THE EFFECT OF ZINC ON CELL DIVISION

by

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B.Sc. (Hons.) (Natal)

Submitted in partial fulfilment
of the requirements for
the degree of

Doctor of Philosophy

in the

Department of Biochemistry
University of Natal

PIETERMARITZBURG MAY 1975

ACKNOWLEDGEMENTS

The author wishes to express his appreciation and thanks to the following persons for their contribution to this thesis:

Dr J. E. Dreosti for his invaluable guidance and supervision throughout the research project, and for his many suggestions and assistance in the preparation of the manuscript.

Professor G. V. Quicke for his continual interest and assistance during the course of the project.

Dr C. Albrecht and Dr D. Balinsky for providing certain materials used in this study.

My colleagues in the Biochemistry Department for their critical interest.

My wife and family for their continual interest and support.

I also wish to express my appreciation to the Council for Scientific and Industrial Research for the financial support which enabled me to undertake this research project.

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CHAPTER 1

INTRODUCTION

Like so many discoveries, the demonstration by Raulin in 1869 (1) that zinc was an essential nutrient for the growth of Aspergillus niger was not followed up until 50 years later when its role in plant life was established (2). Shortly thereafter, it became linked with animal nutrition when Birckner (3) stated "from its constant occurrence in the yolk of eggs, and in human and cows' milk, it is inferred that the element zinc exerts an important nutritive function".

Early attempts to demonstrate the function of zinc met with limited success and it was not until Todd, Elvehjem and Hart (4) in 1934 showed that zinc was essential for the growth of the rat that these studies made any real progress. Since then, evidence on the significance of zinc has been quite dramatic and in the last few years knowledge in this field has made great strides. Possibly one of the reasons for this progress was that previously it was thought that because of the abundance of zinc in the soil, the probability of zinc deficiency developing in man or animals was remote, yet recent evidence has shown that zinc deficiency is responsible for a number of symptoms including retarded growth, skeletal and skin lesions in swine, sheep and cattle (3, 5, 6) and non-development of secondary sexual characteristics and retarded growth in humans (7) (Table 1).

TABLE 1 Zinc deficiency symptoms in animals

SPECIES	MAIN COMPONENT OF DIET	SYMPTOMS	REFERENCE
Pigs	Corn and soyabean meal	Anorexia Growth retardation Parakeratosis Loss of hair	Tucker and Salmon (6)
Dairy cattle	Urea and egg albumin	Skin lesions Poor reproductive performance Low milk production Oedematous swelling of the hind legs	Mills et al. (8)
Lambs	Urea and egg albumin	Anorexia Decreased growth rate Wool growth retarded Production of thin, fragile wool fibres Changes in structure of the hooves and horns	Mills et al. (8)
Rats	Casein, egg albumin or soyabean protein	Anorexia Growth retardation Loss of hair pigmentation Testicular atrophy Hyperkeratosis Teratogenicity of rat foetuses	Prasad (9)
Man	Wheat flour	Short stature Hypogonadism Roughened skin Hepatosphenomegally Poor wound healing Defective connective tissue metabolism Defective hormone regulation	Prasad (7) O'Dell (10) Miller et al. (11) Quarterman et al. (12) Sandstead et al. (13)

With regard to aspects of the zinc-deficient syndrome, Tucker and Salmon (6) have shown that the administration of zinc cured and prevented parakeratosis in swine showing a reversible effect of zinc deficiency on some metabolic processes. Despite this, until a few years ago zinc studies tended to be confined to clinical observations and overt physiological effects. Recently however, much of the effort expended in research upon trace elements and zinc in particular, has been directed towards a detailed study of the metabolic lesion associated with a deficiency of the element. Furthermore, considerable attention was paid to the factors which may influence and modify trace element requirements and the availability of the nutrients for specific physiological functions.

Most information concerning the role of zinc at a molecular level was derived from studies on the effect of a deficiency of this metal on several metabolic processes. The studies have been greatly facilitated due to the rapid onset of a physiological zinc deficiency following dietary depletion (9, 14), which implies that mobilisation of the body reserves does not occur at an adequate rate to meet the demands imposed following a reduced dietary intake (15).

In the last three decades the suggestion has been made that many of the metabolic lesions associated with zinc deficiency are the result of the role of zinc in certain metalloenzyme systems (16, 17), the first of which (carbonic anhydrase) was demonstrated by Keilin and Mann in 1940 (18). Since then the activities of alcohol dehydrogenase (19), carboxypeptidase (20), alkaline phosphatase (21) and several other enzymes (22, 23) (Table 2) have been shown to decrease markedly in certain cells and tissues during states of dietary zinc restrictions.

Despite the fact that the established role of zinc in certain of the metalloenzymes mentioned above may account, at least in part, for the occurrence of certain symptoms of zinc deficiency, the rapid cessation of growth in young rats coupled with the severe teratogenicity accompanying

TABLE 2 Zinc metalloenzymes

Enzyme	Molecular weight	g Atoms Zn/mole	Source
Carbonic anhydrase	29 000	1	Bovine and human erythrocytes
Carboxypeptidase A	34 500	1	Bovine pancreas
	87 000	2	Human liver
Alcohol dehydrogenase	150 000	4	Yeast
Glutamic dehydrogenase	1 000 000	2-6	Beef liver
Lactic dehydrogenase	40 000	1	Beef heart
Alkaline phosphatase	89 000	4	*E. coli*
Aldolase	69 000	1	Yeast, *E. coli*
Phospholipase	-	-	*B. cereus*
Amylase	50 000	0,5	*B. subtilis*
Procarboxypeptidase	67 000	1	Bovine pancreas

short periods of maternal zinc depletion observed by Hurley and Swenerton (24), suggested that lack of zinc was particularly critical during periods of tissue growth and that it may be involved at a primary locus of action more critical than its role in many of the zinc-metalloenzymes. At present little is known concerning this primary lesion although attempts have been made to link it with the emerging role of zinc in nucleic acid and protein synthesis (25 - 27).

A role for zinc in cell multiplication was first proposed by Bertrand and Vladesco (28) based on the presence of large amounts of zinc in the prostate, testes and semen. Fujioka and Lieberman (29) first demonstrated an inhibition of DNA synthesis by zinc deficiency and recently Sandstead and Rinaldi (30), Weser, Seeber and Warnecki (31) and Williams and Chesters (27) have shown a requirement for zinc in DNA synthesis in various tissues. In contrast to these results, Turk (32) and Macapinlac, Pearson, Barney and

Darby (33) found no effect of zinc deficiency on DNA synthesis. It must be stressed, however, that interpretation of these results is difficult because of the variety of tissues used, uncertainty with respect to the precise zinc status of the experimental animals, and the secondary effects of anorexia. Nevertheless, considerable evidence does point to a requirement for zinc during DNA synthesis.

Directly associated with DNA synthesis are RNA and protein synthesis and these two metabolic processes have also recently received a great deal of attention. Again, results have been equivocal and while Williams and Chesters (27), and Macapinlac et al. (33) were unable to detect any differences in the rates of RNA, or protein synthesis between zinc-deficient and control rats, Theuer and Hoekstra (34), Hsu, Anthony and Buchanan (35) and Grey and Dreosti (36) have shown an increase in the rate of protein synthesis in zinc-deficient animals. Others such as Somers and Underwood (26), Schneider and Price (37), Mills, Quarterman, Williams and Dalgarno (38) and Hsu and Anthony (39) have found significant decreases in protein synthesis in deficient animals. As with DNA synthesis results have been difficult to compare because of the variable experimental material used.

A recent and interesting development has been the demonstration of a requirement for zinc during DNA and protein synthesis in rapidly proliferating lymphocytes (40, 41). Since this in vitro system again demonstrates the importance of zinc in cell multiplication it enhances the in vivo evidence discussed earlier. Also emerging from previous investigations has been the suggestion that tumour growth may be affected by zinc levels, since neoplastic tissue generally reflects an increased rate of cell division. In this connection, a number of workers (42 - 45) have studied the development of a spectrum of transplantable animal tumours in mice and rats receiving insufficient dietary zinc, and in most cases a reduced rate of tumour growth and increased survival time was found in deficient animals.

In contrast Poswillo and Cohen (45) found an inhibition of carcinogenesis associated with increased levels of zinc.

The available evidence left little doubt that zinc is intimately involved in many metabolic processes and, in particular, that it plays an important role in nucleic acid metabolism. However, the precise site of action in DNA synthesis was unclear as also was the significance of this association in the production of the characteristic zinc deficiency syndrome. Recent work (27, 36) however, together with data from the present investigation strongly suggests that impaired DNA synthesis represents the primary lesion associated with zinc deficiency, which in turn makes the site of action of zinc in this pathway of particular research interest.

An important aspect in defining the precise role of zinc in DNA synthesis would be to determine the stage of DNA synthesis (i.e. synthesis of precursors, phosphorylation of these compounds or polymerization of the phosphorylated precursors) affected by zinc deficiency. In this regard the incorporation of added precursors such as deoxynucleosides (46, 47) and deoxynucleotides (48, 49) into DNA could provide useful evidence concerning the site of zinc activity in the pathway of DNA synthesis.

The role of zinc in a number of enzyme systems is well established and the view held by the author, which has been supported by recent studies (50, 51), was that zinc acts at an enzymic site in DNA synthesis. Should this be so, once the involvement of zinc in a particular phase of DNA synthesis had been established, the identification of the enzymes involved would follow logically. In this respect, the pathways of DNA synthesis that occur in nuclei and mitochondria provide an interesting comparison since they are known to replicate their DNA independently of one another (52, 53), and it appears that although they have the same mechanism of DNA replication (54 - 56) the systems contain certain component enzymes (thymidine kinase (57) and DNA polymerase (58)) which are genetically different. Accordingly, differences in

DNA synthesis between these sub-cellular organelles as a result of zinc deficiency may reflect a difference in the activity of one or more of the genetically different component enzymes. With regard to this action of zinc on enzymes, two enzymes directly involved in DNA synthesis, aspartate transcarbamylase (59) and DNA polymerase (50), have been identified as zinc metalloenzymes and a third, thymidine kinase (51), has been shown to be zinc-dependent.

The present study was intended to establish the primary locus of action of zinc in a variety of mammalian tissues and to equate these findings with the symptoms associated with the onset of a nutritional zinc deficiency. For this purpose three model systems were employed ((i) regenerating rat liver; (ii) proliferating rat lymphocytes; (iii) rat tumour tissue), each of which reflected rapid cell division while offering a variety of different experimental advantages.

Initially, a study was made of the effect of zinc deficiency on DNA and protein synthesis in regenerating rat livers using EDTA-extracted soyabean as the main ingredient of the deficient diet and using plasma zinc levels as the method of monitoring zinc status in the rats. The timing of the S-phase of DNA synthesis in deficient and control animals was examined, as this important aspect of the cell division cycle has not previously been studied in zinc-deficient tissues. Results showed that zinc deficiency inhibited DNA synthesis in this system and, in addition, delayed the peak of synthesis by 5 - 7½ hours after partial hepatectomy. Protein synthesis was not initially affected in the deficient animals although it did decline later (20 - 48 hours post-operatively).

The second study made use of proliferating rat blood lymphocytes and concerned the influence of varying levels of zinc on DNA and protein synthesis in this system. Such an *in vitro* process offered several advantages over the *in vivo* system of regenerating livers since ambient zinc levels could be finely

controlled and monitored. The effects of EDTA chelation and metal ion supplementation were also studied and the results indicated an inhibition of DNA and protein synthesis by low levels of zinc as was observed with regenerating rat liver tissue, but in addition, high zinc levels were also found to reduce DNA and protein synthesis.

Further work concerned the effect of dietary zinc on the growth of a transplanted hepatoma in rats and on the process of carcinogenesis in mice. Both high ($\geq$ 500 ppm of zinc) and low ($<$ 0,5 ppm of zinc) levels of zinc were found to reduce tumour growth and carcinogenesis and, in addition, similarly to the findings using the lymphocyte system, reduced DNA synthesis was found in the tumours of rats fed the high and low zinc diets.

Because of the genetic differences in certain of the enzymes associated with DNA synthesis in nuclei and mitochondria, a useful experiment was deemed to be a study of the effects of zinc levels on nuclear and mitochondrial DNA synthesis in regenerating rat livers. Differences in the incorporation of ^{3}H-thymidine into DNA between these two sub-cellular fractions may well have provided useful information concerning the site of action of zinc in relation to the genetically distinct enzyme systems. The results showed that zinc deficiency inhibited incorporation of ^{3}H-thymidine in both fractions but that mitochondrial DNA synthesis did not exhibit the shift in peak of DNA synthesis seen in the nuclear fraction. However, very little could be concluded from these findings concerning the site of action of zinc in this process.

Following on this study, an investigation was undertaken in an attempt to locate the stage of DNA synthesis affected by zinc depletion by examining the effect of additional deoxynucleoside and deoxynucleotide precursors on DNA synthesis in nuclei and mitochondria in regenerating rat livers. Data from the nucleoside study indicated that zinc acted at some site beyond the synthesis of the deoxynucleoside precursors, possibly during the phosphorylation of the deoxynucleosides or at the stage of polymerization of the phosphorylated precursors. Little information could be obtained from the

nucleotide experiment since there was uncertainty concerning the mechanism of incorporation of these precursors *in vivo* (60).

Because the earlier studies had pointed to a role of zinc in the later stages of DNA synthesis and since the thymidine kinase "salvage" pathway is reported to be the main pathway of DNA synthesis in rapidly dividing cells (61, 62) it was decided to examine the effects of zinc deficiency on the activities of selected enzymes involved in this pathway. The two enzymes investigated were thymidine kinase and DNA polymerase since these are known zinc-dependent enzymes (50, 51) that are active in the thymidine kinase pathway of DNA synthesis. Results showed both thymidine kinase and DNA polymerase activities to be considerably reduced in regenerating rat livers from zinc-deficient animals which indicated the site of action of zinc in DNA synthesis was on these two enzymes. However, since the role of DNA polymerases in DNA synthesis is in doubt (63, 64) it is probable that the major effect of zinc deficiency was due to a reduction in the activity of the likely rate-determining enzyme in this pathway - thymidine kinase.

It appears then that a number of metabolic irregularities arising out of zinc deficiency are a result of a primary locus of action of zinc on DNA synthesis and that this effect is mediated through an action of zinc on thymidine kinase which is the probable rate-controlling enzyme in DNA synthesis in rapidly dividing cells (65).

CHAPTER 2

THE EFFECT OF ZINC DEFICIENCY ON DNA SYNTHESIS IN REGENERATING RAT LIVER

2.1 Introduction

The rapid cessation of growth in young rats coupled with the severe teratogenicity accompanying short periods of maternal zinc depletion suggested a primary locus of action of zinc that is affected very soon after restriction of adequate dietary zinc. Some workers have suggested that these symptoms may reflect an impairment in nucleic acid metabolism in the deficient animals (66 - 69). A role for zinc in cell multiplication was first suggested by Bertrand and Vladesco (28) who based their suggestion purely on the relatively large amounts of zinc in the prostate, semen and testes. It was not until 1962 that this role of zinc was further studied when Wacker (21) showed that if Euglena gracilis was grown on a zinc-deficient medium cell division was arrested, and the DNA content per cell was doubled in the deficient cells while the cell volume increased four-fold in the same period indicating a mitotic arrest as a result of zinc deficiency. At the same time Lieberman and Ove (66) and Lieberman, Abrams, Hunt and Ove (67) found zinc was essential for DNA synthesis in rat kidney cells cultured in vitro. Similar studies performed by these workers (29) have indicated that zinc reversed the inhibition of DNA synthesis as a result of EDTA perfusion in regenerating rat livers.

Recently, most work has been performed on animals fed a zinc-deficient diet but results from these studies have often been conflicting. While Williams, Mills, Quarterman and Dalgarno (68), Weser, Seeber and Warnecki (31), Williams and Chesters (27), Sandstead and Rinaldi (30) and Buchanan and Hsu (69) have reported a reduced synthesis of DNA in livers of zinc-deficient rats, Turk (32) using chicken livers and Macapinlac et al. (33) using rat testes

were unable to demonstrate an effect of zinc-deficiency on DNA synthesis. Despite the difficulty in comparing results due to the different tissues used in these studies the weight of evidence, including earlier results from this laboratory (36), pointed to a definite effect of zinc deficiency on DNA synthesis. Added to this has been evidence of an involvement of zinc in the enzymes associated with replication (50, 70).

Unfortunately, the animals investigated in all previous studies, except those in this laboratory, were always subjected to prolonged periods of zinc depletion when the secondary effects of deficiency and the accompanying inanition were already apparent. Furthermore, there is at present no data relating to the timing of the S-phase of cell division in zinc-deficient tissues, which if different from normal tissue would complicate the interpretation of data based on identical periods of thymidine incorporation for both control and experimental animals.

The present study was designed to extend the previous findings in this laboratory and to establish the effect of short-term zinc deficiency (a three-day period which resulted in sharply reduced levels of plasma zinc but avoided the complicating influence of inanition) on DNA synthesis in regenerating rat livers. Attention was also paid to the timing of the S-phase of cell division in deficient and control animals. Regenerating liver was selected for use in these studies because it provided an ideal model system that was actively undergoing cell division and cell enlargement.

2.2 Materials and Methods

2.2.1 Reagents

Thymidine (^{3}H-methyl), with a specific radioactivity of 5 mC/mMole was obtained from the Radiochemical Centre, Amersham, Buckinghamshire England. The sample was diluted with physiological saline to give a final activity of 50 mC/ml.

Scintillation fluid. 2,5-Diphenyloxazole (PPO) and 1,4-bis(2-(4-methyl-5-phenyloxazole 1)) benzene (dimethyl POPOP) were obtained from Beckman Instruments Inc., Fullerton, California, U.S.A.

The scintillation fluid was prepared according to the method of Heyes and Gould (71) by dissolving PPO (5,0 g) and dimethyl POPOP (0,1 g) in 500 ml of A.R. toluene and 500 ml of absolute methanol. Toluene was included to facilitate the dissolution of the primary and secondary phosphors whereas methanol was used to increase the solubility of the aqueous sample in the scintillation fluid. The scintillation fluid is extremely photolabile and was accordingly kept in an amber bottle and stored in the dark.

Diphenylamine reagent. Diphenylamine reagent was prepared by dissolving 1,5 g of A.R. diphenylamine (British Drug Houses Ltd., Poole, England) in 100 ml glacial acetic acid and 1,5 ml concentrated sulphuric acid. The reagent was stored in the dark. On the day of use 0,1 ml aqueous acetaldehyde (16 mg/ml) was added to each 20 ml of reagent required.

Standard DNA solutions. A stock solution (1 mg/ml) of highly polymerized calf-thymus DNA (British Drug Houses Ltd., Poole, England) was prepared by dissolving the DNA in isotonic saline. From this solution standards were prepared containing 100 - 500 µg DNA/ml.

2.2.2 Glassware

Because of the danger of contamination all glassware was boiled for 10 min in 2N-nitric acid and rinsed with distilled, deionized water before use.

2.2.3 Rations

Preparation of zinc-deficient soyabean meal. Zinc-deficient soyabean meal was prepared in essentially the manner described by Hurley and Swenerton (24) for soyabean protein. In this procedure 1 kg of soyabean meal was suspended in approximately 8 litres of tap water and the suspension

adjusted to pH 4,3 . EDTA (5 g EDTA/kg) was added to the soyabean and after 30 min stirring the soyabean meal was filtered. The process was repeated three times at which stage it was washed 5 times with distilled, deionized water and once with redistilled ethanol to facilitate the final drying procedure.

Salt-mix. The Salt-mix constituted 4% of the total ration and individual constituents were selected on the basis of their levels of zinc contamination, i.e. brands with the lowest zinc content were used. The salt-mix consisted of KH_2PO_4 (102,93 g), $CaCO_3$ (87,87 g), NaCl (75,18 g), $MgSO_4.7H_2O$ (29,99 g), $Fe(C_6H_5O_7).3H_2O$ (1,87 g), $CaHPO_4.2H_2O$ (1,29 g), $MnSO_4.4H_2O$ (0,36 g), KI (0,002 g), $CuSO_4.5H_2O$ (0,47 g), NH_4 Molybdate (0,008 g) per 300 g.

Diets. The zinc-deficient diet used throughout the investigation reported in this thesis was composed of (g/100 g):-
Sucrose 50; soyabean meal (44% protein) 38,5; corn oil 6,1; salt-mix 4,0; cod-liver oil 0,7; DL-methionine 0,5; choline chloride 0,2.
The ration was analysed and shown to contain less than 0,4 ppm of zinc and 16 - 18% protein.

Control animals received the same ration supplemented with 60 ppm zinc as zinc sulphate (24). A mixture of crystalline vitamins was supplied separately in sucrose three times a week (72).

2.2.4 Animals

Female rats of the Wistar strain (110 - 120 g) were used in all experiments in this thesis except where otherwise stated. The rats were housed three to a stainless steel cage and fed the zinc-deficient and control rations as described. Drinking water was distilled and deionized.

2.2.5 Methods

Collection of blood samples. Blood samples (5 ml) were collected directly from the hearts of rats following ether anaesthesia. 5% Sodium citrate (0,2 ml) was added per ml of blood as an anticoagulent. The plasma

was separated by centrifugation at 900 g for 15 min in a Martin-Christ Piccolo centrifuge and zinc levels of the plasma determined by atomic absorption spectroscopy.

Atomic absorption spectroscopy. This was performed using a Varion Techtron (1000) atomic absorption spectrophotometer, reading at a wavelength of 214,2 nm. Using an acetylene/air flame the reproducibility of the procedure was established by performing twenty individual determinations on a standard zinc solution prepared by dissolving metallic zinc in HCl made up to 2N after dissolution of the metal. Another set of standards, for use with the plasma samples, were made up in 2N-HCl and 14% sucrose. Earlier work in this laboratory (73) has indicated that such solutions have a similar viscosity to that of plasma and in this way differences in sample flow-rate due to varying viscosities can be eliminated. Both standard curves are shown in Appendix 1.

Surgical procedures. Partial hepatectomy (70%) was performed on rats under ether anaesthesia according to the method of Higgins and Anderson (74). A ventral, mid-line incision was made, extending from the xiphisternum posteriorly for approximately 2 cm. The liver was extruded through the incision and the median and left lateral lobes completely excised. The right lateral and caudate lobes were left intact. The lobes to be excised were ligatured at their base before removal, and the abdominal wall and integument were closed separately by individual sutures.

Incorporation of ^{3}H-thymidine. Groups of animals were fed the zinc-deficient and control diets for 3 days, after which they were partially hepatectomised and injected intraperitoneally with 25 µC ^{3}H-thymidine in 0,5 ml physiological saline at various times post-operatively. An incorporation period of 60 min was allowed before the animals were sacrificed by ether anaesthesia and blood and liver samples were removed. Liver samples were wrapped in foil and quick-frozen in liquid air.

Because of the problems associated with the interpretation of incorporation data preliminary studies were performed in which only a 5 minute

incorporation period was allowed 20 hours after partial hepatectomy in control animals. In addition, the specific activity was determined on samples of DNA isolated from rats receiving only a sham operation. An incision was made in these rats but no liver removed and a 60 min incorporation period of ^{3}H-thymidine allowed. In a third experiment, Mitomycin C (1 mg/rat), a specific inhibitor of DNA synthesis (75), was injected intraperitoneally 19 hours after partial hepatectomy and 1 hour before the administration of ^{3}H-thymidine.

The determination of the specific radioactivity of DNA. Two procedures were used in this study:

1. The procedure employed in the first experiment was a slight modification of that used by Fujioka and Lieberman (29), in that the whole regenerating liver was used for the preparation of the homogenate and not merely a portion thereof. The frozen livers were homogenized with a Dounce homogenizer in 10 ml 0,25 M-sucrose solution using 10 passes with the loose plunger followed by 10 passes with the tight plunger, and the homogenate was centrifuged at 10 000 g in a Beckman Model L ultracentrifuge. The pellet was dissolved in 5 ml 0,3 M-NaOH and incubated at 37°C for 18 hours, at which stage 5% TCA (12 ml) was added and the resulting precipitate collected by centrifugation at 10 000 g. The pellet was again dissolved, incubated and reprecipitated as described earlier. The final precipitate was filtered on a Buchner funnel, washed with 25 ml cold 5% TCA and 20 ml cold ethanol. The material was then hydrolysed in 5 ml 1,0 M-HCl in a boiling water bath for 30 min. The insoluble material was removed by centrifugation at 2 500 g in an International centrifuge (PR2) and the supernatant removed and made up to 5 ml with 1,0 M-HCl. An aliquot (0,5 ml) of this hydrolysate was added to 15 ml scintillation fluid for radioactive counting on a Beckman 3-channel liquid scintillation counter. A further 2 ml aliquot was taken for colorimetric determination of the total DNA content of the material isolated.

2. In the second experiment the procedure of Volkin and Cohn (76) was followed. The pellet obtained after centrifugation as described in procedure (1) was suspended in 5 ml 0,25 M-sucrose and 15 ml cold 10% TCA was added. The resulting precipitate was collected by centrifugation at 1 000 g for 10 min and washed serially with 10% TCA, 80% ethanol, 95% ethanol and finally a 3 : 1 ethanol : ether mixture. The washed precipitate was dissolved in 10 ml 0,3 M-NaOH and incubated at 37°C for 18h, at which stage the alkaline solution was neutralised with 6 N-HCl before the addition of 10 ml cold 5% TCA. The precipitate was again collected by centrifugation at 1 000 g for 10 min and washed once with 5% TCA. The final precipitate was hydrolysed in 5 ml 5% TCA in a boiling water bath for 15 min. Insoluble material was removed by centrifugation at 1 000 g, the supernatant was decanted and the precipitate was hydrolysed once more in 2 ml 5% TCA. Insoluble material was again removed by centrifugation and the supernatant pooled with that from the first centrifugation. Duplicate aliquots (0,5 ml) were withdrawn and added to 15 ml of scintillation fluid for radioactive counting. Further aliquots (3 ml) were taken for colorimetric determination of the total DNA content of the material isolated.

Determination of DNA. DNA was determined colorimetrically according to the method described by Burton (77). DNA determinations were performed on duplicate 2 ml aliquots from each hydrolysate and at the same time on the hydrolysed DNA standard solutions. Diphenylamine reagent (4 ml) was added and the colour allowed to develop at room temperature for 17h. The absorbance of the blue-coloured complex was determined in a Spectronic 20 (Bausch and Lamb) at 600 nm. The reagent blank contained 2 ml 1,0 M-HCl and 4,0 ml diphenylamine reagent.

A DNA standard curve was prepared using duplicate 2 ml aliquots of the hydrolysed standards containing 100 - 500 µg DNA/ml (Appendix 2).

2.3 Results

2.3.1 Results of the preliminary study

A preliminary check on the uptake of ^{3}H-thymidine indicated that incorporation into DNA increased by approximately 780% (from 5 864 cpm/mg DNA to 45 879 cpm/mg DNA) 20 hours post-operatively in partially hepatectomised animals when compared with animals subjected to only a sham operation (Table 3). Furthermore, a decrease in the specific activity of 95% (45 879 to 2 236 cpm/mg DNA) was obtained in DNA from rats allowed a 5 min incorporation period, when compared with those receiving a 60 min incorporation period. Addition of Mitomycin C was found to inhibit incorporation of ^{3}H-thymidine by 66% (Table 3) indicating that incorporation of ^{3}H-thymidine was reflective of active DNA synthesis in this study.

TABLE 3 Results of a preliminary investigation into the incorporation ^{3}H-thymidine into the DNA of regenerating livers of stock colony rats 20 hours after surgery. Means of 6 animals and their S.E.'s

Treatment	Incorporation time (min)	Incorporation of ^{3}H-thymidine (cpm/mg DNA)
Partial hepatectomy	60	45 879 ± 4 429
Sham hepatectomy	60	5 864 ± 336
Partial hepatectomy	5	2 236 ± 501
Partial hepatectomy + Mitomycin C	60	6 921 ± 812

2.3.2 The effect of zinc deficiency on the incorporation of ^{3}H-thymidine in regenerating livers 10 to 30 hours after partial hepatectomy

Data concerning the relative incorporation of ^{3}H-thymidine into the DNA of regenerating livers from zinc-deficient and control rats using procedure (1) is presented in Appendix 3 and summarised in Table 4. The data indicate that incorporation was significantly reduced ($p < 0,01$) in the deficient rats 15 to 30 hours post-operatively.

TABLE 4 The effect of zinc deficiency on the incorporation of ^{3}H-thymidine into DNA from regenerating rat livers at various times after partial hepatectomy

Zinc status of rats (1)	No. of rats (2)	Time after partial hepatectomy (h)	Specific activity of DNA (cpm/mg DNA) (3)	Difference of means (cpm/mg DNA)
Control	5	10	10 728 ± 840	419 ± 613
Zinc-deficient	5	10	10 309 ± 481	L.S.D. (0,25) 430
Control	5	15	33 174 ± 2 302	11 174 ± 1 692
Zinc-deficient	5	15	22 000 ± 1 382	L.S.D. (0,01) 4 738
Control	5	17½	46 339 ± 3 841	15 023 ± 3 478
Zinc-deficient	5	17½	31 016 ± 3 975	L.S.D. (0,01) 9 405
Control	9	20	45 879 ± 4 429	15 962 ± 5 182
Zinc-deficient	8	20	29 917 ± 2 696	L.S.D. (0,01) 15 148
Control	6	22½	44 746 ± 2 504	15 719 ± 2 741
Zinc-deficient	5	22½	29 027 ± 3 775	L.S.D. (0,01) 7 674
Control	6	25	41 373 ± 1 682	5 944 ± 1 269
Zinc-deficient	6	25	35 429 ± 1 414	L.S.D. (0,01) 3 942
Control	6	30	36 408 ± 1 063	5 022 ± 1 194
Zinc-deficient	6	30	31 386 ± 1 783	L.S.D. (0,01) 3 243

(1) Mean plasma zinc levels of deficient animals were 0,57 ppm (0,64 - 0,50) and control rats 1,02 ppm (1,13 - 0,92).

(2) Individual results in Appendix 3.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 3.

The data presented in Table 4 point to a substantial reduction in DNA synthesis in the zinc-deficient animals. Moreover, an interesting difference occurred in the timing of the peak of maximum thymidine incorporation between the zinc-deficient and control animals. The difference is highlighted in Table 5 and Figure 1.

TABLE 5 The effect of zinc deficiency on the incorporation of ^{3}H-thymidine into DNA from regenerating rat livers at various times after partial hepatectomy

Zinc status of rats	Time after partial hepatectomy (h)	Specific activity of DNA (cpm/mg DNA)	Difference of means (cpm/mg DNA)
Control	17½	46 339 ± 3 841	4 966 ± 2 538
Control	25	41 373 ± 1 682	L.S.D. (0,05) 4 428
Zinc-deficient	17½	31 016 ± 3 975	4 413 ± 2 571
Zinc-deficient	25	35 429 ± 1 414	L.S.D. (0,05) 4 528
Control	17½	46 339 ± 3 841	10 910 ± 2 478
Zinc-deficient	25	35 429 ± 1 414	L.S.D. (0,01) 9 150

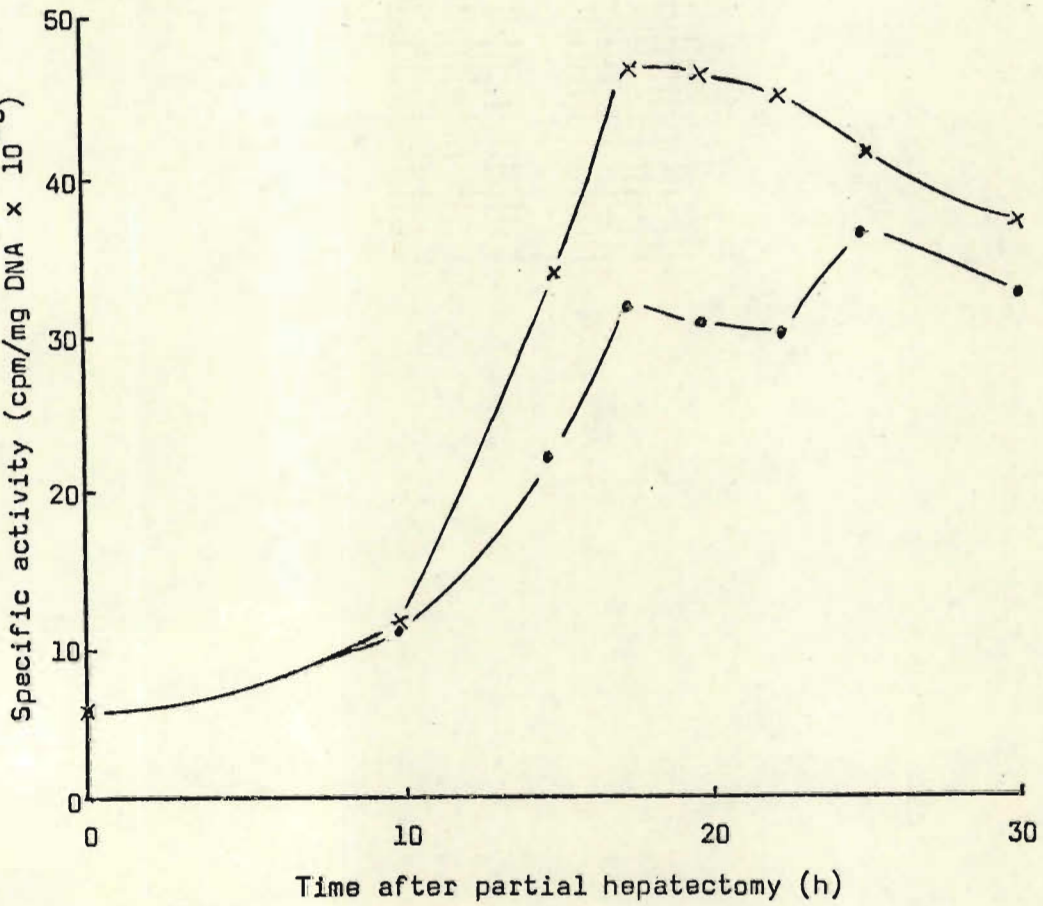

FIGURE 1 Specific activity of DNA isolated from regenerating livers of zinc-deficient and control rats at various times after partial hepatectomy.
× —— × Control; • —— • Zinc-deficient

In the control group maximum DNA synthesis occurred 17½h post-operatively and declined significantly ($p < 0,05$) at 25h. With the zinc-deficient animals however, incorporation of label rose to be higher at 25h than at any other time ($p = 0,05$). Although in the present experiment incorporation of thymidine in the deficient animals was significantly reduced at 17½h ($p < 0,01$) and 25h ($p < 0,01$) after partial hepatectomy, (Table 4), when compared with the corresponding control values, the difference was considerably less (27,3%) when the 17½h control value was compared with the 25h deficient value (Table 5).

Because of the significance of the shift in the peak of DNA synthesis in the zinc-deficient animals the experiment was repeated using a different

technique to determine the specific activity of the isolated DNA (procedure (2)). Use of the second procedure resulted in a higher yield of DNA per liver (Appendix 4) but the specific activity of the DNA was similar to that obtained in the first experiment. Results from the second experiment (Table 6 and Appendix 4) showed DNA synthesis to be significantly reduced ($p < 0,01$) in the zinc-deficient animals and, in addition, indicated a shift in the peak of DNA synthesis from 20h in the control group to 25h in the zinc-deficient group although the incorporation of ^{3}H-thymidine was still significantly lower ($p < 0,01$) in the zinc-deficient rats when the two peaks of incorporation were compared.

TABLE 6 The effect of zinc deficiency on the incorporation of ^{3}H-thymidine into DNA from regenerating rat livers at various times after partial hepatectomy. Means of 6 animals and the S.E.'s

Zinc status[1][2] of rats	Time after partial hepatectomy (h)	Specific activity[3] of DNA (cpm/mg DNA)	Difference of means (cpm/mg DNA)
Control	15	30 066 ± 2 576	7 048 ± 2 052
Zinc-deficient	15	23 018 ± 2 450	L.S.D. (0,01) 6 374
Control	17½	40 009 ± 2 867	11 500 ± 2 109
Zinc-deficient	17½	28 509 ± 2 264	L.S.D. (0,01) 6 551
Control	20	41 165 ± 2 943	11 750 ± 2 147
Zinc-deficient	20	29 415 ± 2 273	L.S.D. (0,01) 6 669
Control	22½	40 321 ± 2 798	9 648 ± 2 159
Zinc-deficient	22½	30 673 ± 2 482	L.S.D. (0,01) 6 706
Control	25	39 509 ± 2 422	6 614 ± 2 352
Zinc-deficient	25	32 895 ± 2 823	L.S.D. (0,05) 5 177
Control	30	36 113 ± 3 061	5 108 ± 2 350
Zinc-deficient	30	31 005 ± 2 106	L.S.D. (0,05) 5 172
Control	20	41 165 ± 2 943	8 270 ± 2 579
Zinc-deficient	25	32 895 ± 2 823	L.S.D. (0,01) 8 010

(1) Mean plasma zinc levels of deficient animals were 0,54 ppm (0,65 - 0,46) and control rats 0,97 ppm (1,03 - 0,91).

(2) Individual results in Appendix 4.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 4.

2.4 Discussion

The nature of past research on the effect of zinc on DNA synthesis has made it difficult to state without qualification that zinc deficiency results in a decrease in DNA synthesis. A number of reasons may be suggested for this uncertainty, one of which is certainly the great variety of tissues and organisms used in these studies, all varying in their zinc requirements, stability of zinc stores, and metabolic turnover rates. However, the fact that a decrease in synthesis with zinc depletion has been shown in a number of tissues active in DNA synthesis does point strongly to a qualitative if not a quantitative effect of zinc on DNA synthesis.

Results by Fujioka and Lieberman (29) using EDTA perfusion to remove zinc must be treated with caution since Becker and Hoekstra (78) have shown that *in vitro*, EDTA removes 70% of the zinc from liver homogenates whereas *in vivo*, zinc-deficient diets result in only 20 - 30% reduction in tissue zinc concentrations. Also, the technique of injecting zinc into deficient animals to provide controls, as used by Becker (79), Weser *et al.* (31) and Williams and Chesters (27) is a doubtful method of control since it may result in transient effects of a different nature to those arising when the element is supplied continuously in the diet. Doubt must also be expressed about controls pair-fed with deficient animals (27, 30) since this method results in partial starvation of the control animals.

In so far as feeding animals on zinc-deficient diets is concerned, again some criticism must be levelled at earlier work. In the first instance, a number of workers described deficient diets as having a level of zinc in excess of 1 ppm. Based on the findings of Dreosti *et al.* (14) the author feels that for a true effect of zinc deficiency to be shown, especially in small laboratory animals such as rats, the diet should contain $< 0{,}5$ ppm Zn before it can be termed a zinc-deficient diet. Also, as mentioned previously, animals were maintained on deficient diets for 4 - 5 weeks at which stage secondary effects of zinc deficiency and inanition are apparent.

Because of these problems the techniques used in the present investigation were designed to eliminate criticism in this respect, i.e. zinc-deficient diets contained 0,35 - 0,4 ppm Zn, controls were fed ad libitum and animals received the experimental diets for only three days prior to surgery. The results indicated that this early zinc deficiency resulted in a significant decrease ($p < 0,01$) in the rate of DNA synthesis in regenerating livers, thus demonstrating the rapid effect of zinc depletion on DNA synthesis.

Another fact which emerges is that DNA synthesis was not only reduced following dietary zinc restriction but, in addition, the peak of maximum thymidine incorporation was delayed by approximately 5 - 7½ hours. This finding, which was doubly checked using two different procedures, is of particular significance with regard to the interpretation of data where identical periods for incorporation have been used, as under these circumstances apparent reductions in DNA synthesis may reflect no more than a delay in the period of maximum thymidine uptake and not reduced nett synthesis. The significance of this observation with regard to past and future studies on the effect of zinc deficiency on DNA synthesis is apparent.

Concerning the reason for the apparent delay in the peak of DNA synthesis a number of suggestions could be made, including a lag in the G_1 period of synthesis preceding the S-phase - possibly due to some enzymic effect - or a prolonged S-phase. From Figure 1 it can be seen that 10 hours after partial hepatectomy the incorporation of thymidine into DNA was almost identical in both deficient and control animals, suggesting that there was no lag before the onset of the S-phase in deficient animals (i.e. no extended G_1-phase). However, the peak of synthesis was reached later in the deficient group than that in the controls indicating an extension of the S-phase before synthesis decreased in the G_2-phase. In this connection, it should be noted that Stern and Hotta (80) have shown that a small amount of DNA synthesis occurs during the G_2-phase of synthesis and it could thus be suggested that in the deficient

rats the peak of synthesis was in fact reached at 17½ hours after partial hepatectomy and that the increase in synthesis at 25 hours was due to this synthesis in the G_2-phase. However, Stern and Hotta observed a steady synthesis in the G_2-phase and not a peak. Also, the peak of synthesis at 25 hours was significantly greater ($p \leq 0,05$) than that at any other time in the deficient rats which militates against any involvement of DNA synthesis during the G_2-phase in these experiments. For these reasons the author proposes that in fact the S-phase of DNA synthesis is lengthened by ± 5 hours in the zinc-deficient rats, i.e. a slower synthesis of DNA. Why this should occur is uncertain but it seems that the answer may be found in the enzymes required for DNA synthesis, i.e. a reduced synthesis or activity of these enzymes in zinc-deficient cells.

Nevertheless, despite this delay, the peak of DNA synthesis at 25h after partial hepatectomy in the deficient animals was still significantly lower ($p < 0,01$) than the peak at 17½h in the control group and was even significantly lower ($p < 0,01$) than at 25h in the controls. Thus it can be stated with some degree of certainty that zinc deficiency reduces DNA synthesis in regenerating rat livers and such impaired DNA synthesis probably represents the primary lesion associated with zinc depletion in growing tissue.

CHAPTER 3

THE EFFECT OF ZINC DEFICIENCY ON PROTEIN SYNTHESIS AND INTERSTITIAL AND INTRACELLULAR FLUIDS IN REGENERATING RAT LIVER

3.1 Introduction

As was discussed in Chapter 2 there is evidence which points to an effect of zinc deficiency on DNA synthesis, yet doubt exists as to the primary lesion associated with the condition. The possibility cannot be excluded that the locus of action may be on RNA or protein synthesis since this would result in an ultimate inhibition of DNA synthesis.

In this connection Schneider and Price (37), Winder and Dennery (81) and Wacker (21) have suggested that the primary locus of action of zinc deficiency is a reduction in RNA synthesis which results in a consequent decrease in protein synthesis. In recent years several studies (26, 34, 38, 39, 69) have provided evidence of defective protein synthesis in zinc-deficient rats. On the other hand, Macapinlac *et al.* (33) and Williams and Chesters (27) were unable to detect any difference in the rate of protein synthesis in various tissues of zinc-deficient rats. Thus, as with DNA synthesis, the evidence for a role of zinc in protein metabolism was uncertain. As mentioned in Chapter 2 this probably arises from the use of a variety of tissues, differing methods for removing zinc, and long periods of feeding on deficient diets. The picture is not made any clearer by results from this laboratory (36) which showed a slightly increased synthesis of protein in zinc-deficient rats in regenerating liver 10h after partial hepatectomy but no difference in synthesis 20h after partial hepatectomy.

Apart from the contradictions in the literature, another problem remains - that of amino acid pools. One of the major problems involved in the use of the technique of injecting labelled amino acids intraperitoneally into animals is the extent to which these compounds are held up in amino acid pools before

being incorporated into protein and whether the availability of amino acids for incorporation from these pools is the same in control and deficient animals. No attempts have, as yet, been made to assess this availability.

In the present investigation, protein synthesis was studied *in vivo* in regenerating livers of zinc-deficient and control rats in an effort to extend earlier findings in this laboratory of the effect of short term (3 day) zinc deficiency on protein synthesis. Attention was also paid to the availability of the labelled amino acid (^{14}C-leucine) for incorporation into protein.

3.2 Materials and Methods

3.2.1 Reagents

(U-^{14}C)L-leucine (10 mC/mMole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. The sample was dissolved in physiological saline to give a concentration of 5 μC/ml.

Scintillation fluid. The scintillation fluid described in Chapter 2 was used in the present investigation.

Microbiuret reagent. Sodium citrate (17,3 g) and sodium carbonate (10 g) were dissolved in approximately 50 ml distilled water by careful warming, to which was added a solution of copper sulphate (1,73 g in 20 ml distilled water). The mixture was cooled and made up to 100 ml in a volumetric flask using distilled water.

3.2.2 Animals and rations

Groups of 3 female rats of the Wistar strain (110 - 120 g) were housed in stainless steel cages and fed the rations described in Chapter 2. Drinking water was distilled and deionized through an Elgastat multibed ion-exchange resin.

3.2.3 Methods

Surgical procedure. Partial hepatectomy was performed, as described in Chapter 2, on animals that had received the experimental rations for 3 days.

Collection of blood samples. Blood samples (5 ml) were collected from the heart and zinc levels were determined by atomic absorption spectroscopy as described in Chapter 2.

Injections and timing. (U-^{14}C)L-leucine (2 μC) was injected intraperitoneally into the experimental animals 10, 20, 48 and 168 hours post-operatively. Thirty minutes later, the animals were killed by chloroform anaesthesia and the livers were removed for incorporation studies.

Because of the difficulty in interpreting amino acid incorporation data, a preliminary study was made on groups of 4 female, stock-colony rats (110 - 120 g) to establish that the data would reflect only active protein synthesis during the incorporation period. Initially, a comparison was made between the amount of incorporation that occurred in 30 min in partially hepatectomised rats 10 and 20 hours post-operatively, with that in animals that had received only a sham operation. In another experiment, the inhibitor puromycin (4,0 mg/rat) (82) was injected intraperitoneally 10h after partial hepatectomy, and 15 min before administration of the ^{14}C-leucine. In a third experiment, protein was isolated 5 min after the injection of the isotope in order to establish the degree of contamination of the labelled, isolated protein by unincorporated ^{14}C-leucine.

Determination of the specific radioactivity of the isolated protein. The procedure adopted was a modification of the methods of Wool and Moyer (83) and Mayne, Barry and Riviera (84) in that different volumes of reagent were necessary due to the larger amounts of liver used. The entire chilled livers were homogenised in 15 ml isotonic saline in an ice-bath using a Dounce homogeniser. Ten passes each were made with the loose- and tight-fitting plungers. Protein was precipitated from the homogenates by the addition of an

equal volume of 20% TCA and the solution allowed to stand in ice for 30 min. Thereafter, the samples were placed in a boiling water bath for 10 min and the protein was separated by centrifugation in a Martin-Christ Piccolo centrifuge (2 500 g for 10 min). The residue was washed three times with 10% TCA and dissolved in 10 ml hot formic acid/hyrdogen peroxide (5 volumes of 90% v/v HCOOH + 1 volume of 30% w/w H_2O_2). The protein was re-precipitated by the addition of 10 ml 10% TCA. After allowing 1 hour for complete precipitation, the protein was separated by centrifugation, washed twice with acetone and dried to constant weight under an infra-red lamp. The dried protein was weighed and dissolved in 10 ml of a solution of 90% HCOOH : 30% H_2O_2 (5 : 1) on a boiling water bath. Duplicate samples (1 ml) of the protein solution were added to 15 ml scintillation fluid for radioactive counting.

Radioactivity was measured in a Beckman three-channel liquid scintillation counter, the period of counting being adjusted so that the standard error of each sample was 1% or less.

Microbiuret protein assay. The microbiuret method of Bailey (85) was used in order to determine the concentration of the isolated protein on a dry weight basis. Samples (10 mg) of protein dried to a constant weight under infra-red irradiation were dissolved in 20 ml 3% v/v sodium hydroxide and duplicate 4 ml aliquots were used in subsequent analyses. Microbiuret reagent (0,2 ml) was added to each 4 ml aliquot and the contents of the tube were mixed by inversion. Thereafter (15 min), the extinction at 330 nm was determined in a Zeiss PMQ II spectrophotometer, using 1 cm quartz cuvettes.

A standard protein curve was prepared using solutions of increasing concentrations of ovalbumin (Appendix 5).

Determination of total free leucine and ^{14}C-leucine in the interstitial and intracellular fluid compartments of regenerating rat liver. The levels of free unlabelled leucine and free ^{14}C-leucine in what essentially comprised the intracellular and interstitial fluid compartments were determine

in the supernatants from homogenates of exsanguinated liver samples after the protein had been precipitated with TCA. Aliquots (0,5 ml) were removed and the leucine concentration determined on a Beckman 120 B amino acid analyser. The radioactivity in aliquots (0,5 ml) withdrawn at the same time was also measured so that both the concentration of leucine and its specific activity in the supernatant could be established. From a standard curve of total activity versus ^{14}C-leucine concentration (Appendix 6) the level of ^{14}C-leucine in the supernatant was determined and expressed as a percentage of the total leucine present.

3.3 Results

3.3.1 Assay of isolated protein

Duplicate determinations were performed on dried (infra-red) protein samples. The protein concentration was found to range from 71 - 74%.

3.3.2 Results from the preliminary investigation

Data from the preliminary investigation on control rats (Table 7) indicates that the incorporation of ^{14}C-leucine increased by approximately 25% and 70% respectively (20 139 cpm/g protein to 25 338 and 39 122 cpm/g protein respectively) in the partially hepatectomised animals 10h and 20h post-operatively when compared with those receiving only a sham operation. Puromycin reduced incorporation of ^{14}C-leucine by approximately 60% (25 338 to 10 740 cpm/g protein) 10h after partial hepatectomy. The specific activity of protein isolated from regenerating liver 5 min after injecting the isotope was 78% lower than that found in rats allowed a 30 min period of incorporation (25 338 to 5 889 cpm/g protein).

TABLE 7 Results of the preliminary investigation on incorporation of ^{14}C-leucine into the protein of regenerating livers of stock-colony rats 10h and 20h post-operatively. Means of 4 animals and their S.E.'s

Treatment	Incorporation time (min)	Post-operative time (h)	Specific activity (cpm/g protein)
Partial hepatectomy	30	10	25 338 ± 3 541
Partial hepatectomy	30	20	39 122 ± 4 542
Sham hepatectomy	30	10	20 139 ± 2 025
Partial hepatectomy + puromycin	30	10	10 740 ± 812
Partial hepatectomy	5	10	5 889 ± 626

3.3.3 The effect of zinc deficiency on the incorporation of ^{14}C-leucine in regenerating rat livers at different times post-operatively

The results of an investigation into the differences in uptake of ^{14}C-leucine by regenerating rat livers at various times after partial hepatectomy are given in Table 8 and Appendix 7. Incorporation was similar in both groups of animals 10h and 20h post-operatively but after 48h and 1 week it was significantly lower ($p < 0,01$ and $p < 0,05$ respectively) in the deficient group. In these experiments the control rats exhibited plasma zinc levels of 0,91 ppm (0,78 - 1,02) compared with the zinc-deficient levels of 0,54 ppm zinc (0,31 - 0,71).

TABLE 8 The effect of zinc deficiency on the incorporation of ^{14}C-leucine into protein of regenerating rat livers at various times after partial hepatectomy

Zinc status of rats	No.(1) of rats	Time after partial hepatectomy	Specific activity of protein (cpm/g protein)	Difference of means (cpm/g protein)
Control	8	10h	25 083 ± 3 728	881 ± 1 910
Zinc-deficient	8	10h	25 964 ± 3 950	L.S.D. (0,01) 3 248
Control	6	20h	34 249 ± 4 147	2 180 ± 4 748
Zinc-deficient	6	20h	36 429 ± 6 656	L.S.D. (0,01) 8 606
Control	8	48h	37 393 ± 5 050	10 377 ± 3 217
Zinc-deficient	8	48h	27 016 ± 3 688	L.S.D. (0,01) 9 620
Control	7	1 week	22 985 ± 3 126	6 554 ± 2 148
Zinc-deficient	7	1 week	16 431 ± 2 267	L.S.D. (0,05) 4 672

(1) Individual results in Appendix 7.

3.3.4 The effect of zinc deficiency on the total free leucine and free ^{14}C-leucine in the intracellular and interstitial fluid compartments of regenerating liver

The data presented in Table 9 indicate that the total concentration of free leucine in the intracellular and interstitial fluid compartments varies with time after partial hepatectomy. So, too, does the concentration of ^{14}C-leucine, although no significant differences could be found in the percentage of labelled leucine available for protein synthesis at any one time between zinc-deficient and control animals. At the time of sacrifice plasma zinc levels in the control animals were 0,96 ppm Zn (0,79 - 1,05) and in the zinc-deficient animals 0,55 ppm (0,50 - 0,61).

TOTAL 9 Total free leucine and free ^{14}C-leucine in the intracellular and interstitial fluid compartments of regenerating livers from rats of differing zinc status at various times after partial hepatectomy. Mean values of 3 animals and their S.E.'s

Zinc status of rats	Time after partial hepatectomy	Total leucine(1) in pool (µM)	Total ^{14}C-leucine(1) in pool (µM)	$\frac{\mu M\ ^{14}C\text{-leu}}{\mu M\text{-leu}} \times 100$
Control	0h	6,74 (6,02 - 7,39)	0,096 (0,087 - 0,103)	1,43 ± 0,25
Control	20h	2,22 (2,21 - 2,24)	0,057 (0,052 - 0,061)	2,56 ± 0,13
Zinc-deficient	20h	2,31 (2,30 - 2,32)	0,053 (0,051 - 0,054)	2,29 ± 0,10
Control	48h	0,43 (0,42 - 0,45)	0,027 (0,024 - 0,028)	6,25 ± 0,70
Zinc-deficient	48h	0,36 (0,35 - 0,37)	0,028 (0,021 - 0,035	7,77 ± 2,0
Control	1 week	5,01 (4,83 - 5,18)	0,098 (0,091 - 0,104)	1,90 ± 0,36
Zinc-deficient	1 week	3,57 (3,46 - 3,68)	0,079 (0,078 - 0,079)	2,19 ± 0,02

(1) Mean value of 3 animals with range in parenthesis.

3.4 Discussion

The data obtained from the preliminary investigation indicated that the incorporation of ^{14}C-leucine into regenerating rat livers provided a satisfactory measure of protein synthesis under the conditions. The zero time study indicated very little contamination of the isolated protein with unincorporated radioactive leucine. Probably increased inhibition of protein synthesis by the addition of puromycin could have been obtained with higher levels of antibiotic. The results nevertheless indicate that the bulk of the incorporation measured in this study reflected active protein synthesis.

The similarity in incorporation of ^{14}C-leucine in the deficient and control animals 10h after partial hepatectomy does not confirm the findings of Grey and Dreosti (36) who demonstrated an increase in protein synthesis at this time in 3-day zinc-deficient rats, although the subsequent decrease in synthesis is

corroborated. The findings are however, in agreement with Weser *et al.* (31), Macapinlac *et al.* (33) and O'Neal, Pla, Fox, Gilson and Fry (86) who all found no difference in protein synthesis in zinc-deficient and control rats using different tissues as their model systems. It must be pointed out that Weser *et al.* used the technique of zinc injection to obtain their controls and doubts as to the validity of this method have already been expressed in Chapter 2.

In contrast to the results obtained at 10 and 20 hours post-operatively, incorporation at 48h and 1 week after partial hepatectomy in the present study indicated significantly decreased protein synthesis in the zinc-deficient animals This supported the findings of Theuer and Hoekstra (34), Somers and Underwood (2 and Williams and Chesters (27) using a number of tissues as model systems. However, the results obtained by these workers could have been due to the effect of prolonged zinc deficiency and even the present results obtained after 1 week on deficient diets were probably influenced to some extent by the secondary effects of zinc deficiency (e.g. inanition) on protein synthesis. Nevertheless, the reduced incorporation which was observed at 48h points to a marked inhibitio by the deficiency *per se*, which is in agreement with the observations of Williams and Chesters (27) also using short periods of zinc deficiency.

The present data suggest that the effect of zinc deficiency on protein synthesis in regenerating rat liver is manifest 20h - 48h post-operatively but that significantly reduced protein synthesis only occurs after 48 hours. Result from periods longer than this could not be attributed exclusively to an effect o zinc deficiency.

Measurement of the rate of uptake of label into protein is generally considered to be a satisfactory index of protein synthesis, provided the specific activity of the precursor amino acid during the experimental period can also be measured (87). In the past, studies on protein synthesis in zinc-deficient animals have consistently failed to take account of possible variations in the availability of the precursor amino acid and the present study represents what i probably the first attempt to assess the amino acid pool size in zinc-deficient

Recently it has been suggested that in rats, the protein of the skeletal muscle was synthesized either directly from extracellular amino acids or from an intracellular pool in rapid equilibrium with the extracellular space (88). Accordingly in the present experiment the specific activity of ^{14}C-leucine was assayed in the fluid compartment comprising in the main, the intracellular and interstitial fluids of regenerating liver. The results indicate no apparent effect on the uptake of intraperitoneally injected ^{14}C-leucine or its transport to the site of protein synthesis in the regenerating liver. Data relating to the concentrations of free labelled and free unlabelled leucine that are available for incorporation into protein, indicate that the reduced synthesis associated with zinc deficiency does not arise from a shortage of this precursor amino acid. It seems that the impairment must arise at some other point in the pathway of synthesis.

The reduction in concentration of total free leucine in the fluid compartments 20h post-operatively was probably due to reduction in the size of these compartments caused by removal of two thirds of the liver. The even greater reduction at 48h was probably a combined effect of partial hepatectomy and the rapid withdrawal of amino acids from the pool for protein synthesis. After 1 week protein synthesis and compartment size had begun reaching normal levels again, thus the increase in total leucine in the pools.

The findings of an effect of zinc deficiency on protein synthesis 20 - 48h post-operatively support the earlier hypothesis that DNA synthesis is the primary locus of action of zinc in the cell since zinc deficiency exhibits an effect on DNA synthesis earlier in the cell cycle (15h) after partial hepatectomy.

CHAPTER 4

THE EFFECT OF ZINC LEVELS ON DNA AND PROTEIN SYNTHESIS IN PHA-STIMULATED RAT LYMPHOCYTES

4.1 Introduction

Lymphocytes are found distributed in a large number of tissues and function mainly in the generation of the immune response (89 - 91). In this particular study however, it is the reaction of these cells to mitogenic agents that was of principal interest. Blood lymphocytes do not normally divide when incubated in a suitable *in vitro* system. In 1960, however, Nowell (92) observed that human peripheral blood lymphocytes could be stimulated to divide in culture by a mucoprotein, phytohaemagglutinin (PHA), extracted from the red kidney bean *Phaseolus vulgaris*. PHA has erythroagglutinating, leukoagglutinating and mitogenic properties, it being the latter property which stimulated the lymphocytes to divide. Other mitogenic agents, such as pokeweed mitogen (93) and streptomycin S (94), have since been isolated but PHA has remained the best experimental agent for lymphocyte studies.

When PHA is added to lymphocytes in culture, they develop a blastoid appearance over the next 48 - 72h after which they divide. These changes are accompanied by striking metabolic alterations in the stimulated cell, the earliest of which include increased RNA synthesis within 24h of exposure to PHA (95) and an increase in both the acetylation of histones and the phosphorylation of histones and lipoproteins (96). A few hours later the cells increase their rate of protein synthesis (97, 98) and produce a variety of cellular enzymes active in glycolysis and oxidative phosphorylation. DNA synthesis begins 30 - 36h after PHA addition (94, 100). Thus the transformation of lymphocytes by PHA may be regarded as an instance of extensive gene activation.

The precise mechanism whereby PHA can initiate this transformation is still unknown. Nowell (92) originally suggested that PHA altered the membrane permeability characteristics of the lymphocyte in a manner which converted the cell

into a mitotically active state, a viewpoint that has been supported by other workers in the field (94, 101). The suggestion has also been made that PHA may attach to some other cellular structure (100) but evidence in this regard is not convincing.

Despite this uncertainty it is well known that a number of factors can influence the *in vitro* transformation of lymphocytes, one of these being chelating agents which remove metal cations from solution. Alford (40) has recently reported that DNA synthesis in transformed lymphocytes was inhibited by the addition of EDTA to the culture. Since DNA synthesis represented the culmination of a series of metabolic changes initiated by the addition of PHA to lymphocytes the EDTA could have acted by inhibiting any of the metabolic steps necessary for this stage to be reached. In fact, Chesters (41) has recently shown that EDTA inhibited protein synthesis in PHA-stimulated pig lymphocytes. However, the fact that metal ions, and zinc in particular, were essential for normal DNA replication in a number of *in vivo* systems (26, 27, 36) as well as in other *in vitro* cell cultures (66, 81, 102) suggested that EDTA acted on these lymphocytes through inhibition of the reactions directly involved in DNA synthesis, probably by chelating the divalent metal ions required for these steps. In all the *in vitro* cases of EDTA inhibition of DNA and protein synthesis zinc ions were found to be the most effective divalent metal ions in reversing this effect (40, 41).

These findings suggest that lymphocyte cell culture offered an ideal *in vitro* system in which to study the effect of zinc on nucleic acid and protein metabolism, since zinc availability could be controlled either by the composition of the culture medium or by addition of chelators. Furthermore, the use of cultured lymphocytes eliminated the problem found in *in vivo* studies of controlling or evaluating redistribution of zinc between the tissues of animals, even when the animals were zinc-deficient.

In this study it was decided to investigate rat blood lymphocytes because of the ease in controlling the level of zinc in the medium by adding serum

collected from rats fed diets varying in zinc content, thus enabling an examination of the effects of zinc levels without the complicating influences of chelating agents. Also, it provided a good *in vitro* system of rapidly dividing cells with which to confirm results from regenerating rat livers without the complicating effects of intracellular and interstitial fluid compartments. In the present investigation a study was made on the effects of zinc status and addition of EDTA and certain metal ions on DNA and protein synthesis in PHA-stimulated lymphocytes.

4.2 Materials and Methods

4.2.1 Reagents

Heparin. Heparin (5 000 units/ml) was obtained from Evans Medica Ltd. and diluted with sterile saline to give a final concentration of 1 000 units/ml.

Hanks salt solution. Hanks basic salt solution (pH 7,2 - 7,3) was made up of NaCl (4,0 g), KH_2PO_4 (0,03 g), $MgSO_4.7H_2O$ (0,1 g), $Na_2HPO_4.12H_2O$ (0,06 g), $CaCl_2$ (0,07 g), glucose (0,5 g), $NaHCO_3$ (0,175 g) and phenol red (0,01 g) in 500 ml distilled water.

White cell diluting fluid. The white cell diluting fluid used in staining the lymphocytes for counting on a haemacytometer consisted of 0,01 g gentian violet in 100 ml 1% glacial acetic acid.

Culture medium. Difco culture medium 199 was obtained from Difco Laboratories, Detroit, Michigan, U.S.A. This medium (11,1 g) together with $NaHCO_3$ (0,35 g) was added to a litre of distilled deionized water and the pH adjusted to 7,2.

Rat serum. Serum used to supplement the culture medium (25%) was obtained from coagulated blood collected directly from the hearts of rats fed either a control (60 ppm of Zn) or a zinc-deficient (0,35 ppm of Zn) diet as described in Chapter 2. Serum was separated from the clotted blood by centrifugation at 1 000 g for 15 min in a Martin-Christ Digesla centrifuge

Phytohaemagglutinin. PHA-P was purchased from Difco Laboratories Detroit, Michigan, U.S.A. and 5 ml sterile water added to each ampoule.

Thymidine (^{3}H-methyl), with a specific radioactivity of 5 mC/mMole was obtained from the Radiochemical Centre, Amersham, Buckinghamshire England. The sample was diluted with physiological saline to give a final activity of 5 µC/ml.

(U-^{14}C) C-leucine (10 mC/mMole) was obtained from the Radiochemical Centre, Amersham. The sample was diluted with physiological saline to give a final activity of 2 µC/ml.

Scintillation fluid. As in Chapter 2.

Giemsa stain solution was purchased from Fluka, Switzerland and diluted to 10% with phosphate buffer, pH 6,8.

May Grunawald stain. Powdered dye was purchased from Fluka, Switzerland. The powder (0,3 g) was dissolved in 100 ml methanol, warmed to 50^{o}C, cooled and filtered.

4.2.2 Animals and rations

Experimental animals were fed zinc-deficient or control diets as described in Chapters 2 and 3.

4.2.3 Glassware and solutions

All glassware used in this investigation was autoclaved and stored under sterile conditions. Sterile plastic containers and syringes were used extensively and were disposed of after use. All solutions were sterilized by filtration through 0,3 µ millipore filters into sterile containers.

4.2.4 Methods

Collection of lymphocytes. The method is essentially that described by Schellekens (102). Rat blood (5 ml) from zinc-deficient or control animals was collected by heart puncture into a syringe containing heparin (500 units) and 12% Dextran (1 ml). The mixture was placed in a sterile plastic

vial and left for 45 min at 37°C. Thereafter, the upper layer of plasma and leucocytes was drawn off with a sterile Pasteur pipette, added to 10 ml Hanks salt solution in another sterile vial and incubated in a column of cotton-wool (in a 30 ml syringe) for 30 min at 37°C. The solution was then pushed through the cotton-wool column into a sterile plastic vial containing 10 ml Hanks salt solution, mixed and centrifuged on an MSE centrifuge at 1 000 g for 15 min. The sedimented cells were resuspended in 20 ml Hanks salt solution and washed as before. The final cell sediment was diluted to 2 ml with Hanks salt solution and 0,1 ml of the suspension was added to 0,9 ml of white cell diluting fluid and the number of cells counted on a Spencer Bright-Line haemacytometer.

Culture and harvesting of cells. Lymphocytes collected from either zinc-deficient or control rats were cultured in culture medium supplemented with either zinc-deficient (deficient conditions) or control serum (control conditions). In some cases varying concentrations of zinc as zinc sulphate were supplied in the medium. In other cases 1 mM-EDTA and various metal ions were added to the culture medium. Cells (10^6) were cultured in 4 ml culture medium and 0,1 ml PHA-P in sterile glass test tubes with plastic caps. PHA-P cultures were performed in triplicate for each animal together with one control containing no PHA. After 48h in culture 2,5 µC ^{3}H-thymidine or 0,5 µC ^{14}C-leucine was added to each tube and a further 24h incubation allowed. The cultures were then centrifuged and the sedimented cells washed twice with 20 ml cold saline, once with 15 ml cold 5% TCA and once with 10 ml methanol. Finally the cells were dissolved in 1 ml Soluene (Packard), added to 15 ml scintillation fluid and counted on a Beckman liquid scintillation counter.

Control experiments. In order to determine the proportion of lymphocytes in the isolated cell suspension, the material was smeared onto a microscope slide and fixed in 95% alcohol for 5 min. Thereafter, the slide was immersed in May Grunawald stain for a further 5 min, washed in running water, placed in the diluted Giesma stain for 10 - 15 min, washed in phosphate buffer

(pH 6,8) and allowed to dry. Cells were subsequently counted under an oil emersion lens of a Zeiss laboratory microscope.

In a further experiment designed to establish whether the incorporation of ^{3}H-thymidine and ^{14}C-leucine was an accurate assessment of active DNA and protein synthesis respectively, 20 µg cycloheximide (Sigma) or 0,1 ml phenylethanol (Sigma), which are known inhibitors of DNA and protein synthesis (103, 104), were added to each culture tube 30 min before the addition of the radioactive labels. In addition, 80 mg of Mitomycin C and puromycin, also known inhibitors of nucleic acid and protein synthesis respectively (75, 82), were added to cultures before addition of radioactive labels.

Another control experiment was carried out to determine the amount of free label in the intracellular pools of the lymphocytes, i.e. unincorporated label. The harvested cells were disrupted in 1 ml butanol (105) with the lipid bound membrane fractions being dispersed in the organic layer and the soluble cell fractions being found in the aqueous layer. DNA and protein were precipitated from these aqueous solutions and their specific activities determined as described in Chapters 2 and 3. The organic solution and soluble aqueous solutio (non-precipitated) were also counted in the same way.

In order to eliminate the possibility that reduced uptake of radioactive label into DNA and protein was due to decreased turnover of this material rather than reduced nett synthesis, a study was made of the total DNA and protein content of the lymphocyte cells 24h after addition of label, together with the specific activity of all isolated DNA and protein fractions. DNA and protein were isolated and purified from the cells and the DNA and protein concentrations determined as described in Chapters 2 and 3 respectively. Aliquots (0,5 ml) of the isolated material were added to scintillation fluid for radioactive counting

4.3 Results

4.3.1 Purity of lymphocyte preparation

The cell count obtained on 15 separate cell suspensions after differential staining was found to comprise on average: 75 - 80% small lymphocytes, 5 - 10% monocytes, 5 - 15% polymorphs, which was considered to be a high degree of purity (102) for the experiments which were performed in this study.

4.3.2 The effect of inhibitors on DNA and protein synthesis in PHA-stimulated lymphocytes

Six determinations were performed in triplicate to test the effec of both cycloheximide and phenylethanol on DNA and protein synthesis, and the effects of Mitomycin C on DNA synthesis and puromycin on protein synthesis. The results (Table 10) indicated that cycloheximide, phenylethanol and Mitomycin C inhibited virtually all uptake of ^{3}H-thymidine into the lymphocytes (99%, 97% and 98% inhibition respectively) but only cycloheximide and puromycin inhibited protein synthesis completely (phenylethanol gave only 63% inhibition since it is not an effective inhibitor of protein synthesis).

TABLE 10 The effect of inhibitors on the incorporation of ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively in cultures of PHA-stimulated rat lymphocytes. Means of 6 animals and their S.E.'s

Treatment of cultures	Incorporation of ^{3}H-thymidine into DNA (cpm/culture)	Incorporation of ^{14}C-leucine into protein (cpm/culture)
Control culture (0,95 ppm Zn)	31 136 ± 4 756	83 198 ± 16 740
Control culture + 20 µg cycloheximide	66 ± 17	1 195 ± 232
Control culture + 0,1 ml phenylethanol	1 014 ± 442	31 531 ± 7 810
Control culture + 80 µg Mitomycin C	640 ± 98	-
Control culture + 80 µg puromycin	-	1 345 ± 137

4.3.3 The distribution of radioactivity in the cell fractions

In Table 11 are summarised the results obtained by disrupting the cells with n-butanol. Clearly very little of the respective labels was not incorporated into DNA and protein (4,9 and 7,1% respectively) showing that the intracellular pool had no effect on the uptake of label. It is interesting that considerably less protein synthesis was found in the membrane fractions when compared with percentage of DNA synthesis found in this fraction (8,3% as opposed to 27,4%). In both cases over 90% of the control count was recovered (Table 11).

TABLE 11 Incorporation of ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively in various fractions of lymphocytes stimulated by PHA. Means of 5 animals and their S.E.'s

Fraction	Incorporation of ^{3}H-thymidine (cpm/fraction)	% of whole cell count		Incorporation of ^{14}C-leucine (cpm/fraction)	% of whole cell count	
TCA precipitated	18 461 ± 4 129	60,0		62 393 ± 4 419	74,7	
Non-precipitated	1 557 ± 348	4,9	92,3	5 830 ± 1 307	7,1	90,1
Butanol phase	8 935 ± 2 005	27,4		6 650 ± 1 489	8,3	

4.3.4 The effect of zinc levels on the specific activity of DNA and protein isolated from PHA-stimulated rat lymphocytes

A reduction in DNA and protein levels was found in the EDTA-treated and deficient cells, together with proportionately reduced specific activities of these fractions (Table 12). This clearly points to reduced nett synthesis in these cultures rather than reduced turnover.

TABLE 12 The concentration and specific activity of DNA and protein isolated from rat lymphocytes after treatment with PHA. Means of 6 animals and their S.E.'s

Culture conditions	Zinc conc. of medium (ppm)	Concentration in culture (µg/ml)	Specific activity (cpm/mg)
(a) DNA			
Control lymphocytes + control serum - PHA	0,95	16,1 ± 2,5	96 103 ± 8 270
Control lymphocytes + control serum + PHA	0,95	34,5 ± 4,1	3 649 593 ± 230 217
Control lymphocytes + control serum + PHA + EDTA (1,0 mM)	0,95	17,9 ± 2,7	189 594 ± 20 615
Zinc-deficient lymphocytes + zinc-deficient serum + PHA	0,35	30,1 ± 3,8	2 759 813 ± 194 422
(b) Protein			
Control lymphocytes + control serum - PHA	0,95	79,5 ± 6,5	48 497 ± 4 393
Control lymphocytes + control serum + PHA	0,95	173,0 ± 11,1	1 982 371 ± 194 679
Control lymphocytes + control serum + PHA + EDTA (1,0 mM)	0,95	85,6 ± 8,2	409 549 ± 34 928
Zinc-deficient lymphocytes + zinc-deficient serum + PHA	0,35	159,0 ± 9,3	1 579 976 ± 119 289

4.3.5 The effect of EDTA and metal ions on the incorporation of ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively, in PHA-stimulated rat lymphocytes

As shown in Table 13 and Appendix 8 the addition of EDTA (1,0 mM) severely reduced (> 90%) the incorporation of both ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively in PHA-treated rat lymphocytes isolated from control cultures (i.e. control lymphocytes plus control serum). Supplementation of the medium (which contains almost no residual metal ions) with a number of divalent metal ions (0,3 mM) after the addition of EDTA, but before incubation, to some extent (5 - 35%) reversed the inhibition by EDTA, but the most effective recovery (80% and 64% respectively) was obtained by the addition of zinc at a concentration of 0,5 mM. Cations other than zinc were added individually at a concentration of 0,3 mM as the reversal of the inhibition by EDTA has been reported to be maximal at these levels (40). Concentrations of zinc above 0,5 mM reduced the uptake of both ^{3}H-thymidine and ^{14}C-leucine to a marked extent (Table 13 and Figure 2). The addition of a combination of Zn^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+} (0,3 mM) to a culture containing 1,0 mM-EDTA restored respectively 93% and 106% of the control activity of DNA and protein synthesis. The findings indicated a far greater requirement for zinc than other metal ions but that other metal ions were necessary for complete reversal of inhibition by EDTA, although it must be borne in mind that when all the metal ions were added together there was 1,8 mM of divalent ions and it is possible that the effect observed was the result of a chelation of EDTA and thus an effective inhibition of its function in the culture.

TABLE 13 The effect of EDTA and metal ions on DNA and protein synthesis in PHA-treated rat lymphocytes. Means of 6 animals and their S.E.'s

Additions to culture	Incorporation of[1] ^{3}H-thymidine (cpm/culture)	% of control	Incorporation of[1] ^{14}C-leucine (cpm/culture)	% of control
None	30 886 ± 4 823	100,0	84 023 ± 6 237	100,0
1 mM-EDTA	833 ± 375	2,7	8 574 ± 1 567	10,3
1 mM-EDTA + 0,3 mM-Zn^{2+}	19 569 ± 3 940	69,9	51 859 ± 3 348	62,5
1 mM-EDTA + 0,5 mM-Zn^{2+}	25 370 ± 3 783	81,5	53 253 ± 4 362	64,0
1 mM-EDTA + 1,0 mM-Zn^{2+}	12 230 ± 2 496	39,1	40 319 ± 2 812	48,5
1 mM-EDTA + 2,0 mM-Zn^{2+}	5 237 ± 385	17,0	24 625 ± 2 469	29,6
1 mM-EDTA + 0,3 mM-Ni^{2+}	11 411 ± 2 291	36,7	30 656 ± 3 994	36,9
1 mM-EDTA + 0,5 mM-Ni^{2+}	9 604 ± 2 480	31,0	-	-
1 mM-EDTA + 0,3 mM-Mn^{2+}	8 746 ± 2 191	27,2	13 640 ± 3 550	16,4
1 mM-EDTA + 0,3 mM-Fe^{2+}	6 684 ± 1 728	21,5	24 981 ± 6 408	30,1
1 mM-EDTA + 0,3 mM-Mg^{2+}	2 589 ± 428	8,2	34 061 ± 2 782	41,0
1 mM-EDTA + 0,3 mM-Ca^{2+}	1 650 ± 671	5,3	15 430 ± 1 269	18,6
1 mM-EDTA + 0,3 mM-Zn^{2+} Ni^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}	29 024 ± 6 481	93,5	88 250 ± 6 633	106,0

(1) Individual results in Appendix 8.

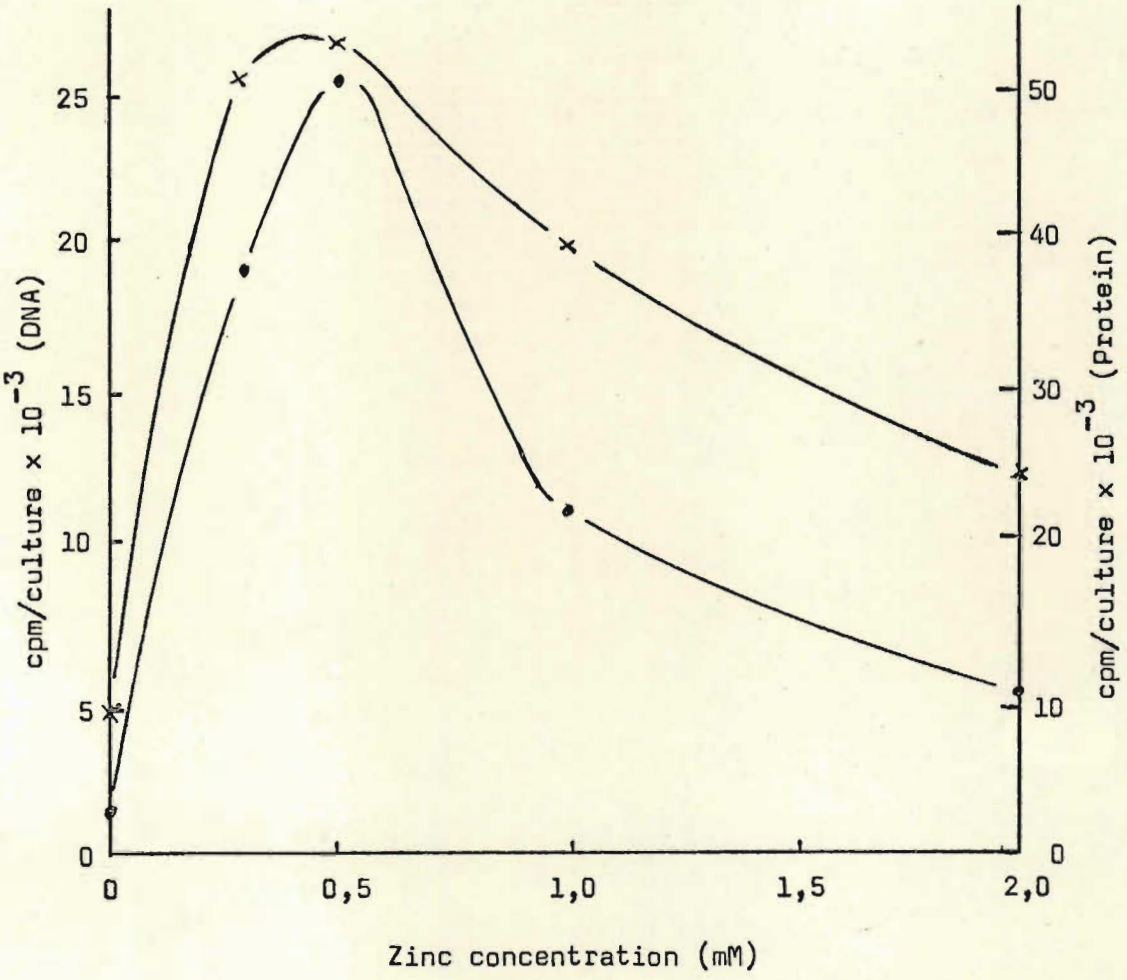

FIGURE 2 The effect of zinc supplementation on the inhibition of DNA and protein synthesis resulting from EDTA addition to PHA-treated rat lymphocytes

• —— • DNA; x —— x Protein

4.3.6 The effect of zinc concentration on the incorporation of ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively in PHA-treated rat lymphocytes

DNA and protein synthesis were significantly reduced ($p < 0,01$) in the zinc-deficient cultures (0,35 ppm of zinc) but the inhibition was effectively reversed by the addition of 2,0 ppm of zinc to these cultures (Table 14 and Appendix 9). Levels of zinc above 2 - 3 ppm reduced incorporation of both ^{3}H-thymidine and ^{14}C-leucine (Table 14 and Figure 3). The addition of zinc

together with a number of other divalent metal ions to the deficient cultures resulted in incorporation of label equivalent to that when zinc was added alone showing that it was an effect of lack of zinc that was seen and not some other metabolic disturbance.

TABLE 14 The effect of the concentration of zinc in the culture medium on the incorporation of ^{3}H-thymidine and ^{14}C-leucine into DNA and protein respectively in PHA-treated rat lymphocytes

Culture conditions	Zinc conc. (ppm)	Incorporation of[1] ^{3}H-thymidine (cpm/culture)	Incorporation of[1] ^{14}C-leucine (cpm/culture)
Control serum + control lymphocytes - PHA	0,95	392 ± 87	937 ± 128
Control serum + control lymphocytes + PHA (control conditions)	0,95	31 136 ± 4 756	83 197 ± 16 740
Zinc-deficient serum + zinc-deficient lymphocytes + PHA (zinc-deficient conditions)	0,35	20 567 ± 3 092	61 294 ± 3 881
Control serum + zinc-deficient lymphocytes + PHA	0,90	30 426 ± 3 902	83 157 ± 7 637
Control conditions + 1,05 ppm Zn^{2+}	2,00	32 555 ± 4 301	88 164 ± 5 561
Control conditions + 2,05 ppm Zn^{2+}	3,00	32 161 ± 4 946	89 173 ± 6 421
Control conditions + 4,05 ppm Zn^{2+}	5,00	30 197 ± 3 780	79 226 ± 4 281
Control conditions + 9,05 ppm Zn^{2+}	10,00	26 363 ± 3 960	67 922 ± 4 318
Control conditions + 1 mM-EDTA	0,95	833 ± 375	8 574 ± 1 567
Deficient conditions + 2,0 ppm Zn^{2+}	2,35	30 927 ± 4 231	87 321 ± 4 231
Deficient conditions + 2,0 ppm Zn^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}	2,35	29 854 ± 3 292	87 824 ± 5 258

(1) Individual results in Appendix 9.

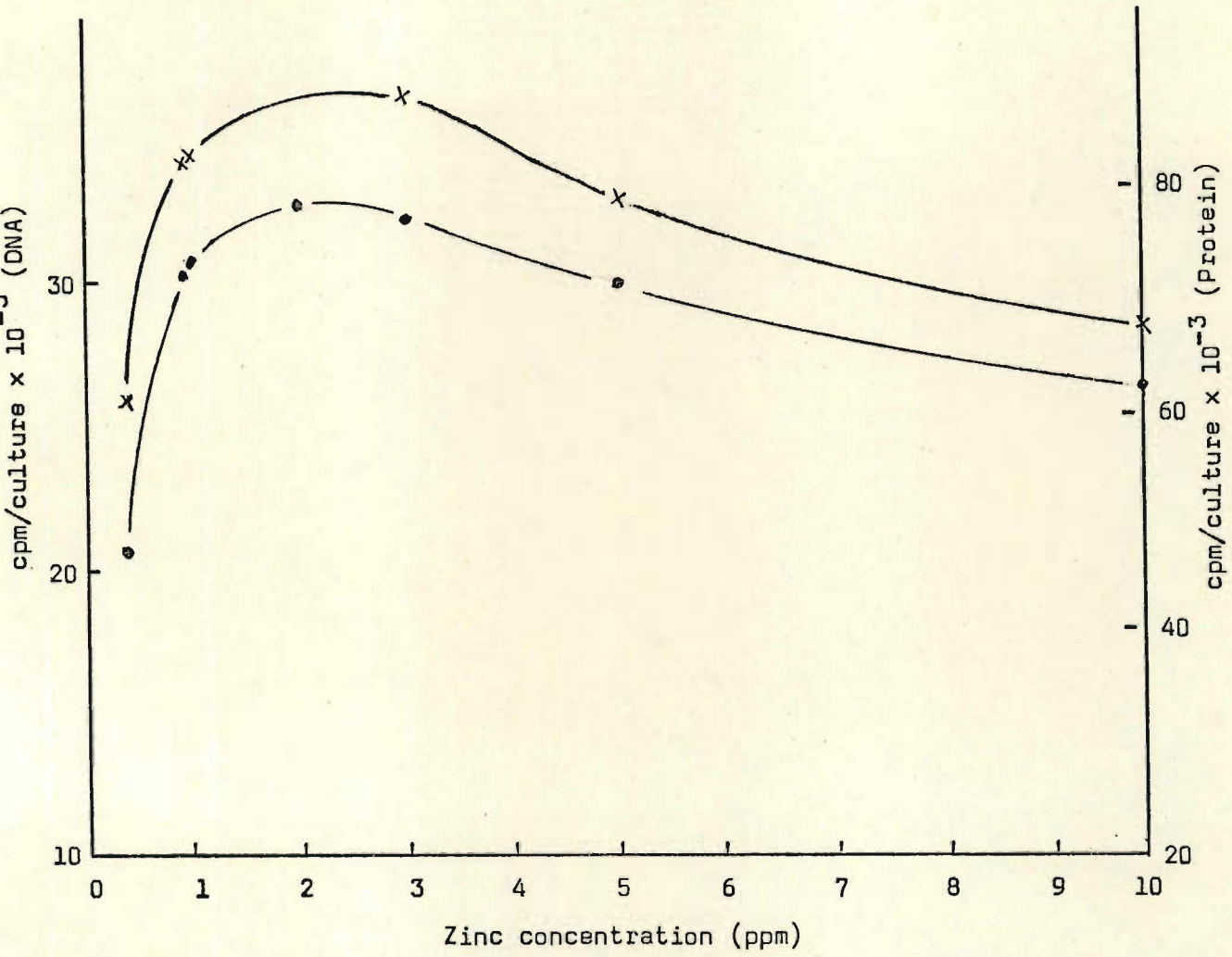

FIGURE 3 The effect of the concentration of zinc in the culture medium on DNA and protein synthesis in PHA-treated rat lymphocytes.
• —— • DNA; x —— x Protein

4.4 Discussion

Lymphocyte transformation is accompanied by an increase in genetic expression (106) which results in a prolonged increase in the rate of protein synthesis (107) and later a synthesis of the enzymes required for DNA replication (108), thus making it an ideal cell culture method for looking at DNA and protein synthesis. One of the numerous advantages of cell and tissue culture methods was the elimination of complications in the uptake of label as a result of metabolic pools. In lymphocyte cultures the only pool present is the intracellular pool and in this study an experiment was performed to test the effect of this pool. As was seen using both ^{14}C-leucine and ^{3}H-thymidine an insignificant proportion of label was held up in the fluid compartment but

was rapidly taken up with other free nucleotides and amino acids to synthesise DNA and protein.

Previously both zinc and ferrous ions had been demonstrated to be essential for growth in tissue culture and for DNA synthesis (66, 102, 109). The present findings suggested a dominant role for zinc in DNA and protein synthesis which confirmed these earlier results and those of Alford (40) and Chesters (41) who used human and pig blood lymphocytes respectively. The essential requirement for zinc also confirmed the earlier findings described in this thesis in connection with regenerating rat liver. With regard to protein synthesis previous work had shown zinc to be essential in regenerating rat livers and for rabbit kidney cells (66) grown in a primary culture. Nevertheless, little was known about its requirements for protein synthesis in blood lymphocytes, the only previous report being that of Chesters (41) who showed that EDTA (0,6 mM) inhibited 50% of the protein synthesis in pig lymphocytes and zinc reversed this effect completely at a level of 0,55 mM. The results from the present study do not entirely agree with these findings in that 90% inhibition of protein synthesis was found with the addition of EDTA (1,0 mM), while addition of zinc (0,5 mM) only gave 64% recovery of the control activity. Also other me ions were almost as effective. However, the results are difficult to compare because of differences in concentrations of EDTA and metal ions used in the two experiments.

At this stage it must be stressed that results obtained using EDTA for removal of zinc have been criticised because of the complexity of the technique in that it removes all other divalent metal ions as well and it was not known what effect this had on the cells' metabolism (78). In addition, it is not known whether EDTA chelates all divalent metal ions to the same extent and it is possible that one or more metal ions remain in solution after EDTA addition thus complicating the explanation of the results obtained. Accordingly, in the present study it was decided to use rat blood lymphocytes as a model system because of the unique opportunity of being able to control the level of zinc

in the cultures by using zinc-deficient rat serum in the preparation of the medium together with zinc-deficient lymphocytes. Additional zinc could be added to the medium at the levels required. In this way the zinc levels could be lowered from 0,95 ppm Zn to the acceptably low concentration of 0,35 ppm while the addition of various concentrations of zinc provided a spectrum of concentrations at which DNA and protein synthesis could be studied. Conclusions could accordingly be drawn concerning the requirements of zinc without the addition of EDTA. In fact this probably represented the first such *in vitro* study which did not necessitate the use of chelating agents. The results obtained in this way reflected those using EDTA chelation in that low and high levels of zinc resulted in reduced DNA and protein synthesis with an optimum synthesis at 2 - 3 ppm Zn. Addition of 2 ppm of Zn to the zinc-deficient cultures resulted in restoration of both DNA and protein synthesis to levels comparable with those of the control cultures. Inhibition of DNA synthesis by high levels of zinc confirmed the results of Alford (40) and in this connection Chesters (41) made the comment that zinc activated two processes - one which stimulated thymidine incorporation and another which inhibited it in high concentrations.

Speculation about the possible molecular basis for the zinc requirement is still uncertain. Kay (110) has suggested that the effect of EDTA on lymphocytes was due to inhibition of the binding of PHA to lymphocytes, a process reversed by zinc and other metal ions due to preferential binding of the metal ions with EDTA, thus releasing PHA. He did, however, conclude that this may not be the only effect and that the divalent metal ions may have some other role in stimulating lymphocyte DNA, RNA and protein synthesis. Kay's results were, however, obtained using very high concentrations of EDTA and metal ions which may have resulted in certain unusual effects. In the present experiments EDTA still permitted morphological transformation of the lymphocytes which would be difficult to reconcile with Kay's suggestions. Furthermore, it would be difficult to account for the great differences in stimulation caused by

the different metal ions as at the pH at which this work was done (pH 7,2) the binding constants of EDTA and a number of the metal ions tested, including zinc, are very similar (111). Other postulations have been put forward, such as EDTA inhibition of the permeability of lymphocyte cell membranes to metal ions (41), but emerging from this speculation it does seem that zinc and other metal ions are required within the cell itself and are not involved in reactions outside the cell, or if they are, it is to a small extent only.

It is reasonable then to suspect that a significant portion of EDTA's effect as an inhibitor of PHA-induced lymphocyte transformation is related to a chelation of zinc and consequent interference with DNA synthesis. These conclusions are enhanced by the results showing reduced DNA synthesis in lymphocytes cultured in media containing low zinc concentrations and by recent findings of zinc involvement in DNA polymerase (50), RNA polymerase (70) and thymidine kinase (51). These *in vitro* experiments confirm the earlier findings using *in vivo* systems, of an essential requirement for zinc in the processes of active DNA and protein synthesis.

CHAPTER 5

THE EFFECT OF ZINC INTAKE ON TUMOUR GROWTH AND CARCINOGENESIS

5.1 Introduction

Evidence implicating zinc in normal cell metabolism (7, 112, 113) and particularly during cell proliferation (15, 114) suggested that this metal may be of particular importance during the development and growth of malignant tissue. However, little attention has been paid to this aspect of zinc metabolism until recently when Dennes, Tupper and Wormall (115) and Davies, Musa and Dormandy (116) reported lowered plasma zinc levels in cancer patients in contrast to normal plasma zinc levels in other cases of serious disease. An investigation by Wright and Dormandy (117) also showed an increase in the concentration of zinc in the livers of patients suffering from hepatic malignancy as well as other malignancies not confined to this organ. These findings, together with recent evidence implicating zinc in nucleic acid metabolism (27, 36, 118), suggested a higher requirement for zinc for the increased DNA synthesis (42) associated with neoplastic growth.

Petering et al. (43) investigated the effects of mineral supplements on the anti-tumour activity of 3-ethoxy-2-oxo-butylaldehyde Bis (thiosemicarbazone) and found that lack of zinc reduced the growth of Walker 256 carcinosarcoma in rats. More recently, De Wys and co-workers (42, 44, 119) have demonstrated reduced growth of a number of transplanted tumours and increased survival time in rats and mice receiving inadequate dietary zinc. Increased levels of zinc, on the other hand, did not appear to effect the growth of a grafted Walker 256 carcinosarcoma in rats (119), although Arachi (120) has shown zinc supplementation resulted in reduced growth of Ehrlich ascites cells.

In contrast to these studies on tumour growth Poswillo and Cohen (45) have examined the effect of zinc levels on the process of carcinogenesis and have

demonstrated an inhibition of DMBA-induced carcinogenesis in the cheek pouches of hamsters given additional zinc in the diet. The effect of zinc deficiency on the process of carcinogenesis has not thus far been investigated. Thus it appears that, whereas low dietary intakes of zinc may inhibit tumour growth, increased levels may inhibit carcinogenesis. Such generalisations are however complicated by differences in the nature of the tumours examined in these various studies.

The present study was designed to compare the previous findings obtained with regenerating livers and proliferating lymphocytes with the effect of zinc on neoplastic tissue and in addition, it was hoped to clarify some of the uncertainty surrounding the role of zinc in neoplastic metabolism. Initially, a study was made of the effect of different levels of dietary zinc on the growth of a transplanted hepatoma in rats. DNA synthesis was also studied in these tissues as it was felt that data obtained from other systems pointed to the primary locus of action of zinc occurring at this point. In addition, the effect of zinc status on methylcholanthrene-induced carcinogenesis in mice was studied. Mice were chosen for this study because a short experimental period was required due to harmful side effects of prolonged zinc deficiency (9) and mice have been shown to be more susceptible to the effects of chemical carcinogenesis than rats (121).

5.2 Materials and Methods

5.2.1 Reagents

Hepatoma. A transplantable hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene (DAB-1) was supplied by Dr C. Albrecht of the National Chemical Research Laboratory of the South African Council for Scientific and Industrial Research.

Chemical carcinogen. 3-methylcholanthrene was purchased from Caliobiochem, San Diego, California and was dissolved in benzene to give a final concentration of 0,5%.

Thymidine (^{3}H-methyl). As described in Chapter 2.

5.2.2 Animals and rations

Female Wistar strain rats (110 - 120 g) were housed three to a stainless steel cage and fed the rations described in Chapter 2, containing levels of zinc ranging from 0,6 ppm to 2 500 ppm, the control diet having 60 ppm of zinc. Drinking water was distilled, deionized water.

Female mice of the Swiss strain (30 - 40 g) were housed individually in stainless steel cages and fed the rations described above.

5.2.3 Treatment of experimental animals

Primary hepatomas were grafted into rats by injecting 150 mg (0,2 ml) of minced DAB-1 hepatoma (122) into the hind leg muscle of a number of experimental animals. The rats were fed the experimental diets for 3 and 5 weeks and thereafter, they were killed by chloroform anaesthesia and tumour and body mass were determined. In a further study, animals were conditioned to the respective experimental diets for 1 week before tumour implantation to evaluate the subsequent viability (3 weeks) of the transplanted tissue in these animals.

In another experiment, animals carrying transplanted tumours for 3 weeks were injected intraperitoneally with 50 µC ^{3}H-thymidine two hours before the tumours were excised and the specific activity of DNA isolated from these tissues was determined as described under procedure (2) in Chapter 2.

In addition, mice were fed the experimental rations for 10 weeks and were painted with 0,5% of a solution of the chemical carcinogen, methylcholanthrene (121, 123), twice weekly in the shaved intrascapular region to assess the effect of dietary zinc levels on carcinogenesis.

5.3 Results

5.3.1 The effect of dietary zinc levels on tumour growth

Tumour growth was markedly reduced after 3 and 5 weeks in animals receiving both the zinc-deficient (0,5 ppm) and zinc-supplemented ($\geq$ 500 ppm) diets after implantation, whether expressed as actual tissue mass or as a percentage of the body weight (Table 15 and Appendix 10). Rats given the maximum zinc supplementation (2 500 ppm) became listless and suffered from alopecia, yet tumour growth was no more retarded in these animals than in the rats receiving 500 - 1 000 ppm of zinc.

TABLE 15 Effect of dietary zinc on the growth of a transplanted tumour in rats

Zinc conc. of diet (ppm)	No.(1) of rats	Body wt. (g)	Tumour wt. (g)	$\frac{\text{Tumour wt.}}{\text{Body wt.}} \times 100$
(a) AFTER 3 WEEKS				
0,5	15	146 ± 3,4	5,05 ± 0,74	3,5
60	15	152 ± 8,5	12,31 ± 2,9	8,1
500	15	154 ± 6,5	9,2 ± 2,4	5,9
1 000	10	155 ± 6,2	8,1 ± 1,9	5,2
2 500	10	150 ± 5,6	8,0 ± 2,0	5,3
(b) AFTER 5 WEEKS				
0,5	15	136 ± 7,1	25,4 ± 2,5	18,7
60	15	161 ± 10,1	39,6 ± 3,7	24,6
500	15	162 ± 7,1	23,5 ± 3,0	14,5
1 000	10	159 ± 7,5	25,1 ± 3,5	15,8
2 500	10	145 ± 7,8	23,5 ± 2,8	16,2

(1) Individual results in Appendix 10.

In the second experiment, to evaluate the viability of the transplanted tumour tissue, tumour growth was again reduced in the zinc-deficient and zinc-supplemented groups but viability of tumours in these animals were no different

from those in animals placed on the experimental diet immediately after implantation (Table 16 and Appendix 11).

TABLE 16 Effect of the prevailing zinc level in viability and growth of transplanted tumour tissue in rats

Zinc conc. of diet (ppm)	No.(1) of rats	Viability of transplant (%)	Tumour wt. after 3 weeks (g)	Body wt. after 3 weeks (g)
0,5	10	100	5,5 ± 1,0	138 ± 14,1
60	10	100	14,6 ± 1,7	155 ± 9,9
500	10	100	7,8 ± 1,8	155 ± 10,8

(1) Individual results in Appendix 11.

5.3.2 The effect of dietary zinc levels on DNA synthesis in transplanted hepatomas

The incorporation of ^{3}H-thymidine into DNA isolated from transplanted hepatomas was significantly reduced ($p < 0,01$) in animals receiving both the zinc-deficient and zinc-supplemented diets when compared with the control animals (Table 17 and Appendix 12).

TABLE 17 Effect of dietary zinc intakes on DNA synthesis in transplanted tumours

Zinc conc. in diet (ppm)	No.(1) of animals	Incorporation of ^{3}H-thymidine (cpm/mg DNA)	Difference of means (cpm/mg DNA)
60	10	4 605 ± 479	1 629 ± 271
0,5	10	2 976 ± 373	L.S.D. (0,01) 775
60	10	4 605 ± 479	1 068 ± 278
500	10	3 537 ± 396	L.S.D. (0,01) 795
60	10	4 605 ± 479	1 878 ± 239
1 000	10	2 727 ± 239	L.S.D. (0,01) 686

(1) Individual results in Appendix 12.

5.3.3 The effect of dietary zinc levels on methylcholanthrene-induced carcinogenesis in mice

In the experiment concerning methylcholanthrene-induced carcinogenesis, both initial papilloma development and the incidence of malignancy at the end of the experimental period (10 weeks) were markedly reduced in the zinc-deficient and zinc-supplemented animals. The zinc-deficient and 500 ppm zinc-supplemented groups, in fact, showed no papilloma development after the 10-week experimental period (Table 18).

TABLE 18 Effect of dietary zinc levels on methylcholanthrene-induced carcinogenesis in mice

Zinc conc. in diet (ppm)	No. of animals	Time before appearance of first papilloma (weeks)	No. of animals with papilloma after 10 weeks
0,5	15	-	0
60	15	4	13
500	15	-	0
1 000	15	6	1
2 500	15	8	1

5.4 Discussion

The suggested importance of zinc in rapidly dividing cancer cells was supported by the results of De Wys and Pories (42, 44, 119) and Petering *et al.* (43) who showed that zinc deficiency inhibited the growth of rapidly growing tumours. The finding was further confirmed by the present study. Detailed comparisons of weight matched controls (42) and an analysis of the relationship between reduced tumour growth and reduced carcass growth in both this study and in that of McQuitty, De Wys, Monaco, Strain, Rob, Apgar and Pories (119) indicated that inhibition of tumour growth was a specific effect of zinc deficiency and not an effect of reduced caloric intake. The requirement for zinc by tumour tissue suggested a possible explanation for the reported decreased levels of zinc in plasma of cancer patients (115, 116) due to a

selective requirement for the element by the neoplasm (117).

Contrary to the findings of McQuitty et al. (119) the present data point to a similar marked inhibition of tumour growth in animals fed high levels of zinc. Clearly, comparisons are difficult because of the widely different tumours used in the two studies. However, the inhibitory effect of high levels of zinc has been confirmed by a recent report (120) of a reduced number of Ehrlich ascites tumour cells in mice given 1 000 ppm of zinc compared with animals given a control diet containing 50 ppm of zinc.

The mechanism of tumour inhibition by both zinc deficiency and zinc supplementation is not clear. As has been pointed out the biochemical lesions associated with zinc deficiency, in particular, are only partially understood but evidence presented in this thesis suggests a primary locus of action associated with DNA synthesis. In this respect, it is of interest that both high and low levels of zinc have been implicated in the inhibition of DNA synthesis in proliferating rat lymphocytes (Chapter 4). Studies concerning the incorporation of ^{3}H-thymidine into DNA in DAB-1 hepatomas pointed to diminished DNA synthesis in the zinc-deficient and zinc-supplemented groups which may account for the accompanying reduced tumour growth in these animals. The reduced DNA synthesis in turn may arise from a reduction in the activities of one or more of the zinc dependent enzymes directly involved in the biosynthesis of DNA (50, 51, 124).

The inhibition of carcinogenesis by zinc supplementation observed in the present study confirms the results of Poswillo and Cohen (45) who found DMBA-induced carcinoma in hamster cheek pouches to be markedly reduced in animals receiving a zinc supplementation of 100 ppm. In contrast to these results Arachi (125) has reported that excess zinc encouraged the production of hepatom by 0,005% N-nitrosodiethylamine. This view cannot be reconciled with the prese findings and it indicates a different role for zinc in different types of tumou The present observation of reduced carcinogenesis in zinc-deficient mice resolv the earlier speculation concerning the effect of low levels of zinc on this process (44) and points to a similar inhibitory role for both low and high

levels of zinc in the process of carcinogenesis, as was found in the case in tumour growth.

An encouraging aspect of the present findings is the possibility of some measure of therapeutic control of cancer growth by the manipulation of the dietary zinc intake. The reduced tumour growth and increased survival time associated with reduced zinc status (42, 44, 119) compares favourably with the effectiveness of many currently used anti-tumour drugs (126). However, the severe adverse effects attributable to prolonged zinc deficiency (9, 10, 12) render this form of therapy unacceptable, and suggests that the inhibition observed at non-toxic levels of zinc supplementation (127) may have greater clinical significance. The findings are encouraging, but considerably more experimentation is required before the therapeutic use of zinc supplementation can be evaluated either in the form of a treatment of cancer or as a prophylactic agent in reducing carcinogenesis.

CHAPTER 6

THE EFFECT OF ZINC DEFICIENCY ON NUCLEAR AND MITOCHONDRIAL DNA SYNTHESIS

6.1 Introduction

The evidence thus far presented in this thesis and by other authors (27, 30, 31, 36) has pointed to an inhibition of DNA synthesis resulting from zinc deficiency in a number of cell types, but in all cases DNA replication has been examined in the whole cell as a unit. No attempt has been made to assess the effect of zinc levels on DNA synthesis in the individual cellular organelles (e.g. nuclei and mitochondria) which are known to replicate their own DNA (52 - 53, 128). The result is that previous investigations have ignored the fact that inhibition of DNA synthesis in zinc-deficient tissues may have been the result of an effect in one organelle only (e.g. the nucleus) which masked a different effect in another organelle (e.g. mitochondrion). Thus, before considering the mechanism of action of zinc in cell division, it was considered important to examine the effect of zinc status on DNA synthesis separately in the nucleus and mitochondrion.

The existence of nuclear DNA has been recognised for several years (129, 130) and its properties are well documented (131 - 133). It was however, only in the last decade that mitochondria from a wide variety of cells were shown to contain a particular species of DNA (mitochondrial DNA) which is replicated within the mitochondrion (134 - 136). Also, it has been demonstrated that mitochondrial DNA differs in physicochemical properties from nuclear DNA (135 - 138). For example, mitochondrial DNA is generally of an open or loosely twisted circular form in contrast to the linear strands of nuclear DNA (139) and it has a different buoyant density to nuclear DNA (139) due mainly to a lower GC content.

In recent years several workers (Nass, Nass and Hennix, (140), Parsons and Simpson, (55), and Wintersberger, (141)) have presented evidence that

mitochondria are able to incorporate DNA precursors into their DNA both *in vitro* and *in vivo*. In addition, Reich and Luck (54) have shown that mitochondrial DNA replicates semi-conservatively. Results from these and other studies (56, 142) have led to the conclusion that mitochondrial DNA synthesis is a self-contained process, i.e. it is capable of self-replication independently of nuclear control. In addition, Budd and Mills (143), Schneider and Kuff (144) a Parsons (145) have shown that mitochondria synthesise DNA continuously unlike nuclei and that mitochondrial DNA synthesis is not synchronous with chromosomal replication. Of particular interest concerning the differences between mitochondrial and nuclear DNA synthesis was the finding of Nass (56) and Khanson, Ivanova, Nikitina, Shutko and Koman (146) who showed that DNA synthesis rose sharply in mitochondria of regenerating rat livers immediately after partial hepatectomy whereas in the nucleus there was a latent period before this increa occurred during the G_1 phase of the cell cycle (65, 147). These findings, together with the previous evidence of asynchronous DNA replication in mitochon dria, point to a lack of periodicity of mitochondrial DNA synthesis. The findings are of particular interest because of the effect of zinc deficiency on the timing of DNA synthesis in whole cells described earlier in this thesis (Chapter 2). It is possible in this case that the shift in the peak of DNA syn thesis observed in zinc-deficient animals was the result of a predominant effec on nuclear DNA synthesis only, and not on mitochondrial DNA synthesis.

Also of interest concerning DNA synthesis in mitochondria was the finding that these organelles from mammalian cells contain DNA polymerase (58) and thymidine kinase (57) which are genetically distinct from the nuclear enzymes. Thus it appears that although nuclei and mitochondria have essentially the same mechanism of DNA replication the systems function independently of one another and contain certain component enzymes which are genetically different. The conclusion is particularly interesting since it has been suggested by the author that the site of action of zinc in DNA synthesis is at an enzymic level (Chapter 2), in which case an investigation into the effect of zinc deficiency

on these two different enzyme systems may provide a useful pointer to the precise role of zinc in DNA synthesis.

Accordingly, in the present investigation DNA synthesis in nuclei and mitochondria was studied individually in an effort to determine whether the effect of zinc-deficiency on this process differed between organelles. Attention was also paid to the effect of zinc status on the timing of nuclear and mitochondrial DNA synthesis.

6.2 Materials and Methods

6.2.1 Reagents

Thymidine (^{3}H-methyl), 5 mC/mM, was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. The sample was diluted with physiological saline to give a final activity of 100 µC/ml.

TMK buffer. All sucrose solutions were made up in TMK buffer which consisted of 0,05 M-Tris-HCl buffer (pH 7,5), 0,025 M-KCl and 0,005 M-$MgCl_2$.

Scintillation fluid. As described in Chapter 2.

Diphenylamine reagent. As described in Chapter 2.

Standard DNA solutions. As described in Chapter 2.

6.2.2 Animals and rations

Groups of 3 female rats of the Wistar strain (100 - 120 g) were housed in stainless steel cages and fed the zinc-deficient and control rations described in Chapter 2. Drinking water was distilled and deionized through an Elgastat multibed ion-exchange resin.

6.2.3 Methods

Surgical procedure. Partial hepatectomy was performed, as described in Chapter 2, on animals that had received the experimental rations for 3 days.

Blood samples. Blood samples (5 ml) were collected directly from the heart at the time the animals were killed and plasma zinc levels determined by atomic absorption spectroscopy as described in Chapter 2.

Injection of ^{3}H-thymidine. (^{3}H-methyl) thymidine (25 µC) was injected intraperitoneally 10 - 40 hours after partial hepatectomy. Sixty minutes later, the animals were killed by ether anaesthesia and the livers were removed and quick-frozen in dry ice.

Preliminary studies concerning the uptake of ^{3}H-thymidine were performed on groups of rats to establish that the data reflected only active DNA synthesis during the incorporation period. Initially, a comparison was made between the amount of incorporation that occurred in a 60 minute incorporation period in partially hepatectomised animals 20 hours post-operatively with animals that had received only a sham operation. In a second experiment, Mitomycin C (1 mg/rat), a specific inhibitor of DNA synthesis (75), was injected intraperitoneally 19 hours after partial hepatectomy and 60 min before the administration of ^{3}H-thymidine. In another experiment, only a 5 min incorporation period was allowed, in order to establish the degree of contamination of labelled isolated DNA with unincorporated ^{3}H-thymidine.

Isolation of nuclear and mitochondrial fractions. The procedure used was essentially that described by Schneider and Hogeboom (148). Frozen livers were homogenised in a Dounce homogeniser in 2 volumes 0,25 M-sucrose solution in TMK buffer. Ten passes were made with the loose plunger followed by 10 passes with the tight plunger. The homogenate was centrifuged at 900 g in a Beckman refrigerated international centrifuge. The sediment of nuclei, unbroken liver cells and red blood cells was resuspended in 4 ml 0,25 M-sucrose in TMK buffer and homogenised in a Dounce homogeniser using 3 passes with the tight plunger. The homogenate was underlayed with 1 ml 0,5 M-sucrose in TMK buffer and centrifuged at 1 000 g for 10 min to provide a sediment which constituted the nuclear fraction. The supernatants of the individual liver homogenates from the two centrifugations were combined, placed in cellulose nitrate

tubes (1,6 mm x 7,5 mm) and centrifuged at 12 000 rpm (10 000 g) for 10 min in a Beckman Model L Ultracentrifuge using the Type 45 rotor. The sediments were washed twice by resuspension in 0,25 M-sucrose and resedimented at 10 000 g. The final sediment represented the mitochondrial fraction.

Purity of nuclear and mitochondrial fractions. The nuclear and mitochondrial fractions were checked for purity by phase contrast microscopy on a Zeiss phase contrast microscope. In addition, samples from each fraction were prepared for electron microscopy on a Hitatchi HU 11 E1 electron microscope. The mitochondrial and nuclear pellets were suspended in 10 volumes 6% glutardialdehyde for 1 - 2 hours and again pelleted at 10 000 g for 10 min. Thereafter they were washed 3 times with 0,05 M-Na-cacodylate buffer for 30 min, centrifuged and treated with 2 ml osmium tetroxide per pellet. After 2 hours most of the osmium tetroxide was drawn off with a Pasteur pipette and the fractions were pelleted, washed twice (30 min) with Na-cacodylate buffer and dehydrated by washings with 50% (10 min), 70% (10 min), 90% (10 min) and absolute (twice for 20 min) ethanol. Finally, the pellets were washed twice (30 min) in propylene oxide. The samples were then embedded in araldite for sectioning and examination under the electron microscope in the Department of Botany, University of Natal, Pietermaritzburg.

Determination of the specific radioactivity of DNA. The procedu of Volkin and Cohn (76), described in Chapter 2 was employed. The final precipitate from the nuclear fraction was hydrolysed in 5 ml 5% TCA in a boiling water bath for 15 min. Insoluble material was removed by centrifugation at 1 000 g, the supernatant was decanted and the precipitate was hydrolysed once more in 2 ml 5% TCA. Precipitates from the mitochondrial fractions were hydrolysed sequentially in 2,5 ml and 1,0 ml 5% TCA. Supernatants from the hydrolyses were pooled for each individual sample. Duplicate aliquots (0,5 ml) of the hydrolyses were withdrawn and added to 15 ml of scintillation fluid for radioactive counting on a Beckman 3-channel liquid scintillation counter.

Further aliquots from the nuclear fraction (3 ml) and from the mitochondrial fraction (2 ml) were taken for colorimetric determination of the total DNA content of the material isolated.

Determination of DNA. As described in Chapter 2.

6.3 Results

6.3.1 Purity of the nuclear and mitochondrial fractions

Both phase and electron microscopy showed that the nuclear and mitochondrial fractions were essentially uncontaminated with each other or with bacteria. Electron micrograph photographs typical of the preparations used in this study are presented in Plates 1 and 2.

6.3.2 Results of the preliminary study

Data from the preliminary investigation indicated that the incorporation of ^{3}H-thymidine increased by approximately 950% (from 4 016 cpm/mg DNA to 42 246 cpm/mg DNA) 20h post-operatively in nuclei of partially hepatectomised control animals when compared with the animals receiving only a sham operation (Table 19). In contrast, incorporation of ^{3}H-thymidine increased by only 120% (from 18 239 cpm/mg DNA to 39 872 cpm/mg DNA) in mitochondria of partially hepatectomised animals 20 hours post-operatively compared with the sham-operated animals, which reflects the higher turnover rate of mitochondrial DNA in normal liver reported by Neubert (149).

Addition of Mitomycin C resulted in a significant inhibition of DNA synthesis in both nuclei and mitochondria (83% and 90% respectively) of partially hepatectomised animals 20 hours post-operatively (Table 18). The specific activity of DNA isolated from regenerating liver 5 min after injection of ^{3}H-thymidine was 91% and 92% lower respectively (42 246 cpm/mg DNA to 3 624 cpm/mg DNA and 39 872 to 3 093 cpm/mg DNA) in nuclei and mitochondria of rats allowed a 60 min incorporation period.

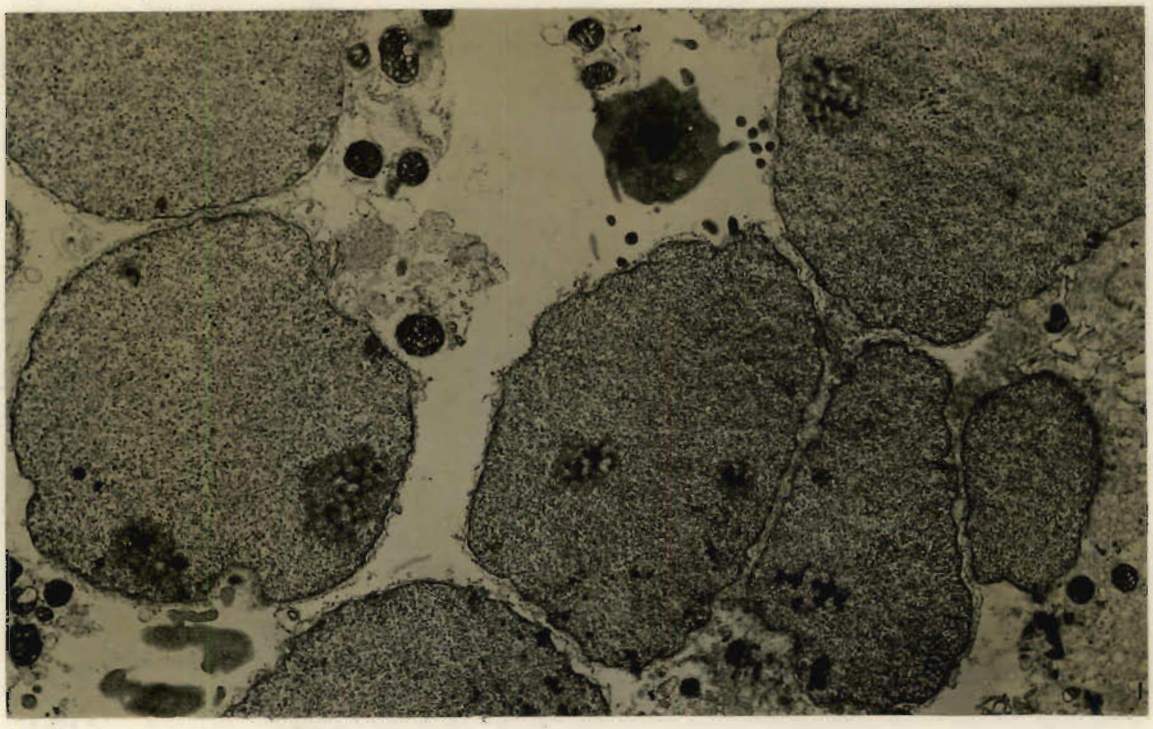

PLATE 1 Electron micrograph of the nuclear fraction of regenerating rat liver (x 7500)

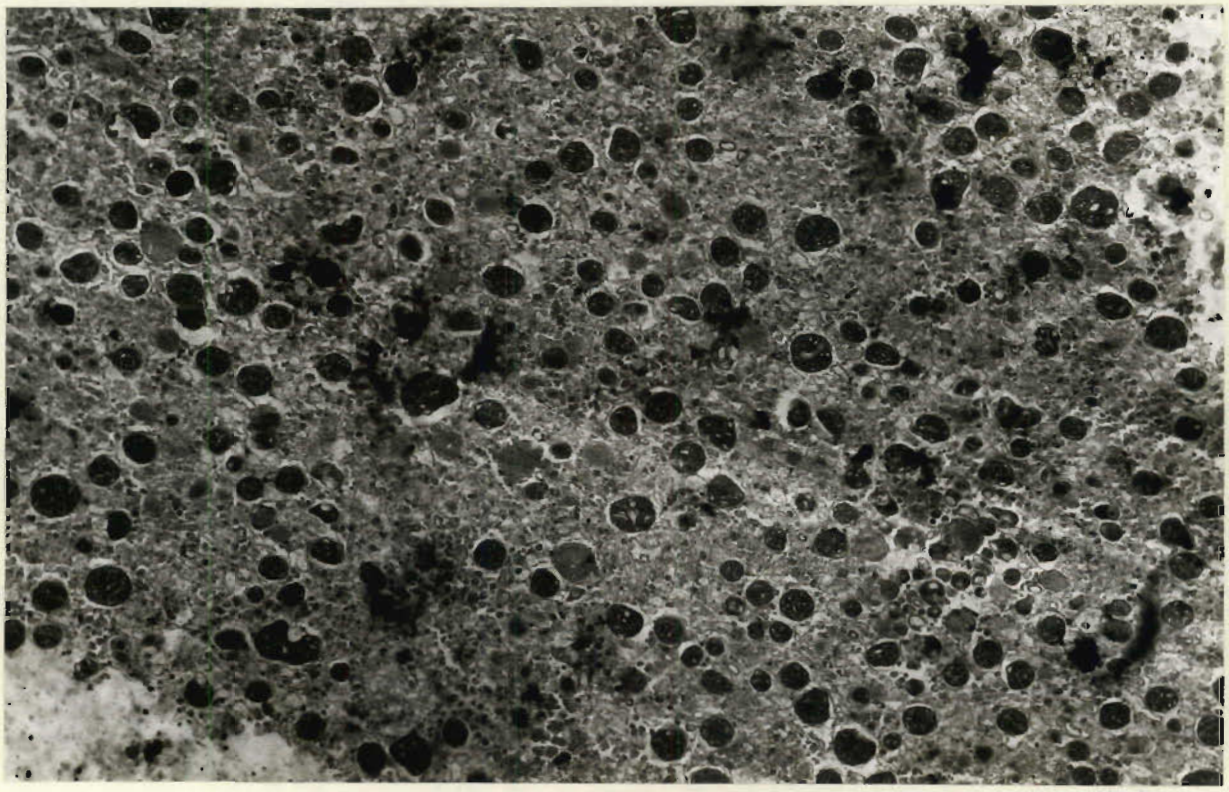

PLATE 2 Electron micrograph of the mitochondrial fraction of regenerating rat liver (x 7500)

TABLE 19 Results of a preliminary investigation into the incorporation of ^{3}H-thymidine into DNA of regenerating livers 20h after surgery. Means of 5 animals and their S.E.'s

Treatment	Incorporation time (min)	Specific activity of DNA (cpm/mg DNA)	
		Nuclear	Mitochondrial
Partial hepatectomy	60	42 246 ± 1 949	39 872 ± 1 775
Sham operation	60	4 016 ± 547	18 239 ± 2 063
Partial hepatectomy + Mitomycin C	60	7 291 ± 803	4 515 ± 742
Partial hepatectomy	5	3 624 ± 581	3 093 ± 437

6.3.3 The effect of zinc deficiency on incorporation of ^{3}H-thymidine into nuclei and mitochondria of regenerating livers 10 - 40h after partial hepatectomy

Detailed results concerning the relative incorporation of ^{3}H-thymidine into nuclear DNA of regenerating livers from zinc-deficient and control animals are presented in Appendix 13 and summarised in Table 20. The data indicate that the incorporation was significantly reduced ($p < 0,01$) between 15 and 40h post-operatively in the zinc-deficient rats compared with the control animals. In addition, a shift was found in the peak of DNA synthesis from 20 hours in the control group to 25 hours in the deficient animals. However, incorporation of thymidine in zinc-deficient animals 25 hours after partial hepatectomy was still significantly less than in the control animals 20 hours post-operatively ($p < 0,01$) but the magnitude of reduction was less than when incorporation of ^{3}H-thymidine into the 2 groups was compared at the same time post-operatively rather than at their respective peaks of incorporation.

TABLE 20 Specific activity of DNA isolated from nuclei of regenerating livers of control and zinc-deficient rats at varying times after partial hepatectomy

Zinc status of rats (1)	No. of rats (2)	Time after partial hepatectomy (h)	Specific activity of DNA (cpm/mg DNA) (3)	Difference of means (cpm/mg DNA)
Control	8	10	9 205 ± 917	165 ± 415
Zinc-deficient	8	10	9 040 ± 824	L.S.D. (0,05) 826
Control	8	15	30 132 ± 2 267	6 792 ± 1 437
Zinc-deficient	8	15	23 340 ± 1 765	L.S.D. (0,01) 3 738
Control	10	17½	40 939 ± 2 163	12 829 ± 1 525
Zinc-deficient	10	17½	28 110 ± 2 639	L.S.D. (0,01) 3 874
Control	10	20	42 246 ± 1 949	13 042 ± 1 179
Zinc-deficient	10	20	29 204 ± 1 775	L.S.D. (0,01) 2 993
Control	10	22½	41 749 ± 2 357	11 597 ± 2 240
Zinc-deficient	10	22½	30 152 ± 2 376	L.S.D. (0,01) 5 688
Control	10	25	41 071 ± 2 268	8 354 ± 1 629
Zinc-deficient	10	25	32 717 ± 2 853	L.S.D. (0,01) 4 138
Control	10	30	38 258 ± 2 025	7 309 ± 1 223
Zinc-deficient	10	30	30 949 ± 1 806	L.S.D. (0,01) 3 080
Control	10	35	36 613 ± 2 049	7 342 ± 1 344
Zinc-deficient	10	35	29 271 ± 2 199	L.S.D. (0,01) 3 413
Control	9	40	35 306 ± 2 743	7 732 ± 1 639
Zinc-deficient	9	40	27 574 ± 2 138	L.S.D. (0,01) 4 208
Control	10	20	42 246 ± 1 949	9 529 ± 1 545
Zinc-deficient	10	25	32 717 ± 2 853	L.S.D. (0,01) 4 418

(1) Mean plasma zinc levels of deficient animals were 0,56 ppm (0,65 - 0,47) and control animals 1,01 ppm (1,09 - 0,93).

(2) Individual results in Appendix 13.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 13.

Data concerning the incorporation of ^{3}H-thymidine into mitochondrial DNA of regenerating livers from zinc-deficient and control animals are summarised in Table 21 and presented in detail in Appendix 14. The results show that DNA synthesis was significantly reduced ($p < 0,01$) between 17½ and 35h after partial hepatectomy in the zinc-deficient group when compared with the control animals. No shift in the peak of DNA synthesis in the zinc-deficient animals was found in mitochondrial fractions and both the control and zinc-deficient rats exhibited the greatest incorporation 35h post-operatively.

TABLE 21 Specific activity of DNA isolated from mitochondria of regenerating livers of control and zinc-deficient rats at varying times after partial hepatectomy

Zinc status of rats (1)	No. of rats (2)	Time after partial hepatectomy (h)	Specific activity of DNA (cpm/mg DNA) (3)	Difference of means (cpm/mg DNA)
Control	8	10	27 808 ± 1 965	1 264 ± 1 339
Zinc-deficient	8	10	26 544 ± 1 820	L.S.D. (0,05) 2 348
Control	8	15	32 613 ± 1 506	2 130 ± 1 424
Zinc-deficient	8	15	30 483 ± 2 419	L.S.D. (0,05) 2 498
Control	10	17½	36 519 ± 2 105	4 850 ± 1 375
Zinc-deficient	10	17½	31 669 ± 2 241	L.S.D. (0,01) 3 491
Control	10	20	39 872 ± 2 699	6 058 ± 1 503
Zinc-deficient	10	20	33 814 ± 2 003	L.S.D. (0,01) 3 816
Control	10	22½	42 439 ± 2 456	6 046 ± 1 465
Zinc-deficient	10	22½	36 393 ± 2 168	L.S.D. (0,01) 3 456
Control	10	25	44 605 ± 2 155	5 783 ± 1 539
Zinc-deficient	10	25	38 822 ± 2 683	L.S.D. (0,01) 3 908
Control	10	30	46 671 ± 1 852	4 918 ± 1 484
Zinc-deficient	10	30	41 753 ± 2 753	L.S.D. (0,01) 3 767
Control	10	35	47 256 ± 2 248	3 652 ± 1 551
Zinc-deficient	10	35	43 604 ± 2 641	L.S.D. (0,05) 2 682
Control	9	40	46 585 ± 2 677	3 425 ± 2 003
Zinc-deficient	9	40	43 160 ± 3 302	L.S.D. (0,05) 3 485

(1) Mean plasma zinc levels of deficient animals were 0,55 ppm (0,63 - 0,48) and control animals 0,99 ppm (1,07 - 0,92).

(2) Individual results in Appendix 14.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 14.

6.4 Discussion

One of the major problems associated with the interpretation of data obtained from studies on the incorporation of labelled precursors into DNA is to determine whether differences in incorporation reflect changes in nett DNA synthesis or merely an altered turnover rate. The problem was particularly important in the present study where an examination of the relative incorporation rates of ^{3}H-thymidine into mitochondrial and nuclear DNA showed that incorporation was 4 - 5 times greater in mitochondria than in nuclei in sham-operated animals, but that this was not the case in partially hepatectomised animals. Interpretation of the present data in terms of zinc deficiency therefore required clarification on the possibility that differences between the two systems might arise from a higher turnover rate in some cases and not from a nett synthesis of new DNA.

In this connection, Schneider and Kuff (144) and Nass *et al.* (140) reported that mitochondria from normal rat liver cells incorporated thymidine ten times more rapidly into DNA than did nuclei. Nass *et al.* (140) showed that this was due to a higher turnover rate of mitochondrial DNA in slow-growing tissues rather than synthesis of new DNA. On the other hand, Neubert (149) and Nass (56) found that the rate of turnover of DNA in mitochondria was considerably reduced in fast-growing tissues such as regenerating liver. Their evidence indicated that almost all the incorporation of labelled precursors into mitochondrial DNA in fast-growing tissues arose from a nett synthesis of DNA rather than from an increased turnover. The results were corroborated by further findings of Nass *et al.* (150) who demonstrated levels of DNA 2 - 3 times higher in mitochondria from regenerating rat liver than from normal liver. Concerning nuclear DNA synthesis, it is well established that increased incorporation of precursors into DNA in regenerating rat liver reflects greater nett synthesis rather than an increased turnover of DNA (151). In the light of the prevailing evidence it was considered safe to conclude that, in this study, increased incorporation of

^{3}H-thymidine following partial hepatectomy reflected a change in nett DNA synthesis as also did differences between the specific activity of mitochondrial and nuclear DNA isolated from zinc-deficient and control animals.

Data from the preliminary investigation in this study further confirmed that the bulk of the incorporation measured in this investigation reflected active DNA synthesis since it was shown that addition of Mitomycin C, a specific inhibitor of DNA synthesis (75), resulted in substantial inhibition (± 90%) of the incorporation of ^{3}H-thymidine in both nuclei and mitochondria. In addition the zero time study indicated little contamination of the isolated DNA with unincorporated ^{3}H-thymidine.

Results from the studies on the incorporation of ^{3}H-thymidine into DNA showed that nuclear DNA synthesis was significantly reduced ($p < 0,01$) 15 - 30 hours after partial hepatectomy in zinc-deficient animals, which was in close agreement with earlier results (Chapter 2) on whole cell DNA synthesis. In addition, mitochondrial DNA synthesis was found to be significantly reduced ($p < 0,01$) between 15 and 35 hours after partial hepatectomy in zinc-deficient rats, but the magnitude of the decrease was considerably less than that in the nucleus. Thus, although zinc deficiency resulted in a decrease in both nuclear and mitochondrial DNA synthesis the major effect appeared to be on nuclear DNA synthesis. It is tempting from these results to postulate that all organelles which replicate their own DNA by a mechanism similar to that found in the nucleus will exhibit a similar inhibition of DNA synthesis as a result of zinc deficiency. However, such a suggestion can only be substantiated by a closer examination of these organelles.

Of further interest concerning the synthesis of DNA in the nucleus and the mitochondria was the response of these organelles to partial hepatectomy. Clearly (Tables 20 and 21) the cycles of mitochondrial and nuclear DNA biosynthesis in the early periods of regeneration of the liver do not coincide. Mitochondrial DNA biosynthesis in the control group increased immediately after surgery to a level significantly higher than that before partial hepatectomy

and reached a maximum 35 hours post-operatively. However, nuclear DNA synthesis increased significantly only after a latent period of 10h and reached a maximum 20 hours after partial hepatectomy in the control groups. The results were in agreement with those of Khanson, Ivanova, Nikitina, Shutko and Koman (146) and Nass (56) and showed that mitochondrial DNA synthesis does not have the distinct synchronization that is characteristic of nuclear DNA in regenerating liver. This data also confirmed the concept of independent nuclear and mitochondrial DNA biosynthesis.

A comparison of the relative specific activities of nuclear DNA in control and zinc-deficient rats, reflected a similar pattern to that seen in whole cell DNA synthesis (Chapter 2). Thus, apart from decreased synthesis, the deficient group also showed a shift in the peak of DNA synthesis from 20 hours (in the control animals) to 25 hours post-operatively. In contrast, in mitochondria both the control and the zinc-deficient group exhibited a peak of DNA synthesis 35 hours after partial hepatectomy. As was suggested for whole cells in Chapter 2 the cause of the delay in maximum DNA synthesis in nuclei in zinc-deficient animals may have been due to a slower synthesis or reduced activity of the enzymes required for DNA synthesis in these cells. This could result fr a direct effect of zinc on these enzymes during the G_1 period of the cell cycle, which in turn could cause a delay in the whole synthetic cycle. In the case of the mitochondria, the asynchronous nature of DNA synthesis in these organell would not allow this effect to be observed although the reduction in DNA synthesis in the zinc-deficient cells probably arose in the same way.

Further speculation on the role of zinc at the enzymic level suggests that the effect of a deficiency on nuclear and mitochondrial DNA synthesis may arise from a direct involvement of zinc in the enzymes associated with the synthetic pathway, since the two systems have essentially the same mechanism of replicati but are distinguished only by differences in the characteristics of the individ enzymes involved (57, 58). In addition, since a deficiency of zinc resulted in

a significant decrease in DNA synthesis in both nuclear and mitochondrial systems it appeared that both systems were probably affected at a similar site. Furthermore, since the decrease in DNA synthesis as a result of zinc deficiency has been found mainly in proliferating tissue it appears that zinc depletion affected some pathway which is mainly active in rapidly dividing cells. An obvious possibility in this regard would be the thymidine kinase "salvage" pathway of DNA synthesis and investigations along these lines are reported in the next chapter.

CHAPTER 7

THE EFFECT OF DEOXYRIBONUCLEOSIDES AND DEOXYRIBONUCLEOSIDE MONOPHOSPHATES ON DNA SYNTHESIS

7.1 Introduction

Despite the previous evidence indicating an involvement of zinc in the processes of cell division and DNA synthesis, the precise role of zinc in these systems remains unclear. As mentioned earlier, it was considered that zin may be involved at an enzymic level, a viewpoint recently shared by certain othe workers (50, 51, 152). To define more precisely the locus of action of zinc it was essential to determine first which phase of DNA synthesis was zinc-dependent i.e. whether zinc deficiency was affecting the synthesis of precursors required for DNA synthesis, the phosphorylation of these precursors to the nucleotides o the polymerization of the phosphorylated derivatives.

It has been established that the major pathway for the production of deoxyribonucleotides in rapidly dividing cells is via the thymidine kinase "salvage" pathway rather than by de novo synthesis (61, 62). Accordingly the present investigation on regenerating liver commenced with a study on the availability of the deoxyribonucleoside precursors required for this pathway since it is possible that zinc may be involved during the synthesis of these precurso Therefore, since it has been reported that deoxyribonucleosides act as effectiv precursors for DNA synthesis in a number of systems (46, 153-160), it was possible to inject unlabelled deoxyribonucleosides into zinc-deficient and control rats in order to establish their effect on the overall rate of DNA synthesis.

The next phase in the synthesis of DNA via the thymidine kinase pathway concerns the phosphorylation of deoxyribonucleosides to deoxyribonucleoside monophosphates. As with nucleosides, deoxyribonucleoside monophosphates have been reported to be effective precursors of DNA synthesis (48, 49, 160, 161)

and it was therefore hoped that the effects of added deoxyribonucleoside monophosphates on DNA synthesis in zinc-deficient tissues might indicate whether zinc was involved at this first phosphorylation step or at some point beyond thi step. Deoxyribonucleoside monophosphates were therefore administered in the sam way as were the deoxyribonucleosides, and their effect on the rate of DNA synthesis in zinc-deficient and control animals was studied.

7.2 Materials and Methods

7.2.1 Reagents

Thymidine (^{3}H-methyl). As described in Chapter 6.

Deoxyribonucleosides and deoxyribonucleoside monophosphates. Deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine and their 5'-monophosphates were purchased from Sigma Chemicals, St Louis, U.S.A. and were dissolved in sterile physiological saline to give a final concentration of 25 mg/ml.

TMK buffer. As described in Chapter 6.

Scintillation fluid. As described in Chapter 2.

Diphenylamine reagent. As described in Chapter 2.

Standard DNA solutions. As described in Chapter 2.

7.2.2 Animals and rations

Animals were fed zinc-deficient and control rations as described in Chapter 2.

7.2.3 Methods

Surgical procedure. As described in Chapter 2.

Collection of blood samples. As described in Chapter 2.

Injections and timing. Deoxyribonucleosides (5 mg in 0,5 ml saline) were injected intraperitoneally either singly or in combinations into experimental animals every 2 hours for 8 hours beginning 15 hours after partial

hepatectomy. ^{3}H-thymidine (25 µC) was injected into the animals 24 hours post-operatively. Sixty minutes later the animals were killed by ether anaesthesia and the livers were removed and quick frozen in dry-ice.

In another experiment the same procedure as described for the nucleosides was adopted using deoxyribonucleoside 5'-monophosphates (5 mg in 0,5 ml saline).

Isolation of nuclear and mitochondrial fractions. As described in Chapter 6.

Determination of the specific radioactivity of DNA. As described in Chapter 6.

Determination of DNA. As described in Chapter 2.

7.3 Results

7.3.1 The effect of added deoxyribonucleosides on nuclear and mitochondrial DNA synthesis in regenerating livers of zinc-deficient and control rats 25 hours after partial hepatectomy

A summary of the effects of additional unlabelled deoxyribonucleosides on the incorporation of ^{3}H-thymidine into nuclear DNA of regenerating rat livers from zinc-deficient and control animals is presented in Table 22 (detailed results in Appendix 15). The data indicated that although the relative incorporation rates differed depending on the deoxyribonucleoside injected, the addition of these free deoxyribonucleosides either singly or in combination had no effect on the inhibition of DNA synthesis accompanying zinc deficiency. In all cases incorporation of ^{3}H-thymidine was significantly reduced ($p < 0,01$) in the zinc-deficient rats compared with the control animals. The specific activity of DNA isolated from animals which had been injected with unlabelled thymidine was lower than that in animals which had received no addition of deoxyribonucleosides, probably due to an isotope dilution effect.

TABLE 22 Effect of added deoxyribonucleosides on nuclear DNA synthesis in regenerating livers of zinc-deficient and control rats 25 hours after partial hepatectomy

Zinc status of rats (1)	No. of rats (2)	Deoxyribonucleoside added	Incorporation of ^{3}H-thymidine (cpm/mg DNA) (3)	Difference of means (cpm/mg DNA)
Control	10	-	41 071 ± 2 268	8 354 ± 1 629
Zinc-deficient	10	-	32 717 ± 2 853	L.S.D. (0,01) 4 138
Control	8	dT	27 445 ± 2 390	6 343 ± 1 633
Zinc-deficient	8	dT	21 102 ± 2 226	L.S.D. (0,01) 4 812
Control	8	dC	35 177 ± 2 535	8 561 ± 1 864
Zinc-deficient	8	dC	26 616 ± 2 735	L.S.D. (0,01) 5 493
Control	8	dA	38 027 ± 2 570	10 891 ± 1 775
Zinc-deficient	8	dA	27 136 ± 2 450	L.S.D. (0,01) 5 231
Control	8	dG	38 716 ± 3 122	11 906 ± 2 026
Zinc-deficient	8	dG	26 810 ± 2 584	L.S.D. (0,01) 5 971
Control	8	dT + dC	26 948 ± 2 184	4 044 ± 1 469
Zinc-deficient	8	dT + dC	22 904 ± 1 964	L.S.D. (0,05) 3 130
Control	8	dA + dG	37 013 ± 1 753	9 980 ± 1 509
Zinc-deficient	8	dA + dG	27 033 ± 2 457	L.S.D. (0,01) 4 447
Control	8	dT + dC	27 510 ± 2 110	4 469 ± 1 438
Zinc-deficient	8	+ dG + dA	23 041 ± 1 955	L.S.D. (0,01) 4 238

(1) Mean plasma zinc levels of deficient animals were 0,54 ppm (0,62 - 0,48) and control animals 1,01 ppm (1,10 - 0,95).

(2) Individual results in Appendix 15.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 15.

Data presented in Table 23, and detailed findings in Appendix 16, indicate that the addition of free deoxyribonucleosides had no effect on the incorporation of ^{3}H-thymidine into mitochondrial DNA of regenerating rat livers and in all cases incorporation of ^{3}H-thymidine was significantly reduced ($p < 0,01$) in zinc-deficient groups.

TABLE 23 Effect of added deoxyribonucleosides on mitochondrial DNA synthesis in regenerating livers of zinc-deficient and control rats 25 hours after partial hepatectomy

Zinc status of rats (1)	No. of animals (2)	Deoxyribonucleoside added	Incorporation of ^{3}H-thymidine (cpm/mg DNA) (3)	Difference of means (cpm/mg DNA)
Control	10	-	44 605 ± 2 155	5 783 ± 1 539
Zinc-deficient	10	-	38 822 ± 2 683	L.S.D. (0,01) 3 908
Control	8	dT	33 042 ± 1 941	5 927 ± 1 477
Zinc-deficient	8	dT	27 115 ± 2 228	L.S.D. (0,01) 4 353
Control	8	dC	38 906 ± 2 468	6 293 ± 1 553
Zinc-deficient	8	dC	32 613 ± 1 885	L.S.D. (0,01) 4 577
Control	8	dA	40 535 ± 2 345	7 142 ± 1 702
Zinc-deficient	8	dA	33 393 ± 2 468	L.S.D. (0,01) 5 016
Control	8	dG	41 083 ± 2 683	8 022 ± 1 801
Zinc-deficient	8	dG	33 061 ± 2 402	L.S.D. (0,01) 5 308
Control	8	dT + dC	32 503 ± 2 113	5 950 ± 1 626
Zinc-deficient	8	dT + dC	26 553 ± 2 471	L.S.D. (0,01) 4 792
Control	8	dA + dG	41 008 ± 2 433	7 949 ± 1 664
Zinc-deficient	8	dA + dG	33 059 ± 2 272	L.S.D. (0,01) 4 904
Control	8	dT + dC	32 041 ± 2 673	5 961 ± 1 904
Zinc-deficient	8	+ dA + dG	26 080 ± 2 712	L.S.D. (0,01) 5 611

(1) Mean plasma zinc levels of deficient animals were 0,60 ppm (0,69 - 0,51) and control animals 1,02 ppm (1,09 - 0,94).

(2) Individual results in Appendix 16.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendix 16.

7.3.2 The effect of added deoxyribonucleoside 5'-monophosphates on nuclear and mitochondrial DNA synthesis in regenerating livers of zinc-deficient and control rats 25 hours after partial hepatectomy

The effect of added deoxyribonucleotides on the incorporation of ^{3}H-thymidine into nuclear and mitochondrial DNA is presented in Table 24 and in detail in Appendices 17 and 18. The results were very similar to those observed using deoxyribonucleosides (Tables 22 and 23) and indicated no significant effect of added deoxyribonucleotides on the inhibition of DNA synthesis by zinc deficie in both nuclei and mitochondria.

TABLE 24 Effect of added deoxyribonucleoside 5'-monophosphates on nuclear and mitochondrial DNA synthesis in regenerating livers of zinc-deficient and control rats 25 hours after partial hepatectomy

Zinc status[1] of rats	No.[2] of rats	Deoxyribo-nucleotide added	Incorporation[3] ^{3}H-thymidine (cpm/mg DNA)	Difference of means (cpm/mg DNA)
(A) Nuclear				
Control	8	dTMP	25 308 ± 2 639	5 256 ± 1 704
Zinc-deficient	8	dTMP	20 052 ± 2 155	L.S.D. (0,01) 4 434
Control	8	dCMP	33 947 ± 2 264	6 899 ± 1 722
Zinc-deficient	8	dCMP	27 048 ± 2 595	L.S.D. (0,01) 4 481
Control	8	dAMP	40 228 ± 3 420	10 160 ± 2 085
Zinc-deficient	8	dAMP	30 068 ± 2 387	L.S.D. (0,01) 5 425
Control	8	dGMP	35 925 ± 2 797	9 671 ± 1 794
Zinc-deficient	8	dGMP	26 254 ± 2 248	L.S.D. (0,01) 4 668
(B) Mitochondrial				
Control	8	dTMP	32 689 ± 2 318	3 559 ± 1 623
Zinc-deficient	8	dTMP	29 130 ± 2 272	L.S.D. (0,05) 2 810
Control	8	dCMP	36 015 ± 2 367	5 209 ± 1 631
Zinc-deficient	8	dCMP	30 806 ± 2 244	L.S.D. (0,01) 4 244
Control	8	dAMP	38 485 ± 2 553	6 339 ± 1 772
Zinc-deficient	8	dAMP	32 146 ± 2 457	L.S.D. (0,01) 4 611
Control	8	dGMP	39 022 ± 2 584	6 604 ± 1 709
Zinc-deficient	8	dGMP	32 418 ± 2 236	L.S.D. (0,01) 4 447

(1) Mean plasma zinc levels of deficient animals were 0,51 ppm (0,59 - 0,43) and control animals 0,95 (0,99 - 0,87).

(2) Individual results in Appendices 17 and 18.

(3) Total DNA in 0,5 ml hydrolysate and cpm of hydrolysate given in Appendices 17 and 18.

7.4 Discussion

The results from this study clearly indicated that the addition of deoxyribonucleosides had no significant effect on the inhibition of DNA synthesi observed in both nuclei and mitochondria following zinc restriction. The fact that these deoxyribonucleosides are incorporated into DNA has been borne out by the evidence of Reichard and Estborn (46), Smellie et al. (47), Brown, Roll and Weinfeld (153) and several other investigators (154, 155) who have shown that labelled thymidine was incorporated into DNA in cells which were synthesising DNA. In addition, Hammarstein, Reichard and Saluste (156) and other workers (157) have shown labelled deoxycytidine to be incorporated into DNA with the label being found in both DNA cytosine and thymine indicating some interconversion of the precursor. Although a number of investigators (158 - 160) have foun the purine nucleoside, deoxyadenosine, to be an effective precursor for DNA synthesis in a number of cell types, the other purine nucleoside, deoxyguanosin was not readily incorporated into DNA in slow growing cells but proved to be an effective precursor in faster growing tissues (158 - 160). Consequently, althou the particular deoxyribonucleoside of interest in the pathway under consideration was thymidine, it was possible to examine the effects of addition of all four deoxyribonucleosides on DNA synthesis in zinc-deficient tissues and thus determine whether a deficiency of one or more of these precursors existed.

It is of interest that the addition of unlabelled thymidine had no effect on the reduction of DNA synthesis in zinc-deficient cells despite the fact that there was a reduction in the specific activity of DNA isolated from zinc-deficient and control rats due to an isotope dilution effect. When deoxycytidi was added a similar but less pronounced effect was seen due to a certain propor tion of this deoxyribonucleoside being converted to thymidine and incorporated into DNA in this form (156, 157). The results from this study suggest that the was no deficiency of deoxyribonucleoside precursors required for DNA synthesis in regenerating livers from zinc-deficient rats. Thus it seems that the site o action of zinc is at some stage (or stages) beyond the synthesis of

deoxyribonucleosides and is possibly involved in the phosphorylation of deoxyribonucleosides or in the polymerization of the phosphorylated derivatives into DNA molecules.

The addition of deoxyribonucleoside 5'-monophosphates to experimental animals showed similar results to those seen when the non-phosphorylated deoxyribonucleoside precursors were added, although in all cases the magnitude of reduction in DNA synthesis in zinc-deficient tissues was less than that found in the deoxyribonucleoside experiments. It was hoped that these results may hav given an indication of whether zinc was involved at the thymidine kinase step. However, the similarity between results from the deoxyribonucleoside and deoxyribonucleotide experiments suggested a dephosphorylation of the deoxyribonucleotides before entry into the cells which supported earlier postulates (60, 162) that deoxyribonucleotides were not incorporated *in toto*. The evidence using ^{32}P-labelled compounds suggested a cleavage of the phosphate bond before entry into the cell (60, 163). As a result very little useful information as to the active site of zinc could be obtained from the addition of deoxyribonucleotides during DNA synthesis.

At this stage in the search for the phase of DNA synthesis affected by zinc deficiency it could only be concluded that the stages affected were either those of phosphorylation or polymerization, or both, as indicated by the deoxyribonucleoside studies. As a result the next logical step would be to examine the effects of zinc levels on the individual enzymes involved in these stages of DNA synthesis and in this case it was logical to make a close examination of the two zinc-dependent enzymes, thymidine kinase (51) and DNA polymerase (50), associated with these phases. In particular, thymidine kinase was of interest since it was thought to be the rate-determining step in DNA synthesis in rapidly dividing cells (133), in which the thymidine kinase "salvage" pathway is the most important pathway for the production of deoxythymidine monophosphate (61, 62). Further evidence for the importance of this pathway was the finding of increased activity of enzymes required for thymidine phosphorylation to

thymidine triphosphate in fast growing cells (65, 164). In contrast, the enzymes required for phosphorylation of the other DNA nucleosides had a similar activity in slow and fast growing tissues (65, 164), and it has been suggested that it is the level of thymidine triphosphate in the cell which controls the rate of DNA synthesis (164, 165).

CHAPTER 8

THE EFFECT OF ZINC LEVELS ON THYMIDINE KINASE AND DNA POLYMERASE ACTIVITY

8.1 Introduction

Two of the most important enzymes involved in DNA synthesis, thymidine kinase and DNA polymerase, have been shown to exhibit greatly enhanced activity in rapidly proliferating tissues such as regenerating liver (62, 166, 167) and various neoplasms (168, 169). In addition, both enzymes have recently been demonstrated to be zinc-dependent (50, 51, 170), thus making them of particular interest with regard to the active site of zinc in DNA synthesis.

Thymidine kinase is almost undetectable in non-proliferating tissues, such as normal liver cells (164, 171, 172), but shortly after partial hepatectomy, and between 5 and 10 hours before DNA synthesis, the activity increases markedly after a short lag period (62, 166, 167, 172 - 174). The activity of kinases that phosphorylate deoxycytidine, deoxyadenosine and deoxyguanosine to the corresponding phosphate esters appear to be approximately the same in normal and regenerating liver (175, 176). Thus it appears that the thymidine kinase salvage pathway for the synthesis of thymine nucleotides becomes predominant in DNA synthesis in rapidly dividing cells whereas *de novo* nucleotide synthesis represents the major pathway in resting cells. Because of this role of thymidine kinase it has been suggested (65, 177) that it may operate as the rate-controlling enzyme in DNA synthesis in proliferating cells. This has been supported by evidence indicating that this enzyme has many of the attributes of a rate-determining enzyme in DNA synthesis (65, 133, 177). For example, thymidine kinase is subject to feedback inhibition by dTTP (178, 179) and it has been shown that the level of deoxyribonucleoside triphosphates in the cell determines the rate of DNA synthesis (180).

The role of the DNA polymerase enzyme in DNA synthesis is less clear because of the existence of multiple molecular weight forms of the enzyme in different

cellular locations in eukaryotic cells (58, 63, 64, 181, 182) and the finding that these enzymes exhibit substantially different characteristics (63, 183, 184). In addition, increases in activity of these enzymes in proliferating cells varies depending on their location (185, 186). In procaryotic cells three DNA polymerase molecules have been suggested to have a role in DNA replication (187 - 190), although one of these enzymes, DNA polymerase I, appears to function only as a repair enzyme. It is in fact DNA polymerase I from E. coli that has been identified as a zinc-containing enzyme (50, 170). Consequently, it is doubtful whether the role of zinc in this enzyme has any effect on actual DNA synthesis, although it has been suggested that all DNA polymerase molecules may be zinc-dependent (170).

Despite the doubt surrounding the roles of the DNA polymerases in DNA synthesis, the importance of these enzymes and of thymidine kinase in the phosphorylation and polymerization of deoxyribonucleosides strongly suggested a study of the effects of zinc deficiency on their activities in regenerating rat livers and transplantable hepatomas and of the effects of addition of metal ions on the activity of these enzymes.

8.2 Materials and Methods

8.2.1 Reagents

DAB-1 hepatoma. As described in Chapter 5.

TMK buffer. As described in Chapter 6.

Triton X-100 was purchased from BDH Chemicals Ltd., Poole, England and diluted to give a final concentration of 25% v/v.

Thymidine (^{3}H-methyl), as described in Chapter 2, was diluted with sterile water to give a final activity of 0,5 mC/ml.

Thymidine 5'-monophosphate (^{3}H-methyl) with a specific activity of 15 C/mM was purchased from Nuclear Dynamics Inc., El Monte, California. The sample was diluted with sterile water to give a final activity of 10 µC/ml.

Thymidine 5'-triphosphate (^{3}H-methyl) with a specific activity of 47 mC/mM was purchased from the Radiochemical Centre Ltd., Amersham, England. The sample was diluted with sterile water to give a final activity of 50 µC/ml.

Adenosine 5'-triphosphate was purchased from Sigma Chemicals, St Louis, U.S.A. and was dissolved in sterile water to give a final concentration of 0,125 mM.

3-Phosphoglycerate was purchased from Sigma Chemicals, St Louis, U.S.A. and was dissolved in sterile water to give a final concentration of 0,15 mM.

Magnesium chloride was purchased from May and Baker Ltd., Dargenham, England and was dissolved in sterile water to give a final concentration of 0,25 mM and 0,075 mM.

5'-triphosphates of 2'-deoxyadenosine, 2'-deoxycytidine and 2'-deoxyguanosine were purchased from Sigma Chemicals, St Louis, U.S.A. and were dissolved in sterile water to give a final concentration of 2,5 µM.

Potassium chloride was purchased from Hopkins and Williams Ltd., Johannesburg, South Africa and was dissolved in sterile water to give a final concentration of 0,075 mM.

2-mercaptoethanol was purchased from BDH Chemicals Ltd., Poole, England and was diluted with sterile water to give a final concentration of 0,025 mM.

DNA. Calf thymus DNA was purchased from BDH Chemicals Ltd., Poole, England and 10 mg was dissolved in 2 ml 0,02 M-Tris-HCl buffer (pH 7,5). The solution was heated to 100°C in a boiling-water bath for 10 min and then rapidly cooled in an ice-bath (191). The resulting heat-denatured DNA solution was used in the enzyme assays.

Perchloric acid was purchased from J. T. Baker Chemicals Co., Phillipsburg, New Jersey, U.S.A. and was diluted with sterile water to give solutions with final concentrations of 1,0 M, 0,5 M and 0,2 M.

Ammonium formate was purchased from BDH Chemicals Ltd., Poole, England and was dissolved in distilled water to give a final concentration of 1 mM.

DEAE-cellulose paper (DE 81) was purchased from Whatman Biochemicals, Kent, England in sheets (46 x 57 cm) and discs of diameter 2,5 cm were cut from the sheets.

Scintillation fluid. As described in Chapter 2.

Folin-Ciocalteu reagent:

Reagent A: 2% Na_2CO_3 in 0,1N-NaOH.

Reagent B: 0,5% $CuSO_4.5H_2O$ in 1% sodium citrate.

Reagent C: 1 ml reagent B mixed with 50 ml reagent A on the day of use.

Reagent D: Folin-Ciocalteu reagent (commercial reagent purchased from BDH Chemicals Ltd., Poole, England) was diluted with water to give a solution 1N in acid.

Standard ovalbumin solutions. A stock solution (1 mg/ml) of ovalbumin (Sigma Chemicals, St Louis, U.S.A.) was prepared. From this solution standards were prepared containing 20 - 200 µg/ml protein.

8.2.2 Glassware

All glassware was washed twice in 2% Contrad solution, rinsed, soaked in 2N-nitric acid for 24 hours, rinsed again and stored in sealed containers to avoid contamination.

8.2.3 Animals and rations

Animals were fed zinc-deficient and control rations as described in Chapter 2.

8.2.4 Methods

Determination of zinc levels in reagents. The zinc concentration in all reagents and solutions was determined by atomic absorption spectroscopy on a Varion Techtron (1000) atomic absorption spectrophotometer.

Surgical procedure. Partial hepatectomy was performed as described in Chapter 2.

Collection of blood samples. As described in Chapter 2.

Hepatoma transplantation. DAB-1 hepatoma was transplanted into rats as described in Chapter 5.

Preparation of enzyme fractions. Nuclear and mitochondrial fractions from regenerating liver, used in the enzyme assays, were isolated as described in Chapter 6 and the post-mitochondrial supernatant retained as the supernatant enzyme fraction. The nuclear and mitochondrial pellets were suspended in 10 and 5 ml 0,25 M-sucrose respectively. Triton X-100 (25%) was added to the suspensions at a final concentration of 1%. The total homogenate was used for determining enzyme activity in DAB-1 hepatomas with Triton X-100 again added at a final concentration of 1%.

Thymidine kinase assay procedure. The procedure followed was a modification of that described by Witschi (167). The reaction mixture contained in a final volume of 0,5 ml 0,12 M-Tris-HCl buffer (pH 8,0): 2,5 µM-ATP; 3,0 µM-3-phosphoglycerate; 5,0 µM-$MgCl_2$; 0,2 ml of one of the enzyme fractions and 5 µC ^{3}H-thymidine. The reaction mixture was incubated at 37^{o}C for 1h and the reaction stopped by immersing the assay tubes in boiling water for 2 min. After cooling, denatured protein was removed by centrifugation at 1000 g for 10 min and 50 µl of the protein-free supernatant was spotted onto a DEAE-cellulose disc. The discs were washed by gentle swirling in 0,1 mM-ammonium formate (20 ml), distilled water (20 ml), ammonium formate and water again and finally in 99% ethanol. The discs were air-dried and placed in a scintillation vial containing 15 ml scintillation fluid, making sure that in each case the disc was firmly seated on the bottom of the vial. The discs were counted for radioactivity in a Beckman 3-channel scintillation counter and the amount of thymidine phosphorylated was calculated from a standard curve of radioactivity versus concentration of ^{3}H-thymidine which was prepared using serial dilutions

of a solution of ^{3}H-thymidine of known specific activity (Appendix 19). Thymidine kinase activity was expressed as pM ^{3}H-thymidine phosphorylated/mg protein/h.

DNA polymerase assay procedure. The procedure was a modification of the methods described by Witschi (167) and Lehman, Bessman, Simms and Kornberg (192). The reaction mixture contained in a final volume of 0,5 ml 0,16 M-Tris-HCl buffer (pH 8,0): 0,05 µM-d ATP; 0,05 µM-d CTP; 0,05 µM-d GTP; 1,5 µM-$MgCl_2$; 1,5 µM-KCl; 0,5 µM-2-mercaptoethanol; 50 µg heat-denatured DNA; 0,2 ml of one of the enzyme fractions and 0,5 µC ^{3}H-d TTP. The reaction mixture was incubated at 37^{o}C for 1h and the reaction stopped by the addition of 0,1 ml ice-cold 1,0 M-$HClO_4$. The precipitate was washed twice with 0,5 M-$HClO_4$, dissolved in 3 ml 0,3 M-KOH, incubated for 60 min at 37^{o}C, reprecipitated with ice-cold 0,5 M-$HClO_4$ (2 ml) and washed once more. The pellet obtained was dissolved in 2 ml 1 M-NaOH and 0,5 ml aliquots were withdrawn for radioactivity determinations. A unit of enzyme activity was defined as the amount required to convert 1 nM of ^{3}H-d TTP into the acid-insoluble product during the period of incubation. The amount of ^{3}H-d TTP incorporated was calculated from a standard curve of radioactivity versus concentration of ^{3}H-d TTP which was prepared using serial dilutions of a solution of ^{3}H-d TTP of known specific activity (Appendix 20). DNA polymerase activity was expressed as nM ^{3}H-d TTP incorporated/mg protein/h.

Addition of metal ions to enzyme fractions. Zinc and other divalent metal ions were added at various concentrations to enzyme fractions from regenerating rat livers of zinc-deficient rats 20h after partial hepatectomy. The solutions were assayed at different times after the addition of zinc and at 60h after addition of other metal ions. In all cases incubations were carried out at 0^{o}C. In addition the stability of the enzymes at 0^{o}C for a period of 60h was investigated by assaying the activity of the enzymes from both control and zinc-deficient animals before and after a 60h incubation period at 0^{o}C.

Determination of protein concentration. The method used was essentially that of Lowry, Rosebrough, Farr and Randall (193). 1 ml of each of the enzyme fractions was mixed with 5 ml of reagent C of the Folin-Ciocalteu reagents and the solution allowed to stand for 10 min at room temperature. 1 ml of reagent D was rapidly pipetted into the mixture with thorough mixing and after 45 min the extinction measured at 750 nm in a Zeiss PMQ II spectrophotometer.

A standard curve was prepared using solutions of ovalbumin ranging in concentration from 20 - 200 µg/ml (Appendix 21).

8.3 Results

8.3.1 Zinc levels in the reagents

Levels of zinc in the reagents and solutions used in this study were all found to be very low (Table 25) and thus were not a source of contamination. The final thymidine kinase assay mixture contained 8×10^{-3} ppm of zinc and the final DNA polymerase assay mixture 10×10^{-3} ppm of zinc.

TABLE 25 Zinc levels in the solutions used in this investigation

Solution	Zinc concentration (ppm x 10^{-3})
Sucrose (0,25 M)	5
Tris-HCl buffer	10
ATP	0
3-phosphoglycerate	0
$MgCl_2$	5
KCl	4
d ATP	0
d CTP	0
d GTP	0
2-mercaptoethanol	2
Heat-denatured DNA	12
Thymidine kinase assay mixture	8
DNA polymerase assay mixture	10

8.3.2 Control experiments

In each assay background samples were prepared by carrying out th reaction substituting water for the radioactively labelled compound. In additio zero time samples were prepared by stopping the reaction as soon as all the com- ponents had been mixed together. The background counts were subtracted from the experimental values in each case.

The efficiency of the DEAE-cellulose disc method of determining the amount of radioactivity in the form of phosphorylated products was tested by a compari- son of the counts obtained when a known concentration of ^{3}H-thymidine 5'-mono- phosphate was spotted onto DEAE-cellulose discs with the counts obtained when the same concentration of the radioactive compound was added to scintillation fluid. The results (Appendix 22) showed that the disc method was ± 20% as effi- cient as that when the radioactive compound was added to the scintillation fluid alone.

The effectiveness of the washing procedure in removing unphosphorylated thymidine but not phosphorylated thymidine from the DEAE-cellulose discs was checked by counting zero time samples before and after washing and also by count ing discs to which a known amount of ^{3}H-thymidine or ^{3}H-d TMP had been added, before and after washing. In all cases (Table 26) it was shown that more than 99% of thymidine was eluted from the paper and > 95% thymidine 5'-monophosphate remained bound to the paper.

TABLE 26 The effectiveness of the washing procedure in removing unphosphorylated thymidine and binding phosphorylated thymidine to DEAE-cellulose discs. Means of 10 readings and their S.E.'s

Sample	cpm before washing	cpm after washing
Zero time	22 141 ± 2 816	104 ± 9
50 pM-^{3}H-thymidine	12 379 ± 1 097	101 ± 8
100 pM-^{3}H-thymidine	23 607 ± 2 402	116 ± 10
50 pM-^{3}H-d TMP	6 928 ± 623	6 591 ± 524
100 pM-^{3}H-d TMP	14 256 ± 1 503	13 553 ± 1 507

8.3.3 The effect of zinc deficiency on thymidine kinase activity in various enzyme fractions from regenerating rat liver

As can be seen from Tables 27 and 28 (and Appendices 23 and 24), in the control group, thymidine kinase activity increased sharply between 10 and 15 hours after partial hepatectomy in the supernatant and nuclear fractions and reached a peak of activity between 15 and 20 hours post-operatively. In the zinc-deficient animals a similar pattern of activity was seen after partial hepatectomy expect that thymidine kinase activity was significantly less ($p < 0,01$) than that in the control group and reached a peak 20 to 25 hours afte partial hepatectomy.

TABLE 27 Effect of zinc deficiency on thymidine kinase activity in the nuclear fraction of regenerating rat liver

Zinc status of rats (1)	No. of animals (2)	Time after partial hepatectomy (h)	Thymidine kinase activity (3) (pM thymidine phosphorylated/mg protein/h)	Difference of means (pM thymidine phosphorylated/ mg protein/h)
Control	10	0	221 ± 19	13 ± 16
Zinc-deficient	9	0	208 ± 28	L.S.D. (0,05) 28
Control	9	10	402 ± 36	147 ± 20
Zinc-deficient	9	10	225 ± 24	L.S.D. (0,01) 51
Control	9	15	1 121 ± 43	609 ± 24
Zinc-deficient	10	15	512 ± 31	L.S.D. (0,01) 61
Control	10	20	1 148 ± 46	396 ± 31
Zinc-deficient	9	20	752 ± 52	L.S.D. (0,01) 79
Control	10	25	1 104 ± 55	350 ± 33
Zinc-deficient	10	25	754 ± 49	L.S.D. (0,01) 84
Control	8	30	997 ± 57	302 ± 37
Zinc-deficient	10	30	695 ± 54	L.S.D. (0,01) 95

(1) Mean plasma zinc levels of deficient animals were 0,55 ppm (0,63 - 0,50) and control animals 0,99 ppm (1,05 - 0,93).

(2) Individual results in Appendix 23.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 50 µl enzyme assay mixture given in Appendix 23.

TABLE 28 Effect of zinc deficiency on thymidine kinase activity in the supernatant fraction of regenerating rat liver

Zinc status[1] of rats	No.[2] of animals	Time after partial hepatectomy (h)	Thymidine kinase[3] activity (pM thymidine phosphorylated/mg protein/h)	Difference of means (pM thymidine phosphorylated/ mg protein/h)
Control	10	0	356 ± 27	9 ± 17
Zinc-deficient	9	0	347 ± 24	L.S.D. (0,05) 29
Control	9	10	599 ± 42	199 ± 21
Zinc-deficient	9	10	400 ± 16	L.S.D. (0,01) 54
Control	9	15	1 853 ± 49	942 ± 28
Zinc-deficient	10	15	911 ± 36	L.S.D. (0,01) 71
Control	10	20	1 896 ± 166	644 ± 82
Zinc-deficient	9	20	1 252 ± 78	L.S.D. (0,01) 208
Control	10	25	1 795 ± 77	570 ± 42
Zinc-deficient	10	25	1 225 ± 54	L.S.D. (0,01) 107
Control	8	30	1 719 ± 63	569 ± 41
Zinc-deficient	10	30	1 150 ± 58	L.S.D. (0,01) 105

(1) Mean plasma zinc levels of deficient animals were 0,53 ppm (0,61 - 0,45) and control animals 1,01 ppm (1,10 - 0,93).

(2) Individual results in Appendix 24.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 50 µl enzyme assay mixture given in Appendix 24.

In the mitochondrial fraction considerably more enzyme activity was found than that in the nuclear and supernatant fractions (Table 29 and Appendix 25). Activity in the sham-operated animals, in particular, was very much higher than in the other two fractions, reflecting the high rate of DNA synthesis in mitochondria of normal liver tissue. In the zinc-deficient animals a similarly significant decrease ($p < 0,01$) in thymidine kinase activity was found as had been noted in the other fractions (Table 29).

TABLE 29 Effect of zinc deficiency on thymidine kinase activity in the mitochondrial fraction of regenerating rat liver

Zinc status of rats (1)	No. of animals (2)	Time after partial hepatectomy (h)	Thymidine kinase activity (pM thymidine phosphorylated/mg protein/h) (3)	Difference of means (pM thymidine phosphorylated/mg protein/h)
Control	10	0	1 142 ± 93	391 ± 47
Zinc-deficient	9	0	751 ± 45	L.S.D. (0,01) 120
Control	9	10	1 656 ± 175	153 ± 87
Zinc-deficient	9	10	1 503 ± 57	L.S.D. (0,05) 151
Control	9	15	2 605 ± 82	795 ± 49
Zinc-deficient	10	15	1 810 ± 68	L.S.D. (0,01) 125
Control	10	20	2 904 ± 70	907 ± 49
Zinc-deficient	9	20	1 997 ± 83	L.S.D. (0,01) 125
Control	10	25	2 850 ± 50	802 ± 28
Zinc-deficient	10	25	2 048 ± 68	L.S.D. (0,01) 96
Control	8	30	2 871 ± 92	876 ± 61
Zinc-deficient	10	30	1 995 ± 91	L.S.D. (0,01) 157

(1) Mean plasma zinc levels of deficient animals were 0,51 ppm (0,59 - 0,45) and control animals 1,05 ppm (1,12 - 0,97).

(2) Individual results in Appendix 25.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 50 µl enzyme assay mixture given in Appendix 25.

8.3.4 The effect of *in vitro* addition of zinc and other metal ions on thymidine kinase activity in zinc-deficient rats

The addition of zinc to enzyme fractions isolated from zinc-deficient animals 20h after partial hepatectomy resulted in an increase in thymidine kinase activity, equivalent to that in the control fractions, if a period of 40 - 60 hours incubation was allowed before assay (Table 30). That the enzyme was stable at the incubation temperature (0°C) for a period of 60h was shown by the finding of very little change in enzyme activity before and after incubation in control and zinc-deficient fractions to which no metal ions had been added (Table 30). Maximum recovery of activity was found when 0,05 - 0,10 mM zinc was added to the enzyme fractions. Increased concentrations of zinc resulted in less recovery of activity.

TABLE 30 Effect of added zinc on thymidine kinase activity in enzyme fractions from regenerating rat livers of zinc-deficient rats 20h after partial hepatectomy. Means of 6 animals and their S.E.'s

Zinc conc. added (mM)	Incubation time (h)	Thymidine kinase activity (pM thymidine phosphorylated/mg protein/h)		
		Nuclear	Supernatant	Mitochondrial
(a) CONTROL FRACTIONS				
0	0	1 103 ± 51	1 902 ± 56	2 895 ± 82
0	60	1 112 ± 45	1 849 ± 42	2 788 ± 91
(b) ZINC-DEFICIENT FRACTIONS				
0	0	758 ± 44	1 303 ± 61	1 974 ± 85
0	60	754 ± 62	1 268 ± 58	1 898 ± 132
0,05	20	795 ± 85	1 401 ± 79	2 209 ± 75
	40	1 084 ± 73	1 769 ± 73	2 630 ± 69
	60	1 103 ± 58	1 872 ± 88	2 864 ± 122
0,10	20	830 ± 63	1 437 ± 104	2 137 ± 56
	40	1 097 ± 111	1 843 ± 109	2 506 ± 117
	60	1 124 ± 92	1 908 ± 129	2 639 ± 129
0,50	20	756 ± 72	1 209 ± 108	1 994 ± 114
	40	707 ± 65	1 196 ± 129	1 991 ± 106
	60	688 ± 84	1 213 ± 96	1 950 ± 151

Addition of other divalent metal ions (0,10 mM) to the zinc-deficient enzyme fractions for a period of 60h prior to assay had very little effect on thymidine kinase activity (Table 31).

TABLE 31 Effect of addition of divalent metal ions on thymidine kinase activity in enzyme fractions from regenerating rat livers of zinc-deficient rats 20h after partial hepatectomy. Means of 6 animals and their S.E.'s

Metal added (0,1 mM)	Incubation time (h)	Thymidine kinase activity (pM thymidine phosphorylated/mg protein/h)		
		Nuclear	Supernatant	Mitochondrial
Control fraction	60	1 112 ± 45	1 849 ± 42	2 788 ± 91
-	60	754 ± 62	1 268 ± 58	1 898 ± 132
Mg^{2+}	60	820 ± 77	1 506 ± 109	2 101 ± 89
Mn^{2+}	60	781 ± 79	1 439 ± 83	1 986 ± 108
Ca^{2+}	60	755 ± 84	1 337 ± 91	2 008 ± 74
Cu^{2+}	60	810 ± 62	1 307 ± 101	2 079 ± 112
Fe^{2+}	60	798 ± 81	1 408 ± 74	1 998 ± 75

8.3.5 The effect of zinc intake on thymidine kinase activity in DAB-1 hepatomas

Thymidine kinase activity in DAB-1 hepatomas 3 weeks after implantation was found to be considerably lower ($p < 0,01$) in the zinc-deficient and zinc-supplemented ($\geq$ 500 ppm) animals when compared with the control group (Table 32 and Appendix 26). In all cases the enzyme activity was greater than that in normal liver tissue indicating a higher rate of cell division.

TABLE 32 Effect of zinc status on thymidine kinase activity in DAB-1 hepatomas. Means of 10 animals and their S.E.'s

Tissue	Zinc conc. in diet (ppm)	(1)(2) Thymidine kinase activity (pM thymidine phosphorylated/mg protein/h)	Difference of means (pM thymidine phosphorylated/ mg protein/h)
Normal adult liver	60	371 ± 34	
Hepatoma	60	2 005 ± 55	679 ± 31
Hepatoma	0,5	1 326 ± 41	L.S.D. (0,01) 78
Hepatoma	60	2 005 ± 55	650 ± 36
Hepatoma	500	1 355 ± 58	L.S.D. (0,01) 91
Hepatoma	60	2 005 ± 55	755 ± 62
Hepatoma	1 000	1 250 ± 128	L.S.D. (0,01) 157

(1) Individual results in Appendix 26.

(2) Total protein in 1,0 ml enzyme fraction and cpm per 50 µl enzyme assay mixture given in Appendix 26.

8.3.6 The effect of zinc deficiency on DNA polymerase activity in various enzyme fractions from regenerating rat liver

DNA polymerase activity did not increase significantly in the nuclear fraction of rat livers after partial hepatectomy (Table 33 and Appendix 27). However, the activity in the zinc-deficient group was significantly lower ($p < 0,01$) than that in the control group at all times after partial hepatectomy.

TABLE 33 Effect of zinc deficiency on DNA polymerase activity in the nuclear fraction of regenerating rat liver

Zinc status of rats (1)	No. of animals (2)	Time after partial hepatectomy (h)	DNA polymerase activity (nM ^{3}H-d TTP incorporated/ mg protein/h) (3)	Difference of means (nM ^{3}H-d TTP incorporated/ mg protein/h)
Control	9	0	1,22 ± 0,10	0,20 ± 0,07
Zinc-deficient	8	0	1,02 ± 0,10	L.S.D. (0,01) 0,1
Control	8	10	1,19 ± 0,09	0,20 ± 0,07
Zinc-deficient	8	10	0,99 ± 0,10	L.S.D. (0,01) 0,1
Control	9	15	1,26 ± 0,09	0,23 ± 0,06
Zinc-deficient	9	15	1,03 ± 0,08	L.S.D. (0,01) 0,1
Control	10	20	1,43 ± 0,08	0,31 ± 0,06
Zinc-deficient	9	20	1,12 ± 0,09	L.S.D. (0,01) 0,1
Control	8	25	1,51 ± 0,20	0,36 ± 0,11
Zinc-deficient	10	25	1,15 ± 0,10	L.S.D. (0,01) 0,2
Control	9	30	1,55 ± 0,18	0,33 ± 0,09
Zinc-deficient	8	30	1,22 ± 0,08	L.S.D. (0,01) 0,2

(1) Mean plasma zinc levels of deficient animals were 0,49 ppm (0,55 - 0,43) and control animals 1,00 ppm (1,08 - 0,93).

(2) Individual results in Appendix 27.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 0,5 ml DNA solution given in Appendix 27.

In the supernatant fraction DNA polymerase activity increased markedly between 10 and 30h after partial hepatectomy in both the control and zinc-deficient groups but the activity in the deficient animals was significantly lower ($p < 0,01$) than that in the control animals between 15 and 30h after partial hepatectomy (Table 34 and Appendix 28).

TABLE 34 Effect of zinc deficiency on DNA polymerase activity in the supernatant fraction of regenerating rat liver

Zinc status[1] of rats	No.[2] of animals	Time after partial hepatectomy (h)	DNA polymerase activity[3] (nM ^{3}H-d TTP incorporated/ mg protein/h)	Difference of means (nM ^{3}H-d TTP incorporated/ mg protein/h)
Control	9	0	0,62 ± 0,06	0,07 ± 0,04
Zinc-deficient	8	0	0,55 ± 0,07	L.S.D. (0,05) 0,07
Control	8	10	0,67 ± 0,07	0,08 ± 0,04
Zinc-deficient	8	10	0,59 ± 0,05	L.S.D. (0,05) 0,07
Control	9	15	1,03 ± 0,07	0,18 ± 0,05
Zinc-deficient	9	15	0,85 ± 0,08	L.S.D. (0,01) 0,13
Control	10	20	1,89 ± 0,07	0,59 ± 0,05
Zinc-deficient	9	20	1,30 ± 0,08	L.S.D. (0,01) 0,13
Control	8	25	2,50 ± 0,13	0,71 ± 0,07
Zinc-deficient	10	25	1,79 ± 0,08	L.S.D. (0,01) 0,18
Control	9	30	3,04 ± 0,12	0,90 ± 0,07
Zinc-deficient	8	30	2,14 ± 0,09	L.S.D. (0,01) 0,18

(1) Mean plasma zinc levels of deficient animals were 0,54 ppm (0,62 - 0,47) and control animals 1,01 ppm (1,10 - 0,93).

(2) Individual results in Appendix 28.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 0,5 ml DNA solution given in Appendix 28.

Mitochondrial DNA polymerase activity only increased two-fold in regenerating rat livers 25 - 30h after partial hepatectomy and as was the case in the nuclear and supernatant fractions the zinc-deficient animals had a significantly lower ($p < 0,01$) activity than the control animals (Table 35 and Appendix 29).

TABLE 35 Effect of zinc deficiency on DNA polymerase activity in the mitochondrial fraction of regenerating rat liver

Zinc status of rats (1)	No. of animals (2)	Time after partial hepatectomy (h)	DNA polymerase activity (3) (nM ^{3}H-d TTP incorporated/ mg protein/h (x 10^2)	Difference of means (nM ^{3}H-d TTP incorporated/mg protein/h (x 10^2)
Control	9	0	8,1 ± 0,5	2,0 ± 0,4
Zinc-deficient	8	0	6,1 ± 0,8	L.S.D. (0,01) 1,0
Control	8	10	8,8 ± 0,6	2,5 ± 0,4
Zinc-deficient	8	10	6,3 ± 0,6	L.S.D. (0,01) 1,1
Control	9	15	11,0 ± 0,7	2,9 ± 0,4
Zinc-deficient	9	15	8,1 ± 0,6	L.S.D. (0,01) 1,0
Control	10	20	13,2 ± 0,6	4,8 ± 0,4
Zinc-deficient	9	20	8,4 ± 0,6	L.S.D. (0,01) 1,0
Control	8	25	15,2 ± 1,8	5,1 ± 0,9
Zinc-deficient	10	25	10,1 ± 0,7	L.S.D. (0,01) 2,3
Control	9	30	18,3 ± 2,0	6,4 ± 1,0
Zinc-deficient	8	30	11,9 ± 0,8	L.S.D. (0,01) 2,6

(1) Mean plasma zinc levels of deficient animals were 0,48 ppm (0,55 - 0,43) and control animals 0,99 ppm (1,05 - 0,92).

(2) Individual results in Appendix 29.

(3) Total protein in 1,0 ml enzyme fraction and cpm per 0,5 ml DNA solution given in Appendix 29.

8.3.7 The effect of *in vitro* addition of zinc and other metal ions on DNA polymerase activity in zinc-deficient rats

The addition of 0,1 mM zinc to enzyme fractions isolated from zinc-deficient animals 20h after partial hepatectomy resulted in an increase in DNA polymerase activity, equivalent to that in the control fractions, after an incubation period of 60h prior to assay (Table 36). Addition of zinc for shorter periods and also at higher concentrations resulted in less pronounced restoration of enzyme activity (Table 36). As was the case with thymidine

kinase the DNA polymerase enzyme was found to be stable for a period of 60h at the incubation temperature of 0°C since there was little change in enzyme activity before and after incubation of the control fractions (Table 36).

TABLE 36 Effect of added zinc on DNA polymerase activity in enzyme fractions from regenerating rat livers of zinc-deficient rats 20h after partial hepatectomy. Means of 7 animals and their S.E.'s

Zinc conc. added (mM)	Incubation time (h)	DNA polymerase activity (nM ^{3}H-d TTP incorporated/mg protein/h)		
		Nuclear	Supernatant	Mitochondrial (x 10^2)
(a) CONTROL FRACTIONS				
0	0	1,39 + 0,09	1,92 + 0,07	13,5 + 1,2
0	60	1,38 + 0,08	1,94 + 0,09	13,1 + 0,9
(b) ZINC-DEFICIENT FRACTIONS				
0	0	1,10 ± 0,08	1,33 ± 0,12	8,5 ± 0,6
0	60	1,08 ± 0,09	1,35 ± 0,13	8,3 ± 0,7
0,05	20	1,17 ± 0,08	1,40 ± 0,07	8,8 ± 0,9
	40	1,25 ± 0,07	1,75 ± 0,08	10,9 ± 0,6
	60	1,30 ± 0,08	1,85 ± 0,08	11,4 ± 0,8
0,10	20	1,19 ± 0,08	1,44 ± 0,08	8,8 ± 0,7
	40	1,26 ± 0,07	1,82 ± 0,06	12,0 ± 0,5
	60	1,33 ± 0,07	1,90 ± 0,06	12,9 ± 0,8
0,50	20	1,20 ± 0,09	1,39 ± 0,07	8,6 ± 0,7
	40	1,22 ± 0,09	1,70 ± 0,07	10,1 ± 0,7
	60	1,28 ± 0,05	1,82 ± 0,08	10,8 ± 0,7

Addition of Mn^{2+} and Mg^{2+} (0,10 mM) to the enzyme fractions for a period of 60h prior to assay resulted in ± 30% recovery of the control activity (Table 37) but other metal ions tested had no effect.

TABLE 37 Effect of addition of divalent metal ions on DNA polymerase activity in enzyme fractions from regenerating rat livers of zinc-deficient animals 20h after partial hepatectomy. Means of 6 animals and their S.E.'s

Metal added (0,1 mM)	Incubation time (h)	DNA polymerase activity (nM ^{3}H-d TTP incorporated/mg protein/h)		
		Nuclear	Supernatant	Mitochondrial (x 10^2)
Control fraction	60	1,38 ± 0,09	1,94 ± 0,09	13,1 ± 0,9
-	60	1,08 ± 0,09	1,35 ± 0,13	8,3 ± 0,7
Mg^{2+}	60	1,21 ± 0,08	1,47 ± 0,07	9,7 ± 0,7
Mn^{2+}	60	1,23 ± 0,07	1,50 ± 0,01	9,9 ± 0,7
Ca^{2+}	60	1,13 ± 0,09	1,34 ± 0,08	8,4 ± 0,7
Cu^{2+}	60	1,10 ± 0,08	1,32 ± 0,09	8,5 ± 0,8
Fe^{2+}	60	1,15 ± 0,09	1,40 ± 0,10	8,7 ± 0,7

8.3.8 The effect of zinc intake on DNA polymerase activity in DAB-1 hepatomas

The activity of DNA polymerase in DAB-1 hepatomas 3 weeks after implantation was found to be considerably lower ($p < 0,01$) in the zinc-deficient (0,05 ppm) and zinc-supplemented ($\geq$ 500 ppm) animals when compared with the control group (Table 38 and Appendix 30). In addition, enzyme activity was found to be 4-fold higher in the hepatomas than in normal rat liver.

TABLE 38 Effect of zinc status on DNA polymerase activity in DAB-1 hepatomas. Means of 10 animals and their S.E.'s

Tissue	Zinc conc. in diet (ppm)	(1)(2) DNA polymerase activity (nM ^{3}H-d TTP incorporated/ mg protein/h)	Difference of means (nM ^{3}H-d TTP incorporated/ mg protein/h)
Normal adult liver	60	0,79 ± 0,07	
Hepatoma	60	2,97 ± 0,07	0,73 ± 0,04
Hepatoma	0,5	2,24 ± 0,07	L.S.D. (0,01) 0,11
Hepatoma	60	2,97 ± 0,07	0,67 ± 0,04
Hepatoma	500	2,30 ± 0,07	L.S.D. (0,01) 0,11
Hepatoma	60	2,97 ± 0,07	0,72 ± 0,04
Hepatoma	1 000	2,25 ± 0,07	L.S.D. (0,01) 0,11

(1) Individual results in Appendix 30.

(2) Total protein in 1,0 ml enzyme fraction and cpm per 0,5 ml DNA solution given in Appendix 30.

8.4 Discussion

The significant decrease in the activity of thymidine kinase following zinc restriction in regenerating rat livers and rat hepatomas observed in this study confirmed and extended the findings of Prasad and Oberleas (51) usin connective tissue as a model system. The results indicate a definite requirement for zinc by thymidine kinase.

A closer examination of thymidine kinase activity in various sub-cellular fractions revealed greatest activity in the post-mitochondrial supernatant whic is in agreement with the findings of a number of other workers (166, 167, 194, 195). The reason for the high level of activity in this fraction was not quite clear although Bollum and Potter (166) have suggested that the enzyme system wa of nuclear origin during DNA synthesis but that it was liberated into the cytoplasm when the nuclear membrane disintegrated during mitosis. However, this does not explain the higher enzyme activity in the supernatant in the present

study as this occurred before mitosis. The higher thymidine kinase activity in the mitochondrial fraction confirmed the findings of Stirpe and La Placa (195) also using regenerating rat liver and enhanced evidence for the independence of the DNA synthetic process in these organelles.

Restoration of thymidine kinase activity in zinc-deficient enzyme fractions following the addition of zinc indicated that reduced thymidine kinase activity was due to a direct effect of zinc on the enzyme rather than an effect on overall protein biosynthesis. The fact that the addition of other divalent metal ions to the zinc-deficient fractions had very little effect on enzyme activity showed that the enzyme was specifically zinc-dependent and not just metal ion-dependent. In addition, the finding that maximum increase in activity was only obtained after 60 hours incubation with zinc indicated that zinc was an integral part of the enzyme and not a readily dissociable metal cofactor. However, the results did show that addition of $>$ 0,1 mM zinc had an inhibitory effect on enzyme activity.

The activity of DNA polymerase in regenerating livers and grafted tumours showed a similar reduction in zinc-deficient tissue to that observed with thymidine kinase. Activity of this enzyme could also be restored to control levels by the addition of 0,1 mM zinc to the fractions, but not by other metal ions, which is in agreement with the results of Springgate, Mildvan, Abramson, Engle and Loeb (170) using a 'zinc-free' DNA polymerase apoenzyme.

However, the results from the present study revealed a DNA polymerase fraction from the nuclei of regenerating rat livers which did not increase substantially in activity after partial hepatectomy. This confirmed the results of Chang and Bollum (186) and Fausto and Van Lancker (194) and is of interest because of the recent findings of Lynch and Lieberman (185) who have shown that rat liver nuclei contain at least 2 DNA polymerases, only one of which increased in activity after partial hepatectomy. It is thus probable that the lack of increase in activity in nuclear fractions after partial hepatectomy, found in this and other studies (185, 186, 194) was due to a predominance of a low

molecular weight DNA polymerase which has a similar role in resting and proliferating cells, and consequently, is unlikely to be the replicative enzyme.

The increase in DNA polymerase activity in mitochondrial fractions after partial hepatectomy, observed in this study, was similar to that found in other laboratories (191, 194) where the activity has been identified as distinct from any other DNA polymerase activity in the cell. In addition, the enzyme itself has been demonstrated to be genetically distinct from the nuclear enzyme (58). Nevertheless, the exact role of this mitochondrial DNA polymerase during DNA synthesis in these organelles is not clear (191, 194).

A several-fold increase in DNA polymerase activity was found after partial hepatectomy in the post-mitochondrial supernatant fraction which is in agreement with other reports (186, 191, 194), but there is much doubt as to the origin of this enzyme activity (184, 186, 191, 196). It has been suggested (186) that a portion of the DNA polymerase activity in mammalian cells may reside in the cytoplasm but it has been speculated that this activity may be of nuclear origin As a result, there is considerable doubt concerning the replicative role of the cytoplasmic enzyme especially since it reaches its peak of activity after the peak of DNA synthesis has been passed (194).

Due to the doubt surrounding the role of DNA polymerase enzymes in DNA replication it is difficult to draw any firm conclusions concerning a site of action of zinc associated with these enzymes despite their zinc-dependence. However, the role of thymidine kinase in DNA replication is clearly established and its importance as a rate-controlling enzyme suggests it would be a favourable site of action for factors which effect the overall rate of DNA synthesis. Thus the decreased activity of thymidine kinase in zinc-deficient tissues observed in this study suggested that it is probably the primary site of action of zinc in DNA synthesis.

It is possible that a number of other enzymes involved in this pathway are also affected by zinc deficiency and it may be that it is the combined effect on these enzymes which results in inhibited DNA synthesis and cell division.

However, no such enzymes have yet been identified as being zinc dependent and present indications are of a primary effect on thymidine kinase not only in nuclear DNA synthesis in regenerating rat livers but also in mitochondrial DNA replication and DNA synthesis in rat hepatomas.

CHAPTER 9

DISCUSSION AND CONCLUSIONS

The serious effect of prolonged zinc impoverishment in animals has been recognised for many years but it is only recently, due to the severity and rapid onset of a number of deficiency symptoms in tissues undergoing rapid cell proliferation (e.g. oesophagus, foetus, testis and liver), that workers have investigated the primary locus of action of zinc in growing tissue. The importance of these studies was accentuated by the recent demonstration of a nutritional zinc problem in children in the Middle East (7), characterised by stunted growth and hypogonadism, as well as by the recent statements by a number of international nutritionalists concerning the question of a widespread marginal zinc deficiency in humans.

Arising from the studies of the effects of zinc deficiency on foetal development a number of investigators (27, 29 - 31, 33, 36, 68) became interested in the possible role of zinc in DNA, RNA and protein metabolism, since these events are directly associated with cell division. However, most of the earlier studies were performed on tissues which were unsatisfactory as models for the study of tissue undergoing rapid cellular growth (31, 66) and many of the methods used for inducing zinc deficiency were not entirely satisfactory (29, 30). Methods such as EDTA chelation and feeding of deficient diets for as long as 100 days have met with considerable criticism (78) because of secondary effects such as chelation of other metal ions and inanition due to prolonged periods of dietary zinc depletion. Furthermore, the common practice (27, 31) of supplementing zinc in the deficient animals by single daily intraperitoneal injections cannot be considered to reflect a normal dietary intake.

With this in mind the present study was designed to provide a means of effecting a reproducible level of zinc deficiency without the introduction of complicating side effects. The diet, prepared from EDTA-extracted soyabean

meal (24), contained less than 0,5 ppm of zinc, and after only 3 days on this diet plasma zinc levels fell by 40 - 50% when compared with the control animals. At this stage no effects of inanition, etc. were evident, thereby enabling a study to be made of certain biochemical systems at clearly defined and uncomplicated levels of zinc deficiency in the body.

Due to the uncertainty surrounding the effect of zinc deficiency on DNA and protein metabolism the problem was studied in a number of model systems which were actively undergoing cell division either *in vivo* or *in vitro*. Foetal tissue, although suitable for this study, was not used because of the difficulty in obtaining sufficient material at the stage of morphogenesis. Instead attention was paid to regenerating rat liver, proliferating lymphocytes and transplantable tumour tissue since all these systems were characterised by a high rate of DNA synthesis and cell division.

In the case of the first system studied - regenerating rat liver - RNA and protein synthesis increase within a few hours after partial hepatectomy and a rapid increase in DNA synthesis follows shortly thereafter (197). In the present study DNA synthesis was found to fall sharply in zinc-deficient rats between 15 and 30 hours after partial hepatectomy while the period of maximum synthesis shifted from 17½ hours post-operatively in the control animals to 25 hours in the deficient group. Protein synthesis became affected by zinc depletion 48 hours after partial hepatectomy. Consequently, the rapidity of the effect on DNA synthesis suggested that this may represent the primary lesion associated with zinc deficiency in rats. Furthermore, the observed lag in DNA synthesis (S-phase) in the deficient animals could have a significant bearing on the interpretation of data in this field where comparisons have been made at the same times post-operatively. Lieberman *et al.* (67) have indicated that in most mammalian cells the length of the G_1-phase of cell division is variable, but the S-phase is constant. It may be then that zinc in fact alters the pattern of cell division which results in an extension of the normal S-phase.

The second model system used in these studies was an *in vitro* culture of PHA-stimulated rat blood lymphocytes. The system offered an opportunity of controlling conditions and examining effects not possible using *in vivo* methods. In addition, by using a medium incorporating plasma obtained from zinc-deficient rats it was possible to study the *in vitro* effects of zinc deficiency for the first time without the addition of chelating agents to remove zinc. The results clearly demonstrated the essentiality of zinc ions for DNA and protein synthesis in these cells although other ions were found to be of lesser importance. The present study does, in fact, represent the first comprehensive investigation into the effects of zinc and protein synthesis *in vitro*.

Due to conflicting reports (44, 119) of the effects of zinc levels on cance growth, it was decided to use this tissue as another model system for the study of the effect of zinc status on cell division. The results showed that low levels of zinc (0,5 ppm) exerted an inhibitory action on both tumour growth and DNA synthesis in the transplanted hepatomas.

Evidence from the present study on three rapidly proliferating tissues clearly pointed to an effect of zinc on the processes of cell division due to a role of zinc in DNA synthesis. However, the exact nature of this involvement was unclear, although a number of enzymes involved in DNA synthesis have been shown to be zinc-dependent (50, 51, 170) and it was the view of the author that the effects of zinc on cell division were due to an involvement of zinc at an enzymic level. Such a situation would explain the reduction in DNA synthesis observed in regenerating rat livers from zinc-deficient animals. Also, since the enzymes associated with DNA synthesis are produced during late G_1-phase (13 and DNA replication occurs in the S-phase, a reduction in activity of these enzymes during G_1-phase would probably result not only in reduced DNA synthesis but also the observed delay in reaching maximum synthesis in zinc-deficient tissues.

A study was accordingly made of the possible involvement of zinc in the pathways associated with DNA synthesis in both regenerating liver and grafted

tumours. An investigation was initially carried out on the nuclear and mitochondrial systems, which differed in their mechanisms of DNA replication in possessing a number of genetically distinct enzymes (57, 58). The study revealed that both systems were similarly affected by zinc deficiency and suggested a common role for zinc in both sub-cellular organelles which was not affected by their complement of genetically distinct enzymes.

Closer examination of the pathways involved in DNA synthesis in rapidly dividing cells revealed that the dominant pathway for the synthesis of nucleoside triphosphates was the thymidine kinase salvage pathway in contrast to the preferred de novo pathway for synthesis of these compounds in resting cells (61, 62). As a result, a possible influence of zinc on the levels of deoxyribonucleoside precursors required in the thymidine kinase pathway in regenerating rat liver was investigated by addition of unlabelled deoxyribonucleosides to control and zinc-deficient animals. The results revealed that the effect of zinc deficiency on DNA synthesis was similar whether deoxyribonucleosides were added or not, indicating a locus of action of zinc either at the stage of phosphorylation of the nucleosides or at the stage of polymerization of the phosphorylated derivatives. Since it was not possible to investigate the influence of zinc levels on the supply of precursors required for the phosphorylation and polymerization phases of DNA synthesis due to a dephosphorylation of these precursors before entry into the cells (60), the next step in the investigation was to examine the effect of dietary zinc intakes on the activity of the individual enzymes in the pathway.

Thymidine kinase has been shown to possess all the attributes of a rate-determining enzyme in DNA synthesis in proliferating tissues (65, 177) and, in addition, has recently been shown to be zinc dependent (51) making it a very likely site of action for zinc. Results from the present study showed a signif cant reduction in thymidine kinase activity in regenerating rat livers and transplanted hepatomas from zinc-deficient animals when compared with the contr

animals. In addition, the observed restoration of thymidine kinase activity in zinc-deficient enzyme fractions by the addition of zinc indicated a direct effec of zinc on the enzyme itself rather than an effect on the synthesis of the enzym Similar results were found in a study of the enzymes involved in polymerization of phosphorylated precursors, i.e. the DNA polymerase enzymes. However, interpretation of the results from the DNA polymerase study was complicated by uncertainty surrounding the exact role of the various polymerases in DNA replication (63, 187). The present evidence therefore indicated an active site for zinc associated with thymidine kinase.

An important consideration in determining the prime focus of action of zinc in cell division is the timing of the various effects associated with zinc deficiency. Figure 4 indicates an effect of zinc deficiency on thymidine kinase activity immediately after partial hepatectomy whereas DNA synthesis was affecte a few hours later, DNA polymerase activity after 15 hours and protein synthesis 20 - 48 hours post-operatively. These observations pointed to a prime focus of action of zinc on thymidine kinase in zinc-deficient tissues.

An interesting finding in the present study was the inhibition of DNA and protein synthesis in proliferating lymphocytes by addition of high concentration of zinc to the cultures, and a similar inhibition of DNA synthesis, together wit reduced tumour growth, in transplanted hepatomas from rats fed zinc-supplemented ($\geq$ 500 ppm) diets. Thymidine kinase was again indicated as the possible prime site of action of zinc associated with the effects of zinc supplementation since enzyme activity was reduced on addition of $>$ 0,1 mM zinc to the enzyme fractions However, the possibility cannot be excluded that the reduction in enzyme activit as a result of high concentrations of zinc was due to a 'poisoning' of the enzym by the metal and thus the effect found may not be the same as that observed in zinc-supplemented lymphocyte cultures and tumour tissue.

It appears then that restricted DNA synthesis represents the primary lesion associated with nutritional zinc deficiency in rats and that in actively dividin tissues this effect is mediated through a site of action of zinc associated with

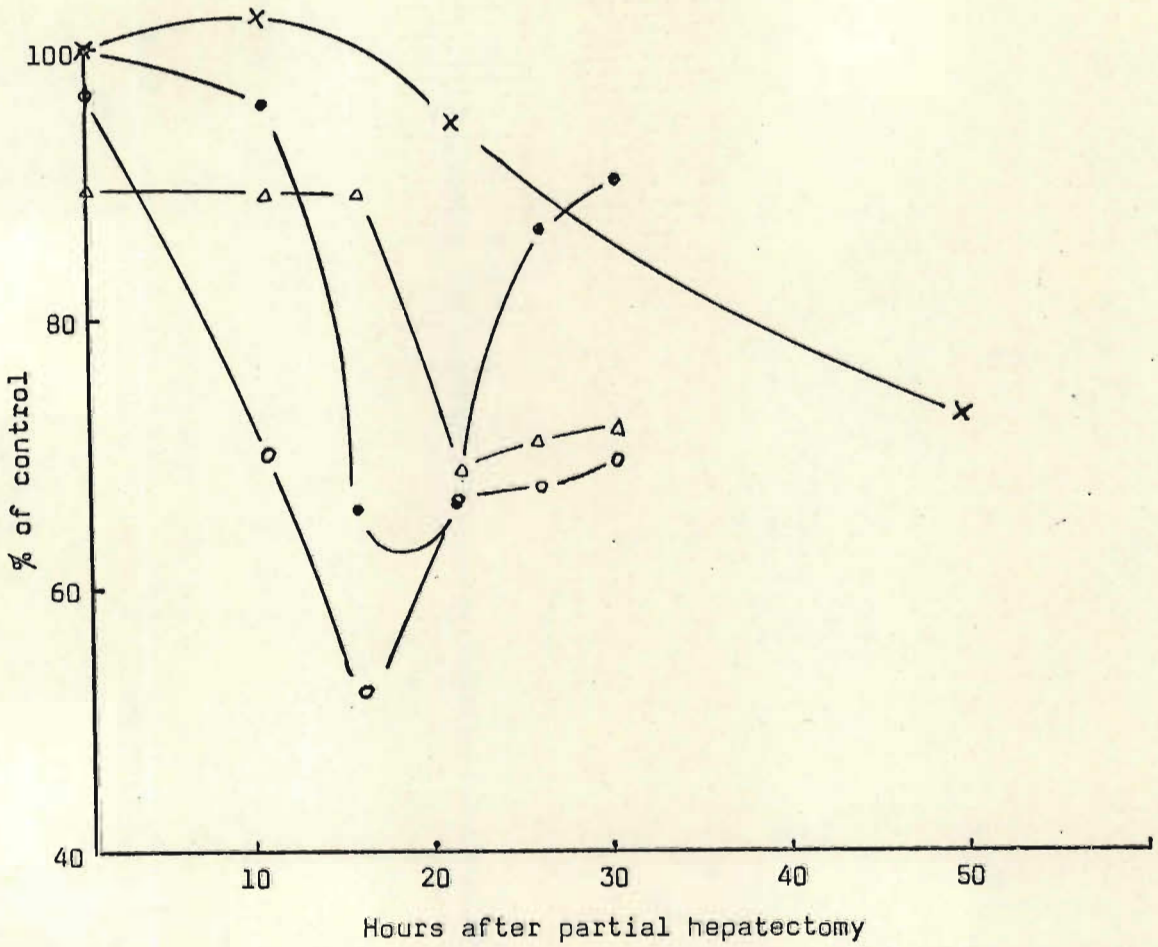

FIGURE 4 Effect of zinc deficiency on different processes of cell division at various times after partial hepatectomy.
•——• DNA synthesis; X——X protein synthesis;
o——o thymidine kinase activity;
Δ——Δ DNA polymerase activity

thymidine kinase. The effect would account for the rapid cessation of growth in zinc-deficient animals as well as the rapid teratogenicity associated with material zinc depletion in rats. In addition, reduced DNA synthesis could also account for certain other zinc deficiency symptoms such as parakeratosis, testicular atrophy and oesophageal lesions, as well as the reduced tumour growth observed in the present investigation. A similar site of action associated with zinc supplementation, indicated by the observed decrease in thymidine kinase activity and DNA synthesis by addition of high levels of zinc, is an area of investigation which requires further examination especially due to the importance of these effects as the possible prime lesions associated with reduced tumour

growth as a result of high dietary intakes of zinc.

Finally, it must be borne in mind that although all evidence points to a locus of action of zinc associated with thymidine kinase it is possible that the effect of zinc levels on DNA synthesis are the result of a combined effect of zinc on a number of enzymes involved in the pathway, some of which may yet be identified as zinc metalloenzymes.

SUMMARY

Because of the established requirement for zinc during tissue growth a study was made to define the primary locus of action of zinc during cell divisio in three actively proliferating rat tissues, viz. regenerating liver, PHA-stimulated lymphocytes and grafted hepatomas.

Initially the effect of a short-term (3-day) dietary zinc deficiency on DNA and protein synthesis in regenerating rat liver was investigated. The findings indicated a significantly reduced ($p < 0,01$) incorporation of ^{3}H-thymidine into the DNA of animals receiving the zinc-deficient rations (0,4 ppm) 10 - 30 hours after partial hepatectomy, when compared with the control animals which received 60 ppm of zinc in their diet. In addition a shift occurred in the timing of the peak of maximum ^{3}H-thymidine incorporation from 17½ hours post-operatively in the control animals to 25 hours post-operatively in the deficient animals. Thus when comparisons were made between incorporation data at the respective peaks of maximum DNA synthesis, the effect of zinc deficiency was considerably reduced ($p < 0,01$) but not eliminated when compared with the data obtained at the same time post-operatively in both groups. Incorporation of ^{14}C-leucine into protein was not affected by zinc deficiency until 48 hours post-operatively whereafter the incorporation was significantly reduced in the deficient animals when compared with the control group. Both the total free leucine and the percentage of ^{14}C-leucine in the regenerating livers varied with time after surgery but did not differ significantly between zinc-deficient and control rats, which suggested that the reduction in protein synthesis in the deficient rats was not due to a shortage of available precursor amino acids. The fact that DNA synthesis was affected before protein synthesis suggested it represented the primary lesion associated with zinc deficiency in rats.

Incorporation of ^{3}H-thymidine into DNA and ^{14}C-leucine into protein was decreased by 90 - 95% in PHA-transformed rat lymphocytes cultured in media to which had been added 1 mM of EDTA. The inhibition was effectively reversed by

the addition of zinc which restored respectively 82% and 64% of the original activities of DNA and protein synthesis. Other divalent ions also reversed the inhibition, especially with regard to protein synthesis, but their effectiveness was considerably less than that of zinc. Concentrations of zinc in excess of 0,5 mM were found to inhibit both DNA and protein synthesis. Incorporation of ^{3}H-thymidine into DNA and ^{14}C-leucine into protein was decreased respectively by 34% and 26,5% in lymphocytes cultured in zinc-deficient (0,35 µg/ml) medium. The inhibition was completely reversed by the addition of zinc to the medium in the range of 2 - 3 µg of zinc/ml. Concentrations of zinc in excess of 5 µg/ml decreased incorporation in both cases. These findings confirm and extend earlie work on EDTA-treated lymphocytes and suggest an important role for zinc in nucleic acid and protein metabolism in these cells.

The growth of a transplanted hepatoma induced by 3'-methyl-4-dimethylamino-azobenzene was significantly reduced ($p < 0,01$) in rats maintained on diets low (0,5 ppm) and high ($\geq$ 500 ppm) in zinc when compared with control animals given 60 ppm of zinc. The viability of the transplanted tumour was not affected by the zinc intake of the animals at tissue implantation. DNA synthesis in these transplanted hepatomas was also reduced ($p < 0,01$) in rats maintained on diets containing low (0,5 ppm) and high ($\geq$ 500 ppm) zinc concentrations and this possi bly represented the primary lesion associated with zinc's effects in these cells Methylcholanthrene-induced carcinogenesis was markedly reduced in mice receiving the same low and high zinc diets during the induction periods, indicating a similar effect of zinc on the processes of carcinogenesis and tumour growth.

In an attempt to elucidate the role of zinc in DNA synthesis, differences were initially sought between the effects of zinc deficiency on nuclear and mitochondrial DNA synthesis in regenerating rat liver. However, zinc deficiency was found to have a similar inhibitory effect on DNA synthesis in both these sub-cellular organelles. Thereafter, since the major pathway of DNA synthesis in rapidly dividing cells is via the thymidine kinase salvage pathway, unlabell

deoxyribonucleosides were injected into partially hepatectomised animals receiving different dietary zinc intakes. Addition of the deoxyribonucleosides either singly or in combination had no significant effect on the inhibition of DNA synthesis caused by zinc deficiency, indicating that zinc was acting at some point later in the pathway.

The effect of zinc levels on thymidine kinase and DNA polymerase, two known zinc-dependent enzymes, was investigated. The activity of both these enzymes was markedly reduced in regenerating livers and transplanted hepatomas from zinc deficient animals when compared with control groups. Control enzyme activity was recovered by addition of 0,1 mM zinc to enzyme fractions from zinc-deficient animals, if an incubation period of 60 hours was allowed before assay. Addition of other metal ions had very little effect on recovery of the control activity. Zinc supplementation ($\geq$ 500 ppm) was also found to reduce both thymidine kinase and DNA polymerase activity in transplanted hepatomas as was the addition of $>$ 0,1 mM zinc to the enzyme fractions from regenerating rat livers of zinc-deficient animals.

Due to the doubt concerning the role of the various DNA polymerase enzymes in DNA synthesis, the results indicated thymidine kinase to be the probable prime locus of action of zinc in DNA synthesis and consequently in cell division since restricted DNA synthesis appeared to be the prime lesion associated with nutritional zinc deficiency in rapidly dividing cells. This effect would account for the rapid teratogenicity, reduced growth, oesophageal lesions and other syndromes associated with zinc depletion in rats.

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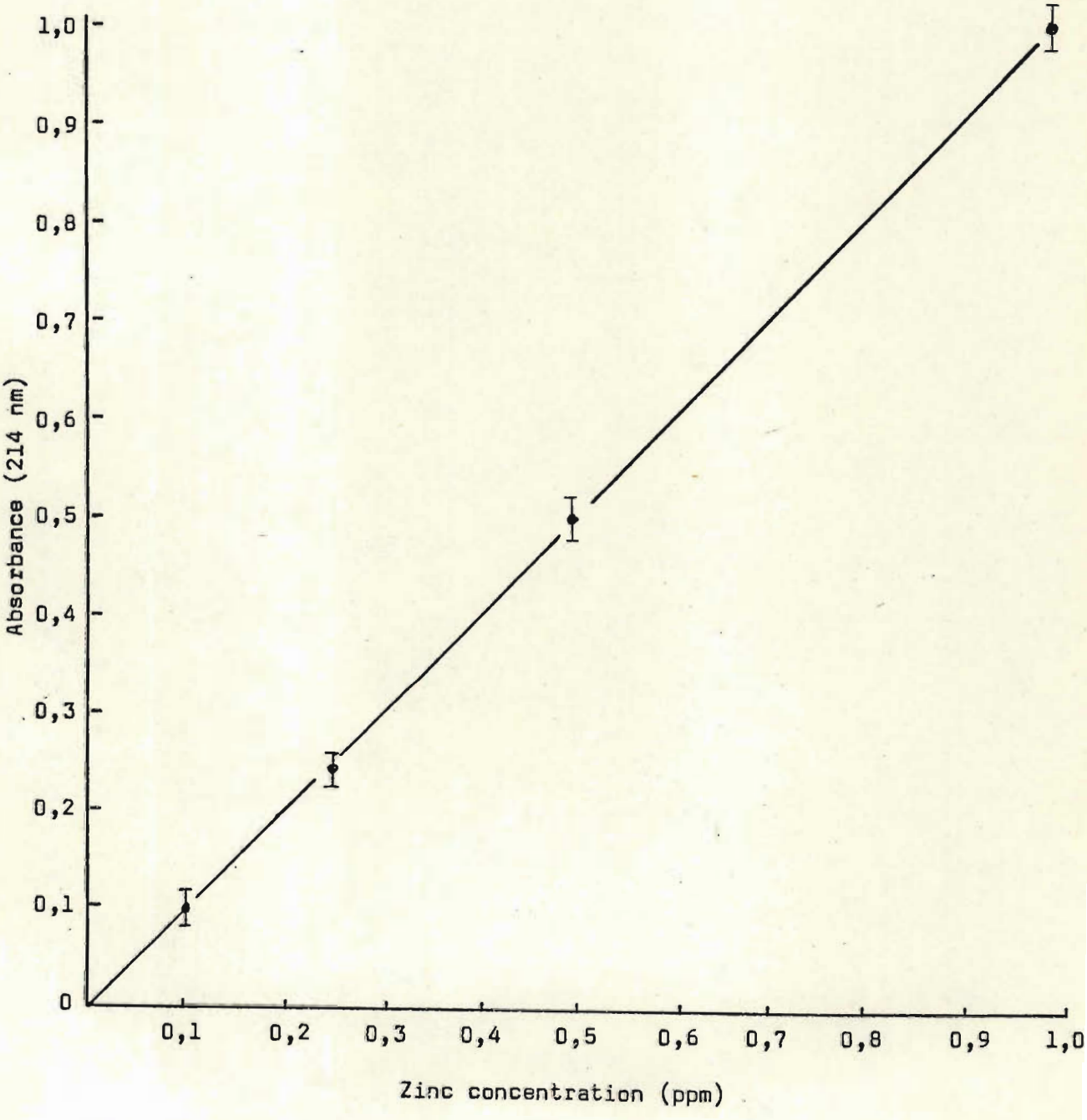
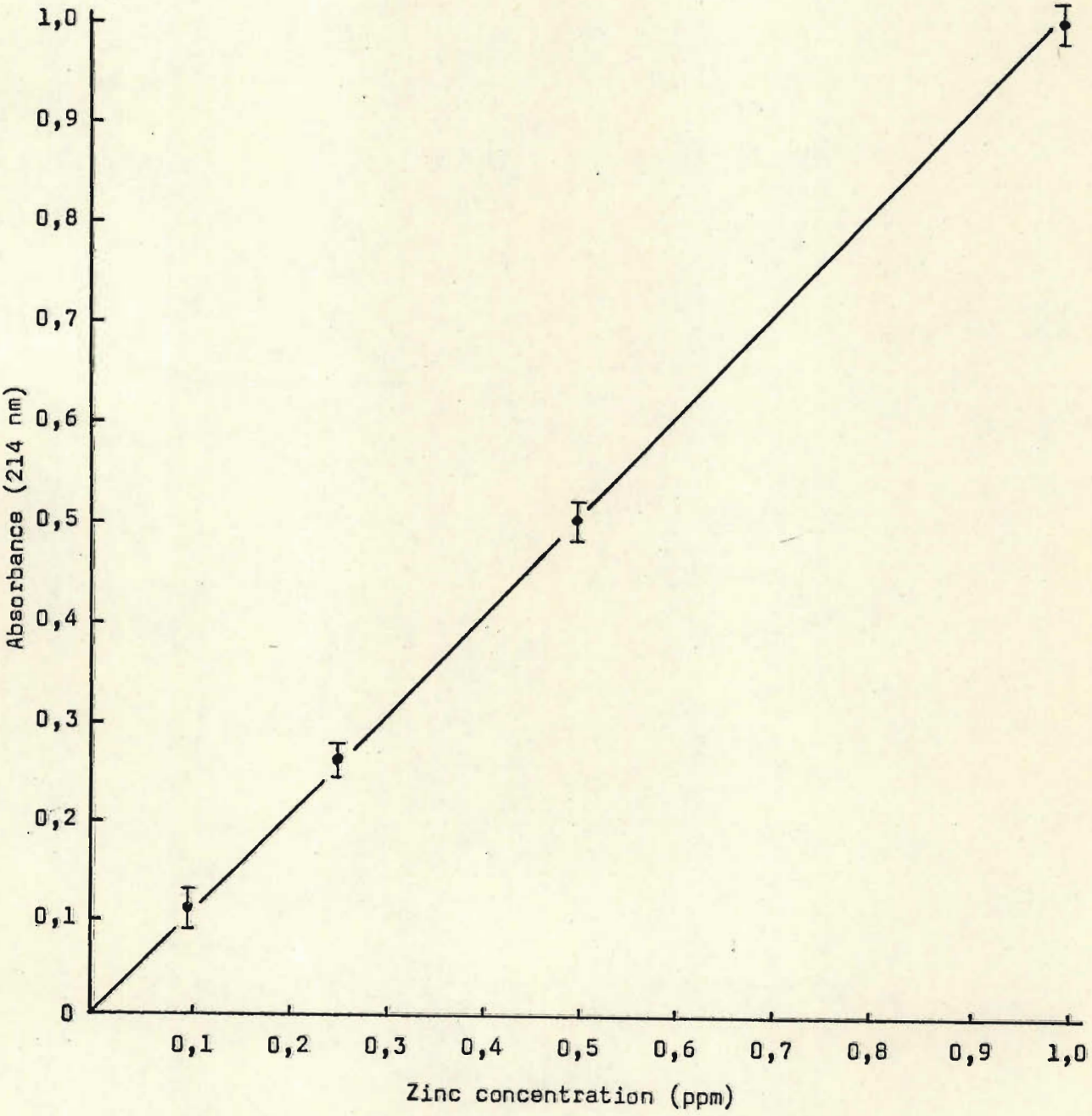
APPENDIX 1

STANDARD CURVE FOR THE DETERMINATION OF ZINC BY ATOMIC ABSORPTION SPECTROSCOPY MEANS OF 20 READINGS AND THEIR S.E.'s

(a) Using aqueous standards

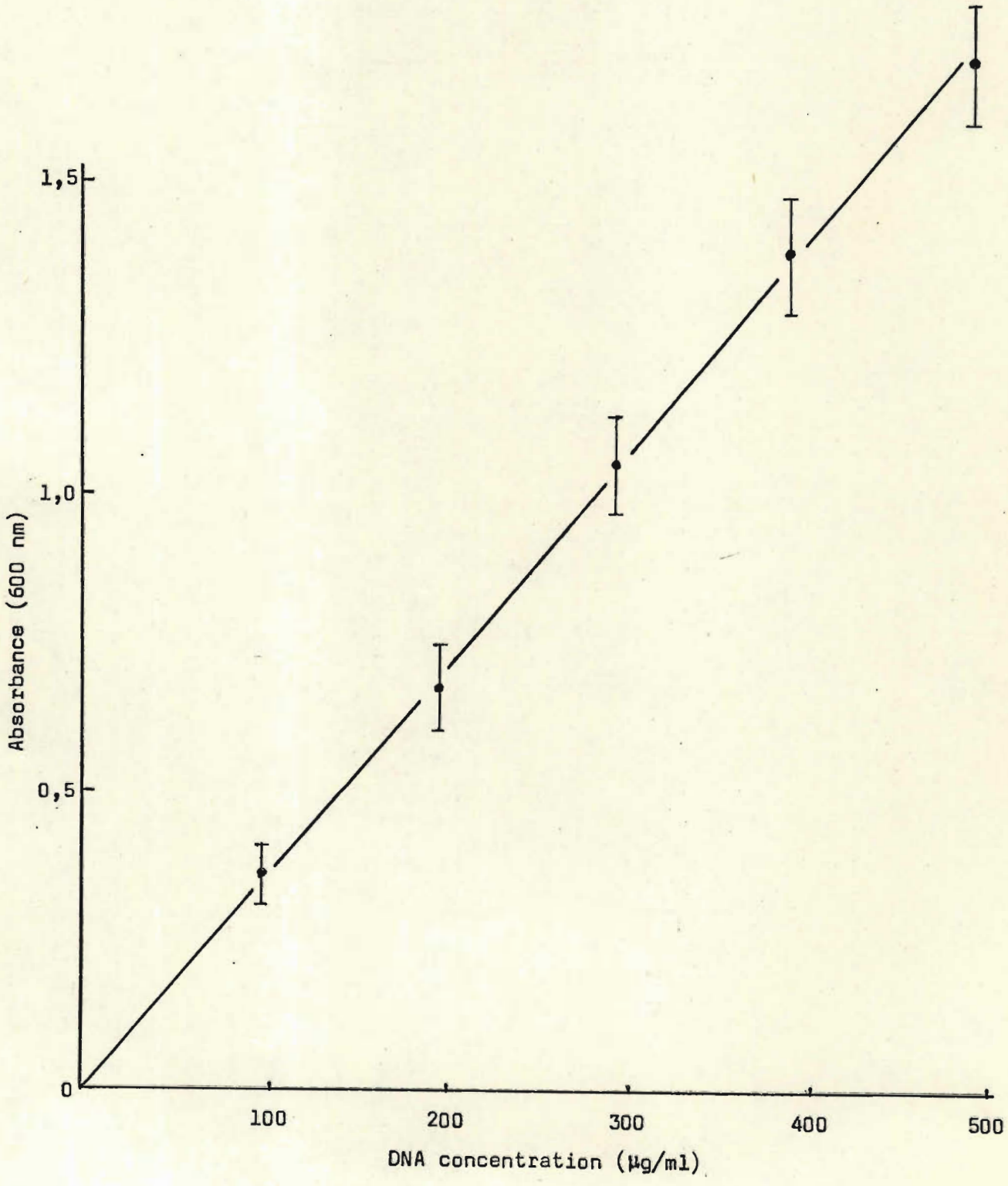


(b) Using sucrose standards



APPENDIX 2

STANDARD CURVE FOR THE DETERMINATION OF DNA
BY THE METHOD OF BURTON (77)
MEANS OF 10 READINGS AND THEIR S.E.'s



APPENDIX 3

THE EFFECT OF ZINC DEFICIENCY ON DNA SYNTHESIS IN REGENERATING RAT LIVER AT VARYING TIMES AFTER PARTIAL HEPATECTOMY

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) 10h					
581,1	112,5	10 377	322,4	102,5	6 284
151,6	25,0	12 234	288,4	92,5	6 230
606,9	196,0	6 495	347,8	57,5	11 990
672,6	97,5	13 725	315,0	90,0	7 000
544,9	100,0	10 827	126,2	12,5	20 042
		Mean 10 728			Mean 10 309
		Difference of means: 419			
		L.S.D. (0,25): 430			
(b) 15h					
1 722,6	123,0	27 031	1 198,9	114,0	21 049
2 088,8	102,1	40 957	1 839,2	160,0	22 987
1 860,4	110,0	33 826	946,7	86,5	21 865
2 400,4	160,5	30 005	1 089,1	114,0	19 115
2 175,9	128,0	34 052	2 013,5	130,0	24 984
		Mean 33 174			Mean 22 000
		Difference of means: 11 174			
		L.S.D. (0,01): 4 738			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) 17½h					
2 460,9	93,3	52 701	2 519,0	152,5	33 005
3 202,5	110,0	58 227	2 237,2	160,0	27 968
801,3	40,0	40,065	1 173,0	90,0	26 102
763,0	39,0	39 128	3 223,1	172,5	37 353
5 755,9	277,0	41 573	1 991,9	130,0	30 651
	Mean	46 339		Mean	31 016
	Difference of means:	15 023			
	L.S.D. (0,01):	9 405			
(d) 20h					
2 719,0	80,0	67 975	2 516,6	151,0	33 332
1 645,9	70,0	47 228	1 505,2	109,0	27 618
1 168,1	73,0	32 003	4 048,8	192,5	42 105
3 433,9	137,5	49 719	627,0	50,0	25 107
1 262,3	115,0	21 953	981,1	114,0	17 215
1 677,4	78,0	42 994	1 621,0	88,0	36 845
2 297,1	102,0	46 045	2 531,4	168,5	30 040
2 272,3	87,5	52 104	1 732,3	128,0	27 051
3 025,3	110,0	54 896			
	Mean	45 879		Mean	29 917
	Difference of means:	15 962			
	L.S.D. (0,01):	15 148			
(e) 22½h					
2 056,8	81,3	50 533	2 569,8	143,8	35 741
2 447,4	103,3	47 582	1 780,0	126,2	28 209
1 595,0	85,0	38 536	2 374,9	197,8	23 011
1 970,8	77,0	51 189	3 194,9	212,5	30 105
1 705,4	93,3	36 582	1 375,0	153,0	28 069
1 850,0	83,0	44 057			
	Mean	44 746		Mean	29 027
	Difference of means:	15 719			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(f) 25h					
1 929,1	106,7	36 109	2 358,7	130,0	36 288
1 318,7	66,0	40 037	1 073,9	61,5	34 972
2 356,9	122,5	38 661	1 891,8	93,0	40 652
3 284,2	146,7	44 798	1 444,5	86,0	33 593
3 017,9	128,0	47 155	2 450,1	132,5	36 968
1 455,2	70,0	41 576	1 386,2	92,0	30 105
	Mean	41 373		Mean	35 429
	Difference of means:	5 944			
	L.S.D. (0,01):	3 942			
(g) 30h					
2 603,9	160,0	32 562	1 925,1	132,5	29 115
2 694,6	170,0	31 718	2 493,4	111,0	46 913
4 347,2	165,0	52 695	976,9	122,3	18 020
2 683,2	148,0	36 254	3 632,8	148,0	51 107
2 271,8	149,0	30 491	917,1	100,0	20 346
3 263,9	187,5	34 727	2 208,7	178,0	26 813
	Mean	36 408		Mean	31 386
	Difference of means:	5 022			
	L.S.D. (0,01):	3 243			

APPENDIX 4

THE EFFECT OF ZINC DEFICIENCY ON DNA SYNTHESIS IN REGENERATING RAT LIVER AT VARYING TIMES AFTER PARTIAL HEPATECTOMY

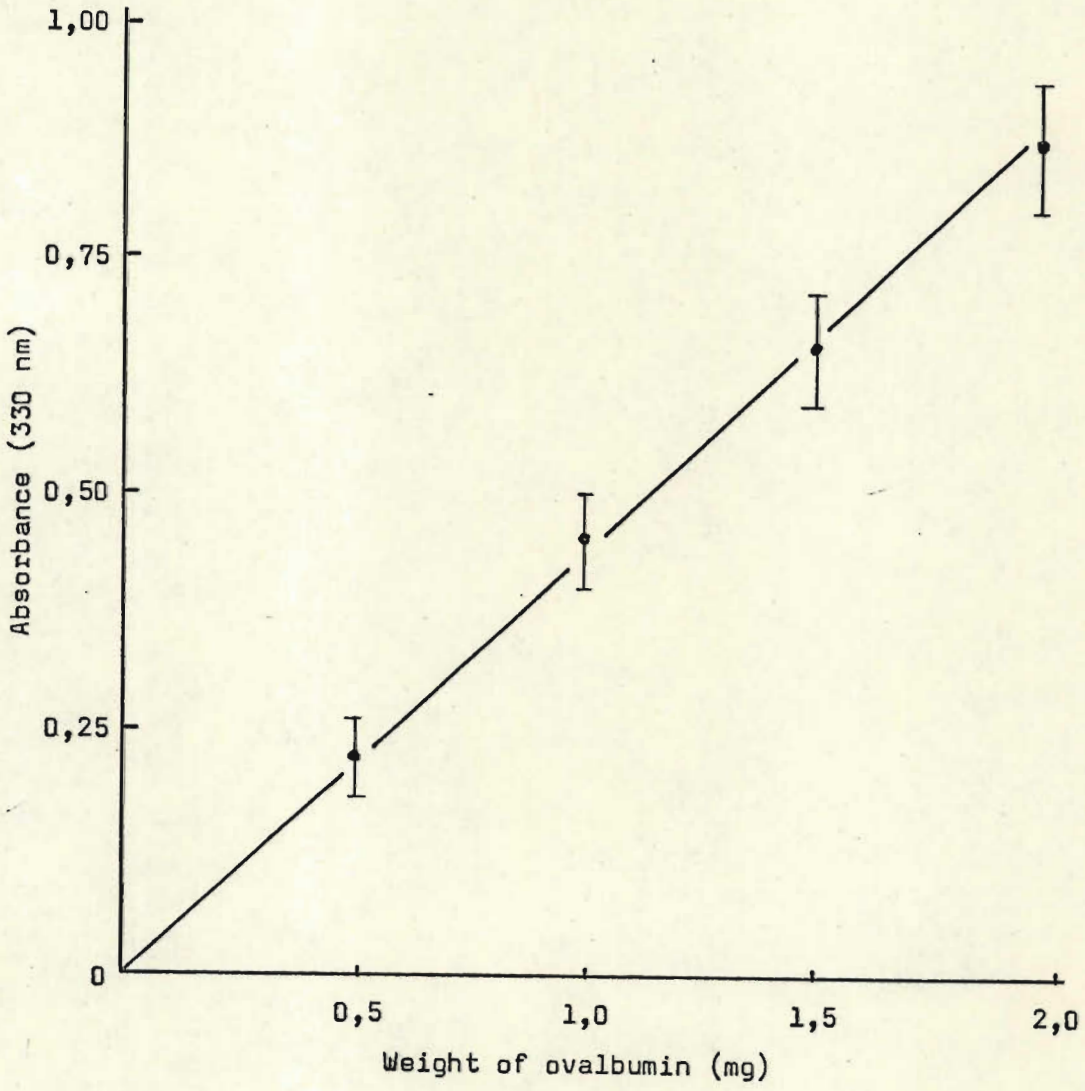
CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) 15h					
11 224	272	41 264	7 370	258	28 567
7 528	258	29 180	5 454	244	22 351
6 569	267	24 605	3 957	250	15 829
8 979	272	33 012	8 601	272	31 620
7 409	272	27 239	6 109	288	21 214
7 429	296	25 097	4 520	244	18 525
	Mean	30 066		Mean	23 018
	Difference of means:	7 048			
	L.S.D. (0,01):	6 374			
(b) 17⅓h					
12 289	288	42 670	6 703	270	24 827
13 243	260	50 934	8 943	255	35 069
8 332	230	36 228	4 561	224	20 362
7 867	267	29 466	8 013	254	31 547
9 600	235	40 852	8 421	258	32 640
10 854	272	39 906	7 984	300	26 613
	Mean	40 009		Mean	28 509
	Difference of means:	11 500			
	L.S.D. (0,01):	6 551			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) 20h					
8 053	224	35 951	7 374	244	30 221
10 407	246	42 306	5 883	288	20 426
7 721	258	29 928	7 462	272	27 434
12 888	280	46 029	8 252	220	37 510
14 477	288	50 267	9 237	296	31 206
10 627	250	42 509	8 077	272	29 694
	Mean	41 165		Mean	29 415
	Difference of means:	11 750			
	L.S.D. (0,01):	6 669			
(d) 22½h					
13 564	270	50 236	11 687	290	40 301
10 199	288	35 415	6 037	230	26 249
10 755	267	40 280	4 569	230	19 864
8 894	280	31 764	8 853	250	35 411
12 589	272	46 284	7 876	244	32 277
9 486	250	37 945	7 416	258	28 743
	Mean	40 321		Mean	30 673
	Difference of means:	9 648			
	L.S.D. (0,01):	6 706			
(e) 25h					
7 479	235	31 825	7 529	247	30 483
10 108	240	42 118	5 517	230	23 986
12 621	258	48 920	6 881	258	26 669
9 568	250	35 175	9 332	260	35 893
10 410	272	38 271	9 607	240	40 031
11 736	288	40 749	11 447	284	40 306
	Mean	39 509		Mean	32 895
	Difference of means:	6 614			
	L.S.D. (0,05):	5 177			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(f) 30h					
10 477	248	42 246	5 906	235	25 132
10 467	233	44 922	6 950	258	26 939
7 248	258	28 094	9 089	272	33 417
8 766	288	30 439	8 477	233	36 382
8 526	288	29 605	9 360	254	36 852
11 253	272	41 371	7 864	288	27 305
	Mean	36 113		Mean	31 005
	Difference of means:	5 108			
	L.S.D. (0,05):	5 172			

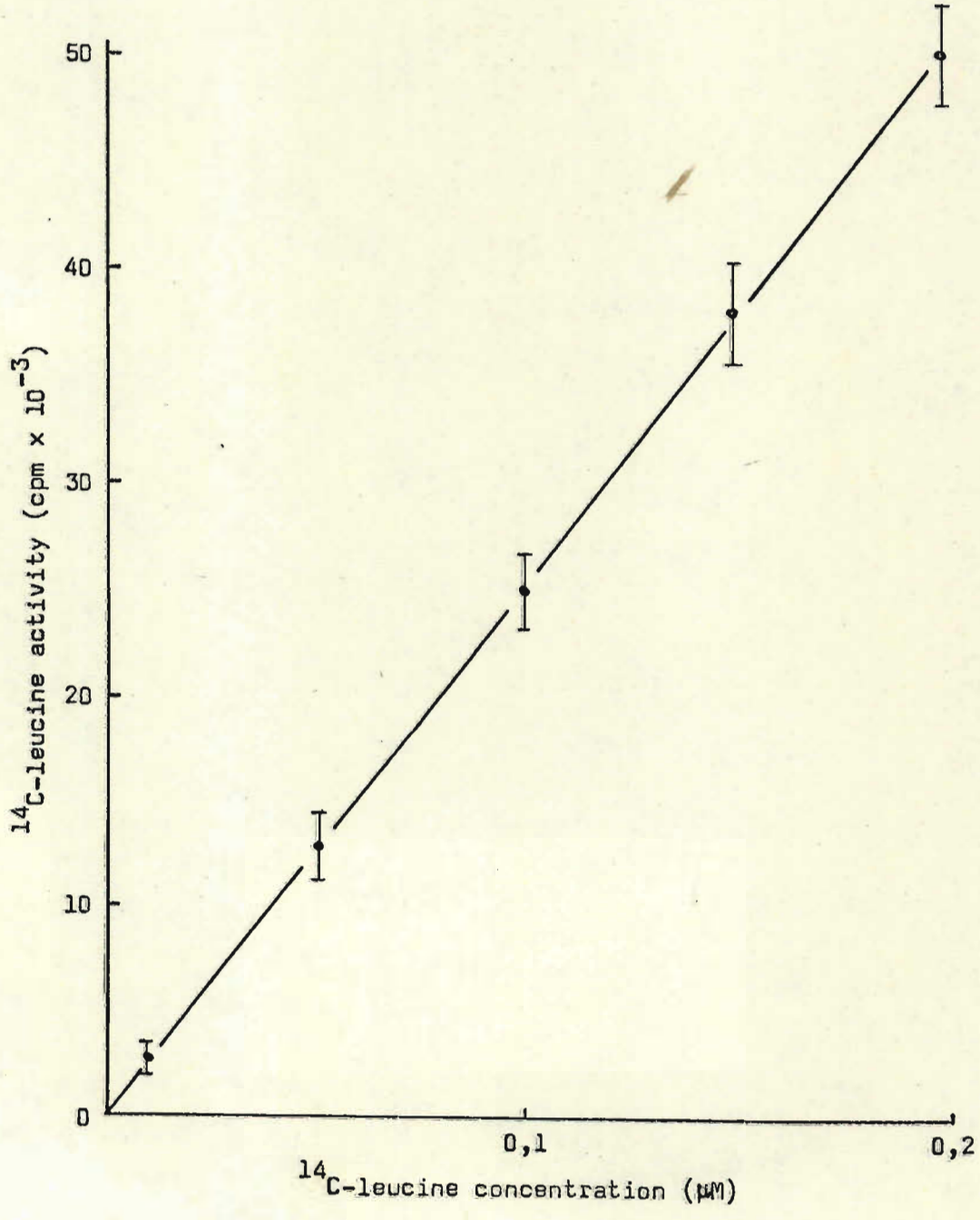
APPENDIX 5

STANDARD CURVE FOR THE DETERMINATION OF PROTEIN
BY THE METHOD OF BAILEY (85)
MEANS OF 10 READINGS AND THEIR S.E.'s



APPENDIX 6

STANDARD CURVE OF ^{14}C-LEUCINE (10 mC/mM)
ACTIVITY VERSUS CONCENTRATION
MEANS OF 8 READINGS AND THEIR S.E.'s



APPENDIX 7

THE EFFECT OF ZINC DEFICIENCY ON THE INCORPORATION OF ^{14}C-LEUCINE INTO PROTEIN IN REGENERATING RAT LIVERS AT VARIOUS TIMES AFTER PARTIAL HEPATECTOMY

CONTROL RATS			ZINC-DEFICIENT RATS		
Weight of protein sample (g)	cpm/ml protein sample	Specific activity (cpm/g protein)	Weight of protein sample (g)	cpm/ml protein sample	Specific activity (cpm/g protein)
(a) 10h					
0,1561	1 839,5	11 982	0,2034	7 051,3	34 671
0,0952	2 066,2	21 024	0,1550	2 590,1	16 718
0,1870	4 159,0	22 195	0,1489	1 995,6	13 415
0,0589	2 600,4	44 151	0,0559	2 642,0	47 267
0,1529	5 616,6	36 673	0,0549	1 633,5	29 758
0,0421	1 077,0	25 582	0,0437	781,2	17 995
0,0232	393,8	16 984	0,0694	1 739,4	25 583
0,0615	1 357,5	22 073	0,0478	1 076,6	22 303
	Mean	25 083		Mean	25 964
	Difference of means:	881			
	L.S.D. (0,10):	3 348			
(b) 20h					
0,0740	3 514,5	47 493	0,0293	1 601,4	54 632
0,1441	4 227,8	29 686	0,0972	2 327,5	23 945
0,0811	2 793,4	34 444	0,0466	2 143,4	45 983
0,1068	2 318,9	21 721	0,030	574,4	19 147
0,0944	4 277,8	44 865	0,0520	1 993,8	38 441
0,0844	2 412,0	27 285			
	Mean	34 249		Mean	36 429
	Difference of means:	2 180			
	L.S.D. (0,10):	8 606			

CONTROL RATS			ZINC-DEFICIENT RATS		
Weight of protein sample (g)	cpm/ml protein sample	Specific activity (cpm/g protein)	Weight of protein sample (g)	cpm/ml protein sample	Specific activity (cpm/g protein)
(c) 48h					
0,2459	8 117,6	33 012	0,1686	5 148,2	30 297
0,1547	4 269,2	26 886	0,1544	7 075,8	45 970
0,1983	8 420,3	42 462	0,2756	6 828,5	24 776
0,2609	8 361,2	70 380	0,1774	4 563,0	25 722
0,0975	3 015,4	31 068	0,1631	2 711,6	16 773
0,1614	5 026,1	37 093	0,0688	1 804,9	26 356
0,1803	6 622,8	30 701	0,1723	3 505,5	19 325
0,1219	3 357,2	27 541			
	Mean	37 393		Mean	27 016
	Difference of means:	10 377			
	L.S.D. (0,01):	9 620			
(d) 1 week					
0,2121	5 137,4	24 235	0,2657	4 281,6	16 125
0,2897	7 325,5	25 283	0,3382	5 661,8	17 955
0,2892	6 726,8	23 271	0,3217	5 709,6	17 753
0,3389	7 776,5	22 807	0,3243	5 814,6	17 931
0,4905	7 166,3	14 862	0,3121	4 216,9	13 511
0,2975	4 557,9	14 645	0,3780	5 787,4	15 309
0,3381	8 064,3	23 852			
	Mean	22 985		Mean	16 431
	Difference of means:	6 554			
	L.S.D. (0,05):	4 672			

APPENDIX 8

THE EFFECT OF EDTA AND METAL IONS ON DNA AND PROTEIN SYNTHESIS IN CONTROL CULTURES OF PHA-TREATED RAT LYMPHOCYTES

Additions to culture	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
None	28 394,0		82 308,0	
	26 828,0		81 293,0	
	39 674,0		79 012,0	
	32 031,0	30 886,0	88 547,0	84 023,0
	28 987,0		87 228,0	
	25 693,0		85 931,0	
	34 595,0		84 842,0	
1 mM-EDTA	674,2		8 323,0	
	705		9 749,0	
	556,8		7 799,3	
	1 149,9		8 319,0	8 573,8
	1 255,1		8 547,1	
	888,1	833,8	8 856,0	
	756,0		8 423,5	
	891,5			
	926,0			
	734,5			
1 mM-EDTA + 0,3 mM-Zn^{2+}	17 575,7		55 307,1	
	26 043,0		50 671,1	
	16 451,2		67 254,9	
	17 285,9	19 569,8	49 876,0	51 895,1
	19 075,7		48 317,0	
	20 987,1		50 014,2	

Additions to culture	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
1 mM-EDTA + 1,0 mM-Zn^{2+}	12 257,4		41 672,3	
	10 671,5		35 462,0	
	9 237,1		30 371,0	40 319,2
	14 376,7	12 230,3	52 163,4	
	13 972,0		41 927,4	
	14 876,1			
1 mM-EDTA + 2,0 mM-Zn^{2+}	6 767,1		22 371,0	
	4 323,2		28 254,6	24 625,6
	4 987,1	5 327,1	23 251,1	
	5 231,0			
1 mM-EDTA + 0,3 mM-Ni^{2+}	13 675,0		31 678,0	
	11 236,1		29 231,1	
	10 345,5		38 693,6	30 656,9
	11 596,4	11 411,0	23 045,1	
	12 237,1			
	9 376,0			
1 mM-EDTA + 0,5 mM-Ni^{2+}	10 367,0			
	9 237,1			
	9 967,0	9 604,7		
	8 847,5			
1 mM-EDTA + 0,3 mM-Mn^{2+}	8 367,1		12 567,3	
	8 325,0		13 245,0	
	7 940,6	8 476,7	17 613,5	13 640,7
	9 224,1		11 137,0	
1 mM-EDTA + 0,3 mM-Fe^{2+}	6 756,1		27 371,0	
	7 231,0		20 237,1	
	6 824,0	6 684,5	34 176,0	24 981,0
	5 927,1		18 139,9	

Additions to culture	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
1 mM-EDTA + 0,3 mM-Mg^{2+}	2 156,1		31 765,4	
	2 527,0		35 371,5	
	2 671,0	2 589,9	38 231,7	34 061,2
	3 005,6		30 876,0	
1 mM-EDTA + 0,3 mM-Ca^{2+}	1 875,1		12 274,0	
	1 361,0		13 451,0	
	1 927,1	1 650,1	17 671,6	15 430,9
	1 437,1		18 327,1	
1 mM-EDTA + 0,3 mM-(Zn^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+})	29 631,0		89 531,8	
	28 437,1		81 673,1	
	25 321,0		84 835,0	
	30 345,7	29 024,1	86 771,1	88 250,4
	30 951,1		94 587,5	
	29 458,9		92 104,0	

APPENDIX 9

THE EFFECT OF THE CONCENTRATION OF ZINC IN THE CULTURE ON THE INCORPORATION OF ^{3}H-THYMIDINE AND ^{14}C-LEUCINE INTO DNA AND PROTEIN RESPECTIVELY IN PHA-TREATED RAT LYMPHOCYTES

Culture conditions	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
Control serum + control lymphocytes - PHA (0,95 ppm of zinc)	380,1	392,0	1 002,1	937,0
	396,4		917,1	
	401,3		884,3	
	390,2		944,5	
Control serum + control lymphocytes + PHA (0,95 ppm of zinc) - Control conditions	26 385,5	31 136,9	110 950,0	83 197,7
	35 787,6		85 271,0	
	39 647,2		80 385,5	
	29 652,0		68 361,7	
	39 400,1		74 947,0	
	26 593,0		79 271,0	
	40 566,0			
	27 403,1			
	25 388,5			
	30 547,6			
Zinc-deficient serum + zinc-deficient lymphocytes + PHA (0,35 ppm of zinc) - Deficient conditions	26 252,1	20 567,1	55 273,0	61 294,6
	22 344,5		61 274,1	
	14 247,6		65 474,1	
	25 896,6		60 571,6	
	21 734,8		66 253,0	
	17 844,7		58 923,5	
	18 691,0			
	19 875,7			
	20 236,5			
	18 547,1			

Culture conditions	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
Control serum + zinc-deficient lymphocytes + PHA (0,90 ppm of zinc)	31 672,1	30 426,4	68 827,1	83 157,4
	30 492,9		88 479,8	
	25 263,4		87 231,3	
	28 921,0		88 091,5	
	32 262,1			
	30 197,1			
	35 231,8			
	29 371,1			
Control conditions + 1,05 ppm Zn^{2+} (2,0 ppm of zinc)	28 927,4	32 555,7	93 137,7	88 164,7
	35 318,0		91 247,1	
	33 289,5		80 567,5	
	32 965,1		89 261,0	
	27 452,1		86 353,6	
	29 389,9		88 421,4	
	36 274,1			
	36 829,4			
Control conditions + 2,05 ppm Zn^{2+} (3,0 ppm of zinc)	25 269,1	32 161,8	85 261,0	89 173,6
	38 231,0		96 381,1	
	29 927,6		88 845,5	
	30 104,1		92 387,1	
	35 289,7		83 471,6	
	34 987,5		88 695,3	
	32 187,1			
	31 298,9			
Control conditions + 4,05 ppm Zn^{2+} (5,0 ppm of zinc)	30 987,7	30 197,5	80 531,7	79 226,4
	24 395,5		74 632,1	
	32 877,1		77 821,0	
	30 287,1		83 428,5	
	30 671,1		82 621,4	
	35 005,6		76 323,9	
	26 428,9			
	30 929,0			

Culture conditions	Incorporation of ^{3}H-thymidine (cpm/culture)	Mean	Incorporation of ^{14}C-leucine (cpm/culture)	Mean
Control conditions + 9,05 ppm Zn^{2+} (10,0 ppm of zinc)	21 624,9	26 363,5	66 655,5	67 932,9
	24 823,3		74 495,7	
	24 163,9		81 688,9	
	20 102,8		50 311,0	
	32 405,5		66 923,1	
	34 435,9		67 523,0	
	30 565,8			
	22 057,5			
Deficient conditions + 2,0 ppm Zn^{2+} (2,35 ppm of zinc)	23 864,5	30 924,7	90 421,1	87 321,1
	27 323,4		75 832,5	
	33 698,1		84 323,8	
	32 228,5		93 672,9	
	34 167,8		96 122,8	
	34 363,9		83 555,3	
Deficient conditions + 2,0 ppm Zn^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Ni^{2+} (2,35 ppm of zinc)	32 133,7	29 854,1	88 220,8	87 824,9
	30 827,1		95 367,4	
	25 222,9		90 856,5	
	26 489,3		82 231,8	
	35 597,5		82 448,0	

APPENDIX 10

THE EFFECT OF ZINC STATUS ON THE GROWTH OF DAB-1 HEPATOMA IN RATS

Zinc conc. in diet (ppm)	Body wt. after 3 weeks (g)	Tumour wt. after 3 weeks (g)	Body wt. after 5 weeks (g)	Tumour wt. after 5 weeks (g)
0,5	141	6,0	141	22,3
	132	3,4	132	22,5
	142	5,5	135	19,3
	145	5,5	152	28,5
	151	4,5	141	42,0
	146	4,6	148	23,5
	148	5,8	141	32,0
	141	4,4	126	15,1
	149	6,5	130	15,6
	147	4,3	120	33,5
	138	4,9	131	17,4
	145	5,1	153	25,1
	144	6,2	129	23,1
	159	4,3	138	25,9
	162	4,7	139	25,5
Mean:	146	5,05	136	25,4
60	163	5,5	148	28,1
	140	11,9	143	28,5
	147	9,2	149	54,2
	159	21,8	187	40,3
	153	12,3	165	45,4
	153	10,3	174	30,3
	148	14,2	169	41,3
	170	12,4	193	45,0
	149	11,0	157	52,1
	148	10,1	154	41,1
	155	12,6	159	38,6
	152	16,2	153	37,2

Zinc conc. in diet (ppm)	Body wt. after 3 weeks (g)	Tumour wt. after 3 weeks (g)	Body wt. after 5 weeks (g)	Tumour wt. after 5 weeks (g)
	153	12,5	159	33,1
	155	11,1	155	38,5
	155	13,4		
Mean:	152	12,31	161	39,6
500	165	10,8	178	19,4
	156	5,4	154	35,9
	155	6,7	143	17,5
	151	5,3	162	53,4
	156	4,5	165	22,0
	134	10,0	140	7,8
	160	7,9	159	8,9
	152	9,8	160	14,2
	157	12,5	159	25,1
	144	13,0	160	26,7
	150	12,1	161	30,0
	156	7,4	173	13,5
	153	8,6	152	19,8
	169	11,5	175	28,9
	166	13,5	181	29,3
Mean:	154	9,2	162	23,5
1000	160	9,0	152	26,4
	152	9,2	155	22,3
	154	8,6	161	36,4
	170	5,0	174	28,5
	162	11,1	170	13,2
	145	6,5	158	20,4
	140	4,2	141	41,9
	152	11,4	147	36,3
	158	7,4	164	18,4
	148	8,0	158	14,2
	152	9,1	152	19,2
	167	7,7	176	24,0
Mean:	155	8,1	159	25,1

Zinc conc. in diet (ppm)	Body wt. after 3 weeks (g)	Tumour wt. after 3 weeks (g)	Body wt. after 5 weeks (g)	Tumour wt. after 5 weeks (g)
2500	152	8,6	142	19,4
	155	11,8	130	16,5
	162	10,1	155	38,2
	171	9,1	148	48,0
	153	5,6	150	12,4
	148	4,3	160	26,3
	144	4,8	161	22,5
	130	6,7	140	26,8
	142	7,8	144	20,4
	156	8,2	151	11,2
	150	8,5	154	16,8
	137	10,6		
Mean:	150	8,0	145	23,5

APPENDIX 11

EFFECT OF THE PREVAILING ZINC LEVEL
ON VIABILITY AND GROWTH OF
TRANSPLANTED TUMOUR TISSUE IN RATS

Zinc concentration in diet (ppm)	Body weight after 3 weeks (g)	Tumour weight after 3 weeks (g)
0,5	156	7,0
	164	4,0
	145	2,0
	104	14,3
	116	8,1
	148	1,3
	145	1,5
	140	5,9
	127	6,8
	139	3,7
	Mean: 138	5,5
60	157	14,1
	150	19,8
	162	13,1
	158	15,1
	151	25,6
	138	10,0
	142	11,5
	158	12,8
	160	5,8
	174	18,2
	Mean: 155	14,6
500	150	20,5
	163	11,4
	161	5,3
	160	19,0
	158	8,6

Zinc concentration in diet (ppm)	Body weight after 3 weeks (g)	Tumour weight after 3 weeks (g)
	141	4,4
	134	7,8
	165	9,5
	170	3,9
	155	7,6
	Mean: 155	9,8

APPENDIX 12

EFFECT OF DIETARY ZINC INTAKES ON DNA SYNTHESIS IN TRANSPLANTED TUMOURS

Zinc concentration in diet (ppm)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,033 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
0,5	2 428	64,0	2 529
	3 423	51,0	4 474
	3 622	54,5	4 431
	2 293	76,0	2 011
	3 615	62,0	3 887
	2 362	68,5	2 299
	1 187	64,0	1 237
	1 757	38,0	3 082
	1 597	61,0	1 745
	3 600	59,0	4 068
			Mean: 2 976
60	2 688	62,0	2 890
	3 622	54,5	4 431
	4 008	51,0	5 239
	4 885	70,0	4 653
	5 006	65,0	5 134
	1 748	45,5	2 561
	3 348	30,5	7 318
	2 405	53,5	2 997
	6 072	64,0	6 325
	5 131	76,0	4 501
			Mean: 4 605
500	3 475	78,5	2 951
	3 136	54,5	3 836
	2 908	64,0	3 029
	4 764	48,5	6 548
	3 245	59,0	3 667

Zinc concentration in diet (ppm)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,033 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
	1 851	43,0	2 871
	1 993	68,5	1 940
	4 223	64,0	4 401
	2 717	54,5	3 323
	3 071	73,0	2 805
			Mean: 3 537
1000	1 574	45,5	2 307
	3 378	59,0	3 817
	1 224	26,7	3 058
	1 384	53,5	1 728
	1 746	45,5	2 558
	2 606	56,0	3 102
	3 421	64,0	3 564
	1 701	68,5	1 655
	2 428	49,0	3 304
	1 487	45,5	2 179
			Mean: 2 727

APPENDIX 13

THE EFFECT OF ZINC DEFICIENCY ON NUCLEAR DNA SYNTHESIS IN REGENERATING RAT LIVERS AT VARYING TIMES AFTER PARTIAL HEPATECTOMY

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) 10h					
1 524	244	6 246	2 423	216	11 216
2 226	205	10 859	2 998	230	13 035
1 743	212	8 224	1 447	235	6 159
1 395	235	5 937	1 776	246	7 220
3 239	256	12 654	2 334	270	8 643
2 666	288	9 258	2 681	272	9 856
2 344	272	8 616	1 669	244	6 841
3 216	267	12 045	2 151	230	9 351
	Mean:	9 205		Mean:	9 040
	Difference of means:	165			
	L.S.D. (0,05):	826			
(b) 15h					
8 181	212	38 591	7 682	272	28 243
9 068	246	36 864	6 760	267	25 319
5 623	278	20 225	6 745	288	23 421
6 924	280	24 730	5 078	258	19 683
6 943	230	30 189	5 153	247	20 864
6 036	230	26 244	4 539	250	18 159
7 286	250	29 145	7 789	230	33 864
9 047	258	35 067	5 835	250	23 340
	Mean:	30 132		Mean:	24 112
	Difference of means:	6 020			
	L.S.D. (0,01):	3 783			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) 17½h					
11 374	280	40 621	6 219	260	23 921
10 451	244	42 831	7 844	256	30 641
8 785	250	35 141	7 370	230	32 045
8 596	246	34 945	5 839	216	27 217
10 605	200	53 023	4 739	254	18 659
10 425	235	44 360	8 486	270	31 428
11 834	244	48 501	8 103	250	32 411
9 025	230	39 237	5 719	233	34 544
6 489	220	29 496	9 027	230	39 247
9 896	240	41 233	5 246	250	20 984
	Mean:	40 939		Mean:	28 110
	Difference of means:	12 829			
	L.S.D. (0,01):	3 874			
(d) 20h					
7 852	200	39 260	4 801	230	20 876
6 731	220	30 959	7 471	247	30 245
7 871	224	35 142	7 602	270	28 156
12 739	285	44 684	9 088	258	35 220
13 634	267	51 026	9 844	246	40 016
12 461	258	48 298	5 637	212	26 591
11 453	250	45 811	4 304	186	23 141
8 836	230	38 418	7 559	188	26 247
9 824	212	46 338	8 140	267	30 487
9 779	230	42 521	7 579	244	31 062
	Mean:	42 246		Mean:	29 204
	Difference of means:	13 042			
	L.S.D. (0,01):	2 993			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(e) 22½h					
11 513	272	42 328	4 885	246	19 859
7 778	260	29 917	7 743	240	32 262
7 925	195	40 642	9 663	220	43 921
8 515	267	31 891	6 591	258	25 548
10 448	244	42 820	4 510	224	20 134
12 449	230	54 125	9 406	250	37 623
9 213	235	39 205	6 583	230	28 623
9 856	250	39 422	6 889	230	29 953
12 388	258	48 017	6 145	205	29 975
12 281	250	49 123	8 203	244	33 618
	Mean:	41 749		Mean:	30 152
	Difference of means:	11 597			
	L.S.D. (0,01):	5 688			
(f) 25h					
8 104	212	38 228	5 391	268	20 115
14 481	280	51 719	5 706	212	26 913
8 345	288	28 975	6 500	224	29 020
8 438	244	34 582	11 250	244	46 107
9 526	235	40 536	11 349	230	49 346
11 392	232	49 104	7 946	235	33 813
8 894	240	37 057	9 129	258	35 386
9 221	258	35 741	7 107	224	31 727
12 728	254	50 109	6 806	267	25 491
11 924	267	44 661	7 957	272	29 254
	Mean:	41 071		Mean:	32 717
	Difference of means:	8 354			
	L.S.D. (0,01):	4 138			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(g) 30h					
5 167	174	29 695	8 346	230	36 288
8 183	258	31 718	8 573	260	32 972
10 517	280	37 562	9 153	272	33 652
11 389	247	46 109	4 389	244	19 593
8 889	240	37 037	7 387	255	28 968
10 272	230	44 661	6 605	235	28 105
7 853	255	30 798	10 528	267	39 429
9 834	272	36 155	6 835	272	25 130
13 217	290	45 576	9 062	258	35 124
12 361	285	43 373	7 194	238	30 225
	Mean:	38 258		Mean:	30 949
	Difference of means:	7 309			
	L.S.D. (0,01):	3 080			
(h) 35h					
11 244	244	46 082	5 092	205	24 839
9 302	233	39 924	9 443	224	42 156
6 853	272	25 195	6 525	260	25 095
9 385	267	35 151	7 072	248	28 516
8 148	235	34 673	9 369	244	38 393
5 635	212	26 582	7 134	235	30 357
8 265	212	38 984	4 847	267	18 152
9 709	255	38 073	7 370	233	31 633
11 176	270	41 393	6 502	258	25 203
11 541	288	40 073	7 942	280	28 366
	Mean:	36 613		Mean:	29 271
	Difference of means:	7 342			
	L.S.D. (0,01):	3 413			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(i) 40h					
8 381	284	29 514	4 373	250	17 493
9 057	250	36 227	4 758	230	20 686
8 977	258	34 793	7 821	267	29 293
8 813	230	38 318	10 444	268	38 972
9 586	238	40 277	8 676	285	30 442
10 419	240	43 412	4 218	164	25 721
8 435	260	32 441	6 510	246	26 465
8 477	244	34 740	6 972	244	28 574
6 168	220	28 037	7 935	260	30 520
	Mean:	35 306		Mean:	25 574
	Difference of means:	7 732			
	L.S.D. (0,0):	4 208			

APPENDIX 14

THE EFFECT OF ZINC DEFICIENCY ON MITOCHONDRIAL DNA SYNTHESIS IN REGENERATING RAT LIVERS AT VARYING TIMES AFTER PARTIAL HEPATECTOMY

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) 10h					
228,7	10,6	21 524	170,4	8,1	21 036
363,0	12,9	28 143	274,5	9,4	29 198
284,6	9,4	30 272	269,1	11,4	33 609
240,6	8,3	28 993	323,2	9,8	32 975
344,8	11,4	30 249	396,8	12,2	32 523
172,9	6,5	26 606	392,0	13,7	28 614
132,7	7,6	17 459	362,6	14,4	25 180
522,7	13,7	38 154	126,8	6,6	19 219
	Mean	27 808		Mean	26 544
	Difference of means:	1 264			
	L.S.D. (0,05):	2 348			
(b) 15h					
262,7	9,8	26 811	412,5	14,0	29 462
391,7	10,8	36 269	287,9	9,8	29 380
358,1	12,6	28 420	307,9	11,4	27 012
452,1	14,0	30 361	199,1	6,6	30 168
274,0	8,3	33 015	285,3	8,2	34 793
593,2	15,2	39 026	280,3	7,6	36 886
284,8	9,3	30 622	324,1	11,8	27 462
399,9	11,0	36 357	281,3	9,8	28 703
	Mean	32 613		Mean	30 483
	Difference of means:	2 130			
	L.S.D. (0,05):	2 498			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) 17½h					
658,5	13,7	48 068	395,2	13,1	30 168
555,5	15,3	36 307	325,4	11,4	28 544
240,5	9,8	24 541	184,5	9,1	20 275
375,5	10,6	35 393	307,3	9,1	33 774
331,0	9,1	36 377	646,2	14,0	46 163
451,8	10,6	42 620	459,8	12,2	37 688
363,6	12,9	28 183	265,9	11,0	24 172
385,7	10,2	37 811	296,2	9,4	31 514
296,2	8,3	35 692	377,5	11,2	33 707
510,6	12,7	40 203	325,2	10,6	30 682
	Mean	36 519		Mean	31 669
	Difference of means:	4 850			
	L.S.D. (0,01):	3 491			
(d) 20h					
628,3	14,8	42 456	345,3	10,1	34 193
389,3	13,3	29 271	235,2	9,4	25 016
255,7	8,2	31 180	256,4	8,2	31 270
238,9	5,9	40 505	611,2	15,2	40 212
500,0	10,8	46 297	601,4	13,7	43 897
287,4	10,1	28 459	406,1	12,2	33 289
546,6	9,8	55 776	395,8	11,2	35 338
633,6	14,0	45 257	268,9	10,8	24 905
316,8	8,3	38 167	513,5	12,9	39 803
376,3	9,1	41 356	296,1	7,8	30 219
	Mean	39 872		Mean	33 814
	Difference of means:	6 058			
	L.S.D. (0,01):	3 816			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(e) 22½h					
338,7	8,3	40 811	338,5	9,4	36 012
341,9	14,0	24 426	329,1	11,4	28 866
499,0	13,7	36 420	449,4	11,4	39 424
446,7	11,4	39 183	534,1	13,8	38 703
511,9	10,6	48 310	129,1	6,5	19 868
616,1	12,2	50 502	284,7	7,1	40 093
437,3	9,8	44 622	492,1	11,8	41 701
416,9	9,4	44 357	397,3	9,8	40 541
473,2	10,2	46 393	477,1	12,2	39 103
572,8	11,6	49 377	412,0	10,6	39 620
	Mean	42 439		Mean	36 393
	Difference of means:	6 046			
	L.S.D. (0,01):	3 456			
(f) 25h					
450,5	10,2	44 168	566,3	11,2	50 563
429,8	10,6	40 544	361,4	9,1	39 711
584,0	12,9	45 275	297,8	7,6	39 180
513,8	12,6	40 774	232,1	9,1	25 505
645,6	11,4	56 631	342,5	10,6	32 309
262,9	8,2	32 068	328,4	12,9	25 457
414,5	8,1	51 172	688,4	13,7	50 246
555,0	13,7	40 514	600,8	14,4	41 722
330,5	6,6	50 075	441,7	10,0	44 167
685,9	15,3	44 828	440,8	11,2	39 356
	Mean	44 605		Mean	38 822
	Difference of means:	5 783			
	L.S.D. (0,01):	3 908			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(g) 30h					
491,9	9,8	50 193	469,9	10,8	43 513
424,1	10,8	39 270	554,6	10,6	52 325
603,7	14,0	43 121	324,6	9,1	35 672
606,1	13,5	44 897	385,0	11,4	33 776
630,9	11,4	55 339	457,0	9,9	46 163
478,4	10,2	46 905	530,7	9,4	56 457
432,6	8,6	50 297	464,4	12,2	38 064
572,9	13,7	41 818	340,6	11,8	28 863
542,8	12,2	44 490	549,2	14,0	39 229
649,9	12,9	50 381	530,3	12,2	43 465
	Mean	46 671		Mean	41 753
	Difference of means:	4 918			
	L.S.D. (0,01):	3 767			
(h) 35h					
411,7	9,1	45 242	693,9	12,8	54 217
437,7	10,8	40 528	648,8	12,4	52 324
424,6	9,8	43 327	365,1	9,1	40 121
803,6	14,4	55 807	175,3	6,8	25 780
473,0	12,4	38 146	559,2	13,7	40 816
527,1	13,5	39 045	565,6	11,4	49 615
475,0	8,6	55 238	422,2	10,8	39 096
465,7	9,4	49 545	591,6	12,8	45 864
707,7	13,7	51 657	416,4	8,6	48 421
659,2	12,2	54 033	632,6	15,9	39 784
	Mean	47 256		Mean	43 604
	Difference of means:	3 652			
	L.S.D. (0,05):	2 682			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(i) 40h					
425,8	10,1	42 161	221,9	7,6	29 199
418,4	8,2	51 025	212,3	5,7	37 238
313,1	8,2	38 179	371,5	8,3	44 754
552,3	9,4	58 753	326,2	10,0	32 621
391,5	10,6	36 931	598,2	9,8	61 045
392,9	8,1	48 511	564,3	11,2	50 381
261,9	5,8	45 153	382,7	8,1	47 241
494,2	9,8	50 431	411,8	9,1	45 258
490,9	10,2	48 123	333,8	8,2	40 704
	Mean	46 585			Mean 43 160
	Difference of means:	3 425			
	L.S.D. (0,05):	3 485			

APPENDIX 15

THE EFFECT OF ADDED DEOXYRIBONUCLEOSIDES ON NUCLEAR DNA SYNTHESIS IN REGENERATING LIVERS FROM ZINC-DEFICIENT AND CONTROL RATS

CONTROL RATS			ZINC-DEFICIENT RATS		
	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a)	+ dT					
	6 681	272	24 562	3 706	202	18 345
	4 788	244	19 621	6 990	285	24 526
	6 188	244	25 359	3 098	250	12 392
	8 102	230	35 228	3 370	212	15 894
	5 741	304	18 884	6 469	230	28 127
	8 283	220	37 651	7 638	247	30 924
	5 939	212	28 015	5 904	288	20 501
	8 075	267	30 243	5 359	296	18 107
		Mean	27 445		Mean	21 102
		Difference of means:	6 343			
		L.S.D. (0,01):	4 812			
(b)	+ dC					
	10 226	254	40 258	4 226	258	16 381
	10 354	250	41 418	4 153	230	18 051
	6 700	230	29 131	5 514	205	26 897
	9 510	200	47 552	6 475	186	34 813
	8 182	304	26 912	8 094	230	35 191
	8 392	244	34 392	7 752	258	30 045
	6 877	220	31 261	5 520	288	19 168
	7 927	260	30 490	7 122	220	32 373
		Mean	35 177		Mean	26 616
		Difference of means:	8 561			
		L.S.D. (0,01):	5 493			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) + dA					
9 250	230	40 219	6 006	304	19 758
7 218	205	35 212	8 185	288	28 421
10 286	288	35 715	7 055	230	30 676
15 299	304	50 326	3 011	200	15 052
6 297	182	34 598	6 915	272	25 421
10 261	220	46 643	9 649	267	36 139
6 905	235	29 382	7 128	250	28 510
7 837	244	32 118	8 542	258	33 109
	Mean	38 027		Mean	27 136
	Difference of means:	10 891			
	L.S.D. (0,01):	5 231			
(d) + dG					
9 496	260	36 524	4 220	230	18 346
10 303	244	42 226	4 785	212	22 573
11 540	230	50 174	10 313	272	37 915
10 895	240	45 395	7 299	235	31 059
7 194	254	28 323	4 810	272	17 683
12 727	267	47 667	10 082	296	34 062
6 026	212	28 423	7 049	250	28 194
6 944	224	30 998	6 015	244	24 650
	Mean	38 716		Mean	26 810
	Difference of means:	11 906			
	L.S.D. (0,01):	5 971			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(e) + dT + dC					
5 432	244	22 264	7 677	272	28 224
9 128	272	33 558	7 612	288	26 431
6 123	235	26 507	5 023	215	23 365
3 823	205	18 648	4 155	250	16 621
9 105	258	35 291	3 798	258	14 719
7 295	258	28 277	7 034	267	27 263
7 784	250	31 136	7 300	258	28 294
4 315	212	20 356	4 470	244	18 318
	Mean	26 948		Mean	22 904
	Difference of means:	4 044			
	L.S.D. (0,05):	3 130			
(f) + dA + dG					
11 609	288	40 312	6 030	200	30 128
8 242	288	28 617	6 138	212	28 952
8 666	258	33 590	8 822	220	40 101
8 621	244	35 330	5 643	212	26 620
9 575	212	45 167	4 439	244	18 192
9 184	250	36 737	8 079	272	29 701
8 535	235	36 318	5 598	250	22 392
9 207	230	40 031	5 490	272	20 182
	Mean	37 013		Mean	27 033
	Difference of means:	9 980			
	L.S.D. (0,01):	4 447			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(g) + dT + dC + dA + dG					
6 697	230	29 116	4 551	186	24 468
8 290	250	33 159	3 458	244	14 172
4 239	230	18 433	6 306	267	23 617
9 952	272	36 589	5 592	288	19 416
8 865	288	30 781	4 716	212	22 245
7 029	280	25 103	6 689	230	29 081
7 146	267	26 765	4 655	235	19 809
5 195	258	20 135	7 962	244	31 526
	Mean	27 510		Mean	23 041
	Difference of means: 4 469				
	L.S.D. (0,01): 4 238				

APPENDIX 16

THE EFFECT OF ADDED DEOXYRIBONUCLEOSIDES ON MITOCHONDRIAL DNA SYNTHESIS IN REGENERATING LIVERS FROM ZINC-DEFICIENT AND CONTROL RATS

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) + dT					
308,6	8,3	37 181	408,7	14,4	38 385
333,8	10,8	30 906	447,1	11,4	39 216
537,0	12,6	42 623	236,9	9,4	25 204
426,1	15,2	28 036	247,6	11,4	21 723
312,0	9,1	34 286	193,8	8,2	23 631
242,3	9,8	24 728	188,6	9,8	19 248
444,2	13,7	32 423	209,4	7,6	27 552
225,4	6,6	34 153	297,2	9,3	31 960
	Mean	33 042		Mean	27 115
	Difference of means:	5 927			
	L.S.D. (0,01):	4 353			
(b) + dC					
377,8	9,1	41 513	387,9	13,3	29 168
640,0	13,7	46 718	252,4	6,6	38 240
488,0	15,2	32 104	206,8	8,3	24 912
288,8	10,6	27 244	265,1	9,1	29 132
480,0	12,9	37 213	465,6	12,2	38 161
526,2	11,0	47 856	303,0	10,8	28 052
418,8	11,2	37 388	360,0	9,8	36 734
576,9	14,0	41 208	299,3	8,2	36 506
	Mean	38 906		Mean	32 613
	Difference of means:	6 293			
	L.S.D. (0,01):	4 577			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) + dA					
477,1	11,0	43 377	337,7	9,4	35 921
586,5	13,7	42 810	247,4	6,5	38 062
373,4	9,8	38 107	339,4	13,7	24 773
427,0	11,4	37 458	295,0	9,8	30 100
380,6	10,6	35 905	317,9	7,6	41 831
610,5	12,2	50 043	320,5	7,6	42 165
403,1	13,7	29 421	376,0	14,4	28 108
594,2	12,6	47 159	316,0	11,2	28 216
	Mean	40 535		Mean	33 393
	Difference of means:	7 142			
	L.S.D. (0,01):	5 016			
(d) + dG					
497,9	10,8	46 104	457,1	11,0	41 558
668,0	13,5	49 481	685,4	15,9	43 106
533,9	10,2	52 345	382,3	12,9	29 639
473,9	12,2	38 843	194,4	6,8	28 585
372,8	10,8	34 526	311,4	12,4	25 114
268,5	8,6	31 221	346,3	9,1	38 057
481,2	13,7	35 127	355,5	12,2	29 137
590,7	14,4	41 019	222,6	7,6	29 290
	Mean	41 083		Mean	33 061
	Difference of means:	8 022			
	L.S.D. (0,01):	5 308			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(e) + dT + dC					
339,1	9,1	37 264	379,9	11,4	33 329
322,9	7,6	42 481	275,0	8,3	33 138
284,9	9,4	30 306	261,3	11,0	23 752
321,8	11,4	28 173	180,0	7,6	23 684
467,6	13,7	34 133	148,0	8,1	18 220
301,5	12,9	23 372	159,3	9,8	16 256
490,9	14,0	35 067	279,0	9,1	30 655
444,3	15,2	29 227	456,8	13,7	33 341
	Mean	32 503		Mean	26 553
	Difference of means:	5 950			
	L.S.D. (0,01):	4 792			
(f) + dA + dG					
666,0	13,7	48 613	379,7	10,2	37 225
456,2	12,2	37 397	448,7	11,4	39 361
683,6	14,4	47 471	444,6	12,6	35 289
326,7	10,6	30 818	362,9	12,2	29 742
292,8	9,1	32 177	224,2	9,1	24 634
335,1	7,6	44 088	448,3	10,6	42 288
350,8	7,6	46 161	299,2	10,6	29 920
504,3	12,2	41 339	184,7	7,1	26 015
	Mean	41 008		Mean	33 059
	Difference of means:	7 949			
	L.S.D. (0,01):	4 904			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(g) $\frac{}{+ dT + dC + dA + dG}$					
232,5	8,1	28 701	222,2	6,5	34 191
312,1	13,0	24 005	217,5	7,6	28 621
481,7	13,7	35 162	253,9	13,7	18 539
342,5	10,6	32 309	253,2	14,0	18 088
504,3	12,2	41 337	363,1	12,2	29 759
671,5	15,3	43 891	346,1	9,1	38 038
261,8	10,6	24 698	188,6	9,8	19 244
257,0	9,8	26 225	234,9	10,6	22 163
	Mean	32 041		Mean	26 080
	Difference of means:	5 961			
	L.S.D. (0,01):	5 611			

APPENDIX 17

THE EFFECT OF ADDED DEOXYRIBONUCLEOTIDES ON NUCLEAR DNA SYNTHESIS IN REGENERATING LIVERS FROM ZINC-DEFICIENT AND CONTROL RATS

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) + dTMP					
7 091	304	23 326	6 263	210	29 822
4 794	212	22 614	3 524	182	19 360
7 906	220	35 937	3 170	186	17 045
8 701	304	28 621	4 878	197	24 761
3 784	205	18 458	3 259	288	11 316
4 850	288	16 839	7 723	304	25 405
4 750	230	20 652	3 959	258	15 346
9 796	272	36 013	4 477	258	17 359
		Mean 25 308			Mean 20 052
		Difference of means: 5 256			
		L.S.D. (0,01): 4 434			
(b) + dCMP					
7 008	232	30 205	5 992	230	26 054
7 505	230	32 630	6 114	258	23 698
10 909	267	40 858	4 531	184	24 626
9 695	230	42 152	4 033	230	17 536
9 969	272	36 651	4 090	205	19 951
5 996	244	24 574	7 513	250	30 050
6 752	250	27 009	9 395	258	36 414
10 199	272	37 496	10 961	288	38 058
		Mean 33 947			Mean 27 048
		Difference of means: 6 899			
		L.S.D. (0,01): 4 481			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) + dAMP					
8 953	192	46 630	8 054	220	36 607
9 473	244	38 824	5 227	205	25 497
9 446	192	49 199	6 815	244	27 932
5 618	186	30 204	7 588	258	29 410
9 049	212	42 688	5 146	250	20 585
5 290	244	21 681	5 259	212	24 807
12 076	272	44 397	7 731	192	40 269
11 086	230	48 200	7 796	220	35 437
		Mean 40 228			Mean 30 068
		Difference of means: 10 160 L.S.D. (0,01): 5 425			
(d) + dGMP					
7 104	260	27 322	6 786	304	22 324
8 598	230	37 384	7 357	288	25 524
10 813	256	42 240	7 694	212	36 292
5 732	220	26 055	6 171	205	30 101
11 397	250	45 589	3 685	182	20 249
9 329	272	34 297	4 280	235	18 211
6 748	230	29 338	8 066	244	33 058
9 573	212	45 157	6 263	258	24 275
		Mean 35 925			Mean 26 254
		Difference of means: 9 671 L.S.D. (0,01): 4 668			

APPENDIX 18

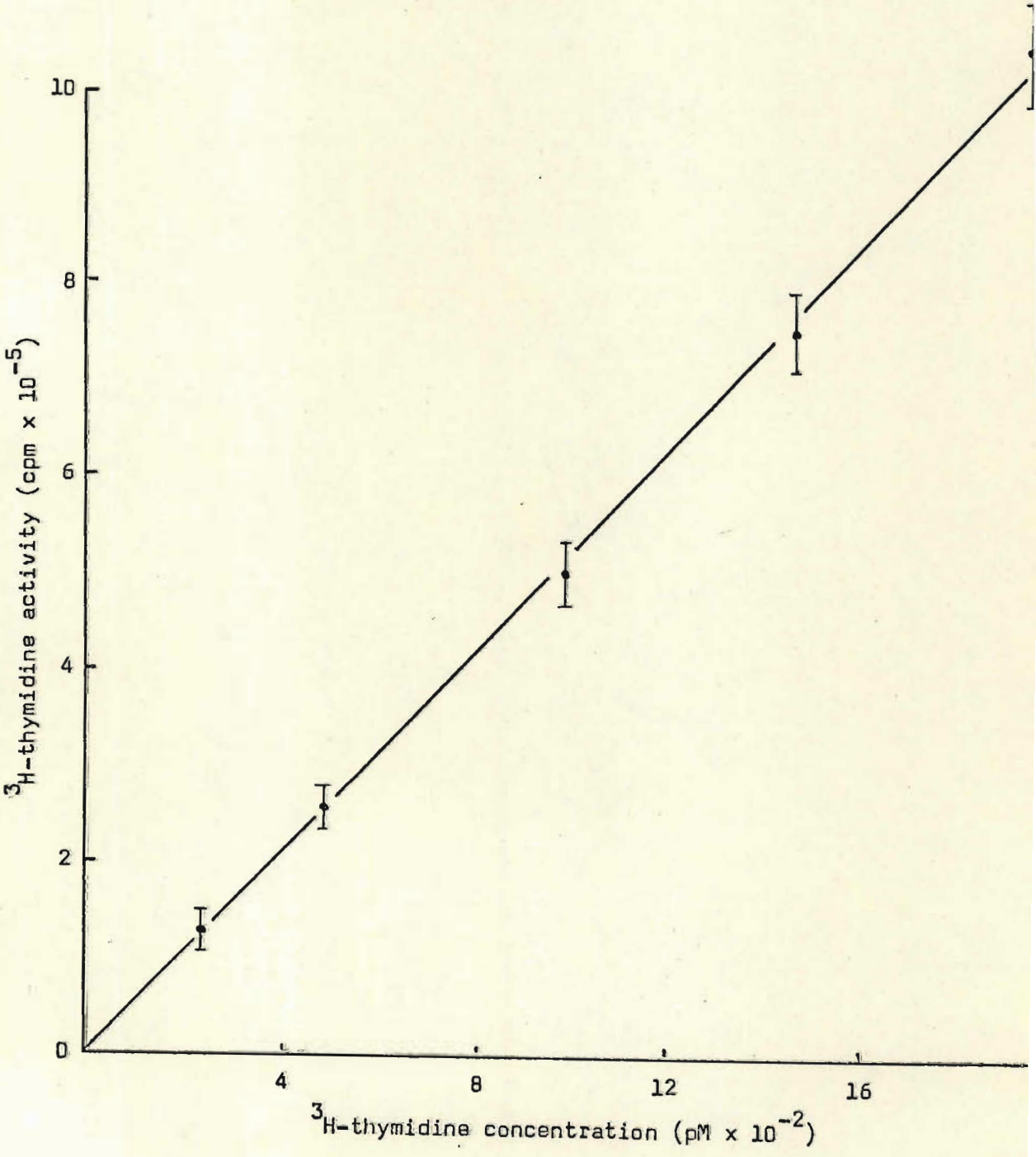
THE EFFECT OF ADDED DEOXYRIBONUCLEOTIDES ON MITOCHONDRIAL DNA SYNTHESIS IN REGENERATING LIVERS FROM ZINC-DEFICIENT AND CONTROL RATS

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(a) + dTMP					
282,9	10,6	26 684	274,9	11,4	24 111
279,6	9,1	30 726	290,0	15,0	19 330
176,2	7,6	23 182	275,5	7,6	36 249
237,9	6,6	36 047	218,3	6,5	33 588
393,2	9,4	41 829	154,4	5,7	27 089
426,2	10,6	40 203	289,6	7,6	38 105
355,7	12,2	29 155	289,1	10,0	28 906
397,5	11,8	33 683	272,0	10,6	25 662
		Mean 32 689			Mean 29 130
		Difference of means: 3 559			
		L.S.D. (0,05): 2 810			
(b) + dCMP					
309,6	9,1	34 017	248,6	6,2	40 101
249,5	7,6	32 835	448,0	12,2	36 723
448,6	9,8	45 775	312,6	10,6	29 491
566,1	14,0	40 438	260,0	11,4	22 806
289,6	11,0	26 324	350,4	10,6	33 053
263,9	9,1	28 998	201,0	7,6	26 441
305,8	8,2	37 290	338,7	9,8	34 561
534,7	12,6	42 439	176,8	7,6	23 263
		Mean 36 015			Mean 30 806
		Difference of means: 5 209			
		L.S.D. (0,01): 4 244			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)	cpm/0,5 ml DNA hydrolysate	Total DNA per 0,5 ml hydrolysate (µg)	Specific activity (cpm/mg DNA)
(c) + dAMP					
356,9	9,8	36 422	368,0	14,0	26 288
434,1	11,4	38 082	265,9	11,4	23 322
423,1	15,0	28 205	249,2	8,2	30 389
313,0	10,6	29 533	259,9	7,6	34 199
470,1	12,2	38 529	415,4	11,8	35 204
514,9	12,2	42 204	337,7	8,3	40 688
363,5	7,6	47 827	525,2	12,6	41 681
310,7	6,6	47 080	231,1	9,1	25 397
		Mean 38 485			Mean 32 146
		Difference of means: 6 339			
		L.S.D. (0,01): 4 611			
(d) + dGMP					
298,0	10,2	29 215	360,6	12,8	28 172
442,0	12,2	36 229	420,1	13,7	30 662
493,2	12,9	38 230	161,8	6,8	23 795
313,8	10,8	29 056	280,7	10,6	26 477
672,3	14,4	46 690	293,7	7,6	38 650
590,7	12,2	48 421	648,4	15,3	42 377
334,7	8,2	40 820	347,2	9,8	35 425
596,1	13,7	43 514	412,2	12,2	33 785
		Mean 39 022			Mean 32 418
		Difference of means: 6 604			
		L.S.D. (0,01): 4 447			

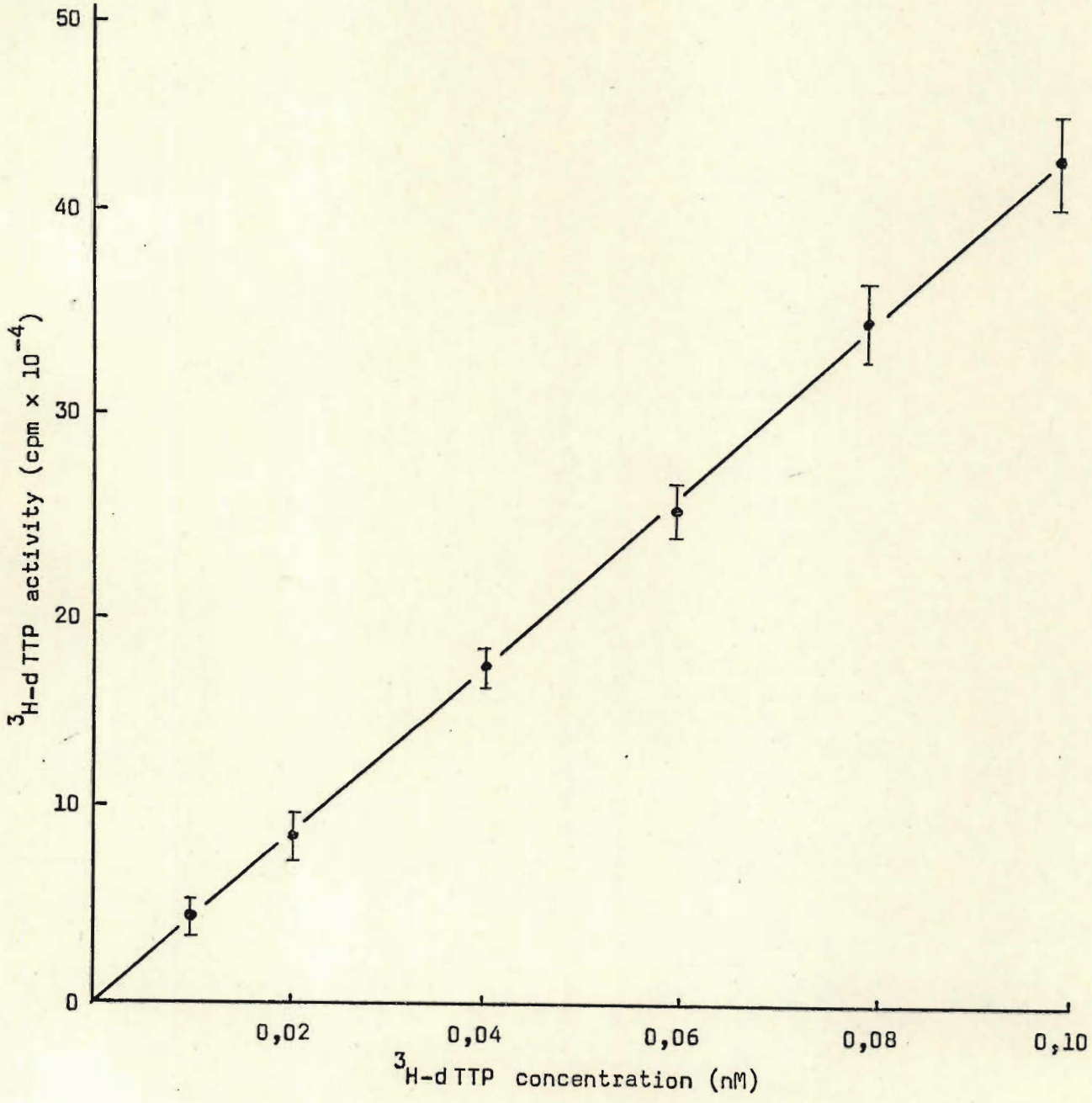
APPENDIX 19

STANDARD CURVE OF ^{3}H-THYMIDINE (5 mC/mM)
ACTIVITY VERSUS CONCENTRATION
MEANS OF 8 READINGS AND THEIR S.E.'s



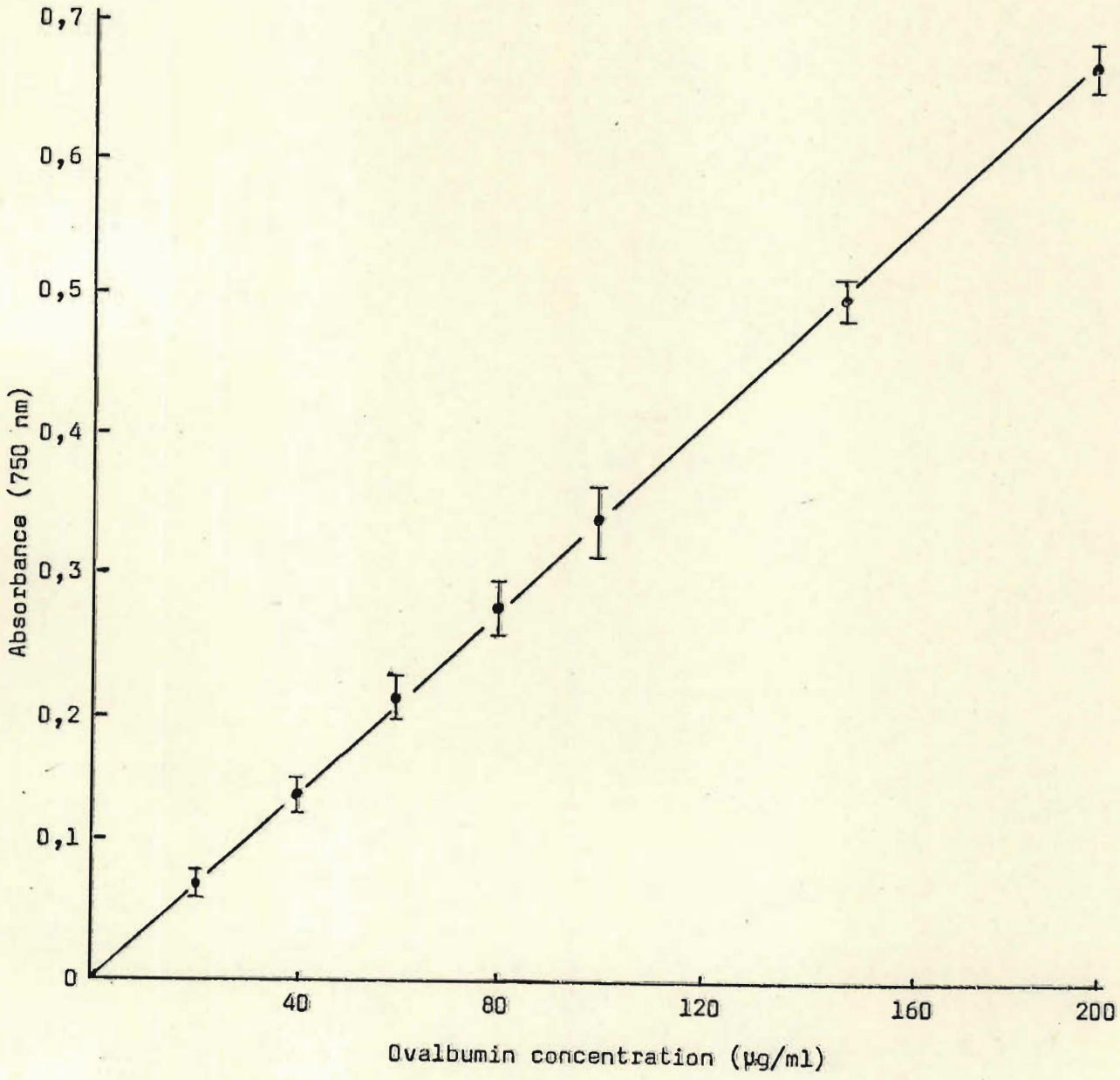
APPENDIX 20

STANDARD CURVE OF ^{3}H-d TTP (47mC/mM)
ACTIVITY VERSUS CONCENTRATION
MEANS OF 10 READINGS AND THEIR S.E.'s



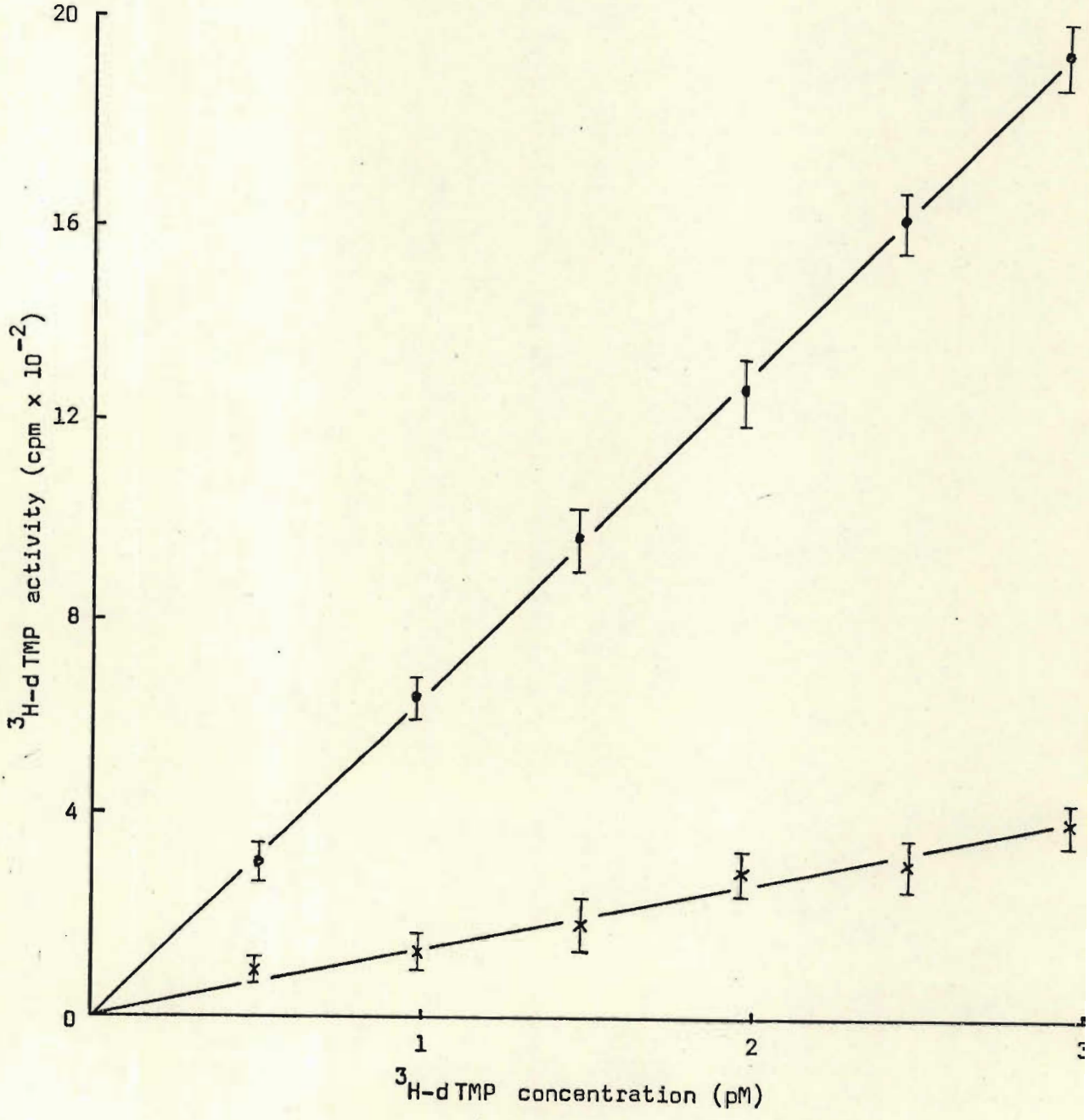
APPENDIX 21

STANDARD CURVE FOR THE DETERMINATION OF PROTEIN
BY THE METHOD OF LOWRY *ET AL.* (193)
MEANS OF 10 READINGS AND THEIR S.E.'s



APPENDIX 22

STANDARD CURVES OF ^{3}H-d TMP (15 C/mM)
ACTIVITY VERSUS CONCENTRATION
MEANS OF 8 READINGS AND THEIR S.E.'s



× —— × Using DEAE-cellulose discs;

• —— • Without DEAE-cellulose discs.

APPENDIX 23

EFFECT OF ZINC DEFICIENCY ON THYMIDINE KINASE ACTIVITY IN THE NUCLEAR FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(a) 0h					
240,8	97	240	234,3	110	206
158,9	92	167	146,9	76	187
124,2	66	182	138,3	88	152
171,2	72	230	96,3	94	99
153,2	57	260	341,6	90	367
295,0	90	317	179,8	92	189
100,0	78	124	286,7	110	252
165,5	64	250	271,5	101	260
210,9	75	272	123,1	74	161
114,7	66	168			
		Mean 221			Mean 208
		Difference of means: 13			
		L.S.D. (0,05): 28			
(b) 10h					
249,3	84	287	332,1	123	261
205,7	61	326	221,2	108	198
258,0	61	409	213,8	68	304
238,2	64	360	258,0	61	409
137,3	58	229	143,1	74	187
462,4	92	486	203,8	88	224
468,2	84	539	199,7	89	217
398,0	75	513	276,9	92	291
320,1	66	469	177,2	84	204
		Mean 402			Mean 255
		Difference of means: 147			
		L.S.D. (0,01): 51			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(c) 15h					
1 230,2	105	1 133	534,4	87	594
1 062,9	90	1 142	621,5	88	683
1 500,4	117	1 240	440,5	92	463
1 728,3	125	1 337	370,2	97	369
1 145,4	106	1 045	344,2	64	520
901,2	91	958	452,9	75	584
1 018,2	86	1 145	582,3	78	722
858,3	86	965	395,6	94	407
1 022,9	88	1 124	384,6	100	372
			394,3	93	410
		Mean 1 121			Mean 512
		Difference of means: 609			
		L.S.D. (0,01): 61			
(d) 20h					
1 164,1	86	1 309	855,4	113	732
1 046,3	87	1 163	919,1	108	823
1 255,8	94	1 292	745,5	96	751
1 465,1	108	1 304	867,2	87	964
986,9	96	994	490,5	88	539
671,1	64	1 014	494,3	76	629
636,1	66	932	425,4	68	605
916,7	74	1 198	664,4	64	1 004
1 010,7	78	1 253	582,3	78	722
865,8	82	1 021			
		Mean 1 148			Mean 752
		Difference of means: 396			
		L.S.D. (0,01): 79			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(e) 25h					
1 182,4	109	1 049	816,5	105	752
949,4	92	998	500,7	76	637
1 265,4	92	1 330	551,5	101	528
1 193,3	96	1 202	700,3	76	891
1 058,1	87	1 176	956,9	96	964
953,9	82	1 125	750,4	99	733
1 072,1	74	1 401	493,7	77	620
671,6	68	955	600,6	96	605
603,6	64	912	796,5	84	917
738,7	86	893	924,6	100	894
		Mean 1 104			Mean 754
		Difference of means: 350 L.S.D. (0,01): 84			
(f) 30h					
1 074,3	87	1 194	738,6	87	821
826,3	88	908	1 113,8	129	835
815,5	94	839	827,4	105	762
990,5	92	1 041	522,0	103	490
842,9	106	769	738,9	123	581
1 586,0	125	1 227	1 198,6	119	974
1 093,8	102	1 037	830,0	96	836
814,9	82	961	475,2	91	505
			556,9	88	612
			464,7	84	535
		Mean 997			Mean 695
		Difference of means: 302 L.S.D. (0,01): 95			

APPENDIX 24

EFFECT OF ZINC DEFICIENCY ON THYMIDINE KINASE ACTIVITY IN THE SUPERNATANT FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(a) 0h					
350,2	118	287	503,4	114	427
362,7	129	272	641,7	116	537
477,3	94	491	240,3	121	192
438,0	110	385	374,5	118	307
450,0	117	372	409,3	94	421
507,9	124	396	437,8	108	392
302,3	116	252	361,6	95	368
594,2	118	487	193,8	86	218
334,3	106	305	347,4	124	271
358,4	110	315			
		Mean 356			Mean 347
		Difference of means: 9			
		L.S.D. (0,05): 29			
(b) 10h					
856,3	120	690	532,5	125	412
772,2	127	588	430,1	128	335
614,2	100	594	735,9	108	659
587,1	94	604	322,9	117	267
425,8	91	410	624,3	112	539
781,4	97	779	237,3	94	244
828,9	116	691	228,9	113	524
644,6	98	636	418,1	109	371
455,0	110	400	224,9	87	250
		Mean 599			Mean 400
		Difference of means: 199			
		L.S.D. (0,01): 54			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphory-lated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphory-lated/mg protein/h
(c) 15h					
1 811,1	102	1 717	1 238,8	128	936
2 094,0	125	1 620	994,5	117	822
2 346,5	118	1 923	1 397,1	118	1 145
2 345,6	123	1 844	1 230,0	118	1 008
2 113,7	117	1 747	1 026,7	103	964
2 204,2	107	1 992	835,3	110	731
2 265,0	108	2 028	896,8	97	894
1 680,1	90	1 805	750,8	88	825
2 007,1	97	2 001	1 046,5	115	880
			1 028,3	110	904
	Mean	1 853		Mean	911
	Difference of means:	942			
	L.S.D. (0,01):	71			
(d) 20h					
1 961,9	102	1 860	1 366,8	104	1 271
2 845,6	124	2 219	1 346,9	98	1 329
2 857,3	116	2 382	1 647,1	97	1 642
2 075,6	118	1 701	1 005,9	86	1 131
2 076,2	109	1 842	1 172,6	105	1 080
2 112,4	110	1 857	1 183,5	114	1 004
1 673,0	94	1 721	1 144,6	118	938
2 023,2	97	2 017	1 450,7	103	1 362
1 442,6	86	1 622	1 968,9	126	1 511
2 052,5	114	1 741			
	Mean	1 896		Mean	1 252
	Difference of means:	644			
	L.S.D. (0,01):	208			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(e) 25h					
1 892,8	104	1 760	1 098,3	75	1 416
1 704,6	98	1 682	1 387,8	110	1 220
1 519,8	97	1 515	1 289,3	120	1 039
1 639,2	84	1 887	1 079,4	105	994
1 622,1	76	2 064	1 082,3	118	887
2 753,0	124	2 147	1 939,6	132	1 421
2 336,5	108	2 092	1 200,6	94	1 235
2 098,9	115	1 765	1 566,7	116	1 306
1 868,2	118	1 531	1 710,9	110	1 504
1 745,4	112	1 507	1 500,9	118	1 230
		Mean 1 795			Mean 1 225
		Difference of means: 570 L.S.D. (0,01): 107			
(f) 30h					
1 561,6	86	1 756	933,9	97	931
1 722,9	117	1 424	1 260,6	105	1 161
2 220,0	118	1 819	1 549,9	99	1 514
1 488,2	94	1 531	1 002,7	101	960
1 800,2	107	1 627	1 297,6	121	1 037
2 134,9	109	1 894	1 290,6	109	1 145
1 522,8	84	1 753	1 566,7	121	1 252
2 497,9	124	1 948	1 756,8	125	1 359
			1 091,2	105	1 005
			1 117,0	95	1 137
		Mean 1 719			Mean 1 150
		Difference of means: 569 L.S.D. (0,01); 105			

APPENDIX 25

EFFECT OF ZINC DEFICIENCY ON THYMIDINE KINASE ACTIVITY IN THE MITOCHONDRIAL FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(a) 0h					
297,9	28	1 029	241,5	34	687
316,2	21	1 456	192,9	30	622
289,1	30	932	232,6	24	937
150,6	20	728	137,6	22	605
297,3	21	1 369	308,6	36	829
276,2	29	921	316,2	42	728
286,2	25	1 107	233,4	38	594
280,2	31	874	290,1	34	825
461,0	28	1 592	269,9	28	932
437,1	30	1 409			
		Mean 1 142			Mean 751
		Difference of means: 391			
		L.S.D. (0,05): 120			
(b) 10h					
589,1	32	1 780	368,5	24	1 485
460,6	29	1 536	781,8	50	1 512
715,5	36	1 122	773,9	48	1 559
636,1	33	1 864	694,1	42	1 598
446,5	30	1 439	739,6	38	1 882
456,9	28	1 578	439,1	32	1 327
352,0	21	1 621	351,9	24	1 418
465,9	30	1 502	528,9	36	1 421
378,1	22	1 662	411,7	30	1 327
		Mean 1 656			Mean 1 503
		Difference of means: 153			
		L.S.D. (0,05): 151			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(c) 15h					
982,7	34	2 795	570,0	34	1 621
920,4	33	2 697	536,1	35	1 481
994,4	37	2 599	804,4	42	1 852
1 319,1	51	2 501	590,4	28	2 039
1 275,4	44	2 803	567,3	26	2 110
1 057,9	34	3 009	767,4	40	1 855
648,5	26	2 412	691,9	38	1 761
712,0	31	2 221	600,8	36	1 614
747,1	30	2 408	613,6	34	1 745
			627,3	30	2 022
	Mean	2 605		Mean	1 810
	Difference of means:	795			
	L.S.D. (0,01):	125			
(d) 20h					
914,9	30	2 949	532,8	28	1 840
748,8	24	3 017	546,9	27	1 959
960,1	36	2 579	662,8	29	2 210
1 405,1	42	3 235	826,5	33	2 422
1 109,4	38	2 823	712,0	34	2 025
1 152,0	40	2 785	553,4	30	1 784
700,7	26	2 606	510,4	30	1 645
614,5	20	2 971	381,8	20	1 846
890,4	30	2 870	648,9	28	2 241
1 193,2	36	3 205			
	Mean	2 904		Mean	1 997
	Difference of means:	907			
	L.S.D. (0,01):	124			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/mg protein/h
(e) 25h					
919,8	34	2 616	835,3	44	1 836
920,9	32	2 783	689,1	36	1 851
1 102,3	38	2 805	922,8	44	2 028
979,1	30	3 156	603,1	27	2 160
758,7	24	3 057	931,3	47	1 916
1 145,0	40	2 768	879,1	42	2 024
1 224,4	42	2 819	489,9	27	1 755
717,1	26	2 667	682,9	30	2 201
810,8	28	2 800	845,9	34	2 406
873,9	28	3 018	792,8	20	2 300
		Mean 2 850			Mean 2 048
		Difference of means: 802 L.S.D. (0,01): 96			
(f) 30h					
975,0	36	2 679	553,4	31	1 726
960,4	37	2 510	840,6	49	1 654
1 342,1	42	3 090	711,1	30	2 292
1 542,2	48	3 107	1 218,6	49	2 405
1 388,1	51	2 632	875,6	42	2 016
705,1	24	2 841	940,5	50	1 819
929,8	30	2 997	816,1	41	1 925
1 050,0	32	3 173	707,1	42	1 628
			659,6	30	2 126
			875,9	36	2 353
		Mean 2 871			Mean 1 995
		Difference of means: 876 L.S.D. (0,01): 157			

APPENDIX 26

EFFECT OF ZINC STATUS ON THYMIDINE KINASE ACTIVITY IN DAB-1 HEPATOMAS

Tissue	Zinc conc. in diet (ppm)	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/ mg protein/h
Whole liver	60	289,0	86	325
		390,9	84	450
		442,3	92	465
		302,7	95	308
		327,8	100	317
		210,6	93	219
		281,9	96	284
		515,9	98	509
		330,7	108	296
		494,5	89	537
				Mean 371
Hepatoma	0,5	1 806,7	120	1 456
		1 788,0	130	1 330
		1 834,2	116	1 529
		1 752,3	118	1 436
		1 461,9	109	1 297
		1 107,4	97	1 104
		1 684,3	117	1 392
		1 346,9	108	1 206
		1 478,8	110	1 300
		1 291,0	103	1 212
				Mean 1 326

Tissue	Zinc conc. in diet (ppm)	cpm/50 µl enzyme assay mixture	Total protein per 1,0 ml enzyme fraction (µg)	pM thymidine phosphorylated/ mg protein/h
Hepatoma	60	1 811,0	98	1 787
		2 048,3	103	1 923
		2 716,0	112	2 345
		2 557,8	118	2 096
		2 589,4	118	2 122
		2 582,5	122	2 047
		1 745,8	94	1 796
		2 144,3	110	1 885
		2 297,6	105	2 116
		2 158,9	108	1 933
				Mean 2 005
Hepatoma	500	1 927,3	104	1 792
		1 434,8	103	1 347
		1 407,3	97	1 403
		1 563,2	114	1 326
		1 436,9	122	1 139
		1 497,8	120	1 207
		1 353,7	110	1 110
		1 805,4	124	1 408
		1 434,3	105	1 321
		1 701,0	116	1 418
				Mean 1 355
Hepatoma	1000	1 191,8	123	937
		1 238,9	96	1 248
		1 701,5	98	1 679
		1 109,1	89	1 205
		1 394,5	98	1 376
		1 224,9	104	1 139
		1 563,1	117	1 292
		1 265,4	118	1 037
		1 431,6	112	1 236
		1 524,0	109	1 352

APPENDIX 27

EFFECT OF ZINC DEFICIENCY ON DNA POLYMERASE ACTIVITY IN THE NUCLEAR FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
(a) 0h					
26 792	88	1,42	16 304	94	0,81
13 509	76	0,83	26 208	90	1,36
27 384	92	1,39	25 783	86	1,40
23 922	98	1,14	10 554	68	0,77
20 966	102	0,96	8 909	64	0,65
30 835	96	1,50	15 879	72	1,03
10 826	64	0,79	18 469	75	1,15
25 856	75	1,61	18 467	88	0,98
17 057	59	1,35			
		Mean 1,22			Mean 1,02
		Difference of means: 0,20 L.S.D. (0,01): 0,18			
(b) 10h					
15 418	75	1,44	26 428	102	5,2
22 064	64	1,61	27 955	96	4,6
18 306	83	1,03	26 269	87	8,2
15 629	82	0,89	18 911	92	9,0
21 868	92	1,11	8 086	64	3,9
22 098	86	1,20	12 024	72	6,5
21 234	74	1,34	13 490	84	7,2
11 684	62	0,88	16 767	90	5,8
		Mean 1,19			Mean 0,99
		Difference of means: 0,20 L.S.D. (0,01): 0,18			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
(c) 15h					
27 201	87	1,46	20 627	86	1,12
19 337	86	1,05	22 440	80	1,31
30 072	93	1,51	11 884	75	0,74
18 468	98	0,88	10 638	72	0,69
30 063	101	1,39	13 542	68	0,93
12 746	64	0,93	28 277	93	1,42
16 650	72	1,08	25 080	96	1,22
22 965	75	1,43	15 830	84	0,88
23 151	68	1,59	16 532	78	0,99
		Mean 1,26			Mean 1,03
		Difference of means: 0,23			
		L.S.D. (0,01): 0,16			
(d) 20h					
36 242	93	1,82	29 619	104	1,33
17 862	86	0,97	16 942	86	0,92
20 757	74	1,31	15 566	92	0,79
29 155	92	1,48	18 706	96	0,91
16 445	64	1,20	18 047	98	0,86
16 394	66	1,16	19 647	74	1,24
23 589	68	1,62	16 453	68	1,13
33 027	97	1,59	21 892	72	1,42
37 713	103	1,71	28 139	90	1,46
22 965	75	1,43			
		Mean 1,43			Mean 1,12
		Difference of means: 0,31			
		L.S.D. (0,01): 0,15			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
(e) 25h					
30 658	103	1,39	26 790	92	1,36
30 739	97	1,48	23 758	86	1,29
29 121	68	2,00	15 469	84	0,86
25 671	74	1,62	15 830	96	0,77
20 709	78	1,24	15 760	80	0,92
30 300	89	1,59	32 068	104	1,44
23 640	80	1,38	23 769	74	1,50
28 987	96	1,41	16 075	102	0,82
			22 131	68	1,52
			19 080	90	0,99
		Mean 1,51			Mean 1,15
		Difference of means: 0,36			
		L.S.D. (0,01): 0,20			
(f) 30h					
25 043	86	1,36	19 972	88	1,06
34 294	88	1,82	24 308	86	1,32
28 985	94	1,44	24 962	94	1,24
33 158	98	1,58	14 614	75	0,91
25 695	100	1,20	15 688	74	0,99
32 401	78	1,94	22 715	68	1,56
34 949	96	1,70	20 209	66	1,43
26 756	88	1,42	20 877	78	1,25
28 904	90	1,50			
		Mean 1,55			Mean 1,22
		Difference of means: 0,33			
		L.S.D. (0,01): 0,23			

APPENDIX 28

EFFECT OF ZINC DEFICIENCY ON DNA POLYMERASE ACTIVITY IN THE SUPERNATANT FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	$nM\ ^3H$-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	$nM\ ^3H$-d TTP incorporated/ mg protein/h
(a) 0h					
16 878	116	0,68	12 168	116	0,49
25 264	118	1,00	13 414	108	0,58
17 429	110	0,74	21 914	119	0,86
10 891	96	0,53	10 131	110	0,43
11 694	103	0,42	23 837	121	0,92
14 235	109	0,61	6 645	97	0,32
14 719	125	0,55	7 484	92	0,38
12 699	121	0,49	10 020	104	0,45
13 669	112	0,57			
		Mean 0,62			Mean 0,55
		Difference of means: 0,07			
		L.S.D. (0,05): 0,07			
(b) 10h					
25 408	113	1,05	15 590	112	0,65
11 630	97	0,56	9 518	117	0,38
9 236	88	0,49	11 859	113	0,49
13 759	126	0,51	17 911	102	0,82
14 997	103	0,68	15 299	94	0,76
20 966	110	0,89	9 984	88	0,53
16 583	121	0,64	14 337	108	0,62
11 123	98	0,53	11 990	112	0,50
		Mean 0,67			Mean 0,59
		Difference of means: 0,08			
		L.S.D. (0,05): 0,07			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
(c) 15h					
20 809	113	0,86	30 315	117	1,21
21 508	108	0,93	28 475	122	1,09
23 384	78	1,40	16 022	86	0,87
22 501	93	1,13	13 085	94	0,65
22 873	98	1,09	13 139	118	0,52
27 583	115	1,12	22 467	106	0,99
18 790	117	0,75	15 957	92	0,81
22 169	119	0,87	13 939	105	0,62
29 958	128	1,10	20 730	110	0,88
		Mean 1,03			Mean 0,85
		Difference of means: 0,18 L.S.D. (0,01): 0,13			
(d) 20h					
44 498	118	1,74	30 834	120	1,20
44 610	112	1,86	22 703	114	0,93
51 392	103	2,33	24 343	116	0,98
29 227	98	2,01	34 066	97	1,64
35 384	96	1,59	36 509	110	1,55
44 342	102	1,62	34 358	113	1,42
45 463	117	1,77	31 914	108	1,38
54 732	110	1,93	30 732	104	1,38
39 878	120	2,13	25 392	98	1,21
42 179	96	1,94			
		Mean 1,89			Mean 1,30
		Difference of means: 0,59 L.S.D. (0,01): 0,13			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
(e) 25h					
53 917	109	2,31	42 476	114	1,74
55 639	116	2,24	45 982	122	1,76
70 549	114	2,89	49 922	124	1,88
53 656	116	2,16	45 432	96	2,21
50 032	118	1,98	42 811	98	2,04
63 584	101	2,94	36 077	108	1,56
53 340	94	2,65	36 760	116	1,48
65 216	108	2,82	47 344	110	2,01
			38 657	118	1,53
			34 949	96	1,70
		Mean 2,50			Mean 1,79
		Difference of means: 0,71			
		L.S.D. (0,01): 0,18			
(f) 30h					
61 316	103	2,78	51 338	108	2,22
55 022	88	2,92	50 285	114	2,06
52 008	96	2,53	37 842	96	1,84
76 266	112	3,18	36 370	88	1,93
70 246	94	3,49	60 194	112	2,51
80 068	114	3,28	50 278	92	2,38
75 046	104	3,37	57 365	114	2,35
69 252	98	3,30	46 203	116	1,86
65 526	120	2,55			
		Mean 3,04			Mean 2,14
		Difference of means: 0,90			
		L.S.D. (0,01): 0,18			

APPENDIX 29

EFFECT OF ZINC DEFICIENCY ON DNA POLYMERASE ACTIVITY IN THE MITOCHONDRIAL FRACTION OF REGENERATING RAT LIVER

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM^3H-d TTP incorporated/ mg protein/h ($\times 10^2$)	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM^3H-d TTP incorporated/ mg protein/h ($\times 10^2$)
(a) 0h					
683	42	7,6	192	28	3,2
515	29	8,3	397	32	5,8
389	20	9,1	398	30	6,2
479	35	6,4	717	36	9,3
697	31	10,5	432	24	8,4
248	20	5,8	353	22	7,5
539	30	8,4	252	28	4,2
401	25	7,5	289	30	4,5
876	44	9,3			
		Mean 8,1			Mean 6,1
		Difference of means: 2,0			
		L.S.D. (0,01): 1,0			
(b) 10h					
507	32	7,4	445	40	5,2
587	33	8,3	356	32	4,6
863	36	11,2	632	36	8,2
649	30	10,1	385	20	9,0
789	38	9,7	234	28	3,9
548	40	6,4	334	24	6,5
518	26	9,3	370	24	7,2
480	28	8,0	350	26	5,8
		Mean 8,8			Mean 6,3
		Difference of means: 2,5			
		L.S.D. (0,01): 1,1			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h (x 10^2)	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h (x 10^2)
(c) 15h					
750	33	10,6	334	30	5,2
632	24	12,3	396	22	8,4
845	42	9,4	516	28	8,6
496	26	8,9	682	36	8,9
887	28	14,8	486	42	5,4
509	22	10,8	437	30	6,8
1 139	40	13,3	706	32	10,3
700	30	10,9	398	20	9,3
491	28	8,2	599	28	10,0
		Mean 11,0			Mean 8,1
		Difference of means: 2,9			
		L.S.D. (0,01): 1,0			
(d) 20h					
532	27	9,2	576	28	9,6
875	28	14,6	457	26	8,2
792	33	11,2	651	32	9,5
866	34	11,9	749	34	10,3
887	30	13,8	334	30	5,2
693	24	13,5	283	22	6,0
659	20	15,4	764	42	8,5
1 295	42	14,4	867	44	9,2
1 203	36	15,6	732	38	9,0
910	34	12,5			
		Mean 13,2			Mean 8,4
		Difference of means: 4,8			
		L.S.D. (0,01): 1,0			

CONTROL RATS			ZINC-DEFICIENT RATS		
cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h (x 10^2)	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h (x 10^2)
(e) 25h					
795	32	11,6	479	26	8,6
848	30	13,2	630	32	9,2
1 103	28	18,4	661	30	10,3
1 519	42	16,9	879	36	11,4
1 041	36	13,5	758	30	11,8
1 286	38	15,8	385	24	7,5
1 229	33	17,4	1 277	42	14,2
638	20	14,9	563	36	7,3
			381	20	8,9
			570	22	12,1
		Mean 15,2			Mean 10,1
		Difference of means: 5,1			
		L.S.D. (0,01): 2,3			
(f) 30h					
1 230	34	16,9	890	40	10,4
1 727	36	22,4	648	24	12,6
962	22	20,2	1 171	36	15,2
1 307	28	21,8	938	30	14,6
725	24	14,1	420	20	9,8
1 029	31	15,5	522	28	8,7
1 326	38	16,3	976	34	13,4
1 573	36	20,4	863	38	10,6
1 019	28	17,0			
		Mean 18,3			Mean 11,9
		Difference of means: 6,4			
		L.S.D. (0,01): 2,6			

APPENDIX 30

EFFECT OF ZINC STATUS ON DNA POLYMERASE ACTIVITY IN DAB-1 HEPATOMAS

Tissue	Zinc conc. in diet (ppm)	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
Whole liver	60	23 837	92	1,21
		15 652	86	0,85
		16 729	84	0,93
		13 541	102	0,62
		13 490	100	0,63
		11 923	96	0,58
		16 577	98	0,79
		20 291	92	1,03
		14 894	94	0,74
		9 799	88	0,52
				Mean 0,79
Hepatoma	0,5	45 326	112	1,89
		47 116	114	1,93
		49 955	108	2,16
		52 836	105	2,35
		60 540	114	2,48
		61 668	120	2,40
		51 802	96	2,52
		51 205	98	2,44
		49 404	103	2,24
		50 787	118	2,01
				Mean 2,24

Tissue	Zinc conc. in diet (ppm)	cpm/0,5 ml DNA solution	Total protein per 1,0 ml enzyme fraction (µg)	nM ^{3}H-d TTP incorporated/ mg protein/h
Hepatoma	60	60 588	94	3,01
		64 069	110	2,72
		61 056	106	2,69
		78 358	114	3,21
		84 609	119	3,32
		75 762	122	2,90
		58 997	96	2,87
		55 352	94	2,75
		74 698	108	3,23
		72 189	112	3,02
			Mean	2,97
Hepatoma	500	49 066	114	2,01
		56 662	108	2,45
		53 562	106	2,36
		54 386	109	2,33
		43 480	94	2,16
		49 644	112	2,07
		51 728	122	1,98
		53 654	96	2,61
		46 354	88	2,46
		65 189	118	2,58
			Mean	2,30
Hepatoma	1000	42 268	105	1,88
		48 514	96	2,36
		52 958	108	2,29
		55 900	114	2,29
		50 032	118	1,98
		49 644	112	2,07
		58 459	105	2,60
		56 198	108	2,43
		49 946	98	2,38
		52 763	112	2,20
			Mean	2,25