36% in

the group fed 5ug Zn/g diet. In general the malformations were very similar to those observed in rats (Chapter 4), although in contrast to rats, which do not respond in this manner until the dietary zinc level falls below 1ug Zn/g (ROGERS *et al* 1985), a dietary zinc concentration of 5ug Zn/g produced a high incidence of developmental anomalies in mice.

Fig. 9.1 Changes in mouse serum zinc levels following supply of a zinc-deficient diet.



In terms of size, the embryos from zinc-deprived dams were extremely small. Those embryos from dams fed 5ug Zn/g were significantly shorter and contained much less protein than the control embryos, although the somite number was not greatly affected. Photographs of the representative embryos are presented in Figures 9.2a,b,c.

Embryos from each group were also sectioned and examined under the light microscope. In common with the zinc-deficient rats, embryos from both the zinc-deprived and low zinc dams had extensive areas of necrosis, unlike the control embryos which showed no such effect (Fig. 9.2c,d,e)

Figure 9.2.Embryos from mice fed the zinc-replete diet (a) or (b)5ug Zn/g or (c) zinc-deficient diet. Embryo a is normal, embryo b is poorly rotated, anophthalmic and has an open neural tube (arrow). Embryo c has not started to rotate, the neural tube is open and there are cranio-facial defects. Bar=1mm.
Figures 9.2 d-f show sections (1um) through the cranial regions of embryos similar to those in a,b and c. Section d shows a normal neural epithelium and mesoderm, whereas e shows limited evidence of cell death and large extra-cellular spaces. Section f shows extensive cell necrosis in both the neural epithelium and mesoderm. Bar=25um.

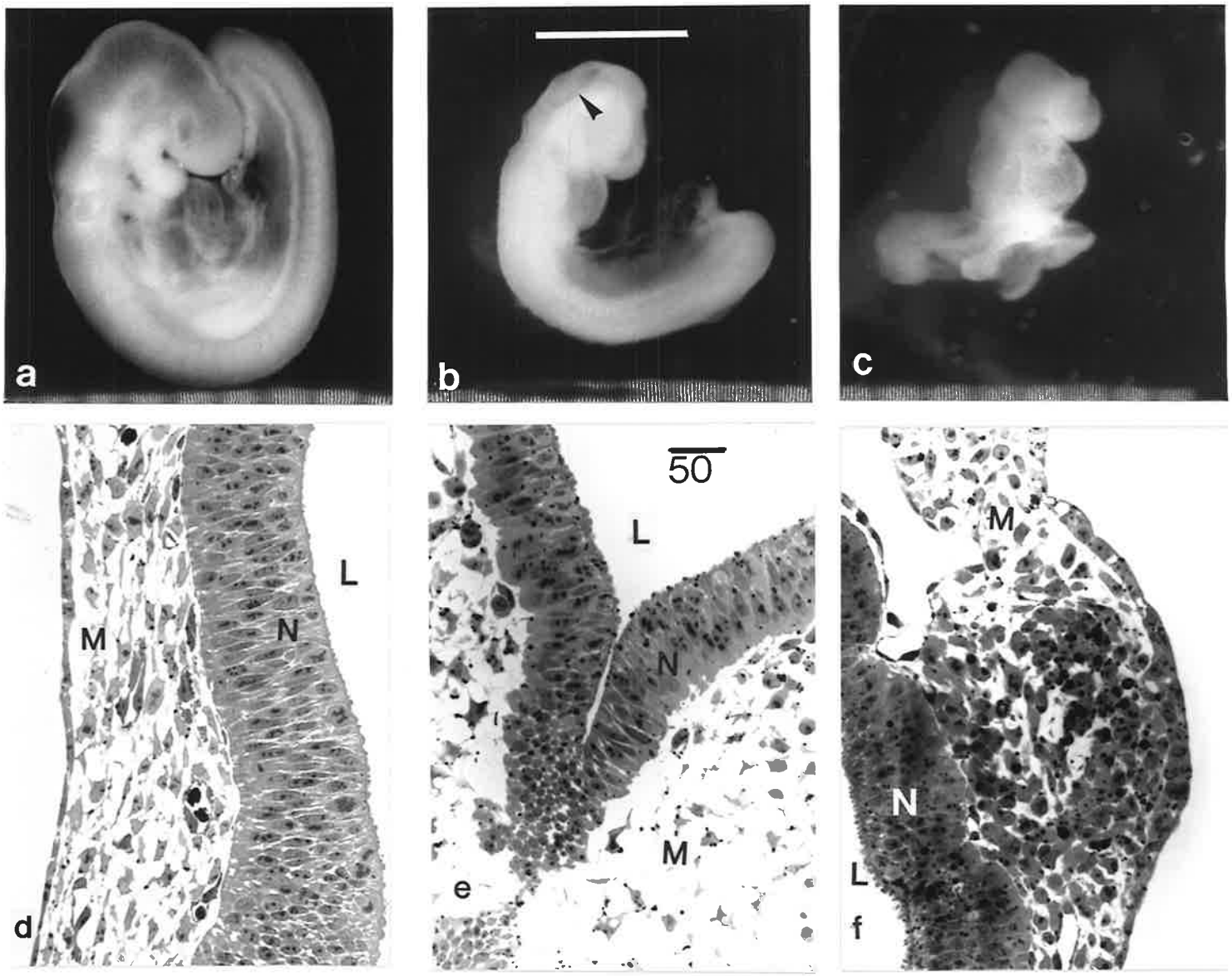

a
b
c
50
L
N
M
d
e
f

Table 9.1 Effects of feeding diets containing different levels of zinc on embryonic development in pregnant mice.

	Dietary zinc level (ug/g)		
	< 0.5	5.0	100
Dams with corpora luteae	6	11	6
Dams with embryos	3	6	5
Maternal serum zinc (ug/ml)	0.41±0.09[b]	0.80±0.03[b]	0.96±0.03
Total number of embryos	16	47	34
Dead embryos	5	5	10
Malformed embryos	11	15	1
Defects:			
Chorio-allantoic fusion	11	0	1
Rotation	1	1	1
Open neural tube	11	9	0
Other (anophthalmia, distorted head, neural tube)	(a)	5	0
Crown-rump length (mm)	0.71±0.08[b]	2.09±0.07[b]	2.51±0.07
Somite number	(a)	22.4±0.4	23.6±0.4
Protein (ug/embryo)	25±16[b]	107±7[b]	152±10

(a) : Due to the severity of the malformations, these values could not be determined.
(b) : Significantly less than control group (100 ug/g) $P<0.05$.

9.4 DISCUSSION.

As with rats, the serum zinc level of mice fed a zinc-deficient diet fell rapidly. However, as has been shown previously, the food intake of mice does not fluctuate in a cyclical manner (DREOSTI *et al* 1986). This absence of food cycling implies that the serum zinc levels do not fluctuate as they do in rats. Indeed in this study there was no evidence of significant fluctuations in serum zinc levels (Fig. 9.1), even though food deprivation following a period of zinc deficiency did lead to an increase in serum zinc levels. It has been suggested (DREOSTI *et al* 1986) that the continued exposure of the embryos to low maternal serum zinc levels is a major factor in the increased sensitivity of this strain to low maternal dietary zinc levels which have been observed in a number of other studies (HACKMAN & HURLEY 1981a; MILLER *et al* 1983; KEPPEN *et al* 1985; SATO *et al*

1985). The large litter loss prior to day 9 suggests that had similar studies to those conducted on rats and discussed in Chapter 8 been performed using mice, indications as to any effects of zinc deficiency on the process of implantation might have been observed.

It is also of interest to note that the serum zinc levels of the dams fed 5ug Zn/g were only marginally below those allowed the zinc-replete ration, and the embryonic size was only slightly reduced, yet the incidence of malformations was high. Whether the increased sensitivity of this strain was due to a poor transfer of the metal from the dam to the embryo, or to some other metabolic factor remains unclear. The observation that cell necrosis in the mouse was found in similar embryonic regions to the rat embryo also provides additional evidence to support the contention that a maternal zinc deficit has a similar mode of action in both species.

9.5 CONCLUSIONS.

The current study has not only confirmed these findings, and has demonstrated that zinc deficiency in mice can severely affect the embryo before implantation, but has also provided evidence to show that the cellular necrosis induced by zinc deficiency in rats (Chapter 5) also occurs in the mouse embryo. This demonstration that the cytotoxic effects of zinc deprivation leading to the observed terata are not species-specific, and indeed even occur at dietary zinc levels of 5ug Zn/g could be interpreted to suggest that this model might more suitably represent the human situation where (in Western societies) frank zinc deficiency is rare, but marginal dietary zinc intakes are common (RECORD *et al* 1985a)

CHAPTER 10

GENERAL DISCUSSION

10.1 INTRODUCTION.

Since the initial observations that a maternal dietary zinc deficiency was highly teratogenic in rats (HURLEY & SWENERTON 1966) there have been many studies into both the occurrence and the reasons for the wide variety of defects observed. The most detailed study of the dysmorphologies (HURLEY AND SHRADER 1972) illustrates the range of terata and also the rapidity with which they can be produced. Despite the immense number of biochemical studies conducted on zinc-deficient tissue, it has not been possible to isolate the primary locus of action whereby zinc - or a deficit of the metal - exerts its effects on cell replication or embryonic and fetal growth and development.

In this chapter it is the intention of the the author to draw together the various aspects of the effects of a dietary zinc deficiency which have been described in this thesis, as well as in writings by other workers, firstly to describe the events leading to the production of teratogenic abnormalities, secondly to propose biochemical or physiological explanations for the phenomena and finally to suggest how these observations might be of relevance the human situation.

10.2 DEVELOPMENT OF THE TERATA INDUCED BY MATERNAL ZINC DEPRIVATION.

The studies reported in Chapter 3 of this thesis demonstrated that it was not possible to induce a teratogenic zinc deficiency *in vitro* by simply culturing embryos in serum obtained from zinc-deficient dams . There was however a very significant finding that some, but not all, embryos removed from zinc-deficient dams after 9.5 days of gestation were malformed at that stage. It was also discovered that these embryos continued to develop

abnormally, even in zinc-sufficient medium, over the next 48h. On the other hand, apparently normal embryos removed from zinc-deficient dams continued to develop normally, and indeed grew to the same extent as the zinc-replete embryos.

This diversity in appearance of 9.5-day embryos prompted the studies reported in the next two chapters (Chapters 4 and 5) of this thesis. In Chapter 4 it was demonstrated that the phenomenon of cyclical feeding which becomes apparent after about three days of consumption of a zinc-deficient diet was intimately linked with maternal serum zinc levels and the appearance of the embryos at day 11.5 of gestation. It is of interest to note that, although this eating pattern has been observed and studied by several other groups of workers (CHESTERS & QUARTERMAN 1970; WILLIAMS & MILLS 1970; GRIFFITH & ALEXANDER 1972; CHESTERS & WILL 1973; WALLWORK *et al* 1981,1982;WALLWORK & SANDSTEAD 1983), this is, to the author's knowledge the first instance where the effect has been linked to embryonic or fetal dysmorphologies. From these studies it can be suggested that, as the dam feeds and fasts, alternating between anabolic and catabolic states, so the circulating zinc levels alter, thus exposing the embryo or fetus to approximately two days of zinc deprivation followed by two days of comparative zinc repletion. Indeed reports from other workers suggest that such rats do fluctuate between anabolic and catabolic states (GIUGLIANO & MILLWARD 1983,1984). Furthermore it has been shown (MASTERS *et al* 1983; HURLEY 1985) that force-feeding of pregnant zinc-deficient dams late in gestation adversely affects the developing litter, and conversely, fasting can ameliorate the problems. Similar effects have also been reported to occur in monkeys (GOLUB *et al* 1982; HURLEY 1985).

Histological examination of zinc-deficient and control embryos taken during the period from day 9.5 to day 11.5 yielded more valuable information about the teratogenic response. From the information presented in Chapter 5, it can be seen that periods of low circulating maternal zinc

levels were associated with the appearance of unscheduled cell death within the embryonic tissues.

Although cell death has been reported to occur in the intestines and pancreas of zinc-deficient animals (ELMS 1977; KOO & TURK 1977; ELMS & JONES 1980) and other subcellular changes such as testicular atrophy (DIAMOND & HURLEY 1970), chromosomal abnormalities in sperm and ova (WATANABE *et al* 1983) and also in fetal liver and bone marrow (BELL *et al* 1975) have been described, to the author's knowledge the experiments reported in Chapters 5 and 9 and elsewhere (RECORD *et al* 1986a,b) are the first to describe unscheduled cell death in the zinc-deficient embryo of any species. The pathologies observed are similar to those described by ELMS (1977), KOO & TURK (1977) and ELMS & JONES (1980) for zinc deficiency, and also to those described for many other teratogens e.g. alcohol (BANNIGAN & BURKE 1982) arsenic (MORRISSEY & MOTTET 1983) cadmium (MESSERLE & WEBSTER 1980) ethylnitrosourea (FUJIWARA 1980) as well as many others (reviewed by SCOTT 1977). Although in this case it seems reasonable to propose that the cellular necrosis is largely responsible for the terata, as the foci of necrosis correlate well with subsequently observed malformations, SCOTT (1977) has pointed out that not all embryonic necroses preceed dysmorphologies, nor do all teratogens induce necrosis. The mechanisms of cell necrosis in this study are not clear, however as discussed later, the author feels that membrane lipid peroxidation might be a factor.

The increase in the mitotic index of cells in the neuroepithelium and limb-buds of morphologically normal embryos (take when the maternal serum zinc levels were high) was in agreement with the observations of others (HURLEY & SHRADER 1972; ECKHERT & HURLEY 1977). However, there did not appear to be an accumulation of cells at any particular stage of mitosis as might be expected if there was a blockage in this stage of the cell cycle. Although it is recognised that this increase in the mitotic index might

might reflect a general lenghthening of the cell cycle time, this author feels that this increased mitotic rate is a real expression of a "catch-up" growth phase occurring as a response to a greater availability of circulating zinc or of material salvaged from engulfed cells and cellular debris.

The information accumulated in these chapters formed the basis for further studies into the effects of a maternal zinc deprivation during other periods of gestation. In Chapter 6, the ability of the embryos to recover after an 11-day period of zinc deficiency was studied. Apart from a large litter loss in one group of zinc-deficient animals, the most striking observation in this study was the inverse correlation between the surviving conceptual mass on day 20 and the maternal serum zinc levels at that time. In this study the dams were returned to a zinc-replete diet after 11 days of gestation, thus it is unlikely that a transient zinc deprivation could have exerted a direct influence on the circulating zinc levels after this period. Indeed the only conclusion which can be drawn from this study is that the size of the litter has a profound influence on the maternal serum zinc levels when the dietary zinc intake is marginal. This aspect will be discussed in the context of human zinc metabolism late in this chapter.

Chapter 7 provides further evidence to support the hypothesis espoused here and elsewhere (MASTERS *et al* 1983; RECORD *et al* 1985b,f,1986; HURLEY 1985) that the maternal metabolic state is of great importance in both embryonic and fetal development. The studies reported here demonstrate that younger, growing dams have a greater zinc requirement for their own anabolism, thus limiting the amount of zinc available to the litter. Older dams, on the other hand, do not require as much zinc for their own tissues and are more able to meet the needs of the embryos. As pregnancy progressed, the grossly malformed embryos from the young dams died and were resorbed, whereas those of the older dams continued development, albeit

more slowly, giving rise to a large number of grossly malformed embryos at the end of gestation.

In Chapter 4 it was noted that several zinc-deficient dams allowed their food cyclically to induce low serum zinc levels at about the time of implantation had no observable implantation sites, and it was proposed that the process of implantation might be affected adversely. This possibility was addressed in Chapter 8. Institution of a zinc-deficient regime shortly after the time of mating did not reduce the number of ova fertilised, as judged by the number of embryos recovered on day 4. In this study the earlier findings of HURLEY & SHRADER (1975) were confirmed in that it was apparent that embryos recovered on day 3 were abnormal and fragmenting. The pluripotent nature of the blastomeres and early embryos was illustrated by the ability of embryos exposed to a zinc-deficient environment to recover by day 11 of gestation. Indeed it was demonstrated in this study that the majority of dysmorphologies did not arise until after day 7. The reasons for the peri-implantation loss observed in Chapter 4 remain unclear.

In order to ascertain whether or not the embryonic cell necrosis was confined to only this one species, similar studies were repeated on mice (Chapter 9). In this strain of mice, which does not consume the zinc-deficient diet in a cyclical manner (DREOSTI *et al* 1986), there was a high incidence of pre-implantation loss in the group fed diet containing less than 0.5ug Zn/g. The few surviving embryos from this group, as well as those recovered from dams fed diets containing 5ug Zn/g were grossly malformed and showed areas of necrosis similar to that observed in rats. These observations provide strong evidence to support the contention that these necroses observed as a result of maternal zinc deprivation are not peculiar to one species.

The sequence of events leading to the production of congenital abnormalities and intra-uterine growth retardation by maternal zinc

deprivation can be summarised in the following manner:

It is apparent that after consumption of a zinc-deficient diet for 2-3 days, rats become anorexic and reduce their food consumption. While the food consumption is normal, the dam is still in an anabolic state. Apparently due to the maternal demand for zinc and the requirement for digestive enzymes, the serum zinc level falls rapidly. If the onset of the zinc-deficient regime coincides with the time of conception, then the developing embryo (which derives its nutrients from the uterine fluid) is also subjected to a reduction in the amount of available zinc, resulting in fragmentation of the blastomeres.

As is the case with other teratogens a transient zinc deficiency at this point does not appear to have any lasting influence on embryonic development. This is most probably due to the pluripotent nature of the embryonic cells whose fate does not appear to be determined until the time of cavitation. In the rat, however, food consumption declines and the maternal serum zinc level increases, presumably due to maternal tissue catabolism causing the uterine fluid zinc concentration to increase and allowing embryonic growth and differentiation to continue.

Once implanted in the uterine wall, the embryo undergoes a phase of rapid growth and differentiation when the organ anlagen appear and the embryo is most sensitive to teratogenic stimuli (BEAUDOIN 1979). During this time (in particular before day 11 or 12 when the placenta becomes functional) periods of low circulating zinc levels are associated with the death of dividing and differentiating embryonic cells . Fragments of dead cells are engulfed by healthier neighbours. Cell death at this stage can result in both a reduction in the final size of the organ and dysmorphologies, depending upon the cells affected (SCOTT 1977). The remnants of the dying cells, together with any zinc they contain, will undoubtedly be available for re-utilisation by healthy cells, thus allowing

those to proliferate, possibly at an increased rate .

It is perhaps most likely that this is the critical time for embryonic survival. If the chorion and the allantois fail to fuse to form the chorio-allantoic placenta, or if the vitelline vessels connecting the embryo to the visceral yolk sac do not form, then the embryo will perish due to lack of nutrients in general. Other gross defects, such as open neural tubes may also contribute to embryonic death.

In the fetal period cellular necrosis is not as obvious, although isolated foci of necrotic cells are detectable until just prior to delivery (DREOSTI *et al*, unpublished results). This apparent reduction in cell death is probably due to several factors. Firstly the proportion of cells within the fetus undergoing division and differentiation decreases. As will be discussed later, this appears to be the critical stage for the survival of the cell. It is also possible that other established cells within the fetus have accumulated zinc during the previous maternal catabolic phase, and that these cells can, to some extent, meet the requirements of the neighbouring cells. Over the period from day 11 to 15 the decidual tissue surrounding the embryo regresses and disappears. It is not unlikely that the zinc contained in this tissue can be re-utilised by the fetus to supplement that derived directly from the maternal circulation. In this way the embryo can obtain sufficient zinc to support limited growth until term. As the conceptual mass increases so does the demand on the maternal zinc supply. If the zinc supply to the dam is limited, then she must rely on tissue catabolism to meet the needs of the litter. Smaller dams have lesser zinc stores than larger dams and therefore are more likely to lose their litters. The larger dams are more able to meet the demands, and carry their fetuses to term.

It can be appreciated that, in zinc-deficient pregnant rats, the exact timing of the peaks and troughs of the feeding cycle have a profound effect on the development of individual organs. In studies involving

zinc-deficient rats it is not sufficient to use only pair-fed controls, but attention must be paid to ensure that such animals are in comparable stages of the feeding cycle.

In contrast to rats, zinc-deficient mice do not consume their food cyclically, thus the dams to not alternate between anabolic and catabolic states. Therefore at no stage is the mouse embryo or fetus able to enjoy an adequate zinc supply.

10.3 MODE OF ACTION OF ZINC DEFICIENCY.

While the reasons for the death of the cells remain unclear, it is tempting to speculate that the cause might lie in the vulnerability of cell membranes to peroxidative damage in zinc-deficient tissue. It has long been suggested (CHVAPIL 1976; BETTGER & O'DELL 1981) that zinc ions play an important role in the structure and functions of biomembranes, and studies from this laboratory have shown that free superoxide radicle production is increased as a result of zinc deficiency (DREOSTI & RECORD 1978) and the levels of malondialdehyde (an indicator of membrane damage) in fetal rat liver microsomes were elevated as a result of both zinc deficiency and alcohol (DREOSTI *et al* 1985).

It can be suggested therefore that the picture of intracellular damage and subsequent cell death which is a feature of both zinc deficiency and alcohol damage arises as a result of intracellular membrane damage. There is also a mounting body of evidence from other workers to support the suggestion that zinc deficiency can affect biomembranes. It has been shown (BETTGER *et al* 1978) that erythrocytes from zinc-deficient rats showed increased osmotic fragility and that supraphysiological levels of zinc protect normal erythrocytes from such damage (CHVAPIL *et al* 1974). As mentioned in Chapter 1, the antioxidant vitamin E reduces the degree of lipid peroxidation in zinc-deficient chickens (BETTGER *et al* 1980) although supplementation with this vitamin failed to reduce the incidence of

teratology in zinc-deficient rats (HURLEY *et al* 1983). It has been suggested (BETTGER & O'DELL 1981a) that institution of a zinc-deficient regime firstly depresses the extracellular zinc concentration, then circulating zinc-depleted ligands re-extract zinc from the cellular plasma membranes resulting in a reduction in membrane integrity, specifically in an increased sensitivity to peroxidative damage and other functional deficits followed by a need to increase energy expenditure to maintain cellular integrity, decreased macromolecular biosynthesis and altered lipid, carbohydrate and protein metabolism and water balance.

The suggestion that zinc ions are of critical importance in cell replication has also been made (CHESTERS 1978,1982). As has been discussed in Chapter 1, zinc plays an important role in every facet of cell division, including protein, RNA and DNA synthesis. Much attention has been placed on the observation that the incorporation of [^{3}H]-thymidine into newly synthesised fetal DNA (SWENERTON *et al* 1969; ECKHERT & HURLEY 1977; DREOSTI *et al* 1985) and the activities of two metalloenzymes thymidine kinase (DREOSTI & HURLEY 1975; DUNCAN & HURLEY 1978; RECORD & DREOSTI 1979) and DNA polymerase (DUNCAN & HURLEY 1978) are significantly reduced as a result of a dietary zinc deficiency. It has been demonstrated (RECORD 1980; RECORD *et al* 1980; DREOSTI 1985) that the activity of thymidine kinase, the enzyme responsible for re-utilising preformed thymidine to synthesise new DNA was not the rate-limiting step in DNA synthesis and that the activity of some other enzyme e.g. DNA polymerase might be of greater significance. Indeed, as CHESTERS (1982) has pointed out, the reduction in thymidine incorporation induced by zinc deficiency is paralleled by the decrease in the number of cells with labelled DNA (FUJIOKA & LIEBERMAN 1964; RUBIN 1972; SARYAN *et al* 1979). This would not be expected if a general reduction in the synthesis of zinc metalloenzymes necessary for DNA synthesis had occurred. In fact it would appear that zinc-deficient cells either synthesise new DNA or they do not. Inspection of the micrographs in

Chapter 5 also shows apparently healthy, normal mitotic cells next to fragmenting or dying cells, which tends to support this view.

There is also good evidence to show that zinc deficiency delays the cells at the G2/M boundary, although other transitions such as G1 to S and S to G2 are also delayed (FALCHUK *et al* 1975). It is possible that, in the case of the embryonic rat, zinc released from dying cells may allow nearby cells to continue their cycle. Other cells might accumulate at a boundary and be released once the maternal serum zinc level rises, resulting in a more synchronous passage through mitosis and producing an increased mitotic index (HURLEY & SHRADER 1975; RECORD *et al* 1985; Chapter 5) rather than a blockage in mitosis as was proposed earlier (SWENERTON & HURLEY 1969; HURLEY & SHRADER 1972). Although the possibility cannot be discounted that the cells are merely progressing through their cycle at a slower rate, this would appear unlikely as the dams do have normal circulating zinc levels at this stage.

As has been discussed in Chapter 1, it can be argued that there exists a role for free zinc ions in the reversible denaturation of DNA (EICHORN 1973) and also the initial phase of thermal unwinding (SUBIRANA 1973) probably relating to the removal of the H1 histone (BARRETT 1976) and phosphorylation which is related to DNA synthesis (SLUYSER 1977). More recent evidence shows that zinc ions might be a specific second messenger of mitogenic induction (GRUMMT *et al* 1986). Indeed these observations might explain the observed chromosomal abnormalities in zinc-deficient sperm and ova (WATANABE *et al* 1983) and fetal liver and bone marrow (BELL *et al* 1975). Although the above studies were carried out *in vitro*, if these processes require free or loosely bound (e.g. metallothionine-bound) zinc, it would explain the major effects of zinc deficiency which are accompanied by only small changes in tissue zinc concentrations.

Indeed the requirement for zinc ions would explain why, under certain circumstances other divalent metals such as cadmium can substitute for

zinc, and also why these elements are antagonistic towards zinc.

The precise role of zinc in cell division and differentiation remains unclear. If, however, zinc ions or at least readily exchangeable zinc is essential for these processes described above it is tempting to speculate that in the cell undergoing division or differentiation zinc is required for DNA unwinding, replication and re-association, and in the absence of sufficient free zinc, sequesters the metal from various membranes. As a consequence of the metabolic activities, the membranes undergo peroxidative damage and the cell perishes. In the case of a quiescent cell, there is little need for zinc in the replicative process, except to allow normal protein synthesis. Should such a cell be stimulated to divide in some way (e.g. by lectin activation or partial hepatectomy) then, the process leading to cell division cannot commence until sufficient zinc is acquired by the cells. This hypothesis would explain the cell death occurring in normally rapidly dividing tissues such as in the embryo, the testes and small intestine, and the lack of cell death in other non-dividing tissues.

10.4 ZINC DEFICIENCY IN THE HUMAN SITUATION.

Although extrapolation of animal studies to humans is fraught with danger, it seems appropriate to attempt to place the observations detailed in this thesis in a human context. It has been stated in Chapter 1 that the majority of human zinc deficiences are conditioned rather than frank, even though the dietary zinc intake of many women is marginal. It is also apparent that about 60% of all human conceptions do not give rise to healthy, normal children. In the view of the author, it is possible that many instances of human reproductive failure, particularly in early gestation might result from a low to marginal zinc intake, exacerbated by one or more of the factors listed in Table 1.2. Other factors having a bearing on the materno-fetal relationship have been described in Chapters 6 and 7.

In the first of these (Chapter 6) it was described how, in the presence of limited dietary zinc intake, the demands of the conceptual mass had a profound effect on the maternal serum zinc levels which provided an explanation for the observed inverse relationship between birthweight and maternal serum zinc levels previously observed (JAMESON 1976; McMICHAEL *et al* 1982; METCOFF 1983; FEHILY *et al* 1986). Although in this study zinc deficiency prior to day 11 produced the variation in fetal number and weight necessary to obtain a correlation, it is more than likely that any such effect in early pregnancy unrelated to zinc metabolism, could produce similar results. It can therefore be proposed that elevated maternal zinc levels associated with a small fetus need not be indicative of some defect in the transport of zinc from the mother to the fetus.

The second study (Chapter 7) provided good evidence to show that the size of the mother, and hence her zinc stores, could profoundly affect the development of the embryo and fetus. In the first facet young (180g) zinc-deficient females had a far greater incidence of embryonic abnormalities than the older (310g) females, although those younger dams lost their litters shortly afterwards. The older dams on the other hand managed to retain their litters, although the majority of fetuses were malformed. It is possible that human studies would show that either young, growing women are more at risk of having complications of pregnancy if the dietary is marginal than older women who are not growing, or subject to a weight loss during pregnancy, but consume similar amounts of zinc. To the knowledge of the author these factors during pregnancy have not yet been investigated.

In addition there is no evidence that humans fed a zinc-deficient diet consume their food cyclically. In this respect perhaps mice provide a more appropriate model. From the information in Chapter 9 it can be seen that mice are much more sensitive to zinc deficiency than rats, and that the incidence of embryonic and fetal malformations is high even when the diets

contain 5ug Zn/g. On a daily basis this is equivalent to about 0.5mg Zn/Kg body weight. The human RDA is about 0.2mg/Kg body weight. Even allowing for the differences in conceptual mass at term between the species (the mouse litter weight being about 25% of the total body weight at term versus about 10% for the human) it can be suggested that the human fetus is only just being supplied with sufficient zinc. If the human mother is herself in an anabolic state, or has limited zinc reserves, then fetal growth and development may be at risk.

In summary then, it can be suggested that the human female most at risk of having complications at any stage of pregnancy due to a deficit of zinc (a) has a marginal zinc intake (b) is at risk from an induced zinc deficiency (c) is unable to catabolise her own tissues to release zinc.

10.5 CONCLUSIONS

The studies reported in this thesis have shown for the first time that, in pregnant rats and mice, maternal dietary zinc deprivation induces abnormal cell death in embryonic tissues during the critical periods of organogenesis. Furthermore the importance of the maternal feeding cycle in the teratological response has also been elucidated. As a corollary to these studies, some interactions between maternal and fetal metabolism in the latter part of gestation have been studied.

CHAPTER 11

BIBLIOGRAPHY

Alvarez, OF. & Meyer, J. (1968). Regional Differences in Parakeratotic Response to Mild Zinc Deficiency. Arch. Dermatol. 98,191-201.

Alvarez, OF. & Meyer, J. (1973). Thymidine Uptake and Cell Migration in Cheek Epithelium of Zinc Deficient rats. J. Oral Pathol. 2,86-94.

Apgar, J. (1970). Effect of Zinc Deficiency on Maintainance of Pregnancy in the Rat. J. Nutr. 100,470-476.

Apgar, J. (1973). Effect of Zinc Repletion Late in Gestation on Parturition in the Zinc-Deficient Rat. J. Nutr. 103,973-981.

Apgar, J. (1975). Effect of Some Nutritional Deficiencies in Parturition in Rats. J. Nutr. 105,1553-1561.

Australian Bureau of Statistics. (1985a). Causes of Death in Australia, 1983. Commonwealth of Australia, Canberra.

Australian Bureau of Statistics. (1985b). Perinatal Deaths in Australia, 1983. Commonwealth of Australia, Canberra.

Bannigan, J. & Burke, P. (1982). Ethanol Teratogenicity in Mice: A Light Microscopic Study. Teratology. 26,247-254.

Barney, GH., Macapinlac, MP., Pearson, WN. & Darby, WJ. (1967). Parakeratosis of the Tongue - A Unique Histopathologic Lesion in the Zinc-Deficient Squirrel Monkey. J. Nutr. 93,511-517.

Barney, GH., Orgebin-Crist, MC. & Macapinlac, MP. (1969). Genesis of Oesophogeal Parakeratosis and Histologic Changes in the Testes of the Zinc-Deficient Rat and Their Reversal by Zinc Repletion. J. Nutr. 95,526-534.

Barr, M. (1973). The Teratogenicity of Cadmium Chloride in Two Stocks of Wistar Rats. Teratology. 7,237-242.

Barrett, T. (1976). Role of Histone H1 in Chromatin Condensation. Nature. 260,576-578.

Bassiouni, BA., Foda, AL. & Rafe, AA. (1979). Maternal and Fetal Plasma Zinc in Pre-Eclampsia. Europ. J Obstet. Gynec. Reprod. Biol. 9,75-80.

Bauld, R., Sutherland, GR., & Bain, AD. (1974). Chromosome Studies in Investigations of Stillbirths and Neonatal Deaths. Arch. Dis. Child. 49,782-788.

Beaudoin, AR. (1979). Embryology and Teratology. In "The Laboratory Rat. Vol. 2. Research Applications". (Baker, HJ., Lindsey, JR. & Weisbroth, SH., eds.) Academic Press, New York.

Beck, F. (1981). Induced Cell Injury and Cell Death as a Cause of Congenital Malformations in Rats. Histochem. J. 13,677-679.

Becker, WM. & Hoekstra, WG. (1971). The Intestinal Absorption of Zinc. In "Intestinal Absorption of Metal Ions, Trace Elements and

Radionuclides".(Skoryna, SC. & Waldron-Edward, D., eds.) Pergamon, Oxford.

Beckman, DA. & Brent, RL. (1984). Mechanisms of Teratogenesis. Ann. Rev. Pharmacol. Toxicol. 24,483-500.

Bell, LT., Branstrator, M., Roux, C. & Hurley, LS. (1975). Chromosomal Abnormalities in Maternal and Fetal Tissues of Magnesium- or Zinc-Deficient Rats. Teratology. 12,221-226.

Berglund, T. (1984). Zinc - Biological Effects. University of Stockholm. Institute of Physics Report #84-12.

Bergman, R. & Soremark, R. (1968). Autoradiographic Studies on the Distribution of zinc-65 in Mice. J. Nutr. 94,6-12.

Bergmann, KE., Makosch, G. & Tews, KH. (1980). Abnormalities of Hair Zinc Concentration in Mothers of Newborn Infants with Spina Bifida. Am. J. Clin. Nutr. 33,2145-2150.

Bertrand, G. & Bhattacherjee RC. (1934). L'Action Combinee du Zinc et des Vitamins dans l'Alimentation des Animaux. Cr. Acad. Sci. 198, 1823.

Bettger, WJ., Fish, TJ. & O'Dell, BL. (1978). Effects of Copper and Zinc Status of Rats on Erythrocyte Stability and Superoxide Dismutase Activity. Proc. Soc. Exp. Biol. Med. Sci. 158,279-282.

Bettger, WJ., Reeves, PG., Savage, JE. & O'Dell, BL. (1980). Interaction of Zinc and Vitamin E in the Chick. Proc. Soc. Exp. Biol. Med. Sci. 163,432-436.

Bettger, WJ. & O'Dell, BL. (1981). A Critical Physiological Role of Zinc in the Structure and Function of Biomembranes. Life Sci. 28,1425-1438.

Blamberg, DL., Blackwood, WB., Supplee, WC. & Combs, CF. (1960). Effect of Zinc Deficiency in Hens on Hatchability and Embryonic Development. Proc. Soc. Exp. Biol. Med. 104,217-220.

Brown NA. & Fabro, S. (1981). Quantitation of Rat embryonic Development *In Vitro*: A Morphological Scoring System. Teratology. 24,65-78.

Burnet, FM. (1981). A Possible Role of Zinc in the Pathology of Dementia. Lancet. 1,186-188.

Cavdar, AO., Arcasoy, A., Baycu, T. & Himmetoglu, O. (1980). Zinc Deficiency and Anencephaly in Turkey. Teratology. 22,141.

Casey, CE. & Hambidge, KM. (1980) Epidemiological Aspects of Human Zinc Deficiency. In "Zinc in the Environment." (Nriagu, JO., ed.) Wiley, New York. 1-28.

Catalanotto, FA. (1978). The Trace Metal Zinc and Taste. Am. J. Clin. Nutr. 31,1098-1103.

Cerklewski, FL. & Forbes, RM. (1976). Influence of Dietary Zinc on Lead Toxicity in the Rat. J. Nutr. 106,689-696.

Cherry, FF., Bennett, EA., Bazzano, GS., Johnson, LK., Fosmire, GJ. & Bateson, HK. (1981). Plasma Zinc in Hypertension/Toxaemia and Other Reproductive Variables in Adolescent Pregnancy. Am. J. Clin. Nutr.

34,2367-2375.

Chesters, JK. (1972). The Role of Zinc Ions in the Transformation of Lymphocytes by Phytohaemagglutinin. Biochem. J. 130,133-139.

Chesters, JK. (1975). Comparison of the Effects of Zinc Deficiency and Actinomycin D on Ribonucleic Acid Synthesis by Stimulated Lymphocytes. Biochem. J. 150,211-218.

Chesters, JK. (1978). Biochemical Functions of Zinc in Animals. World Rev. Nutr. Diet. 32,135-164.

Chesters, JK. (1982). Metabolism and Biochemistry of Zinc. In "Clinical, Biochemical and Nutritional Aspects of Trace Elements."(Prasad, AS., ed.) Liss, New York. pp 221-238.

Chesters, JK. & Quarterman, J. (1970). Effects of Zinc Deficiency on Food Intake and Feeding Patterns of Rats. Br. J. Nutr. 24,1061-1069.

Chesters, JK. & Will, M. (1973). Some Factors Controlling Food Intake by Zinc-Deficient Rats. Br. J. Nutr. 30,555-566.

Chesters, JK. & Will, M. (1978). Effects of Age, Weight and Adequacy of Zinc Intake on the Balance Between Alkaline Ribonuclease and Ribonuclease Inhibitor in Various Tissues Of the Rat. Br. J. Nutr. 39,375-382.

Chvapil, M. (1976). Effect of Zinc on Cells and Biomembranes. Med. Clin. North Am. 60,799-812.

Chvapil M., Peng, YM., Aronson, AR. & Zukoski, C. (1974). Effect of Zinc on Lipid Peroxidation and Metal Content in Some Tissues of Rats J. Nutr. 104,434-443.

Cockroft DL. (1979). Nutrient Requirements of Rat Embryos Undergoing Organogenesis *In Vitro*. J. Reprod. Fert. 57,505-510.

Cohen, IK., Schechter, PJ. & Henkin, RI. (1973). Hypoguesia, Anorexia, and Altered Zinc Metabolism Following Thermal Burn. J. Am. Med. Assoc. 223,914-916.

Crosby, WM., Metcoff, J., Costiloe, JP. et al. (1977). Fetal Malnutrition. An Appraisal of Correlated Factors. Am. J. Obstet. Gynecol. 128,22-31.

Damyanov, I. & Dutz, W. (1971). Anencephaly in Shiraz, Iran. Lancet. 1,82.

Davies, NT. (1980). Studies on the Absorbtion of Zinc by Rat Intestine. Br. J. Nutr. 43,189-203.

Davis, GK. (1980). Micronutrient Interactions of Zinc, Copper and Iron in Mammalian Species. In "Micronutrient Interactions: Vitamins. Minerals and Hazardous Elements." (Levander, OA & Cheng, L., eds.) Ann. NY. Acad. Sci. 355,130-137.

Day, HG. & McCollum, EV. (1940). Effects of Acute Dietary Zinc Deficiency in the Rat. Soc. Exp. Biol. Med. Proc. 45,282-284.

Diamond, I. & Hurley, LS. (1970). Histopathology of Zinc-Deficient Fetal Rats. J. Nutr. 100,325-329.

Diamond, I., Swenerton, H. & Hurley, LS. (1971). Testicular and Esophogeal Lesions in Zinc Deficient Rats and Their Reversibility. J. Nutr. 101,77-84.

Dickmann, Z. & Dey, SK. (1974). Steroidogenisis in the Pre-implantation Rat Embryo and its Possible Influence on Morula-Blastocyst Transformation and Implantation. J. Reprod. Fertil. 37,91-93.

Dreosti, IE. (1982). Zinc. J. Food Nutr. 39,167-173

Dreosti, IE. (1984). Interactions Between Trace Elements and Alcohol in Rats. In " Mechanisms of Alcohol Damage *In Utero*" Pitman, London. (CIBA Foundation Symposium 105.) pp103-123.

Dreosti, IE. Tao, S. & Hurley, LS. (1968). Plasma zinc and Leukocyte Changes in Weaning and Pregnant Rats During Zinc Deficiency. Proc. Soc. Exp. Biol. Med. 128,169-174.

Dreosti, IE. & Hurley, LS. (1975). Depressed Thymidine Kinase Activity in Zinc-Deficient Rat Embryos. Proc. Soc. Exp. Biol. Med. 150,161-165.

Dreosti, IE. & Record, IR. (1978). Lysosomal Stability, Superoxide Dismutase and Zinc Deficiency in Regenerating Rat Liver. Br. J. Nutr. 40,133-137.

Dreosti, IE., Record, IR. & Manuel, SJ. (1980a). Incorporation of 3H-Thymidine into DNA and the Activity of Alkaline Phosphatase in Zinc-Deficient Fetal Rat Brains. Biol. Trace Element Res. 2,21-29.

Dreosti, IE., Buckley, RA. & Record, IR. (1980b). The Effect of Ethanol on Zinc and Copper Metabolism in Rats. Biol. Trace Element Res. 2,31-39.

Dreosti, IE., Manuel, SJ., Buckley, RA., Fraser, FJ. & Record, IR. (1981). The Effect of Late Prenatal and/or Early Postnatal Zinc Deficiency on the Development and some Biochemical Aspects of the Cerebellum and Hippocampus in Rats. Life Sci. 28,2133-2141.

Dreosti, IE. & Fraser, FJ. (1984). The Effect of Ethanol and/or Zinc Deficiency on Hippocampal Development in the Fetal Rat. In "The Neurobiology of Zinc. Part B." (Frederickson, CJ., Howell, GA. & Kasarkis, EJ., eds.)Liss, New York. pp223-233.

Dreosti, IE. & Record, IR. (1984). Accumulation of Zinc in the Hippocampus of the Neonatal Rat. In "Neurobiology of Zinc. Part A." (Frederickson, CJ., Howell, GA. & Kasarkis. EJ., eds.). Liss, New York. pp319-326.

Dreosti, IE. Record, IR. & Manuel, SJ. (1985). Zinc Deficiency and the Developing Embryo. Biol. Tr. Element Res. 7,103-122.

Dreosti, IE., Buckley, RA. & Record, IR. (1986). The Teratogenic Effect of Zinc Deficiency and Accompanying Feeding Patterns in Mice. Nutr. Res. 6,159-166.

Drum, DE., Harrison, JH., LI, T-K., Bethune, JL. & Vallee, BL. (1967). Structural and Functional Zinc in Liver Alcohol Dehydrogenase. Proc. Natl. Acad. Sci. USA., 57, 1434-1440.

Ducibella, T., Albertini, DF., Anderson, E. & Biggers, JD. (1975). The Pre-implantation Mammalian Embryo: Characterisation of Intercellular Junctions and their Appearance During Development. Dev. Biol.

45,231-250.

Duncan, JR. & Dreosti, IE. (1973). Deoxyribonucleic Acid Synthesis and Protein Synthesis in Zinc-Deficient Rats. Agrochemophysica. 5,51-56.

Duncan, JR. & Dreosti, IE. (1974). The Effect of Zinc Deficiency on the Timing of Deoxyribonucleic Acid Synthesis in the Regenerating Rat Liver. S. Afr. Med. J. 48,1697-1699.

Duncan, JR. & Dreosti, IE. (1975). The Effect of Zinc on DNA and Protein Synthesis in Rat Lymphocytes Stimulated by Phytohaemagglutinin. Agrochemophysica. 5,1-4.

Duncan, JR. & Hurley, LS. (1978a). Thymidine Kinase and DNA Polymerase Activity in Normal and Zinc Deficient Rat Embryos. Soc. Exp. Biol. Med. Proc. 159,39-43.

Eckhert, CD. & Hurley, LS. (1977). Reduced DNA Synthesis in Zinc Deficiency: Regional Differences in Embryonic Rats. J. Nutr. 107,855-861.

Eichorn, GL. (1973). Complexes of Polynucleotides and Nucleic Acids. In "Inorganic Biochemistry" Elsevier, Amsterdam. Vol. 2. pp1210-1243.

Eklund, H., Nordstrom, B. & Zeppezauer, E. (1974). The Structure of Horse Liver Alcohol Dehydrogenase. FEBS. Lett. 44,200-204.

Elmes, ME. (1977). Apoptosis in the Small Intestine of Zinc-Deficient and Fasted Rats. J. Path. 123,219-223.

Elmes, ME. & Jones, JG. (1980). Ultrastructural Changes in the Small Intestine of the Zinc-Deficient Rat. J. Path. 130,37-43.

Fairweather-Tait, SJ., Payne, V. & Williams, CM.(1984) The Effect of Iron Supplements on Pregnancy in Rats Fed a Low-Zinc Diet. Br. J. Nutr. 52,79-86.

Fairweather-Tait, SJ., Wright, AJA., Cooke, J. & Franklin, J. (1985). Studies of Zinc Metabolism in Pregnant and Lactating Rats. Br. J. Nutr. 54,401-413.

Falchuk, KH., Fawcett, DW., & Vallee, BL. (1975). Role of Zinc in Cell Division of *Euglena Gracilis*. J. Cell. Sci. 17,57-78.

Falchuk, KH., Krishnan, A. & Vallee, BL. (1975). DNA Distribution in the Cell Cycle of *Euglena Gracilis*. Cytofluorimetry of Zinc-Deficient Cells. Biochemistry. 14,3439-3444.

Falchuk, KH., Hardy, C., Ulpino, L. & Vallee, BL. (1977). RNA Polymerase, Manganese and RNA Metabolism of Zinc Sufficient and Deficient *E. Gracilis*. Biochem. Biophys. Res. Comm. 77,314-319.

Fehily, D., Fitzsimmons, B., Jenkins, D., Cremin, FM., Flynn, A. & Soltan, MH. (1986). Association of Fetal Growth with Elevated Maternal Plasma Zinc Concentration in Human Pregnancy. Human Nutrition:Applied Nutrition. 40C, 221-227.

Fell, BF., Leigh, LC. & Williams, RB. (1973). the Cytology of Various Organs in Zinc-Deficient Rats with Particular Reference to the Frequency of Cell Division. Res. Vet. Sci. 14,317-325.

Fernandez-Madrid, F., Prasad, AS. & Oberleas, D. (1973). Effect of Zinc Deficiency on Nucleic Acids, Collagen and Non-Collagenous Protein of the Connective Tissue. J. Lab. Clin. Med. 82,951-961.

Flynn, A., Pories, WJ., Strain, WH., Hill, OA. & Fratianne, RB. (1971). Rapid Serum Zinc depletion Associated with Corticosterone Therapy. Lancet. 2,1169.

Follis, RH., Day, HG. & McCollum, EV. (1941). Histological Studies of the Tissues of Rats Fed a Diet Extremely Low in Zinc. J. Nutr. 22,223-237.

Fong, LYY., Sivak, A. & Newberne PM. (1978). Zinc Deficiency and Methylbenzylnitrosamine Induced Oesophogeal Cancer in Rats. J. Natl. Cancer Inst. 61,145-150.

Fraser, FJ. (1982). A Selective Stain for Mitotic Figures Particularly in the Developing Brain. Stain Technol. 57,219-224.

Frazier, JM. (1981). Effect of Excess Zinc on Cadmium Disappearance From Rat Plasma Following Intravenous Injection. Fund. Appl. Toxicol. 1,452-456.

Freier, S., Faber, J., Goldstein, R. & Mayer, M. (1973). Treatment of Acrodermatitis Enteropathica by Intravenous Amino Acid Hydrolysate. J. Pediat. 82,109-112.

Frommer, DJ. (1975). The Healing of Gastric Ulcers by Zinc Sulphate. Med. J. Aust. 2,793-796.

Fujioka, M. & Lieberman, I. (1964). A Zinc Requirement for Synthesis of DNA by Rat Liver. J. biol. Chem. 239,1164-1167.

Fujiwara, H. (1980). Cytotoxic Effects of Ethylnitrosourea on Central Nervous System of Rat Embryos. Acta Pathol. Jpn. 30,375-387.

Gallagher, D. & Hurley, LS. (1980). Low Zinc Concentration in Rat Uterine Fluid after 4 Days of Dietary Deficiency. J. Nutr. 110,591-593.

Geelan, JAG. & Langman, J. (1977). Closure of the Neural Tube In the Cephalic Region of the Mouse Embryo. Anat. Res. 189,625-640.

Gershoff, SN. (1968). Effects of Vitamin B6 and B1 Deficiencies and Restricted Calorie Intake on Tissue Zinc Levels. Proc. Soc. Exp. Biol. Med. 127,1207-1210.

Giroud, A., LeFebvres, J. Prost, H. & Dupuis, R. (1955). Malformation des Membres Dues a des Lesions Vasculaires chez le Foetus de Rat Deficient en Acide Pantothenique. J. Embryol. exp. Morphol. 3,1-12.

Giugliano, G. & Millward, DJ. (1983). Muscle Protein Turnover in the Severely Zinc Deficient Rat. Proc. Nutr. Soc. 43,77a.

Giugliano, G. & Millward, DJ. (1984). Growth and Zinc Homeostasis in the Severely Zinc Deficient Rat. Br. J. Nutr. 52,545-560.

Greeley, SJ. (1984). Zinc Repletion During Late Gestation Following Chronic Suboptimal Zinc Intake. Nutr. Rep. Int. 30,389-395.

Golub, MS., Gershwin, ME., Hurley, LS., Hendrickx, AG. & Baly, DL. (1982).

Induction of Marginal Zinc Deficiency in Female Rhesus Monkeys. Am. J. Primatology 3,299-305.

Grey, PC., & Dreosti, IE.(1972). Deoxyribonucleic Acid and Protein Metabolism in Zinc-Deficient Rats. J. Comp. Path. 82,223-228.

Griffith, PR., & Alexander, JC. (1972). Effect of Zinc Deficiency on Amino Acid Metabolism of the Rat. Nutr. Rep. Int. 6,9-20.

Gross, RL., Osdin, N., Fong, L. & Newberne, P. (1979). Depressed Immunological Function in Zinc-Deficient Rats as Measured by Mitogen Response of Spleen, Thymus and Peripheral Blood. Am. J. Clin. Nutr. 32,1260-1265.

Grummt, F., Weinmann-Dorsch, C., Schneider-Schaulies J. & Lux A. (1986). Zinc as a Second Messenger of Mitogenic Induction. Exp. Cell Res. 163,191-200.

Hackman, RM. & Hurley, LS. (1981a). Interaction of Dietary Zinc, Genetic Strain and Acetazolamide in Teratogenesis in Mice. Teratology. 28,355-368.

Hackman, RM. & Hurley, LS. (1981b). Interactions of Salicylate, Dietary Zinc and Genetic Strain in Teratogenesis in Rats. Teratology. 30,225-236.

Halas, ES., Wallwork, JC. & Sandstead, HH. (1982). Mild Zinc Deficiency and Undernutrition During the Prenatal and Postnatal Periods in Rats: Effects on Weight, Food Consumption and Brain Catecholamine Concentrations. J. Nutr. 112,542-551.

Hallbook, T. & Lanner, E. (1972). Serum-Zinc and the Healing of Leg Ulcers. Lancet. 2,780-782.

Hambidge, KM., Neldner, KH. & Walravens, PA. (1975). Zinc, Acrodermatitis Enteropathica and Congenital Malformations. Lancet. 1,577-578.

Henkin, RI., Patten, BM., Re, PK. & Bronzert, DA. (1975). A Syndrome of Acute Zinc Loss. Arch. Neurol. 32,745-752.

Henzel, JH., De Veese, MS. & Lichti, EL. (1970). Zinc concentrations Within Healing Wounds. Arch. Surg. (Chicago).100,349-356.

Hertig, AT. (1967). The Overall Problem in Man. In "Comparative Aspects of Reproductive Failure." (Benirschke, K., ed.) Springer-Verlag, New York. pp 12-41.

Herzfeld, GA. Reynolds, LA. & Ritchie, SJ. (1985). Zinc in Maternal and Fetal Tissues of rats Fed Two Levels of Zinc During Gestation. Nutr. Rep. Int. 31,849-856.

Hickory, W., Nanda, R. & Catalanotto, FA. (1979). Fetal Skeletal Malformations Associated with Moderate Zinc Deficiency During Pregnancy. J. Nutr. 109,883-891.

Hirsch, KS. & Hurley, LS. (1978). Relationship of Dietary Zinc to

6-Mercaptopurine Teratogenesis and DNA Metabolism in the Rat. Teratology. 17,303-314.

Holden, JM., Wolf, WR. & Mertz, W. (1979). Zinc and Copper in Self-Selected Diets. J. Am. Diet. Assoc. 75,23-28.

Hsu, JM., Anilane, JK. & Scanlan, DE. (1966). Pancreatic Carboxypeptidases: Activities in Zinc-Deficient Rats. Science. 153,882-8823.

Hsu, JM., Anthony, WL. & Buchanan, PJ. (1969). Zinc Deficiency and Incorporation of ^{14}c-Labelled Methionine into Tissue Proteins in Rats. J. Nutr. 99,425-432.

Hsu, JM., Kim, KM. & Anthony, WL. (1974). Biochemical and Electron Microscopic Studies of Rat Skin During Zinc Deficiency. Adv. exp. Med. Biol. 48,347-388.

Hunt, IF., Murphy, NJ., Gomez, J. & Smith, JC. (1979). Dietary Zinc Intake of Low-Income Pregnant Women of Mexican Descent. Am. J. Clin. Nutr. 32,1511-1518.

Hurd, RW., Wilder, BJ. & Van Rinsvelt, HA. (1983). Valproate, Birth Defects and Zinc. Lancet. 1,181.

Hurley, LS. (1985). Maternal Catabolism and Fetal Zinc Status. Nutr. Res. Suppl. 1,300-305.

Hurley, LS. & Swenerton, H. (1966). Congenital Malformations Resulting from Zinc Deficiency in Rats. Proc. Soc. Exp. Biol. Med. 123,692-696.

Hurley, LS. & Swenerton, H. (1971). Lack of Mobilization of Bone and Liver Zinc Under Teratogenic Conditions of Zinc Deficiency in Rats. J. Nutr. 101,597-604.

Hurley, LS., Gowan, J. & Swenerton, H. (1971). Teratogenic Effects of Short-Term and Transitory Zinc Deficiency in Rats. Teratology. 4,199-204.

Hurley, LS. & Shrader, RE. (1972). Congenital Malformations of the Nervous System in Zinc Deficient Rats. Int. Rev. Neurobiol. Suppl. 1 (Pfeiffer, CC. ed) pp 7-51.

Hurley, LS. & Shrader, RE. (1975). Abnormal Development of Pre-Implantation Rat Embryos After Three Days of Maternal Dietary Zinc deficiency. Nature. 254,427-429.

Hurley, LS., Gordon, P., Keen, CL. & Merkhofer, L. (1982). Circadian Variation in Rat Plasma Zinc and Rapid Effect of Dietary Zinc Deficiency. Proc. Soc. Exp. biol. Med. Sci. 170,48-52.

Hurley, LS., Dungan, DD., Keen, CL. & Lonnerdal, B. (1983). The Effect of Vitamin E on Zinc Deficiency Teratogenesis in Rats. J. Nutr. 113,1875-1877.

Ikeda, M., Hosotani, T., Ueda, T., Kotake, Y. & Sakakibara, B. (1979). Observations of the Concentration of Zinc and Iron in Tissues of Vitamin B6-Deficient Germ-Free Rats. J. Nutr. Sci. Vitaminol. 25,151-158.

Iniguez, C., Casas, J. & Carreres, J. (1978). Effects of Zinc Deficiency on the Chick Embryo Blastoderm. Acta Anat. 101,120-129.

Iqbal, M. (1971). Activity of Alkaline Phosphatase and Carbonic Anhydrase in Male and Female Zinc-Deficient Rats. Enzyme, 12,33-40.

Jackson, AJ. & Schumacher, HJ. (1979). The Teratogenic Activity of a Thalidomide Analogus EM12 in Rats on a Low Zinc Diet. Teratology. 19,341-344.

Jameson, S. (1976). Effects of Zinc Deficiency in Human Reproduction. Acta Med. Scand.(Suppl. 593).

Jensen, LS. (1975). Precipitation of a Selenium Deficiency by High Levels of Copper and Zinc. Proc. Soc. Exp. Biol. Med. 149,113-116.

Johnson, EM. (1964). Effects of Maternal Folic Acid Deficiency on Cytologic Phenomena in the rat Embryo. Anat. Rec. 149,49-56.

Kang, YJ., Olson, MOJ. & Busch, H. (1974). Phosphorylation of Acid-Soluble Proteins in Isolated Nuclei of Novkoff Hepatoma Ascites Cells. J. biol. Chem. 249,5580-5585.

Kasarkis, EJ., Sparks, DL. & Slevin, JT. (1986). Changes in Hypothalamic Noradrenergic Systems During the Anorexia of Zinc Deficiency. Biol. Trace Element Res. 9,25-35.

Keilin, D. & Mann, T. (1940). Carbonic Anhydrase. Purification and Nature of the Enzyme. Biochem. J. 34,1163-1176.

Keppen, LD., Pysher, T. & Rennert, OM. (1985). Zinc Deficiency Acts as a Co-Teratogen with Alcohol in Fetal Alcohol Syndrome. Pediat. Res. 19,944-947.

Kirchgessner, M., Reichlmayr-Lais, AM. & Schwarz, FJ. (1981). Interactions of Trace Elements in Human Nutrition. In "Nutrition in Health and Disease and International Development". Symposia from the xii International Nutrition. 189-197.

Klein, NW., Vogler, MA., Chatot, CL. & Pierro, LJ. (1980). The Use of Cultured Rat Embryos to Evaluate the Teratogenic Activity of Serum: Cadmium and Cyclophosphamide. Teratology. 21,199-208.

Klingberg, WG., Prasad, AS. & Oberleas, D. (1976). Zinc Deficiency Following Penacillamine Therapy. In "Trace Elements in Human Health and Disease Vol. 1." (Prasad, AS., ed.).Academic Press, New York. pp51-65.

Kohler, E., Merker, H-J., Ehmke, W. & Wojnorowicz, F. (1972). Growth Kinetics of Mammalian Embryos During the stage of Differentiation. Arch. Pharmacol. 272,169-181.

Koo, SI. & Turk. DE. (1977). Effect of Zinc Deficiency on the Ultrastructure of the Pancreatic Acinar Cells and Intestinal Epithelium of the Rat. J. Nutr. 107,896-908.

Lamy & Frezal, (1961). The Frequency of Congenital Malformations. In "1st International Congress on Congenital Malformations." (Fishbein, M., ed.) Lippincott, Philadelphia. pp 34-44.

Lavy, UI. (1972). The Effect of Oral Supplementation of Zinc Sulphate on Primary Wound Healing in Rats. Br. J. Surg. 59,194-196.

Levander, OA. & Cheng, L.,(eds) (1980). Micronutrient Interactions: Vitamins, Minerals and Hazardous Elements. Ann. N.Y. Acad. Sci. 355.

Lieberman, I. & Ove, P. (1962). Deoxyribonucleic Acid Synthesis and its Inhibition in Mammalian Cells Cultured from the Animal. J. biol. Chem. 237,1634-1642.

Lindeman, RD., Baxter, DJ., Yunice, AA. & Kraikitpanitch,S. (1978). Serum Concentrations and Urinary Excretion of Zinc in Cirrhosis, Nephrotic Syndrome and Renal Insufficiency. Am. J. Med. Sci. 275,17-31.

Lonnerdal, B., Ken, CL. & Hurley, LS. (1984). Zinc Binding Ligands and Complexes in Zinc Metabolism. Adv. Nutr. Res. Vol. 6. (Draper, HH., ed.) Plenum Press, New York. pp139-167.

Lowry, OH., Rosebrough, NJ., Farr, AL. & Randall, RJ. (1951). Protein Measurement with the Folin-Phenol Reagent. J. Biol. Chem. 193,265-275.

Lowry, SF., Goodgame, JT., Smith, JC., Maher, MM., Makuch, RW., Henkin, RI. & Brennan, MF. (1979). Abnormalities of Zinc and Copper During Total Parenteral Nutrition. Ann. Surg. 189,120-128.

Lyon, TDB., Smith, H. & Smith, LB. (1979). Zinc Deficiency in the West of Scotland? A Dietary Intake Study. Br. J. Nutr. 42,413-416.

MacIntosh, JEA. & Lutwak-Mann, C. (1972). Zinc Transport in Rabbit Tissues. Biochem. J. 126,869-876.

McKenzie, JM., Fosmire, GJ. & Sandstead, HH. (1975). Zinc Deficiency During the Latter Third of Pregnancy: Effects on Fetal Brain, Liver and Placenta. J. Nutr. 105,1466-1475.

McMichael, AJ., Dreosti, IE., Gibson, GT., Hartshorne, JM., Buckley, RA. & Colley, DP. (1982). A Prospective Study of Serial Maternal Serum Zinc Levels and Pregnancy Outcome. Early Human Dev. 7,59-69.

Macapinlac, MP., Pearson, WN., Barney, GH. & Darby, WJ. (1968). Protein and Nucleic acid Metabolism in the Testes of Zinc Deficient Rats. J. Nutr. 95,569-577.

Machin, GA. (1975). A Perinatal Mortality Survey in South-East London 1970-73. The Pathological Findings in 726 Necropsies. J. Clin. Pathol. 28,428-434.

Marden, PM., Smith, DW. & McDonald, MJ. (1964). Congenital Anomalies in the Newborn Infant Including Minor Variations. J. Pediat. 64,357-371.

Masters, DG., Keen, CL., Lonnerdal, B. & Hurley, LS. (1983). Zinc Deficiency Teratology: The Protective Role of Maternal Tissue Catabolism. J. Nutr. 113,905-912.

Meadows, NJ., Ruse, W., Smith, MF., Day, J., Keeling, PWN., Scopes, JW., Thompson, RPH. & Bloxham, DL. (1981). Zinc and Small Babies. Lancet. 2,1135-1137.

Metcoff, J., Cottilo, JP. & Crosby, W. (1981). Maternal Nutrition and Fetal Outcome. Am. J. Clin. Nutr. 34,708-721.

Methfessel, AH. & Spencer, H. (1973). Zinc Metabolism in the Rat II. Secretion of Zinc into Intestines. J. Appl. Physiol. 34,63-67.

Miller, JK. & Miller, WJ. (1962). Experimental Zinc Deficiency and Recovery of Calves. J. Nutr. 76,467-474.

Miller, SI., Del Villano, BC., Flynn, A. & Krumhansl, M. (1983). Interaction of Alcohol and Zinc in Fetal Dysmorphogenesis. Pharmacol. Biochem. Behav. 18,311-315.

Miller, WJ. (1971). Zinc Metabolism in Farm Animals. In "Trace Mineral Studies With Isotopes in Domestic Animals". FAO/IAEA. Vienna. pp23-41.

Mills, CF., Quaterman, J., Chesters, JK., Williams, RB. & Dalgarno, AC. (1969). Metabolic role of Zinc. Am. J. Clin. Nutr. 22,1240-1249.

Millward, DJ., Bates, PC. & Rosochack, IS. (1981). The Extent and Natur of Protein Degradation in the Tissues During Development. Reprod. Nutr. Develop. 21,265-277.

Milne, DB., Canfield, WK., Mahalko, JR. & Sandstead, HH. (1983). Effect of Dietary Zinc on Whole Body Surface Loss of Zinc: Impact on Estimation of Zinc Retention by Balance Method. Am. J. Clin. Nutr. 38,181-186.

Moore, AT., Williams, KE. & Lloyd, JB. (1978). The Effect of Chemical Treatments of Albumin and Orosomucoid on Rate of Clearance from the Rat Bloodstream and Rate of Pinocytic Capture by Rat Yolk Sacs Cultured *In Vitro*. Biochem. J. 164,607-616.

Morrissey, RE. & Mottet, NK. (1983). Arsenic-Induced Exencephaly in the Mouse and Associated Lesions Occuring During Neurulation. Teratology. 28,399-411.

Nalbandov, AV. (1971). Endocrine Control of Implantation. In "The Biology of the Blastocyst". (Blandau, RJ., ed.). Univ. Chicago Press, Chicago. pp383-392

National Acadamy of Sciences. (1978). Nutrient Requirements of Domestic Animals No. 10. Nutrient Requirements of Laboratory Animals. 3rd. ed. National Acadamy of Sciences, Washington D.C. pp7-37.

Neathery, MW., Miller, WJ., Blackman, DM., Pate, FM. & Gentry, RP. (1973). Effects of Long Term Zinc Deficiency on Feed Utilisation, Reproductive Characteristics and Hair Growth in the Sexually Mature Male Goat. J. Dairy Sci. 56,98-105.

New, DAT. (1966). The Culture of Vertebrate Embryos. Academic Press, New York. pp33-46.

New, DAT. (1978). Whole Embryo Culture and the Study of Mammalian Embryos During Organogenesis. Biol. Rev. 53,81-122.

New, DAT., Coppola, PT. & Cockroft, DL. (1976). Comparison of Growth *In Vitro* and *In Vivo* of Post-Implantation Rat Embryos. J. Embryol. exp. Morph. 36,133-144.

Nicholas, SJ. (1962). Experimental Methods and Rat Embryos. In "The Rat in Laboratory Investigation". (Farris, EJ. & Griffith, JO., eds.). Hafner, New York. pp 51-67.

Nie, NH., Hull, CH., Jenkins, JG., Steinbrenner, K. & Bent, DH. (1975). Statistical Package for the Social Sciences. McGraw-Hill, New York.

Norman, JN., Rahmat, A. & Smith, G. (1975). Effect of Supplementation of Zinc Salts on the Healing of Granulating Wounds in the Rat and Guinea Pig. J. Nutr. 105,815-822.

Oberleas, D., Muhrer, ME. & O'Dell, BL. (1966). Dietary Metal Complexing Agents and Zinc Availability in the Rat. J. Nutr. 90,56-62.

Ohlsson, A. (1981). Acrodermatitis Enteropathica-Reversibility of Cerebral Atrophy with Zinc Therapy. Acta. Pediat. Scand. 70,269-273.

Palludan, B. (1966). A-Avitaminosis in Swine. Munksgaard, Copenhagen.

Parisi, AF. & Vallee, BL. (1970). Isolation of a Zinc alpha-2-Macroglobulin from Human Serum. Biochemistry. 9,2421-2426.

Parizek, J. (1957). The Destructive Effect of Cadmium Ion on Testicular Tissue and its Prevention by Zinc. J. Endocrinol. 15,56-63.

Park, JHY., Grandjean, CJ., Antonson, DL. & Vanderhoof, JA. (1985). Effect of Short-Term Isolated Zinc-Deficiency on Intestinal Growth and Activties of Several Brush Border Enzymes in Weanling Rats. Pediat. Res. 19,1333-1336.

Parry, WH. (1977). Distribution of Protein Bound Zinc in Normal and Zinc-Deficient Lamb Plasma. Nutr. Metab. 21(Suppl. 1), 48-49.

Patrick, J., Dervish, C. & Gillieson, M. (1982). Zinc and Small Babies. Lancet. 2,169-170.

Pearson, WN., Schwink, T. & Reich, M. (1966). *In Vitro* Studies of Zinc Absorption in the Rat. In "Zinc Metabolism." (Prasad, AS., ed.)Thomas, Springfield, Ill. pp 239-249.

Pecaud, A., Donzel, P. & Schelling, JL. (1975). Effect of Foodstuffs on the Absorbtion of Zinc Sulphate. Clin. Pharmacol. Ther. 17,469-474.

Perinatal Statics Unit. (1980-85). Congenital Malformations Monitoring Report. University of Sydney, Sydney.

Phillips, GD. (1982). Zinc in Total Parenteral Nutrition. In "Clinical Applications of Recent Advances in Zinc Metabolism." (Prasad, AS., Dreosti, IE. & Hetzel, BS., eds.).Liss. New York. pp169-180.

Popov, VB., Vaisman, BL., Puchkov, VF. & Ignateva, TV. (1981). Toxic Action of Ethanol and its Biotransformation Products on Postimplantation Rat embryos in Culture. Bull. Eksp. Biol. Med. 92,725-728.

Pories, WJ., Henzel, JH., Rob, CG. & Strain, WH. (1967). Acceleration of Wound Healing in Man With Zinc Sulphate Given by Mouth. Lancet. 1,121-124.

Prasad, AS. (1979). Zinc in Human Nutrition. CRC Press, Boca Raton. pp66-67.

Prasad, AS., Halsted, JA. & Nadimi, M. (1961). Syndrome of Iron Deficiency, Anaemia, Hepatosplenomegaly, Hypogonadism, Dwarfism and Geophagia. Am. J. Med. 31,532-546.

Prasad, AS., Miale, A., Farid, Z., Sandstead, HH. & Darby, WJ. (1963). Biochemical Studies on Dwarfism, Hypogonadism, and Anaemia. Arch.

Intern. Med. 111,407-428

Prasad, AS., Oberleas, D. & Halstead, JA. (1965). Determination of Zinc in Biological Fluids by Atomic Absorbtion Spectrophotometry in Normal and Cirrhotic Subjects. J. Lab. Clin. Med. 66,508-516.

Prasad, AS. & Oberleas, D. (1970). Binding of Zinc to Amino Acids and Serum Proteins In Vitro. J. Lab. Clin. Med. 76,416-425.

Prasad, AS. & Oberleas, D. (1971). Changes in Activities of Zinc-Dependent Enzymes in Zinc-Deficient Tissues of Rats. J. Appl. Physiol. 31,842-846.

Prasad, AS. & Oberleas, D. (1974). Thymidine Kinase Activity and Incorporation of Thymidine into DNA in Zinc-Deficient Tissue. J. Lab. Clin. Med. 83,634-425.

Prohaska, JR., Leuke, RW. & Jasinski, R. (1974). Effect of Zinc Deficiency from Day 18 of Gestation and/or During Lactation on the Development of Some Rat Brain Enzymes. J. Nutr. 104,1525-1531.

Rabbani, P. & Prasad, AS. (1978). Plasma Ammonia and Liver Ornithine Transcarbamoylase in Zinc-Deficient Rats. Am. J. Physiol. 235,E203-E206.

Rama, R. & Planes, J. (1981). Effects of Dietary Zinc on Iron Metabolism in Chickens. Biol. Trace Element Res. 3,287-299.

Raulin, J. (1869). Etudes Cliniques sur la Vegetation. Ann. Sci. Natl. Botan. Biol. Vegetale. 11,93.

Record, IR. (1980). Zinc and Fetal Development. M. App. Sc. Thesis. South Australian Institute of Technology, Adelaide , S. Australia.

Record, IR. & Dreosti, IE. (1979). Effects of Zinc Deficiency on Liver and Brain Thymidine Kinase in the Fetal Rat. Nutr. Rep. Int. 20,749-755.

Record, IR., Dreosti, IE., Manuel, SJ. & Buckley, RA. (1980a). Compositional Development of the Zinc Deficient Fetal Rat. Proc. Nutr. Soc. Aust. 5,157.

Record, IR., Dreosti, IE. & Smith, RM. (1980b). The Influence of Maternal Zinc Deficiency on the Salvage and De Novo Pathways of DNA Synthesis in the Fetal Rat. Proc. Aust. Biochem. Soc. 13,98.

Record, IR., Dreosti, IE., Manuel, SJ. & Buckley, RA. (1982a). Interaction of Cadmium and Zinc in Cultured Rat Embryos. Life Sci. 31,2735-2743.

Record, IR., Dreosti, IE. & Manuel, SJ. (1982b). Inhibition of Rat Yolk Sac Pinocytosis By Cadmium and its Reversal by Zinc. J. Nutr. 192,1994-1998.

Record, IR., Dreosti, IE., Fraser, FJ., Buckley, RA. & Manuel, SJ. (1982c). Postnatal Accumulation of Zinc by the Rat Hippocampus. Biol. Trace Element Res. 4,279-288.

Record, IR., Dreosti, IE., Tulsi, RS. & Manuel, SJ. (1984a). Growth of Zinc-Deficient Rat Embryos In Vitro. Teratology. 29,53A.

Record, IR., Record, SJ., Dreosti, IE. & Rohan, TE. (1985a). Dietary Zinc Intakes of Premenopausal Women. Human Nutrition: Applied Nutrition.

39A,363-369.

Record, IR., Dreosti, IE., Manuel, SJ. & Tulsi, RS. (1985b). Teratological Influence of the Feeding Cycle in Zinc-Deficient Rats. In "Trace Element Metabolism in Man and Animals 5 ". (Mills, CF., Bremner, I. & Chesters, JK., eds.) Commonwealth Agricultural Bureaux, Slough, UK. pp210-213.

Record, IR., Tulsi, RS., Dreosti, IE. & Manuel, SJ. (1985c). Induction of Teratogenic Abnormalities in Rats by Maternal Dietary Zinc Deficiency. J. Anat. 142,223.

Record, IR., Dreosti, IE & Tulsi, RS. (1985d). Fetal Outcome Following Transient Zinc Deficiency in the Pregnant Rat. Genes, Environment and Mankind. 1,41.

Record, IR., Dreosti, IE. & Tulsi, RS. (1985e). In Vitro Development of Zinc-Deficient and Replete Rat Embryos. Aust. J. Exp. Biol. Med. Sci. 63,65-71.

Record, IR., Tulsi, RS., Dreosti, IE & Fraser, FJ. (1985f). Cellular Necrosis in Zinc-Deficient Rat Embryos. Teratology. 32,397-405.

Record, IR., Dreosti, IE., Tulsi, RS. & Manuel, SJ. (1986). Maternal Metabolism and Teratogenesis in Zinc-Deficient Rats. Teratology. 33,311-317.

Reinhold, JG. (1971). High Phytate content of Rural Iranian Bread: A Possible Cause of Human Zinc Deficiency. Am. J. Clin. Nutr. 24,1204-1206.

Reinhold, JG., Faradji, B., Abadi,P. & Ismail-Beigi, F. (1976). Binding of Zinc to Fibre and Other Solids of Wholemeal Bread: With a Preliminary Examination of the Effects of Cellulose Consumption upon the Metabolism of Zinc, Calcium and Phosphorus in Man. In "Trace Elements in Human Health and Disease. Vol. 1. "(Prasad, AS., ed.). Academic Press, New York. pp163-180.

Reinstein, NH., Lonnerdal, B., Keen, CL. & Hurley, LS. (1984). Zinc-Copper Interactions in the Pregnant Rat: Fetal Outcome and Maternal and Fetal Zinc, Copper and Iron. J. Nutr. 114,1266-1279.

Robinson, LK. & Hurley, LS. (1981a). Effect of Maternal Zinc Deficiency or Food Restriction on rat Fetal Pancreas. 1. Procarboxypeptidase A and Chymotrypsinogen. J. Nutr. 111,858-868.

Robinson, LK. & Hurley, LS. (1981b). Effect of Maternal Zinc deficiency or food restriction on Rat Fetal Pancreas. 2. Insulin and Glucagon. J. Nutr. 111,869-877.

Robinson, MP., McKenzie, JM., Thomson, CD. & Van Rij, AL. (1973). Metabolic Balance of Zinc, Copper, Cadmium, Iron, Molybdenum and Selenium in Young New Zealand women. Br. J. Nutr. 30,195-205.

Rogers, JM., Keen, CL. & Hurley, LS. (1985). Zinc Deficiency in Pregnant Long-Evans Rats: Teratogenicity and Tissue Trace Elements. Teratology. 31,89-100.

Rubin, H. (1972). Inhibition of DNA Synthesis in Animal Cells by EDTA and its Reversal by Zinc. Proc. Nat. Acad. Sci. US. 69,712-716.

Rubin, H. & Koide, T. (1973). Inhibition of DNA Synthesis in Chick Embryo

Cultures by Deprivation of Either Serum or Zinc. J. Cell. Biol. 56,777-786.

Ruth, RE. & Goldsmith, SK. (1981). Interaction Between Zinc Deprivation and Acute Ethanol Intoxication During Pregnancy in Rats. J. Nutr. 111,2034-2038.

Sahagain, BM., Harding-Barlow, I. & Perry, HM. (1966). Uptakes of Zinc, Manganese, Cadmium and Mercury by Intact Strips of Rat Intestine. J. Nutr. 90,259-267.

Sahgal, A. (1980). Functions of the Hippocampal System. Trends Neurosci. 3,116-119.

Samarawickrama, GP. & Webb, M. (1979). Acute Effects of Cadmium on the Pregnant Rat and Embryo: Fetal Development. Environ. Health Perspect. 28,245-249.

Sandstead, HH. (1982). Availability of Zinc and its Requirements in Human Subjects. In "Current Topics n Nutrition and Disease Vol. 6" (Prasad, As., ed.) Liss, New York. pp83-102.

Sandstead, HH., Lanier, VC., Shepard, GH. & Gillispie, DD. (1970). Zinc and Wound Healing. Am. J. Clin. Nutr. 23,514-519.

Sandstead, HH., Holloway, WL. & Baume, V. (1971). Zinc Deficiency: Effects on Polysomes. Fed. Proc. Fed. Am. Soc. Exp. Biol. 30,517.

Sandstead, HH., Vo-Khactu, KP. & Solomons, N. (1976). Conditioned Zinc Deficiencies. In "Trace Elements in Human Health and Disease Vol. 2" (Prasad, AS. & Oberleas, D. eds.) Academic Press, New York. Ch. 2.

Sandstrom, B. & Cederblad, A. (1980). Zinc Absorption from Composite Meals II. Influence of Main Protein Sources. Am. J. Clin. Nutr. 33,1778-1783.

Saryan, LA., Minkel, DT., Dolhun, PJ., Calhoun, BL., Weilgus, S., Schaller, MS. & Petering, DH. (1979). Effect of Zinc Deficiency on Cellular Processes and Morphology in Ehrlich Ascites Tumour Cells. Cancer Res. 39,2457-2465.

Sato, F., Watanabe, T., Hoshi, E. & Endo, A. (1985). Teratogenic Effect of Maternal Zinc Deficiency and its Co-Teratogenic Effect With Cadmium. Teratology. 31,13-18.

Schluter, G. (1973). Ultrastructural Observations on Cell Necrosis During Formation of the Neural Tube in Mouse Embryos. Z. Anat. Entwickl. Gesch. 141,251-264.

Schmid, BP. (1985). Xenobiotic Influences on Embryonic Differentiation, Growth and Morphology In Vitro. Xenobiotica. 15,719-726.

Schwarz, FJ. & Kirchgessner, M. (1974). Absorption von Zinc-65 und Kupfer-64 in Zinkmangel. Int. Z. Vit. Ernforshung. 44,258-266.

Scott, HJ. (1977). Cell Death and Reduced Proliferative rate. In "Handbook of Teratology. Vol. 2." (Wilson, JG. & Fraser, FD., eds.) Plenum Press, New York, pp81-98.

Sever, LE. & Emmanuel, I. (1973). Is There a Connection Between Maternal Zinc Deficiency and Congenital Malformations of the Central Nervous System in Man? Teratology. 7,117-118.

Shepard, TH., Lemire, RJ., Akso, O. & Mackler, B. (1968). Studies on the Development of Congenital Anomalies in Embryos of Riboflavin-Deficient, Galactoflavin-Fed rats. 1. Growth and Embryonic Pathology. Teratology. 1,75-92.

Simmer, K. & Thompson, RPH. (1985). Maternal Zinc and Intrauterine Growth Retardation. Clin. Sci. 68,395-399.

Sivasubramanian, KN. & Henkin, RI. (1978). Behavioural and Dermatoligic Changes and Low Serum Zinc and Copper Concentrations in Two Premature Infants After Parenteral Alimentation. J. Pediat. 93,847-851.

Sluyser, M. (1977). The H1 Histones. Trends Biochem. Sci. 2,202-204.

Smith, JC. (1980). Inter-Relationship of Zinc and Vitamin A Metabolism in Human and Animal Nutrition. A Review. In "Clinical, Biochemical and Nutritional Aspects of Trace Elements".(Prasad, AS. ed.) Liss, New York. pp239-259.

Soltan, MH. & Jenkins, DM. (1982). Maternal and Foetal Plasma Zinc Concentration and Foetal Abnormality. Br. J. Obstet. Gynaecol. 89,56-58.

Somers, M. & Underwood, EJ. (1969). Ribonuclease Activity and Nucleic Acid and Protein Metabolism in the Testes of Zinc-Deficient Rats. Aust. J. biol. Sci. 22,1277-1282.

Spencer, H., Osis, D., Kramer, L. & Norris, C. (1978). Studies of Zinc Metabolism in Man. In "Trace Substances in Environmental Health-5". (Hemphill, DD., ed.) University of Missouri, Columbia. p193-203.

Steele, CE., Jeffrey, EH. & Diplock, AT. (1974). The Effect of Vitamin E and Synthetic Oxidants on the Growth In Vitro of Explanted Rat Embryos. J. Reprod. Fert. 38,115-123.

Steele, CE. & New DAT. (1974). Serum Variants Causing the Formation of Double Hearts and other Abnormalities in Explanted Rat Embryos. J. Embryol. exp. Morphol. 31,707-719.

Stewart, C., Katchan, B., Collipp, PJ., Clejan, S., Pudalov, S. & Chen, SY. (1981). Zinc and Birth Defects. Pediat. Res. 15,515.

Subirana, JA. (1973). Studies on the Thermal Denaturation of Nucleohistones. J. Molec. Biol. 74,336-386.

Sullivan, JF. & Lankford, HE. (1962). Urinary Excretion of Zinc in Alcoholism and Postalcoholic Cirrhosis. Am J. Clin. Nutr. 10,153-157.

Swanson, CA. & King, JC. (1982). Zinc Utilisation in Pregnant and Nonpregnant Women Fed Controlled Diets Providing the Zinc RDA. J. Nutr. 112,697-707.

Swenerton, H., Shrader, RE. & Hurley, LS. (1969). Zinc Deficient Embryos: Reduced Thymidine Incorporation. Science. 166,1014-1015.

Swenerton, H. & Hurley, LS. (1980). Zinc Deficiency in Rhesus and Bonnet Monkeys, Including Effects on Reproduction. J. Nutr. 110,575-583.

Terhune, MW. & Sandstead, HH. (1972). Decreased RNA Polymerase Activity in Mammalian Zinc Deficiency. Science. 177,68-69.

Thyresson, N. (1974). Acrodermatitis Enteropathica. Acta derm-vener. Stockholm. 54,283-285.

Todd, WR., Elvehjem, CA. & Hart, EB. (1934). Zinc in the Nutrition of the Rat. Am. J. Physiol. 107,146-156.

Tucker, HF. & Salmon, WD. (1955). Parakeratosis or Zinc Deficiency Disease in Pigs. Soc. Exp. Biol. Med. Proc. 88,613-616.

Tulsi, RS. (1983). Neural Elements Associated with the Subcommissural Organ of the Brush-Tailed Possum (*Trichosuras vulpecula*). Cell Tissue Res. 235,335-350.

Underwood, EJ. (1977). Trace Elements in Human and Animal Nutrition. 4th ed. Academic Press, New York. pp196-242.

Underwood, EJ. & Somers, M. (1969). Studies on Zinc Nutrition in Sheep 1. The Relation of Zinc to Growth,Testicular Development and Spermatogenesis in Young Rams. Aust. J. Agric. Res. 20,889-897.

Vallee, BL. (1983). Zinc in Biology and Biochemistry. In " Zinc Enzymes." (Spiro, TG., ed.) Wiley, New York. pp1-24.

Van Campen, DR. (1969). Copper Interference with the Intestinal Absorption of Zinc-65 by Rats. J. Nutr. 97,104-108.

Van Campen, D. & House, WA. (1974). Effect of a Low Protein Diet on Retention of an Oral Dose of ^{65}Zn and on Tissue Concentrations of Zinc, Iron and Copper in Rats. J. Nutr. 104,84-90.

Vojnik, C. & Hurley, LS. (1977). Abnormal Prenatal Lung Development Resulting from Maternal Zinc deficiency in Rats. J. Nutr. 107,862-872.

Vormann, J., Hollriegl, V., Merker, H-J. & Gunther, T. (1986). Effect of Salicylate on Zinc Metabolism in Fetal and Maternal Rats Fed Normal and Zinc-Deficient Diets. Biol. Trace Element Res. 9,55-64.

Wallwork, JC., Fosmire, GJ. & Sandstead, HH. (1981). Effect of Zinc Deficiency on Appetite and Plasma Amino Acid concentrations. Br. J. Nutr. 45,127-136.

Wallwork, JC., Botnen, JH. & Sandstead, HH. (1982). Influence of Dietary Zinc on rat Brain Catecholamines. J. Nutr. 112,514-519.

Wallwork, JC., Milne, DB., Sims, RL. & Sandstead, HH. (1983). Severe Zinc deficiency: Effects on the Distribution of Nine Elements (Potassium, Phosphorus, Sodium, Magnesium, Calcium, Iron, Zinc, Copper and Manganese) in Regions of the Rat Brain. J. Nutr. 113,1895-1905.

Wallwork, JC. & Sandstead, HH. (1983). Effect of Zinc Deficiency on Appetite and Free Amino Acid Concentrations in Rat Brain. J. Nutr. 113,47-54.

Walravens, PA., Van Doorninck, WJ. & Hambidge, KM. (1978a). Metals and Mental Function. J. Pediat. 93,535.

Walravens, PA., Hambidge, KM. Neldner, KH., Silverman, A., van Doorninck, WJ., Mieran, G. & Favana, B. (1978b). Zinc Metabolism in Acrodermatitis Enteropathica. J. Pediat. 93,71-73.

Walsh, DA. & Christian, ZH. (1984). The Effects of Phenylalanine on Cultured Rat Embryos. Terat. Carc. Mutag. 4,505-513.

Warkany, J. & Petering, HG. (1972). Congenital Malformations of the Central Nervous System in Rats Produced by Maternal Zinc Deficiency. Teratology. 5,319-334.

Watanabe, T., Sato, F. & Endo, A. (1983). Cytogenic Effects of Zinc Deficiency on Oogenesis and spermatogenesis in Mice. Yamagata Med. J. 1,13-19.

Webster, WS. & Messerle, K. (1980). Changes in the Mouse Neuroepithelium Associated with Cadmium-Induced Neural tube Defects. Teratology. 21,79-88.

Wenk, GL. & Stemmer, KL. (1982). Activity of the Enzymes Dopamine-Beta-Hydroxylase and Phenylethanolamine-N-Methyl Transferase in Discrete Regions of the Copper-Zinc deficient rat Following Aluminium Ingestion. Neurotox. 3,93-99.

Whanger, PD., Ridlington, JW. & Holcomb, CL. (1980). Interaction of Zinc and Selenium on the Binding of Cadmium to Rat Tissue Proteins. In " Micronutrient Interactions: Vitamins, Minerals and Toxic Elements." (Levander, OA. & Cheng, L., eds.) Ann. N. Y. Acad. Sci. 355,333-345.

White, HS. (1979). Zinc Content and the Zinc-to-Calorie Ratio of Weighed Diets. J. Am. Diet. Assoc. 68,243-245.

W.H.O.: See World Health Organization.

Wilkins, PJ., Grey, PC. & Dreosti, IE. (1972). Plasma Zinc as an Indicator of Zinc Status in Rats. Br. J. Nutr. 27,113-120.

Williams, KE., Kidston, EM., Beck, F. & Lloyd, JB. (1975). Quantitative Studies of Pinocytosis 1. Kinetics of Uptake of [^{125}I]-Polyvinylpyrrolidone by Rat Yolk Sacs Cultured *In Vitro*. J. Cell Biol. 64,113-122.

Williams, KE., Roberts, G., Kidstone, ME., Beck, F. & Lloyd, JB. (1976). Inhibition of Pinocytosis in the Rat Yolk Sac by Trypan Blue. Teratology. 14,343-354.

Williams, RB. & Chesters, JK. (1970). The Effects of Early Zinc Deficiency on DNA and Protein Synthesis in the Rat. Br. J. Nutr. 24,1053-1059.

Williams, RB. & Mills, CF, (1970). The Experimental Production of Zinc Deficiency in the Rat. Br. J. Nutr. 24,939-1003.

Williams, RB., Davies, NT. & McDonald, I. (1977). The Effect of Pregnancy and Lactation on Copper and Zinc Retention in the Rat. Br, J. Nutr. 38,407-416.

Williams, RO. & Loeb, LA. (1973). Zinc Requirement for DNA Replication in Stimulated Human Lymphocytes. J. Cell. Biol. 58,594-601.

Wilson, JG. (1965). Enbryological Considerations in Teratology. In "Teratology: Principles and Techniques. (Wilson, JG. & Warkany, J., eds.)University of Chicago Press, Chicago, Ill. p271.

World Health Organization. (1970). Spontaneous and Induced Abortions. Tech. Rep. Ser. 461.

Wynter, JM., Walsh, DA., Webster, WS., McEwan, SE. & Lipson, AH. (1983). Teratogenesis After Acute Alcohol Exposure in Cultured Rat Embryos. Terat. Carcin. Mutag. 3,421-428.

APPENDIX 1

PUBLICATIONS AND ABSTRACTS

Record,I.R., Dreosti, I.E. and Tulsi, R.S. (1985). In vitro development of zinc-deficient and replete rat embryos. *Australian Journal of Experimental Biology and Medical Science,* 63(1), 65–71.

I.R. Record, I.E. Dreosti, S.J. Manuel, R.A. Buckley and R.S. Tulsi. Teratological influence of the feeding cycle in zinc-deficient rats. In "Trace Element Metabolism in Man and Animals 5" (Mills, Bremmer & Chesters, ed) Commonwealth Agricultural Bureaux, Slough. pp. 210-213.

Teratological Influence of the Feeding Cycle in Zinc-deficient Rats

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Maternal dietary Zn deficiency during the whole or part of gestation has been shown to be highly teratogenic in rats (Hurley & Shrader, 1972). Zn deficiency also results in the establishment of a cyclical feeding pattern with a period of about 4 days, over which the food intake fluctuates dramatically (Williams & Mills, 1970) and is accompanied by changes in the plasma Zn concentration (Wallwork *et al.*, 1981). In a previous study (Record *et al.*, 1983) we noted an apparently anomalous inverse relationship between maternal serum Zn levels and embryonic development mid-way through pregnancy, as well as large

differences between the growth and development of individual Zn-deficient litters. In order to help explain some of these observations we have investigated aspects of cyclical feeding and maternal Zn metabolism in Zn-deficient rats during the first half of pregnancy.

I. Materials and Methods

Virgin female Sprague-Dawley rats (200-230g) were confined overnight with males of the same strain. From the day of detection of sperm (day 0.5 of gestation) they were fed a soya-bean based diet containing either less than 0.5 μg Zn/g or 100 μg Zn/g. In the first experiment Zn-deficient dams were allowed the diet *ad-lib.*, but the control animals were fed only 12 g/day (the average daily amount consumed by the Zn-deficient group). Food intake was monitored daily. In the second study, Zn-deficient animals and their controls were presented with their diets in a systematic, cyclical manner designed to reproduce complementary patterns of the feeding cycle observed in the first experiment. Over the 11 d period food intake averaged 12 g/d although daily intakes ranged from 1.4 to 19.6 g.

After 11.5 d of gestation, all animals were anaesthetised with ether, serum samples obtained after cardiac puncture, and the embryos removed for morphological examination and protein determination to evaluate embryonic growth.

II. Results

In the first experiment, Zn-deficient rats rapidly entered individual cyclical feeding patterns, each with a period of about 4 d, but without any real degree of synchrony. Maternal serum Zn levels on the morning of day 11 were inversely correlated with the dietary intake during the previous night ($r = -0.95$; $P<0.001$). There was a high degree of variability between the embryos of different litters, both in terms of embryonic size and development, however variation within the individual litters was less. The coefficient of variation (CV) of embryonic protein between the litters was 36%, however, within the litters this was reduced to 25%. The CV between control litters was 16% and within the litters 13%. Overall, the protein content of the Zn-deficient embryos was significantly less ($P<0.001$) than the Zn-replete embryos (Table 1). In the Zn-deficient group the incidence of major malformations (including anophthalmia and neural tube defects) ranged from 100% in one litter to nil in two litters, with an overall rate of 30%. Comparison of the feeding cycles with the known time of development of individual organ systems suggested that a period of high food intake (and hence low maternal serum Zn levels) was related to the production of specific organ malformations and the overall prevalence of terata.

Table 1. Growth and development of Zn-deficient and replete rat embryos[a]

	Zn-deficient	Zn-replete
Total embryos (dams)	112 (8)	65 (6)
Protein (μg)	204 ± 9.9[b]	351 ± 9.6
Open neural tubes	10 (9%)	1 (1.5%)
Other major defects	24 (21%)	2 (3%)
Total major defects	34 (30%)	3 (4.5%)
Maternal serum Zn (μg/ml)	0.63 ± 0.10[b]	1.07 ± 0.03

a: Values are means ± SEM. Zn-deficient dams were allowed free access to the diet. Zn-replete dams were limited to 12 g diet/day.
b: $P<0.001$

This suggestion received experimental support when, in a separate study, pregnant dams were fed the Zn-deficient diet systematically to represent the cycle of feeding which induced a low maternal serum Zn level on days 8 and 9 of gestation. Another pattern was induced to produce adequate levels on these days, but decreased levels on days 6, 7, 10 and 11 (Fig. 1a, 1b).

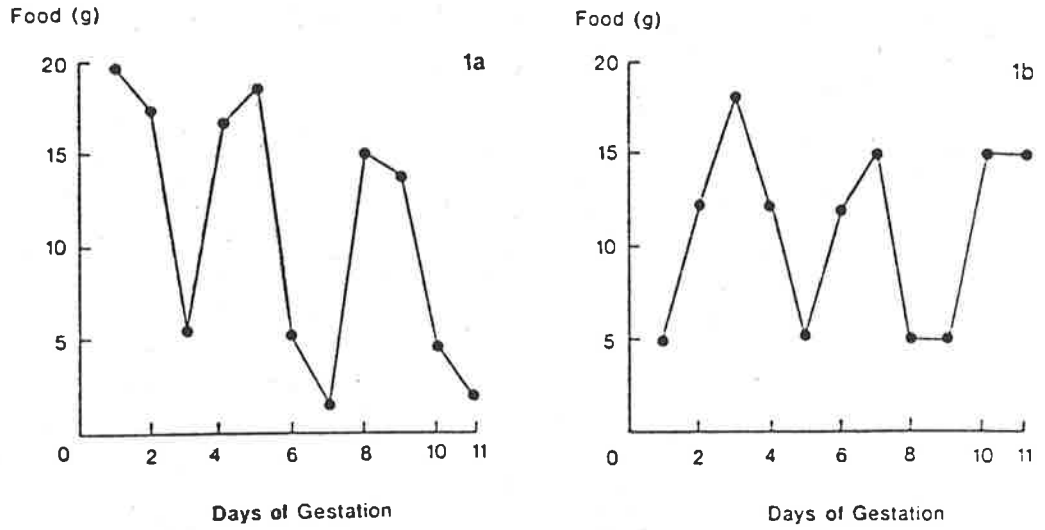

Fig.1. *Feeding cycle presented to dams to induce peak consumption and hence low serum zinc levels on (a) days 8 and 9, (b) days 6, 7, 10, and 11.*

Induction of low maternal serum Zn levels on gestational days 8 and 9 further decreased the size of the embryos and increased the incidence of open neural tubes to 32%, with a total of 72% of the embryos exhibiting major malformations (Table 2). The dams fed the Zn-deficient diet in the reverse cycle had larger embryos with only a 2% malformation rate, although there was an increased loss of embryos close to the time of implantation (Table 2).

III. Discussion

Recent work (Masters *et al.*, 1983) has demonstrated that force-feeding of anorexic, pregnant Zn-deficient rats produces deleterious effects on both the dam and developing fetus by inducing an anabolic state in the force-fed animals. In this study it is probable that during the feeding cycle of *ad-lib* fed Zn-deficient animals, the dams alternate between a fasting, catabolic state, and an anabolic state following eating. While fasting, the maternal tissues would release Zn, raising the serum Zn concentration and making Zn available to the embryo. During the feeding part of the cycle, circulating Zn will be lost in digestive enzymes and may be required for maternal tissue function or repair, thus diminishing the availability of Zn to the conceptus. Other factors such as the level and lability of maternal body Zn stores probably influence the rapidity of onset of the feeding cycle, and hence the timing of the fluctuations in the maternal serum Zn concentrations.

During gestation, the Zn-deficient litter will be exposed to 4 or 5 waves of Zn deficiency, each exerting an effect at a particular time of development. The biochemical and teratological effects of single or multiple waves of Zn deficiency during other stages of organogenesis are currently under investigation.

References

Hurley, L.S.; Shrader, R.E. (1972) in *Neurobiology of the Trace Elements Zinc and Copper* (Pfeiffer, C.C. ed.), pp. 7-52, Academic Press, New York

Hurley, L.S.; Shrader, R.E. (1975) *Nature* **254**, 427-429

Masters, D.G.; Keen, C.L.; Lonnerdal, B.; Hurley, L.S. (1983) *J. Nutr.* **113**, 905-912

Record, I.R.; Dreosti, I.E.; Tulsi, R.S.; Manuel, S.J.; Fraser, F.J. (1983) *Proc. Aust. Teratol. Soc.* **1**, 7

Wallwork, J.C.; Fosmire, G.J.; Sandstead, H.H. (1981) *Br. J. Nutr.* **45**, 127-136

Williams, R.B.; Mills, C.F. (1970) *Br. J. Nutr.* **24**, 989-1003

Table 2. Growth and development of Zn-deficient and replete rat embryos of cyclically-fed dams[a]

	Peak food intakes on days			
	8 and 9		6, 7, 10, 11	
	Deficient	Replete	Deficient	Replete
No. dams with total resorptions	0/8	0/4	4/9	0/4
No. embryos	105	42	76	52
Protein (μg)	128 ± 24[b]	216 ± 7	175 ± 13[b]	251 ± 6
Open neural tubes	34 (32%)	0	1 (1.3%)	0
Other major defects	42 (40%)	0	1 (1.3%)	2 (3.8%)
Total major defects	76 (72%)	0	2 (2.6%)	2 (3.8%)
Maternal serum Zn (day 11.5) ug/ml	1.13 ± 0.04	1.04 ± 0.05	0.51 ± 0.06[b]	1.06 ± 0.03

a: Values are means ± SEM.
b: $P < 0.001$ vs the respective Zn-replete group.

Discussion

WHITE (Perth, W.A.): Did you measure the Zn content of embryos from the two treatments to see if it was getting through.

RECORD (Adelaide): No, but we are looking at this by radioisotope methods.

M. GOLDEN (Kingston, Jamaica): What was the protein content of your diets and did you try an experiment with constantly restricted amounts of Zn-deficient diet so that animals received the mean intakes of other low-Zn treatments.

RECORD (Adelaide): No, we wanted to get the fluctuations in daily food intake characteristic of Zn deficiency that we feel are important in its teratogenic effects. Dietary protein was 20-25%.

Record,I.R., Tulsi, R.S., Dreosti, I.E. and Fraser, F.J. (1985). Cellular necrosis in zinc-deficient rat embryos. *Teratology (Currently known as: Birth Defects Research Part A: Clinical and Molecular Teratology),* 32(3), 397–405.

Record,I.R., Dreosti, I.E., Tulsi, R.S. and Manuel, S.J. (1986). Maternal metabolism and teratogenesis in zinc-deficient rats. *Teratology (Currently known as: Birth Defects Research Part A: Clinical and Molecular Teratology),* 33(3), 311–317.

Nutr. Res. (submitted)

GROWTH AND DEVELOPMENT OF THE ZINC-DEFICIENT MOUSE EMBRYO

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ABSTRACT

The teratogenic effects of a dietary zinc deficiency in rats are well established. There are comparatively few reports regarding similar effects on mice, however it has been established that mice are much more sensitive to a dietary deficit of the metal than rats.

Recent studies from this laboratory have shown that, in rats, during the periods of organogenesis, cellular death arising as a result of low zinc levels is responsible for the observed terata. The present studies using C57BL/6J mice demonstrate that necrosis also occurs in the embryo of this species and is probably associated with the observed dysmorphologies.

Key words: zinc deficiency, mice, teratogenesis, cell necrosis

INTRODUCTION

Despite the extensive investigations into the effects of a maternal dietary zinc deficiency on the in utero development of the rat (1), surprisingly little is known about a deficit of this metal on the mouse. Demonstrations of the teratogenicity of zinc deficiency in this species have been limited to interactions with other teratogens such as acetazolamide (2), alcohol (3,4) and cadmium (5).

More recently a report from this laboratory (6) has established that the strain of mice used (C57BL/6J) were highly sensitive to zinc deficiency and required at least 5 µg Zn/g diet to retain any fetuses and more than 10 µg Zn/g diet to avoid the occurrence of malformations. In addition, this latter study (6) showed clearly that, unlike rats, mice consumed their zinc-deficient diet on a constant basis, with no cyclical feeding pattern. The suggestion was made that due to the absence of fluctuations in food intake and the transient rises in maternal serum zinc levels associated with a maternal fast, the mouse embryos were subjected to extended periods of zinc impoverishment leading to an increased incidence of fetal dysmorphologies. However the etiology of the terata produced in mice by a deficit of the metal has not previously been investigated.

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The purpose of this current study was to confirm both the earlier findings (6) and to assess the morphological and histological appearance of mouse embryos at the time of organogenesis to discover whether cellular necrosis attributed to maternal zinc deprivation in rats (7) also occurred in this species.

MATERIALS AND METHODS

Animals

Mice of the C57Bl/6J strain were obtained at 8-10 weeks of age from the Gilles Plains Animal Resource Centre, Adelaide, South Australia, and allowed free access to food and water. Female mice were placed in a darkened cage with males (4 females/ 2 males) for 2h from 08.30 to 10.30 each morning and examined hourly for the presence of a vaginal plug. Thereafter they were housed individually in stainless steel and plastic cages and allowed free access to various soy-flour-based diets containing either <0.5, 5 or 100 µg Zn/g diet (8). On the 9th day of gestation (after 220hr gestation) dams were anaesthetised with ether, maternal blood samples collected by cardiac puncture and the ovaries and uteri excised. The ovaries were examined for the presence of corpora lutea and the embryos removed from the uterus and decidual tissue for assessment (8) and protein determination (9). Representative embryos were also fixed in Karnovsky's fixative for histological assessment.

RESULTS

In common with rats, the serum zinc level of mice decreased rapidly following institution of a zinc-deficient regime and rose after an imposed overnight fast (Fig. 1). However no evidence was seen of significant fluctuations in serum zinc levels accompanying feeding cycles as has been reported in rats (7,8) which supports the notion (5) that mouse embryos are exposed to continuous low circulating zinc levels.

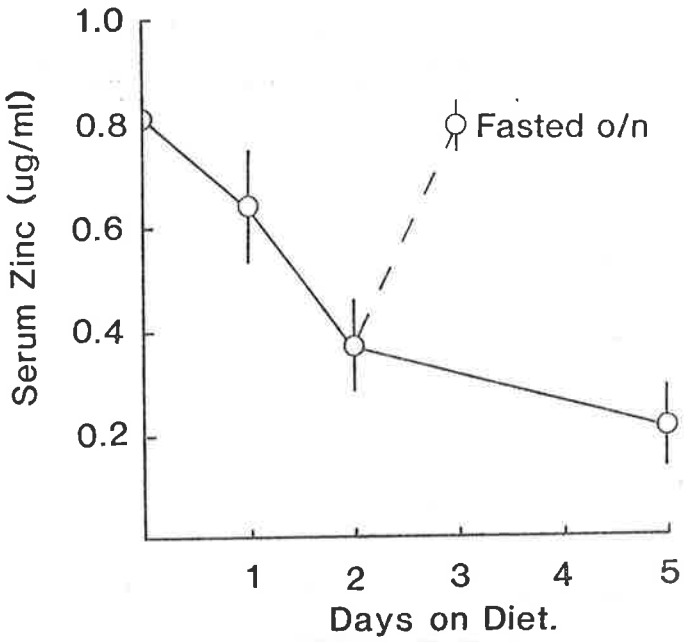

FIG. 1
Changes in mouse serum zinc levels following supply of a zinc-deficient diet and the response to an overnight fast. Means ± SEM of 4 animals/group.

In the mice fed the lowest level of zinc recovery of embryos was substantially decreased (Table 1). This was primarily due to the failure of a large proportion of pregnant dams (as judged by the presence of corpora lutea) to carry their embryos until day 9. The absence of implantation sites suggested that this loss occurred in early gestation. There was a surprisingly large proportionof dead embryos in the control (100 ppm zinc group) however the incidence of malformations was considerably higher in the groups fed low levels of zinc and ranged from 100% in zinc-deficient group to 36% in the group fed 5 µg Zn/g diet. In general the malformations were very similar to those observed in rats fed diets devoid of zinc (7), although a level of only 5 µg Zn/g diet proved to be highly teratogenic in this species. Indeed, due to the degree of the deformities it is unlikely that many of the embryos in this group would have survived until the end of gestation. In terms of size, the embryos from zinc-deprived dams were extremely small. Those embryos from dams fed 5 µg Zn/g were significantly smaller and contained much less protein than the control embryos, although the somite number was not greatly affected (Table 1). Photographs of the representative embryos are presented in Fig. 2A,B,C.

TABLE 1
Effects of feeding diets containing different levels of zinc on on embryonic development in pregnant mice

Outcome	Dietary zinc level (µg/g)		
	<0.5	5.0	100
Dams with corpora luteae	6	11	6
Dams with embryos	3	6	5
Total number of embryos	16	47	34
Dead embryos	5	5	10
Malformed embryos	11	15	1
Defects:			
Chorio-allantoic fusion	11	0	1
Rotation	1	1	1
Open neural tube	11	9	0
Other (anophthalmia, distorted head, neural tube)	(a)	5	0
Maternal serum zinc (µg/ml)	0.41±0.09[b]	0.80±0.03[b]	0.96±0.03
Grown-rump length (mm)	0.71±0.08[b]	2.09±0.07[b]	2.51±0.07
Somite number	(a)	22.4±0.4	23.6±0.4
Protein (µg/embryo)	25±16[b]	107±7[b]	152±10

(a) : Due to the severity of the malformations, these values could not be determined.

(b) : Significantly less than control (100 µg/g) group $P<0.05$.

Embryos from each group were also sectioned and examined under the light microscope. While control animals were entirely normal histologically, (Fig. 2D), in common with the zinc-deficient rats, embryos from both the zinc-deprived and low zinc dams were grossly abnormal (Figs. 2E, F). In the latter groups there were extensive areas of cells containing dark-staining inclusions representative of engulfed cellular material which were identical with the necrosis previously described for zinc-deficiency (7) and other (10, 11) teratogens. Regions of both the neural epithelium and mesoderm showed some degree of involvement. Large extra-cellular spaces, probably arising as a result of the removal of dead cells were also observed, particularly in the 5 µg/g group (Fig. 2E).

DISCUSSION

As with rats, the serum zinc level of mice fed a zinc-deficient diet fell rapidly. However, as has been shown previously, the food intake of zinc-deficient mice remains relatively constant, at least for the 9-day period of the study, although food deprivation following a period of zinc deficiency does lead to an increase in serum zinc levels. This absence of food cycling would imply that the serum zinc levels do not fluctuate as they do in rats, a suggestion supported by the data in the present study. Thus it appears that the continued exposure of mouse embryos to a level of maternal serum zinc restriction may be a major factor in the increased sensitivity of this species to low maternal dietary zinc levels which would not be teratogenic to rats.

The current study confirms that the mouse embryo is extremely sensitive to maternal zinc deprivation, and also provides evidence that the cellular necrosis induced by zinc deficiency in rats also occurs in the mouse embryo. This demonstration that the cytotoxic effects of zinc deprivation leading to the observed terata are not species-specific, and indeed even occur at dietary zinc levels of 5 µg Zn/g could be interpreted to suggest that this model might more suitably represent the human situation where (in the Western society) frank zinc-deficiency is rare, but marginal dietary zinc intakes are more widespread (1).

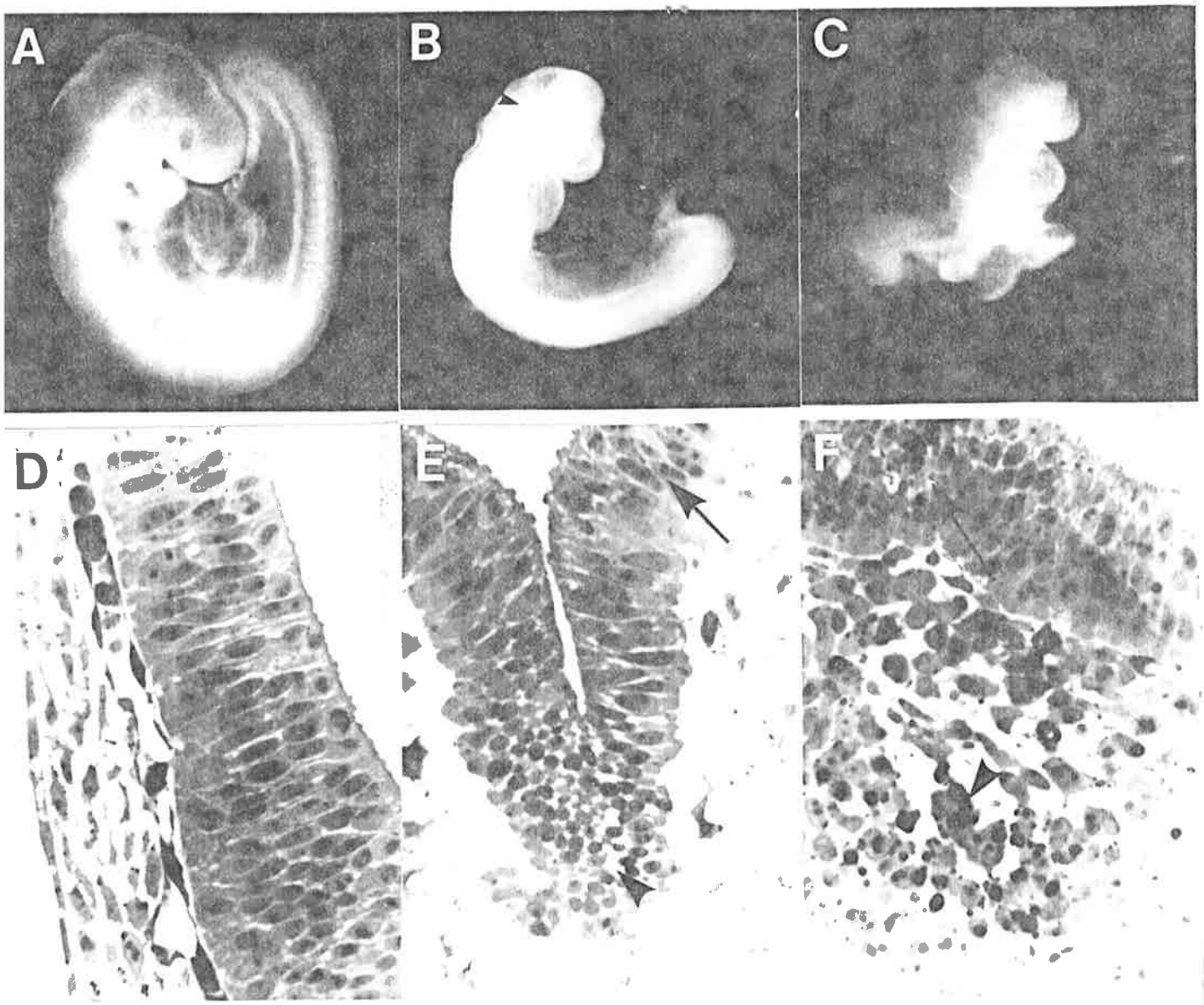

FIG. 2

Terata in zinc-deficient mouse embryos. A - control showing normal station and growth. B - (5μg Zn/g diet) showing open cranial neural tube (arrow) and poor rotation. C - (<0.5 μg Zn/g) showing multiple defects. D-F 1μM sections through the cranial regions of embryos similar to those in A-C. D - control. E - (5μg Zn/g diet) with limited areas of necrosis (arrow) and large extracellular spaces (arrowhead). F - (<0.5 μg Zn/g) showing extensive necrosis in both the neural ectoderm (arrow) and mesoderm (arrowhead).

REFERENCES

1. Dreosti IE, Record IR, Manuel SJ. Zinc deficiency and the developing embryo. Biol. Trace Element Res. 1985; 7: 103-122.

2. Hackman RM, Hurley LS. Interaction of dietary zinc genetic strain and acetazolamide in teratogenesis in mice. Teratology 1983; 28: 355-368.

3. Miller SI, Del Villano BC, Flynn A, Krumahansl M. Interaction of alcohol and zinc in fetal dysmorphogenesis. Pharmacol Biochem. Behav. 1983; 18 Suppl. 1: 311-315.

4. Keppen LD, Pysher T, Rennert DM. Zinc deficiency acts as a co-teratogen with alcohol in fetal alcohol syndrome. Pediat. Res. 1985; 19: 944-947.

5. Sato F, Watanabe T, Hoshi E, Endo A. Teratogenic effect of maternal zinc deficiency and its co-teratogenic effect with cadmium. Teratology. 1985; 31: 13-18.

6. Dreosti IE, Buckley RA, Record IR. The teratogenic effect of zinc deficiency and accompanying feeding patterns in mice. Nutr. Res. 1986; 6: 159-166.

7. Record IR, Tulsi RS, Dreosti IF, Fraser FJ. Cellular necrosis in zinc-deficient rat embryos. Teratology 1985; 32: 397-405.

8. Record IR, Dreosti IE, Tulsi RS, Manuel SJ. Maternal metabolism and teratogenesis in zinc-deficient rats. Teratology 1986; 33: 311-317.

9. Lowry OH, Rosebrough NS, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagent J. Biol. Chem. 1951; 193: 265-272.

10. Webster WS, Messerle K. Changes in the mouse neuroepithelium associated with cadmium-induced nural tube defects. Teratology 1980; 21: 79-88.

11. Morrissey RE, Mottet NK. Arsenic-induced exencephaly in the mouse and associated lesions occurring during neurulation. Teratology; 28: 399-411.

12. Record IR, Record SJ, Dreosti IE, Rohan TE. Dietary zinc intake of pre-menopausal women. Human Nutrition: Applied Nutrition 1985; 39A: 363-369.

Record,I.R., Dreosti, I.E., Tulsi, R.S. and Manuel, S.J. (1984). The zinc deficient rat embryo: morphological and biochemical observations. *Teratology (Currently known as: Birth Defects Research Part A: Clinical and Molecular Teratology)*, 30(1), 49A.

DEVELOPMENT OF ZINC-DEFICIENT RAT EMBRYOS IN CULTURE

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In rats, a maternal dietary zinc deficiency has been shown to be highly teratogenic, resulting in developmental abnormalities in every organ system. Many of these abnormalities arise during the critical periods of organogenesis, especially during the time of closure of the neural tube (about d 11 in the rat). Advances in technique have now made it possible to remove rat embryos from the dam after 9.5 days gestation, and to culture egg cylinders in vitro for up to 48 h, during which time development proceeds at the same rate as in vivo. Use has been made of this technique to study the development of the zinc deficient rat embryo during the time when the embryo is most susceptible to teratogenic stimuli.

Normal 9.5 d embryos obtained from Sprague-Dawley rats grew and developed normally in sera obtained from both zinc-deficient and replete rats. Embryos from some dams fed the zinc-deficient diet since mating were found to be morphologically normal, whilst other dams provide embryos which appeared stunted and/or malformed. These latter embryos developed abnormally in culture, regardless of the serum in which they were incubated. Morphologically normal zinc-deficient embryos, however, grew and developed to the same extent as control embryos, even in zinc-deficient sera. Inclusion of ^{65}Zinc in the medium showed that all embryos were able to obtain sufficient zinc from the media to maintain growth over this period. The results of these studies suggest that abnormal embryonic development due to zinc deficiency cannot be induced in vitro, however, it would appear that a maternal zinc deficiency can exert its effect prior to day 9.5 of gestation, and that these effects are not easily reversible.

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CYCLICAL FEEDING PATTERNS AND TERATOGENESIS IN ZINC-DEFICIENT RATS

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In rats, zinc deficiency results in the establishment of individual 4-day cyclical feeding patterns which are inversely related to fluctuations in the maternal serum zinc levels. A maternal dietary zinc deficiency has also been shown to be highly teratogenic in rats, producing abnormalities in every organ system. Taken together, these observations suggest that during development, the zinc-deficient rat embryo might be exposed to several waves of zinc deficiency, interspersed with adequate supplies of zinc. We have examined aspects of cyclical feeding in zinc-deficient and control rats, and the effects on the morphological development of the embryo over the first 11.5 days of gestation.

Pregnant animals fed the zinc-deficient diet since mating produced 11.5 day litters with varying degrees of malformation, at an overall rate of about 30%. Presentation of the diet in a systematic fashion intended to induce a low maternal serum zinc concentration on days 8 and 9 of gestation increased the rate of major malformations observed on day 11.5 to about 70%. When low maternal serum zinc levels were induced on days 6, 7, 10 and 11, the number of implantation sites was decreased, as was the incidence of major and minor malformations.

Further studies were performed in which single feeding cycles were timed to provide peak intakes of food on days 7 and 8 of pregnancy in one case and on days 9 and 10 in another. While in both cases the prevalence of terata was high (49% and 67% respectively) at the end of gestation, the type of abnormality differed between groups from a predominance of skeletal malformations in the day 7 and 8 group to mainly palatal and soft tissue defects in the day 9 and 10 animals.

These results demonstrated that judicious regulation of the food intake of zinc-deficient rats can influence the maternal serum zinc levels, and hence the availability of zinc to the embryo at selected stages of development, thus producing specific malformations of the organs developing at that time.

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Record,I.R., Dreosti, I.E., Tulsi, R.S. and Manuel, S.J. (1984). Growth of zinc-deficient rats. *Teratology (Currently known as: Birth Defects Research Part A: Clinical and Molecular Teratology),* 29(2), 53A.

THE INFLUENCE OF MATERNAL AGE ON THE TERATOGENICITY OF ZINC DEFICIENCY IN THE RAT

I.R. RECORD*, I.E. DREOSTI*, R.S. TULSI** AND S. LEOPARDI*

Cyclical feeding of a zinc-deficient diet to pregnant rats in order to induce low maternal zinc levels on days 8 and 9 of gestation has been shown to produce a high degree of embryonic dysmorphogenesis (Record et al. 1985). It would appear from these, and other studies (Masters et al. 1983) that during the feeding cycle, the zinc-deficient dam alternates between a catabolic state (when the dam reduces her food intake), and an anabolic state (when the dam is feeding). It is this latter phase, which is associated with the onset of developmental anomalies. These observations suggested to us that the litters of young, growing rats would be more susceptible to the teratogenic effects of zinc than older dams of more stable weight.

In order to establish the validity of this hypothesis, young (175 g) rats were fed zinc-deficient diets to produce a maximal teratogenic response, and at 11.5 days of gestation the litters were compared with those from similarly treated older (315 g) rats.

Embryos from the older zinc-deficient dams were smaller than those from the pair-fed zinc-replete dams, but were morphologically normal. The embryos from the younger zinc-deficient dams were significantly smaller than any from zinc-replete controls or even those from the older zinc-deficient dams. In addition, 18% of the embryos were dead and a further 68% of the live embryos malformed.

These results indicate that the ability of the growing dam to apportion stores of labile zinc between her own tissues and those of the developing litter is a major determinant of embryonic development, and is probably of even more significance in later gestation when the fetuses acquire most of their zinc.

MASTERS, D.G., KEEN, C.L., LONNERDAL, B. and HURLEY, L.S. (1983). J. Nutr. 10: 905.

RECORD, I.R., TULSI, R.S., DREOSTI, I.E. and FRASER, F.J. (1985). Teratology (in press).

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Record,I.R., Dreosti, I.E. and Tulsi, R.S. (1985). Fetal outcome following transient zinc deficiency in the pregnant rat. In *Genes, environment and mankind : a joint scientific meeting and annual scientific meetings of Australia New Zealand Environmental Mutagen Society, Australian Teratology Society, Human Genetics Society of Australasia, Genetics Society of Australia : programme and abstracts, University of Sydney, August 26th-30th, 1985*.

Record,I.R., Tulsi, R.S. and Dreosti, I.E. (1985). Dietary zinc deficiency induces cell necrosis in rat embryos. In *Genes, environment and mankind : a joint scientific meeting and annual scientific meetings of Australia New Zealand Environmental Mutagen Society, Australian Teratology Society, Human Genetics Society of Australasia, Genetics Society of Australia : programme and abstracts, University of Sydney, August 26th-30th, 1985*.

Record,I.R., Tulsi, R.S. and Dreosti, I.E. (1985). Abnormal cell death in zinc-deficient rat embryos. *Journal of Anatomy,* 143, 221-222.

Record,I.R., Tulsi, R.S. and Dreosti, I.E. and Manuel, S.J. (1985). Induction of teratogenic abnormalities in rats by maternal dietary zinc deficiency. *Journal of Anatomy,* 142, 223.