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STUDIES ON THE ZINC STATUS
OF THE OB/OB MOUSE

By

Joanne Rowe

Thesis submitted to the School of
Graduate Studies of the University
of Ottawa in partial fulfilment of
the requirements for the degree of
Masters of Science

Department of Biochemistry
Faculties of Health Sciences
and Science and Engineering
University of Ottawa
Ottawa, Ontario

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DEDICATION

This thesis is dedicated to my son Christopher and to my parents who have always supported and encouraged all my endeavors.

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SUMMARY

Studies were carried out on the zinc status of the obese (ob/ob) mouse , an animal model of obesity and diabetes. The ob/ob mouse exhibits certain similiarities to a zinc deficient mouse. Both the obese mice and zinc deficient mice have impaired glucose tolerance, immune dysfunctions, hypogonadism, and impaired thermogenesis. A working hypothesis was established that the ob/ob mouse may be zinc deficient. The effect of zinc supplementation of the ob/ob mouse (1,000 ppm, for a period of three weeks) was examined.

It was found that tissue levels of zinc of the obese mice on a control diet were different than those of the lean mice. The obese mice had higher zinc levels in certain soft tissues and lower zinc levels in bone. Zinc supplementation had no significant effect on tissue zinc levels in either lean or obese mice.

Zinc-dependent physiological parameters were then measured to assess their response to zinc supplementation. Peripheral glucose handling, as measured by a glucose tolerance test, was not improved by zinc supplementation of the obese mice. The response of lymphocytes in certain immune assays was enhanced in the zinc-supplemented obese mice but it was also enhanced in lean mice. Finally, adenosine deaminase activity, a purine nucleotide degradation enzyme associated with immune dysfunction, was not affected by zinc supplementation in the tissues measured in either lean or obese mice. These results indicate certain quantitative differences in accumulation of zinc between lean and obese mice. Zinc supplementation did not specifically benefit any parameter measured in the ob/ob mice.

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INTRODUCTION

The effect of diabetes and obesity on zinc status is now beginning to be studied. In human diabetics plasma zinc has been reported to be elevated (Mateo et al, 1978), reduced (Alexander, 1974), and normal (Tarui et al, 1963; Pidduck et al, 1970). In an attempt to clarify the role of zinc in the pathogenesis of diabetes and obesity several researchers have turned to experimental animal models. This review will first describe certain pertinent aspects of the ob/ob mouse, the experimental model used in these studies, and then briefly discuss zinc studies performed with experimental animals.

I) The Obese (ob/ob) Mouse

The ob/ob mouse is one of a number of rodent strains characterized by genetically transmitted obesity. It is transmitted as an autosomal recessive trait with the mutation on chromosome 6 (Bray and York, 1971). The mice used in our laboratory are an inbred stock on the C57BL/6J background. There are other colonies of the ob/ob mouse on different backgrounds which alter the severity of expression of the ob/ob gene with respect to onset and severity of hyperglycemia, hyperinsulinemia, hyperphagia, islet cell morphology, and obesity (Herberg and Coleman, 1977).

Unless otherwise stated all further discussion will be confined to the C57Bl/6J ob/ob mouse. This mouse has been extensively studied in the past thirty years and its life history is well established (Bray and York, 1979). While many aspects of the ob/ob syndrome have been investigated I would like to concentrate the discussion on various areas which can be affected by zinc i.e. immunity, glucose homeostatic mechanisms, and body composition.

Immunity

The ob/ob mouse has been studied to some extent with respect to immunity. Distubances in both humoral and cell mediated immunity have been discovered. Some groups have reported decreased spleen and thymus weights in the ob/ob mouse as opposed to their lean littermates (Chandra, 1980a; Meade et al, 1979). However Finger et al (1971) did not find a difference in spleen and thymus weights which might be explained by the fact that the animals were older and that with age many of the hormonal variations have attained a plateau.

Evidence for defective cell mediated immunity are an impaired ability of the ob/ob mouse to reject skin grafts and to react to a contact sensitising agent (Sheena et al, 1978). The spleen cells of the ob/ob mouse also have a decreased cytotoxic response when the assay is performed in vivo but not when it is performed in vitro (Meade et al, 1979; Chandra et al, 1980b).

Obese mice were found to have an increased number of plaque forming cells in both the direct (IgM) and indirect (IgG) assays. when expressed per 10^6 cells (Chandra, 1981a). This probably represents an alteration in T-cell function rather than of B-cell function as the formation of antibodies by B-lymphocytes was found to be normal in the obese mouse (Finger et al, 1971).

Obese mice have significantly elevated levels of thymic hormone activity as compared to their lean littermates (Chandra et al, 1981b). This finding suggested the possibility that the ob/ob mouse might perhaps demonstrate enhanced immunocompetence in certain situations. Melanoma tumors grew much more slowly and were more resistant to metastasis in obese mice compare to lean mice (Thompson et al, 1983). Obese mice were reported to have three times the proliferative response to a T-cell mitogen by one group (Thompson et al, 1983) but no difference was found by another group (Nichols et al, 1981).

To summarize, skin grafts, contact sensitizing agents, and possibly spleen and thymus weights would indicate diminished immunocompetence whereas plaque cell assays, thymic hormone activity, tumour growth, and possibly the response to a T-cell mitogen would indicate enhanced immunocompetence. Thus, depending on the assay used to test immunocompetence the conclusions reached were different. This could be explained by various subpopulations of lymphocytes in the ob/ob responding differently to their internal milieu.

Experiments have been reported in which an attempt had been made to distinguish if the differences seen between lean and obese mice are due to the lymphocyte itself or to the hormonal/metabolic environment in which the lymphocyte finds itself. There was no difference in graft versus host reactions when spleen cells from obese or lean littermates were injected into a common F1 environemnt (Meade et al, 1979). Also, as cited above, cytotoxicity assays demonstrate a difference in vivo but not in vitro. These findings indicated that the problem is probably due to the hormonal/metabolic environment of the obese mouse.

Various explanations have been proposed to explain the immune dysfunctions of the ob/ob mouse. The most interesting and popular explanation utilizes the observation that the ob/ob mouse has a hypertrophied adrenal cortex (Naesar, 1975) and elevated plasma corticosterone levels from 4-17X higher than those of its lean counterpart (Dubuc et al, 1975; Herberg et al, 1975; Garthwaite et al, 1980).

The exact mechanism whereby adrenal hyperfunction originates is uncertain. It appears to be secondary to the obese-hyperglycemic syndrome and not a primary event. Measurements of basal corticosterone levels of weanling 17-day old ob/ob and lean mice showed only marginal differences (Dubuc, 1976), whereas hyperglycemia and hyperinsulinemia are clearly established by this age.

ACTH levels are elevated in the pituitary of the obese mouse but similiar hypothalmic CRF activity has been found in lean and obese mice (Edwardson et al, 1975). High ACTH levels are not a prerequisite for hypercorticosteroidism as food restriction can normalize ACTH levels without normalizing corticosteroid levels (Dubuc, 1977). High levels of steroids are detrimental to the lymphocyte and can directly influence immune function (Dougherty et al, 1945; Kimoshita et al, 1974).

Hyperinsulinemia could be a factor influencing immune function either, indirectly by increasing corticosteroid levels, or by a direct action on lymphocytes. Thymic lymphocytes of the ob/ob mouse have a decreased number of insulin receptors (Soll et al, 1974). This may be associated with decreased cell responsiveness to the hormone. Some in vitro experiments suggest a role for insulin in lymphocyte function. In certain circumstances, insulin can enhance T-cell mediated cytotoxicity (Strom et al, 1975). Insulin has been shown to influence glucose uptake and plasma membrame ATPase activity in lymphocytes (Hadden et al, 1972). In addition, insulin receptors were found to be present on alloimmune T cells but not upon nonimmune T-enriched cells (Helderman et al, 1977). It thus appears that certain subpopulations of T cells may be dependent upon insulin for their function.

Recently other hypotheses have also been advanced explaining the immune alterations of the ob/ob mouse. The obese mouse has increased endogenous opioids (Ferguson-Segall, et al, 1982) and reduced body temperature (Trayhurn et al, 1977) both of which are associated with reduced cancer growth or increased immunocompetence (Thompson et al, 1983). These have been used to explain the enhanced immunocompetence of the ob/ob mouse.

Glucose homeostasis

The obese mouse is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. Both plasma insulin and glucose are elevated up to fourfold in 5-6 week old obese mice (Dubuc, 1976). Much work in the past years has attempted to clarify and explain the evolution and pathophysiology of these events.

The pancreatic islets of Langerhans of the obese mouse are hypertrophied and hyperplastic (Herberg and Coleman, 1977). The B-cells are severely degranulated, suggesting a high secretory activity and not surprisingly there is an excessive response of insuliin secretion to glucose stimulation both in vitro and in vivo (Cameron et al, 1972). The etiology of insulin hypersecretion remains unclear. Various pituitary or pancreatic factors have been implicated but no definitive results have yet been obtained (Bray and York, 1979). It is known that hyperinsulinemia is a primary problem of the obese mouse and not simply a response to the hyperphagia. Food restriction of an obese mouse will lower insulin levels but will never reduce to them levels seen in a lean mouse (Mordes et al, 1981).

The events occurring after insulin secretion become more complicated to interpret. The insulin itself is biologically potent (Stauffacher et al, 1967; Genuth, 1969) and is degraded at equal or faster rates than in lean mice (Coore et al, 1970). The high insulin levels are therefore not due to production of an ineffective insulin or a slow rate of degradation.

Liver, adipose tissue, and muscle cell membranes all have decreased numbers of insulin receptors. It is not certain whether the reduced number of receptors itself is sufficient to alter the physiological effect of insulin but all these tissues have also been shown to be almost unresponsive to the action of insulin in the obese mouse both in vivo and in vitro (Freychet et al, 1972; Bégin-Heick et al, 1976; Le Marchand-Brustel et al, 1978). It has been proposed then that the insulin resistance may be at the level of the receptor, the hormone-receptor complex, or possibly a post-receptor intracellular event (Jeanrenaud, 1978).

Whatever the molecular mechanism involved in insulin resistance, in the obese mouse, it results in an impaired glucose tolerance curve when obese mice are administered a glucose load intraperitoneally, intravenously, or orally. Fasting glucose levels are much higher, there is an exaggerated response to a glucose dose, and a slow return to basal glucose levels (Genuth et al, 1971; Cameron et al, 1972; Bégin-Heick et al, 1974, 1979).

Body composition

The obese mouse is also characterized by obesity and hyperphagia. As diet can alter body composition it is interesting to note how feeding regimens can affect the obesity and hyperphagia.

The obese mouse begins depositing extra body fat at 10-12 days of age by both hyperplastic and hypertrophic growth of adipocytes (Assimacoplouos-Jeannet, 1976; Dubuc, 1976). Metabolically, there is an increased rate of lipogenesis in adipose and hepatic tissue.

Although the obese mouse consumes more food than the lean mouse, restricting the food intake does not prevent the adiposity. The carcass of the obese mouse always contains a higher percentage of fat than that of lean mice of the same size (Dubuc, 1976). There is a corresponding decrease in protein deposition (Alonso et al, 1955; Chlouverakis, 1972).

It has been postulated that the hyperphagia may be the result of a hypothalmic lesion. These mice do not regulate their food intake normally. Obese mice are less able to adapt to alterations intake when their diet was diluted with cellulose or made bitter with quinine. They overcompensated in response to a high fat diet (Fuller et al, 1955). In addition obese mice eat continuously as opposed to most rodents which exhibit a diurnal variation of consumption patterns. There was no time in a 24 hour period when obese mice consumed more or less (Bailey et al, 1975). These findings are important to consider when altering the feeding regimen of the obese mouse.

II) Zinc

Trace minerals, and especially zinc, have received enormous attention, in the past few years as their importance in nutrition is becoming increasingly evident. Fifty years ago, zinc was shown to be essential for rats (Todd et al, 1934) and since then many different species including man have been shown to require this metal in their diets. It is only in the last fifteen years that better methods of detection have allowed quantitation and understanding of the diverse role zinc has in the body. Zinc is now believed to be important in a number of biological functions: wound healing, taste acuity, fetal growth and development, immunity, and fertility are some of the most commonly mentionned.

Re-examining zinc at a molecular level one begins to appreciate the reason it seems to possess such a wide variety of functions. Zinc has been found to be an essential component of 80-90 metalloenzymes. Key enzymes involved in nucleic acid, protein, lipid, and carbohydrate metabolism are zinc dependent (Solomon, 1981). Although a very small proportion of body zinc is necessary to maintain the activity of these enzymes the importance of zinc in the body can be appreciated by virtue of its ubiquitous nature.

It has been proposed that the primary function of zinc in the body is to act as a membrame stabilizer and not only as a constituent of metalloenzymes (Chvapil et al,1973; Bettger et al, 1981). This would help explain the rapid beneficial effects of zinc on deficient subjects, which could not be explained by enzyme induction.

As it is not possible to review the enormous body of information on the subject of zinc this discussion will focus on the experimental animal and the effects of deficiency or toxicity of zinc.

Tolerable levels of zinc in the diet

Zinc requirements of the mouse have not been well documented. One study determined the zinc requirement of two different strains of growing mice to be 5.9 ug Zn/g diet (Luecke et al, 1979). They used body weight gain and antibody forming ability as measured by Jerne's plaque assay as indices of adequate zinc. This figure compares to estimated requirements for the rat of 12 ug/g (Forbes et al, 1960), 45-50 ug/g for pigs (Miller et al, 1970), and 12 ug/g for guinea pigs (Alberts et al, 1975).

Zinc is a relatively non-toxic substance to mammals. On a standard laboratory chow rats consumed up to 2000 ppm without any discernible deleterious effects (Stake et al, 1975). There was no difference obtained whether the zinc was ingested as the metal, chloride, or carbonate. Similiar results have been obtained with pigs (Lewis et al, 1969). Sheep and cattle are slightly less tolerant and at levels greater than 500 ppm deleterious effects begin to appear (Ott et al, 1966).

A zinc intoxicated animal will demonstrate subnormal growth, anorexia, bone abnormalities, and severe anemia. These effects are mainly due to the interaction of zinc with other divalent cations such as copper, calcium, and iron in the process of absorption and utilization. For example, the anemia accompanying zinc toxicity is largely overcome with copper supplements (Cox et al, 1960). Toxicity in the experimental animal requires levels hundreds of times greater than those normally consumed.

Deficiency of dietary zinc can be produced in the mouse within 4-8 weeks provided care is taken to avoid sources of zinc contamination. Mice fed a zinc deficient diet will show growth failure and loss of appetite within 2 weeks of withholding zinc from the diet. The mice will develop hypogonadism, acrodermatitis-like skin lesions, gastrointestinal malfunction, and immune dysfunctions (Underwood, 1977).

Indices of zinc status

Tissue levels of zinc, zinc-dependent metalloenzymes, and various immune parameters have all been utilized as indices of zinc status. The validity of the use of these various measurements in clinical medicine has been reviewed by both Solomons (1979) and Patrick (1983). Little work is available concerning evaluation of zinc status of the experimental animal where availability of tissues and lifespan are not constraining factors.

Analysis of these studies is confounded by the following factors. 1) One must distinguish between zinc depletion and associated weight loss. 2) Interpretation will differ depending whether the animals are young and growing or adults. 3) Finally, since little is known about interaction of various zinc pools in the body it is difficult to label an animal absolutely zinc deficient especially when there are metabolic and/or hormonal derangements.

Tissue levels of zinc

Animal data is limited for levels of zinc in various tissues. Underwood (1977) published values of normal levels of zinc in many tissues in rat, pig, monkey and man. Zinc analysis of tissues to determine their responses to zinc deficiency or toxicity has been performed in a few studies.

Muscle

This is an important tissue to consider since it is the major component of lean body mass. Zinc content of muscle varies with color and functional activity. The zinc content of red muscle is three time the zinc content of white muscle, therefore one must only compare values of zinc content in muscle with caution (Cassens et al, 1967).

Zinc deficiency did not alter the zinc content of muscle in calves or goats (Miller, 1969). In rats, Jackson et al (1982) found no difference in levels of zinc in red or white muscle in the deficient or control group whereas Prasad et al (1967) found a significant decrease in the zinc deficient group. Another group studied a variety of muscles and found only the soleus (a primarily red muscle) to reduce its zinc concentration upon zinc deficiency (O'Leary et al, 1979). Zinc supplementation of rats (1,000 ppm) did not alter the zinc content of muscle (Chvapil et al, 1974).

Bone

Zinc is necessary for proper bone formation and many animals develop skeletal abnormalities when they are zinc deficient. Bone has one of the highest zinc concentrations of all tissues. Studies on turnover rates of zinc indicate that the skeleton does not function as a reservoir of zinc in times of dietary inadequacy (Murray et al, 1981). Release of zinc from the bone seems to be secondary to normal bone turnover.

The bone does seem to be a good indicator of zinc status in growing animals. Femur zinc declined significantly in zinc deficient rats (Prasad et al, 1967; Brown et al, 1975; Jackson et al, 1982) and correlated well with zinc level of the diet (from 0-20 ppm) in rats (Luecke et al, 1969). Tibia zinc declined significantly within two weeks on a zinc deficient diet in growing mice (Prasad, 1982b) and rats (Meydani et al, 1982). Maternal tibia zinc declined throughout gestation for rats on a zinc deficient diet (Herzfeld et al, 1983).

Liver

Liver appears to be the major organ for the metabolism of newly absorbed zinc. Studies have shown zinc will induce hepatic metallothionein synthesis (Menard et al, 1981) and accumulate within (Whanger et al, 1981). This protein binds 6-7 metal ions per molecule and acts in the absorption and storage of many essential and toxic metals including copper, zinc and cadmium (Brady, 1979).

In zinc deficient pigs (Burch, 1974), calves, goats, (Miller, 1969) there was a significant decrease in liver zinc content. Liver zinc was reported declined in zinc depleted rats by one group (Jackson et al, 1982) but not by another (Prasad et al, 1969). Rats supplemented 1,000 or 2,000 ppm zinc for two weeks showed the greatest accumulation of zinc in the liver (Chen et al, 1974; Chvapil et al, 1974).

Testes

Zinc deficiency results in hypogonadism and it appears that zinc is essential for spermatogenesis and testosterone steroidgenesis (Prasad, 1982a). The weight and zinc content of the testes of zinc deficient rats declined to approximately half that of normal rats (Prasad et al, 1967; Williams et al, 1970; Jackson et al, 1982). Supplementation of rats up to 2,000 ppm did not increase zinc levels of the testes (Chen et al 1974; Chvapil et al, 1974). This indicates that zinc levels of testes may be be a useful measurement in deficiency states.

Plasma

Plasma zinc falls quite early in the event of dietary deficiency in the experimental animal. It has often been used as an indicator of zinc deficiency but one must interpret plasma levels with caution. In a simple dietary restriction of zinc from the diet, low plasma levels can indicate the beginning of negative zinc balance. However, only partial repletion of body zinc will bring plasma zinc levels back to normal (Jackson et al, 1982). Also in several stress states low plasma zinc often reflects ~~a~~ redistribution of zinc to other tissues of the body (Prasad, 1982). As an isolated finding abnormal plasma zinc will not provide much information.

Pancreas

The zinc content of the pancreas has been shown to reflect the available insulin content. The zinc content of the pancreas declined in pigs (Miller et al, 1970) and rats (Miller et al, 1967) in zinc deficiency. Zinc deficiency also results in B-cell degranulation (Boquist et al, 1969). Maternal zinc deficiency resulted in a lower insulin content and a decreased number of B-cells in fetal pancreata (Robinson et al, 1981).

Other tissues

Other tissues such as hair, skin, erythrocyte, and leucocyte have been utilized as indices of zinc status with the latter seeming the most promising at the moment (Jones et al, 1981).

Zinc and glucose homeostasis

Zinc has an integral role in storage, secretion, and synthesis of insulin via mechanisms not yet well elucidated. Zinc is necessary for insulin crystallization in most mammalian species (Scott, 1934), and the amount of zinc in B-cells corresponds with levels of insulin present (Boquist et al, 1969).

Zinc deficient animals have impaired glucose tolerance. Both intraperitoneal and intravenous glucose tolerance curves were elevated in zinc deficient hamsters (Boquist et al, 1969). There is an impaired intravenous (Huber et al, 1973) and intraperitoneal (Quarterman et al, 1966) glucose clearance in zinc deficient rats. Oral glucose tolerance is not impaired (Hendricks et al, 972; Brown et al, 1975) which may be due to a greater stimulation of insulin secretion by glucose given orally.

It has been proposed that impaired glucose tolerance in zinc deficiency may be due to a decrease in insulin secretion or an increased rate of insulin degradation (Hendricks et al 1972). Zinc deficient rats have lowered plasma insulin levels after glucose stimulation (Hendricks et al, 1972; Quarterman et al, 1972). As of yet, it is difficult to ascertain if zinc acts at the level of the pancreas , peripherally, or both.

Zinc and immunity

The presence of zinc is necessary for cell differentiation and replication. As many immunological events are associated with these two functions, deficiency of zinc results in immune dysfunction. Zinc deficiency produces thymic hypoplasia resulting mainly from involution of the cortex. This first begins at around 2 weeks, and if deficiency is prolonged, only thymic remnants will be seen (Fraker et al, 1978). Zinc deficiency has also been shown to produce a markedly atrophied thymus in pigs (Miller et al, 1968), rats (Quarterman, 1973), and calves (Brummerstedt, 1971). Cellular depletion and atrophy of T-cell dependent areas of lymph nodes is observed in zinc deficient mice (Tanaka et al, 1978).

The thymus is the site of maturation of the thymus dependent T-lymphocytes which are essential for cell mediated responses and which also serve as helper T-cells for most humoral responses. Clearly involution of the thymus in zinc deficiency could express itself as T-cell dysfuntion. Many studies in the zinc deficient animals have validated this assumption.

There are decreased levels of thymic hormone in zinc deficient mice (Iwata et al, 1979) and rats (Chandra et al, 1980c). This hormone is required for precursor thymocytes to develop into mature lymphocytes. T-cell mediated functions such cytotoxicity, and natural killer cell activity, are reduced in zinc deficient mice (Fernandes et al, 1979). Zinc deficient mice develop a marked inability to generate T-killer cells in the face of tumour challenge (Fernandes et al, 1979; Frost et al, 1981) and to react to a contact sensitizing agent (Fraker et al, 1979). They are more vulnerable to certain pathogens (Fraker et al, 1982a) and have impaired delayed type hypersensitivity reactions (Fraker et al, 1982b). Most importantly, T-cell function is restored upon refeeding with a zinc adequate diet, providing good evidence of a link between zinc and immune function (Fraker et al, 1978; Frost et al, 1981).

Zinc deficiency is a stress situation and as such zinc deficient rats (Quarterman, 1973) and mice (De Pasquale-Jardieu et al, 1979) will exhibit adrenal hypertrophy and elevated glucocorticoid levels. This high glucocorticoid level is not solely responsible for changes in lymphoid organs. Adrenalectomized, zinc deficient mice displayed a large decrease in humoral immune reponse (DePasquale-Jardieu et al, 1980). Histologically, a zinc deficient mouse displays differences from one given hydrocortisone (Iwata et al, 1979). In zinc deficiency, the germinal centers of the lymph nodes are not involuted and only T-cell dependent areas are affected (Schloen et al, 1979). A high steroid dose leads to involution of the germinal centers of lymph nodes and no particular predilection for T-cell dependent areas. The effects of zinc deficiency on immune function are no doubt partly due to high glucocorticoid levels but other factors are involved as well.

Some mechanisms have been proposed to explain the underlying molecular mechanism of immune dysfuntion in zinc deficiency. Enzyme defects, associated with immune dysfunction, are found in purine nucleotide degradation pathways. 5'nucleotidase is an enzyme which catalyzes the dephosphorylation of adenosine monophosphate (AMP), inosine monophosphate (IMP), and guanosine monophosphate (GMP) to adenosine, inosine, and guanosine respectively. Deficiency of this enzyme has been observed in patients with hypogammaglobinemia (Edwards et al, 1978). The next step in this pathway is the deamination of adenosine to inosine by adenosine deaminase (ADA). Deficiency of ADA has been reported in several children with impairment of T-cell and B-cell immunological function (Osborne, 1981). The next step in the purine degradation pathway is catalyzed by purine nucleoside phosphorylase (PNP). Guanosine or inosine react with organic phosphate to form guanine or hypoxanthine respectively, with the release of ribose-1-phosphate. Deficiency of this enzyme is associated with severe T-cell immunodeficiency (Giblett et al, 1975).

Recent reports indicate that zinc may play a regulatory role in purine metabolism (Prasad et al, 1981). Zinc deficiency results in severe impairment of T-lymphocyte function (Fraker et al, 1978; Fernandes et al, 1979). Altered activity of purine nucleotide degradation enzymes may therefore be the biochemical basis for immune dysfunction of the zinc deficient animal. Nucleoside phosphorylase activity was decreased in zinc deficient mice (Prasad et al, 1981). Increased activity of adenosine monophosphate deaminase was reported by Brody et al, 1977 in zinc deficient rats. The increased plasma ammonia levels found in zinc deficiency may thus be the result of increased deamination of adenosine (Rabbani et al, 1978). Zinc appears to inhibit AMP deaminase in vitro (Yoshino et al, 1978).

Adenosine deaminase catalyzes the irreversible deamination of adenosine to inosine as well as deoxyadenosine to deoxyinosine. It appears that adenosine deaminase is the product of a single gene although many isoenzymes exist (Giblett, 1980). These "tissue specific" isoenzymes are believed to be formed by a complex of the catalytically active adenosine deaminase (M.Wt. 35,000) and a catalytically inactive tissue specific combining protein (M.Wt. 250,000) (Swallow et al, 1977).

A number of mechanisms have been proposed to account for lymphocyte dysfunction in ADA deficiency. 1) Deoxyadenosine is a substrate for ADA and elevated concentrations of dATP are present in blood cells of ADA deficient patients (Coleman et al, 1978). Ribonucleotide reductase is subject to allosteric inhibition by dATP which may lead to decreased DNA synthesis detrimental to the dividing lymphocyte (Fox et al, 1979). 2) Increased intracellular cAMP concentration caused by accumulation of adenosine may be the basis of immunodeficiency. Adenosine can increase cAMP concentrations in lymphocytes by direct stimulation of adenylate cylcase (Uberti et al, 1979). 3) It is possible that accumulation of adenosine and deoxyadenosine causes an accumulation of S-adenosyl homocysteine.

Accumulation of this compound can be directly toxic to the cell and can also inhibit methylation reactions leading to a decreased concentration of S-adenosyl methionine, a methyl donor to DNA and RNA. These hypotheses offer some speculation as to the underlying molecular disturbance of immune dysfunction in zinc deficiency.

This review has shown that certain zinc dependent physiological functions are aberrant in the ob/ob mouse. It was therefore of interest to investigate further the zinc status of the ob/ob mouse by measuring tissue levels of zinc and to determine the effect of zinc supplementation on tissue levels of zinc in the ob/ob mouse as well as other physiological parameters.

MATERIALS

Special chemicals and reagents were obtained as follows:

Sigma Chemical Co. (St.-Louis, Mo.) supplied the the following: penecillin, streptomycin, and mitomycin C.

New Enland Nuclear (Lachine, Que.) supplied the following: [^{3}H]-thymidine (Specific Activity= 20.0 Ci/mmol); [2-^{3}H]-adenosine (Specific Activity= 16.3 Ci/mmol); [8-^{14}C]-adenosine (Specific Activity= 45.5 mCi/mmol; [^{125}I]-labelled pork insulin (Specific Activity= 100 uCi/ug).

Concanavalin A was obtained from Calbiochem. Sheep red blood cells were obtained from Qualicam Laboratories, Ottawa. Calf serum, fetal calf serum and RMPI 1640 medium were obtained from Flow Laboratories. Ultrex nitric acid was obtained from J.T. Baker Chemical Company. Zinc standard solution (1,000ul/l) was obtained from Canlab. Eastman Chromagram Sheets were obtained from Fisher Laboratory. Rat insulin standard (25 U/mg) was obtained from Novo Research Laboratories.

All other chemicals were reagent grade of the highest purity available commercially.

METHODS

1) Animals

Male C57BL/6J ob/ob mice and their lean counterparts were obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A., at 7-8 weeks of age. They were used in various experiments at 9-12 weeks of age. The animals were kept in a temperature controlled room at $24 \pm 1^{\circ}C$ with 12 h light cycles. The animals had free access to food and water.

2) Treatments

The animals were housed in individual cages during the treatment periods. Mice of the same age (9-12 weeks old) and approximately the same weight were assigned to the following experimental groups; (1) lean control, (2) Zn-supplemented lean mice, (3) obese control, (4) Zn-supplemented obese mice.

The supplemented diet consisted of ground rat Purina chow with 1,000 ppm Zn added in the form of zinc carbonate. The control diet consisted of ground rat Purina chow with a reported zinc content of 30.3 ppm zinc. All the diets were analyzed for zinc by atomic absorption spectrophotometry. The samples were prepared for analysis by wet ashing in a nitric-perchloric acid mixture. (Horowitz 1965), see below.

Food consumption was monitored daily to ensure that the amount of food consumed by the zinc supplemented and control groups was not significantly different. These values were also compared with those of mice consuming pelleted rather than ground chow to determine if the change in consistency was interfering with consumption patterns.

The mice were weighed every 2-3 days and were maintained on the specified diet 21-25 days prior to sacrifice, as specified in the legends to tables and figures.

3) Sacrifice and dissection of mice

Mice were killed by decapitation between 9-11h. If blood was taken the decapitated mice were bled directly into 12 ml. polyethylene tubes. All other required organs were quickly dissected and placed in pre-weighed polyethylene bags. The organs were weighed then frozen at -20° C until zinc analysis was performed. In the case of the thymus, pancreas, and brown adipose tissue the surrounding fat tissue was removed under the dissecting microscope prior to weighing and storage.

4) Body moisture and fat composition

The carcasses were first cut up into small pieces. Body moisture was determined by freeze drying the carcasses until constant weight was obtained. The weight of the dried material was obtained and the loss of weight assumed to be moisture.

Body fat composition was determined by extraction of the dried carcass for 10-12 h with petroleum ether in the Soxhlet apparatus. The bulk of the solvent was recovered in the upper portion of the apparatus after removing the extraction thimble. The fat in the flask was brought to constant weight by overnight drying in an oven (100°C). The weight of the fat residue in the flask was taken to represent the fat content.

Fat free dry weights of liver and brown adipose tissue of lean and obese mice was determined. The tissues were dissected free of adhering fat and connective tissue, then weighed. Each tissue was homegenized in 5 ml of choroform;methanol (2:1) in a Polytron homogenizer at one-half maximal speed for approximately one minute. The homegenates were filtered through previously washed and dried filter papers and rinsed with 5 ml chlorofrom methanol (2:1). The filter paper with the dried, defatted tissue was placed in a dessicator overnight and weighed the next morning.

5) Glucose tolerance test

Mice which had been on a specified diet for 21 days were fasted overnight (21:00 h to 9:00 h) prior to the test. After removal of a 0-time sample, mice were injected intraperitoneally with a 25% glucose solution at a dosage of 1g/kg body weight. Blood samples were obtained from the tail vein at the times indicated and collected in heparinized capillary tubes. The plasma was separated by centrifugation and the glucose and insulin were measured.

6) Glucose Determinations

Plasma glucose was measured by a glucose oxidase method using a Beckman Glucose analyzer (Beckman Instruments, Fullerton, California). After separation of the plasma by centrifugation, a 5 ul portion of plasma was diluted with 10 ul of distilled water prior to analysis.

7) Insulin Determinations

Insulin was measured by a double antibody radioimmunoassay according to the method of Dalpé-Scott et al (1982). All assays were done at 4°C in 0.04 M sodium borate buffer (pH 8.0), containing 1% bovine serum albumin. Briefly, the antibodies [antiinsulin serum (AIS) and anti-guinea pig globulin (AGG)] combined with guinea pig serum were incubated for 24h at 0°C. 25-50 ul of the plasma sample or standards were then added and this was incubated for a further 6h. A known quantity of [^{125}I]insulin was added to each tube and the mixture was incubated a further 18h. The tubes were centrifuged at 2,000g for 30 min, the supernatant decanted and the tubes wiped. The precipitate was counted in a gamma counter (Beckman) for 10 min.

8) Zinc Analysis of Tissues

Zinc analysis was performed by atomic absorption spectrophotometry. Appropriate dilutions of a 1,000 ug/l Zn stock solution (Canlab) were made to obtain standards containing 0.100, 0.200, and 0.300 ppm Zn. The standards were made up in deionized water and slightly acidified with 11.6 M HCl to be in the same range of acidity as the samples. Unknowns were diluted to be within the working range of the standards. The Instrumentation Laboratory Model 457 atomic abosorption spectrophotometer (Fisher Scientific, Missisauga, Ontario) was used with the following settings: wavelength 213.9 nm, and current 3.5 mA.

9) Preparation of samples for zinc analysis

A)muscle, liver, femur, thymus, brown adipose tissue, testes

The tissues were freeze dried and their dry weight obtained. They were prepared for analysis by one of the following two methods.

Method I-Samples were ashed overnight(16-18 h.) in an oven at 400° C in acid soaked crucibles. They were then digested 60-90 minutes with 3-5 mls 16M nitric acid (Ultrex Grade, Baker Laboratories). The acid was evaporated off and the samples were again placed in the oven at 400°C for one hour. The resulting white ash was diluted appropriately with 1.0 N HCl and the zinc concentration was determined.

Method II- The tissues were prepared for analysis by the method of Gaffin, 1979. Weighed samples (0.2-0.3 g) were placed in the Teflon cup of a stainless steel decomposition vessel (Uniseal Decomposition Vessels, Ltd.) and 2.5 ml of 16M nitric acid was added. The Teflon lined cap of the vessel was screwed down and the unit placed in a preheated laboratory oven or a thermostatically controlled heating block at 135°C for 120 min. After cooling the samples were quantitatively transferred into 25 ml flasks, evaporated to dryness on an electric hot plate. The residue was diluted appropriately with 1.0N HCl and the zinc concentration determined on the spectrophotometer.

B) plasma

Plasma was obtained by centrifugation of whole blood, pipetted into a clean Eppendorf tube, diluted 1:10 with 0.5N HCL and the zinc concentration determined directly.

10) Cunningham Assay- Plaque Cell Assay

This method is a modification of the classical Jerne plaque method which uses a supporting agar medium for the detection of antibody forming cells (Jerne et al 1963). In the Cunningham method lymphoid cells and target erythrocytes are incubated together as a monolayer without any supporting medium (Cunningham 1965). This can be accomplished by placing three pieces of double sided tape each 1 cm. wide on a clean microscope slide (75 x 25 mm) dividing it into two equal areas. Another clean slide is pressed firmly on top to form two shallow chambers . The reaction mixture is pipetted in. This method allows greater sensitivity and easier visualization of plaque forming cells than the Jerne method (Cunningham 1967).

A) Preparation for assay

i) Mice: Each mouse was injected intraperitoneally with $1x10^8$ cell/ml of sheep red blood cells (Qualicum Scientific) and on Day 5 postinjection the spleens were removed and used for assay.

Preparation of spleen cell suspension; Spleens were chopped finely in Hank's medium and this suspension was filtered and squeezed through a double layer of gauze into a 12 ml conical polyethylene tube. The cells were washed three times in 5-6 ml of isotonic saline and centrifuged at 1500-1700 rpm for 10 min between washings. The washed cells were resuspended in Hanks medium at a concentration of 1×10^7 cells/ml. The composition of Hank's medium was as follows (in g/l); KCl; 0.4 ; KH_2PO_4; 0.06; $MgCl_2 \cdot 6H_2O$;0.10; $MgSO_4 \cdot 7H_2O$;0.10 ;NaCl; 8; $NaHCO_3$;0.35; Na_2HPO_4 ;0.065; glucose;1.0 ;$CaCl_2$;0.2.

ii) Preparation of sheep red blood cells (SRBC); The cells were washed two to three times in 4-5 ml isotonic saline, spun at 1600-1900 rpm for 10 min until the supernatant was clear. The final pellet was resuspended in Hank's medium at a concentration of 2.0-2.4 x 10^8 cells/ml.

iii) Preparation of complement: Guinea pig serum was absorbed with SRBC at a volume of 10:1. This was placed at 4°C for one hour then centrifuged at 1500 rpm for 10 min. For the assay the complement was diluted 1:3 in Hank's solution.

B) Assay

A reaction mixture of 20 ul SRBC, 20 ul complement, 20 ul Hanks medium, and 20 ul lymphoid cells was well mixed in a microliter plate. Each measure was made in duplicate. Blanks consisted of either no lymphoid cells or no complement. The reaction mixture was pipetted into slide chambers, sealed with Vaseline-parafilm and incubated 1 hr at 37° C. Plaque forming cells were visualized and counted under the microscope.

11) Mitogen assay (Concanavalin A)

A) Preparation of cells for culture

Spleens were removed aseptically and placed on a sterile meshed screen in a Petri dish and covered with RMPI 1640 medium (Flow Laboratories). The spleen was squeezed through the screen with a rubber stopper and the screen well rinsed with medium. The contents of the Petri dish were transferred into a 15 ml sterile conical tube and RMPI 1640 medium was added to 10-12 ml. This was underlayered with 2-3 ml calf serum and allowed to stand 5 min tp precipitate clumps. The top layer was transferred to a clean 15 ml sterile conical tube and centrifuged 5 min at 1500-1700 rpm. The resultant pellet was resuspended in medium and approximately 2 ml of sterile NH4Cl (.17M) was added to lyse the red blood cells. This cell suspension was allowed to stand 5 min. The medium was made up to 10-12 ml, underlayered with calf serum and centrifuged as above. The cells were washed once more with RMPI 1640 medium and resuspended to the desired concentration. All cell counts were done with gentian violet dye.

B) Assay

The cell density was adjusted to 3 x 10^6 cells/ml in RMPI 1640 complete medium (RMPI 1640 medium supplemented with 5% fetal calf serum, 2mM glutamine, 1,00 U/ml penecillin G, and 1,00 ug/ml of streptomycin, and buffered with 20 mM Hepes pH 7.2). Concanavalin A was dissolved in RMPI 1640 complete media and added to the cell cultures at a concentration of 4 ug/ml cells. The cultures were prepared in triplicate and incubated for 1,2, or 3 days at 37°C, 95% humidity.

The cultures were pulsed with 2 uCi [methyl ^{3}H] thymidine and incubated a further 30 min. The cells were harvested using a Millipore apparatus and filtered through glass fiber filters (Whatman 2.4 cm). The filters were first washed with 10 ml of isotonic saline and then 10 ml of 5% trichloro acetic acid. The filters were dried overnight then counted 2 min in 10 ml Scintiverse in an Isacap liquid scintillation counter (Searle Instruments).

12) One-way mixed lymphocyte reaction

A) Cell preparation

The spleen cell suspensions were prepared as for the mitogen assay except the red blood cells were not lysed. Responder cells were obtained from spleens of C57BL/6J ob/ob and +/? mice. The cell concentration was adjusted to 4 x 10^6 cells/ml.

The stimulator cells were obtained from spleens of BalbC mice. They were treated with Mitomycin C (40 ug/ml cells) and incubated 30 min at 37°C. The cells were washed twice with RMPI 1640 medium and the cell concentration readjusted to 4 x 10^6 cells/ml in complete medium (as defined above).

B) Assay

Each culture was prepared in triplicate. For each single spleen cell suspension the following conditions were set up;

a) 0.25 ml stimulator cells + 0.25 ml responder cells

b) 0.50 ml stimulator cells alone

c) 0.50 ml responder cells alone

Cultures were incubated for 1, 2, 3, 4, or 5 days at 37°C. In the last 8 h of culture they were pulsed with 2uCi of [^{3}H]-thymidine. The cells were harvested as described for the mitogen assay. The filters were counted 2 min in 10 ml Scintiverse.

13) Adenosine Deaminase Assay

A) Preparation of cell extract

The tissues were dissected out, weighed and placed on ice in 50 mM potassium phosphate buffer,. pH 7.0. They were well homogenized in a Potter- Elvejhem homogenizer in the same buffer at approximately 10% w/v. The homogenate was centrifuged at 20,000 g for 20 min and the supernatant fraction was quick frozen in dry ice/ethanol and assayed within one month for adenosine deaminase activity. Protein was measured by the Coomasie Blue method of Bradford (1976).

B) Enzyme assay

The assay is based on the chromatographic separation of adenosine from inosine and all its by products. Adenosine deaminase activity was assayed by conversion of adenosine to inosine. Either [8-^{14}C] adenosine or or [2-^{3}H] adenosine was used; as specified in individual tables and figures. The radioactivity was diluted to obtain a specific activity of either 5 mCi/mmol for [^{14}C] adenosine or 16.3 mCi/mmol for [^{3}H] adenosine. The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, containing 200 uM adenosine.

For the assay, 100 ul of supernatant was added to 50 ul of reaction mixture to yield a final volume of 150 ul. After incubation at 37^{o}, the reaction was terminated by adding 10 ul of 5M perchloric acid and placing on ice for 15 min. 10 ul of 5M KOH was then added to neutralize and precipitate the perchlorate. This was left on ice for a further 15-30 min and centrifuged at 100g for one minute in an Eppendorf 3240 centrifuge. Zero time values were obtained by the addition of perchloric acid to the reaction mixture prior to incubation.

A 20 ul aliquot from each reaction mixture was spotted on an Eastman Chromagram cellulose sheet (20x20 cm). These sheets were divided into 9 strips with a 1 cm margin on each side of the sheet. Each spot had 3-4 ul of a standard solution containing 5 mM each of AMP, xanthine, hypoxanthine, inosine, adenine, and adenosine to aid in the visualization of the separated compounds. The chromatographic plates were developed in acetonitrile: NH4 acetate (1.0 M ,pH 7.0): NH3 60:30:10 v/v/v. This step was completed in 45- 60 min. The plates were left to dry at room temperature for 1 hr and the spots were visualized by ultra violet light. This method allows separation of compounds into five main bands 1) nucleotides 2) xanthine and uric acid 3) inosine and hypoxanthine 4) adenine and 5) adenosine. These five spots were cut out and were each counted separately in 10 ml of Aquasol for 10 min.

Activity of adenosine deaminase was expressed as nmol of adenosine converted/ μg protein/min.

14) Statistical analysis

The data was analyzed using the Students t-Test or analysis of variance and the results are expressed as mean ±SEM. Differences between means were considered significant when p was smaller than 0.05.

RESULTS

Preliminary examination of the literature revealed various zinc dependent physiological functions were aberrant in the ob/ob mouse. Disorders of immunity had been reported in the ob/ob mouse (Sheena et al, 1979; Chandra et al, 1980a); decreased spleen and thymus weights reported in the ob/ob mouse were reminiscent of those found in zinc deficient mice (Fraker et al, 1978). The insulin resistance and impaired glucose tolerance of the ob/ob mouse (Bray and York, 1979) were similiar to that of zinc deficient mice (Huber et al, 1973). Infertility in the obese mouse manifested itself as reduced size and weight of gonads and decreased plasma levels of sex hormones (Bray and York, 1979). Zinc deficient animals also exhibit hypogonadism and reduced concentrations of sex hormones (Underwood, 1977). The obese mouse has reduced body temperature (Bray and York, 1979), as do zinc deficient animals (Topping et al, 1982). Other symptoms of zinc deficiency such as skin or hair abnormalities, gastrointestinal malfunction or collagen disturbances had never been reported in the obese mouse. Finally, the obese mouse is hyperphagic while zinc deficient animals are anorectic. To assess the zinc status of the ob/ob mouse it was decided to place the ob/ob mouse and its lean counterpart on a control or zinc-supplemented diet (1,000 ppm) for a period of three weeks and to then measure a variety of zinc-related parameters.

1) Effect of Zinc supplementation on growth, food consumption, and tissue levels of zinc

When establishing the protocol for these experiments it was first necessary to ascertain that high levels of zinc in the diet did not interfere with food consumption or growth patterns. Table 1 show that the zinc content of the supplemented diet was essentially as calculated by addition of zinc carbonate i.e. 1,000 ppm. The zinc content of the control diet (Purina ground rat chow) was measured to be 50% higher than the value published by Purina company (45.7 vs. 30.3 ppm). This , however, represents a twenty-fold difference in the zinc content of the control versus zinc supplemented diets.

It has been reported that both zinc deficiency or toxicity can cause anorexia in animals (Underwood ,1977) In the lean mice the high zinc diet had no effect on amount consumed as compared to the lower zinc diet. The obese mice consumed moderately less food on the high zinc that on the control diet. They still consumed twice the amount of food of lean mice. It has been the experience in our laboratory that the obese mouse placed on a ground chow diet rather that pelleted chow immediately increases its food intake. The amount of food consumed by the obese mouse on the high zinc diet was therefore more comparable to the amount it consumes on a pellet diet.

The lower food consumption of the obese-zinc supplemented mouse did not reflect itself in any differences in weight gain or body composition (See Table 1). The results do illustrate the extent of adiposity of the ob/ob mouse. At 9-12 weeks of age lean mice have 11% body fat while obese mice have 52% body fat. These results agree with previously published values for fat content of lean and obese mice at this age (Dubuc, 1976). The high % of body fat of the obese mouse results in a proportional decrease in moisture content.

In attempting to document the zinc status of the ob/ob mouse a number of tissues were analyzed for zinc content. Tables 2-7 report the results of tissue analysis for zinc.

Bone) The obese mice had significantly lower levels of zinc in the femur than lean mice (See Table 2). Zinc supplementation did not increase either the weight or zinc content of the femur in the obese mouse but resulted in increased weight of the femur in the lean mouse.

Reduced skeletal growth has been reported in the ob/ob mouse (Dubuc, 1975). There was, however, no difference found in the weight of the femurs between lean and obese mice in these experiments.

Muscle) The muscle weights of the obese mice are 30% lower than those of lean mice. Obese mice display low locomotor activity from 3-4 weeks of age (Yen et al, 1972; Joosten et al, 1974). Decreased activity, insulin resistance, and reduced protein deposition of lean tissue in the obese mouse all combine to explain the reduced muscle mass (See Table 3). The zinc content in muscle were not different between the four groups. There was tremendous variation within each group and whether this was due to contamination (especially by hair) or individual variation is uncertain at present.

Liver) The obese mouse has a much larger liver than the lean mouse (See Table 4). This is mainly due to increased hepatic lipogenesis and subsequent fat infiltration (Hems et al, 1975). Obese mice had a much higher zinc content in the whole tissue and if zinc content is expressed on a fat free dry weight basis, eliminating the large difference in fat between the lean and obese liver, the high zinc content in the liver of the obese mouse was quite evident. Zinc supplementation did not change the zinc content of the liver significantly in either lean or obese mice, however, in both lean and obese mice receiving zinc supplemented diets, there was a trend to higher zinc levels.

<u>Plasma)</u> The results indicate no significant differences in plasma levels of zinc between lean and obese mice whether they were on a control or zinc supplemented diet although again there was a trend towards higher levels in supplemented groups (See Table 5). The excess zinc in the supplemented diets was thus either not well absorbed and/or quickly redistributed to other body compartments. As both lean and obese mice showed the same behaviour, the fate of ingested zinc appears to be the same in both groups.

<u>Testes)</u> The testes of ob/ob mouse have been reported to be smaller that those of lean mice (Bray and York, 1971) but this was not found to be the case in the present experiments (See Table 6). The zinc content displayed wide variation but was not significantly different between lean and obese mice. It would probably be useful to study larger groups of mice as well as the effect of zinc supplementation on the weight and zinc content of the testes.

<u>Brown adipose tissue (BAT))</u> The brown adipose tissue of the obese mouse contained five times as much zinc as that in its lean counterpart (See Table 7). As in the liver; the fat content of BAT in obese mice is much higher than of lean mice and thus expressing the zinc content on a fat free dry weight emphasized the enormous quantity of zinc in the BAT of the obese mice.

Table 1- Zinc Content of Diet, Food Consumption, Body Weight, Body Fat, and Body Water for a 3-Week Feeding Period

	lean-control	lean-Zn-suppl.	ob/ob-control-	ob/ob-Zn-suppl.
Zinc Content of Diet (ppm)	45.7 ± 0.5	964 ± 100	45.7 ± 0.5	964 ± 100
Food Consumption (g/day)	$3.9 \pm .08^{a}$	$3.7 \pm .08^{a}$	$9.4 \pm .21^{b}$	$7.2 \pm .30^{c}$
Body Weight (g)				
Before	23.1 ± 0.9^{a}	$22.9 \pm 0,7^{a}$	45.1 ± 0.3^{b}	44.5 ± 0.7^{b}
After	24.3 ± 0.8^{a}	24.7 ± 0.6^{a}	48.9 ± 0.5^{b}	46.7 ± 0.4^{b}
Body Fat(%)	11.3 ± 0.9^{a}	9.1 ± 1.0^{a}	52.1 ± 1.0^{b}	51.2 ± 0.7^{b}
Body H_2O(%)	62.1 ± 0.9	64.7 ± 0.8	33.6 ± 0.8	33.7 ± 0.6

The experiments were performed as described in Methods. Body fat and body water are expressed as % of total wet weight of the carcass. The results are averages±SEM. The zinc content of the diet was measured three times. All other values represent a minimum of 5 animals. Values in each horizontal line followed by different letters are significantly different from each other ($P < 0.05$).

Table 2- Weight and Zinc Content of the Femur

Group	Weight (mg)	Zn/tissue(ug)	Zn/Dry Wt (ppm)
lean-control	34.5 ± 1.3^{a}	$4.24 \pm .21^{a}$	164.5 ± 3.9^{a}
lean-Zn suppl.	44.2 ± 3.7^{b}	$4.94 \pm .20^{a}$	165.8 ± 3.5^{a}
ob/ob-control	37.1 ± 2.7^{a}	$3.32 \pm .24^{b}$	128.4 ± 1.9^{b}
ob/ob-Zn suppl.	42.2 ± 2.1^{a}	$3.83 \pm .24^{b}$	132.8 ± 1.8^{b}

The tissues were obtained as described in the Methods section. The control and supplemented animals received ground rat chow with zinc concentrations as shown in Table 1. The animals were on a specified diet 21-25 days. Zinc/tissue refers to the total zinc content of the tissue. Zinc/dry wt. refers to mg zinc/ kg dry wt. of tissue. The results are expressed as mean ±SEM for at least 4 animals. Values in each column followed by different letters are significantly different from each other ($P < 0.05$). Data in each column not followed by letters are not significantly different from each other.

Table 3- Weight and Zinc Content of the Triceps Muscle

Group	Weight (mg)	Zn/Tissue(ug)	Zn/Dry Wt (ppm)
lean-control	181.1±11^{a}	4.29+±.33	77.6±1.7
lean-Zn suppl.	206.0±11^{a}	4.91±.64	75.9±2.3
ob/ob-control	126.5±10^{b}	4.26±.65	113±6.4
ob/ob-Zn suppl.	136.8±10^{b}	4.29±.90	100±7.7

The tissues were obtained as described in the Methods section. The results are expressed as means ±SEM for at least 6 animals. For other details see Legend to Table 2.

Table 4- Weight and Zinc Content of the Liver

Group	Weight (g)	Zn/Tissue (ug)	Zn/Dry Wt (ppm)
lean-control	1.27±0.7^{a}	31.4±1.1^{a}	118.2±2.8^{a}
lean-Zn suppl.	1.32±0.4^{a}	41.4±4.0^{a}	130.5±9.5^{a}
ob/ob control	3.26±0.3^{b}	72.3±5.1^{b}	158.6±8.1^{b}
ob/ob-Zn suppl.	2.80±0.3^{b}	72.0±5.3^{b}	174.1±9.0^{b}

The tissues were obtained as described in the Methods section. The fat free dry weight was .21 g /g wet wt; and .15 g /g wet wt. for lean and obese mice, respectively. The results are expressed as means ±SEM for at least 6 animals. For other details see Legend to Table 2.

Table 5- Zinc Concentration of Plasma

Group	Zinc (ppm)
lean-control	1.35±.09
lean-zinc suppl.	1.60±.17
ob/ob-control	1.28±.08
ob/ob-zinc suppl	1.47±.09

The plasma was obtained as described in the Methods section. The results are expressed as means ±SEM for a minimum of 10 animals. For other details see Legend to Table 2.

Table 6- Weight and Zinc Content of the Testes

Group	Weight (mg)	Zn/tissue	Zn/Dry wt.
lean-control	168.0±14.3	8.96±1.2	287±25
ob/ob-control	175.9±5.7	11.6±3.8	449±107

The tissues were obtained as described in the Methods section. Results are expressed as means ±SEM for a minimum of 4 animals. For other details see legend to Table 2.

Table 7- Weight and Zinc Content of BAT

Group	Weight (mg)	Zn/Tissue (ug)	Zn/Dry Wt (ppm)
lean-control	84.8±5.6^b	9.0±1.3^a	124.2±14.5^a
ob/ob-control	283±24^a	52.4±5.6^b	1062±160^b

The tissues were obtained as described in Methods. The fat free dry weight was .86 g/g wet wt; and .18 g/g wet wt. for lean and obese mice respectively. The results are means ±SEM for 5 animals. For other details see Legend to Table 2.

2) Effect of Zinc Supplementation on Glucose Tolerance

Glucose tolerance has been shown to be impaired in zinc-deficient animals (Quarterman, 1966; Huber et al, 1973). Obese mice also exhibit defective glucose tolerance and because of the role zinc plays in insulin secretion and storage the efffect of dietary zinc supplementation on in vivo glucose tolerance was assessed. The results of the glucose tolerance test on the four groups of mice are given in Figure 1. No differences in glucose tolerance were found between control or zinc supplemented groups for both lean and obese mice. As previously described, the ob/ob mouse had a high fasting plasma glucose level, an exaggerated response to an intraperitoneal glucose load, and sustained hyperglycemia during the rest of the test (Herberg et al, 1970; Bégin-Heick et al, 1974; Dubuc, 1976).

The plasma insulin values following the glucose load in the obese groups are presented in Table 8. There was no difference in plasma insulin in the control or zinc supplemented group for the first 60 minutes of the test. At 90 min, the obese zinc-supplemented group exhibited an elevated insulin level as compared to the obese-control group. This did not coincide with a concomitant reduction in plasma glucose levels. Zinc supplementation did not significantly alter peripheral glucose utilization in the ob/ob mouse.

Fig. 1. Glucose tolerance tests in lean and obese mice on a control or zinc-supplemented diet

The mice were fasted overnight , as described in Methods. After removal of a 0-time sample, glucose (1g/kg body wt) was injected intraperitoneally and blood samples obtained at the times indicated. Each point represents the mean ± SEM for five animals.

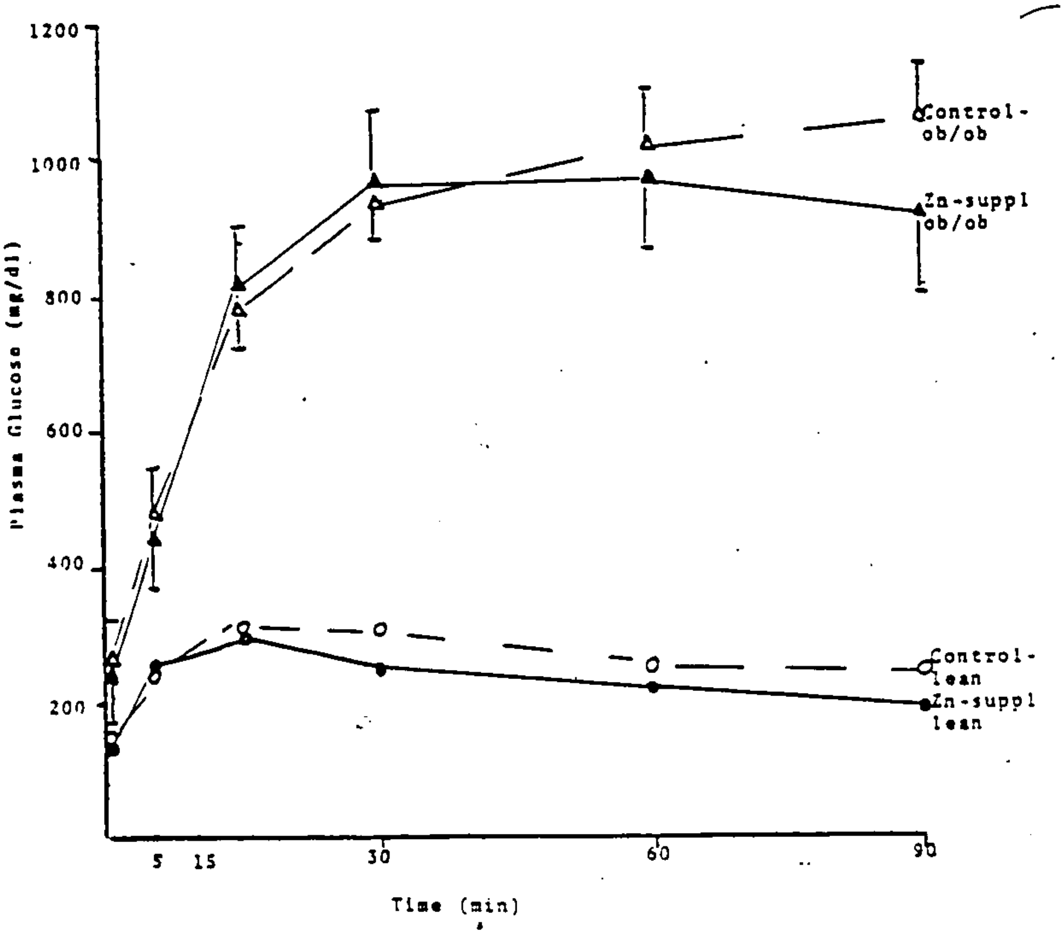

Table 8- Plasma Insulin Levels in Obese Control and Zinc Supplemented Mice Following a Glucose Load (1g/kg)

Time (min)	Plasma Insulin (ng/ml plasma)	
	ob/ob control	ob/ob Zn-suppl.
0	8.4±2.6	8.4±1.2
5	6.3±1.6	8.3±2.4
15	7.4±1.5	7.6±1.4
30	11.7±1.9	8.8±2.7
60	5.8±1.1	9.5±1.7
90	5.5±1.0[a]	10.3±1.6[b]

Plasma insulin was measured as described in the Methods section. Results are given as means ±SEM for 5 animals. Values in each horizontal line followed by different letters are significantly different from each other ($P<0.05$).

3) Immune Assays on lean and obese mice

An adequate zinc status is necessary for proper immune function (Prasad, 1978). A number of assays have been developed to test the immunocompetence of an individual and many investigators have utilized immune assays as direct indices of zinc status. Spleen cells from lean and obese mice were tested in various assays to determine their ability to proliferate and to produce antibodies.

Blastogenic Responses of Lean and Obese Mice:

Lymphocytes of one mice strain, when cultured with lymphocytes of another mice strain respond with blastogenesis and mitosis to the alloantigens on the latter cells. A good response in the mixed lymphocyte reaction (MLR) is one of the criteria of general T-cell immunocompetence. The proliferative response of splenic lymphocytes from lean and and obese mice was tested in a one-way mixed lymphocyte reaction (MLR) against Mitomycin-C treated Balb-C spleen cells using a 1:1 responder to stimulator cell ratio.

Figure 2 presents the results of the experiment. In the lean mice, the response was typical of that seen in normal immunocompetent animals. There was a sharp increase in [^{3}H]-thymidine incorporation with the peak on day 2 of culture and a decrease in incorporation thereafter. In the control obese mice, the peak activity was delayed, maximal activity occuring at 3 days, and the blastogenic activity was only 1/3 that found in lean mice. The response of ob/ob spleen cells was reduced significantly (60-70%) in an mixed lymphocyte reaction. This has been previously reported (Nichols et al, 1981), however, this group found only a slight 15-20% decrease in reponse in their experiments.

Zinc supplementation of both lean and obese mice improved the response on Day 2 of culture which coincides with the peak of response but had no effect on other days of culture. The improved response of the zinc-supplemented obese mouse on Day 2 of culture was still 50% lower than that exhibited by the lean control mouse. These results suggest the splenic lymphocytes from the lean mouse respond better in a MLR than those from an obese mouse and also that zinc supplementation can enhance the response.

Certain plant constituents, termed mitogens, are capable of inducing transformation and proliferation of lymphocytes in vitro. Concanavalin A induces mitosis of T-lymphocytes. The response of spleen cells of lean and obese mice to the mitogen Concanavalin A is presented in Figure 3. Spleen cells from obese mice exhibited three times the proliferative response than those of lean mice to this dosage of Con A. This effect began to appear on Day 2 of culture and continued through Day 3. These results show that in this assay splenic lymophocytes from the obese mouse respond better than those from lean mice.

Antibody producing ability of lean and obese mice:

Humoral response was assessed by the number of antibody producing cells in reponse to an antigen challenge. Table 9 presents the dat on the number of plaque forming cells (PFC) in lean and obese mice on either control or zinc-supplemented diets in response to a challenge with sheep red blood cells. The production of antibodies against sheep red blood cell (SRBC) antigens by the plasma cells of the spleen (B-cells) requires the cooperation of the thymus dependent cells (T-cells). PFC responses to immunization with SRBC were increased in the spleen of the obese mouse, as previously reported (Chandra et al, 1980a). Obese mice on a control diet exhibited a response twice as great as the lean mouse. Both lean and obese mice which were zinc supplemented showed a higher proportion of antibody-forming cells. Zinc supplementation of the lean mouse gave a PFC response in the same range as that of the obese mouse on a control diet. Zinc supplementation of the obese mouse doubled the response over that exhibited by these mice on a control diet. In this assay, zinc supplementation of the diet did appear to have a stimulatory effect for both lean and obese mice.

Fig. 2. Mixed lymphocyte responses of spleen cells from lean (o) and obese (▵) mice

The mice were either on a control (○,▵) or zinc-supplemented diet (●,▲). Spleen cells for each condition were obtained from one mouse. Values are expressed as the mean cpm of triplicate cultures for [^{3}H]-thymidine incorporation in the last 8h of culture. For further details see the Methods section.

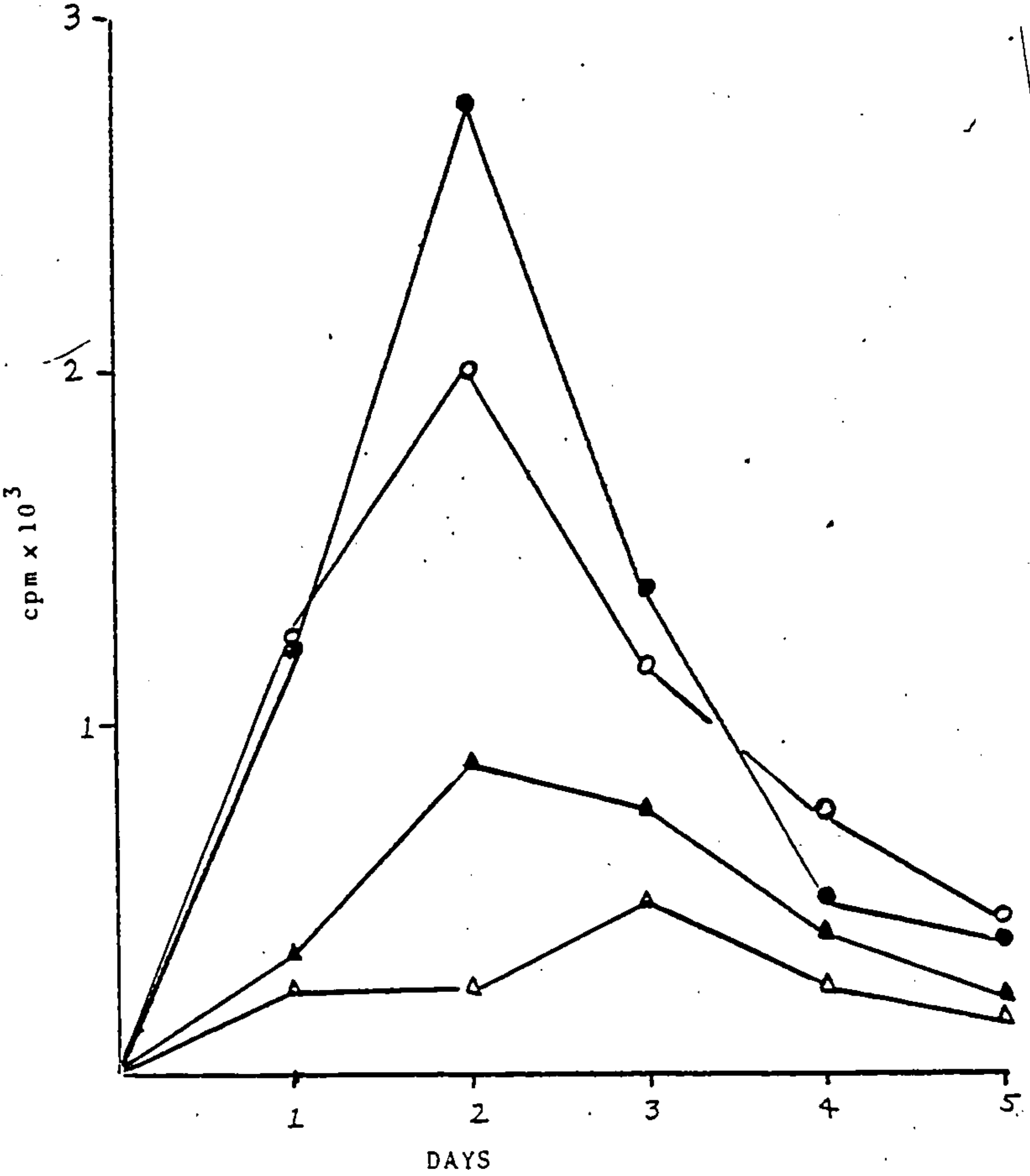

Fig.3. Mitogen response of spleen cells
from lean (o) and obese (△) mice

The assay was performed as described in Methods. The spleen cells were either control (o △) or stimulated with the mitogen Concanavalin A at a dosage of 4 ug/ml cells (● ▲). For each condition, spleen cells were obtained from one mouse. Values represent the mean cpm of triplicate cultures for [^{3}H]-thymidine incorporated in the last 30 min of culture.

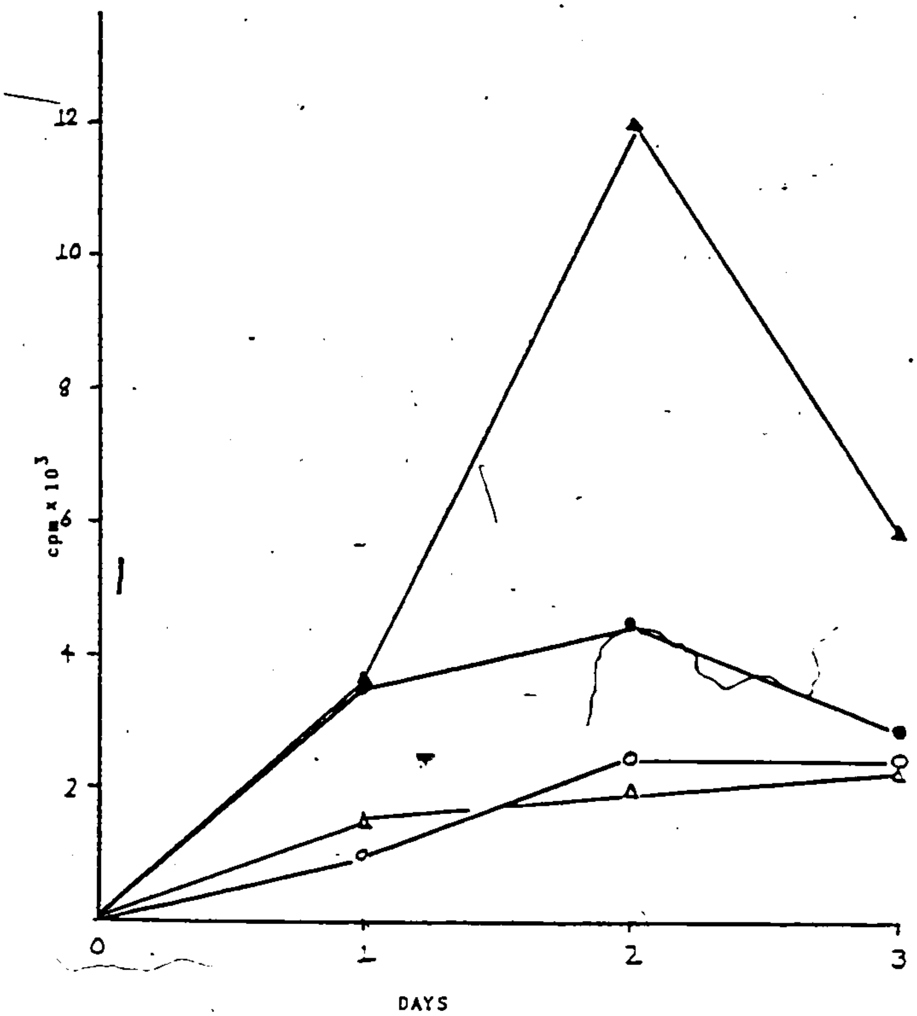

Table 9. Plaque Forming Cells (PFC) in Control and Zinc Supplemented Animals

Group	PFC/10^6 cells
lean-control	150 ± 22^{a}
lean-zinc suppl.	281 ± 68^{b}
ob/ob-control	318 ± 11^{b}
ob/ob-zinc suppl.	748 ± 68^{c}

The assay was performed as described in the Methods section. 5 days prior to the assay, the mice were immunized with 1×10^8 cell/ml of sheep red blood cells. The results are expressed as means ±SEM for 5 animals per group. Values followed by different letters are significantly different from each other ($P < 0.05$).

4) Adenosine Deaminase Activity in Lean and Obese Mice

Adenosine deaminase catalyzes the deamination of adenosine to inosine; a step in purine degradation. Deficiency of this enzyme in humans results in severe combined immunodeficiency (Giblett et al, 1979). Some evidence was available indicating the activity of this enzyme was dependent on the zinc status of the individual (Prasad et al, 1981). As the zinc status of the ob/ob mouse was under investigation and immune alterations of the ob/ob mouse had previously been reported (Chandra et al, 1980a), it seemed of interest to measure the activity of adenosine deaminase in the ob/ob mouse. The effect of zinc supplementation of the mice on the activity of this enzyme was also measured.

Some preliminary experiments were performed to ascertain assay conditions were proper for measuring adenosine deaminase activity in our mouse strain. Figure 4a presents a time course for adenosine deaminase activity in liver cell extracts of lean mice. Under the experimental conditions the activity of the enzyme increased proportionally with time up to the maximum incubation period of 60 min. A 10 min incubation period was then decided upon as the standard assay condition for the remaining experiments.

Optimum protein concentrations for the assay of adenosine deaminase in liver, spleen , and testes were then determined. Figure 4b shows that in liver adenosine conversion to inosine increases proportionally as protein was increased from 20-60 ug. In spleen and testes, up to 100 ug of protein in the assay tube maintained the activity in the linear portion of the curve (See figures 5 and 6).

The activity of adenosine deaminase in various tissues of lean and obese mice which had been maintained on a control or zinc-supplemented diet is presented in Table 10. Lean and obese mice displayed remarkably similiar adenosine deaminase activity in spleen and testes. In liver, the activity of this enzyme was 50% lower in the obese mice as compared to the lean mice. Zinc supplementation did not alter the activity of adenosine deaminase significantly in any of these tissues.

Fig 4A. Time course of liver adenosine deaminase activity

Adenosine deaminase activity was assayed as described in Methods with 40 ug protein and [^{3}H]-adenosine as the substrate. The liver extract was from a lean mouse. Other details were as given in the Methods section.

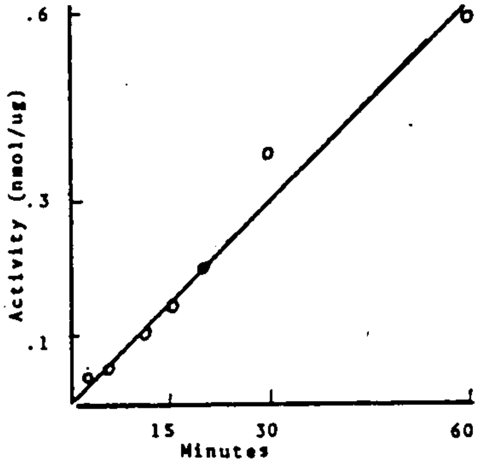

Fig. 4B. The effect of protein concentration on liver adenosine deaminase activity

The assay was done at the concetrations of protein indicated on the abscissa for 10 minutes. For other details refer to the legend to Fig 4A and the Methods section.

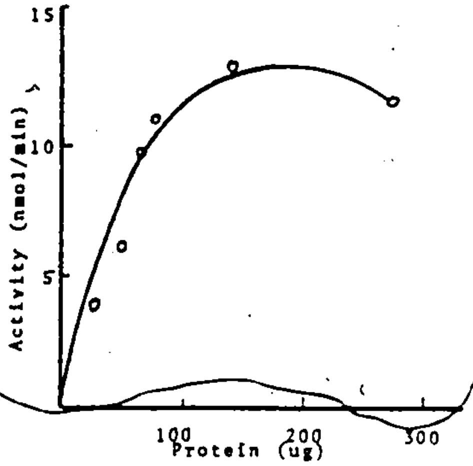

Fig. 5. The effect of enzyme concentration on spleen adenosine deaminase activity

For details, refer to the Methods section and the Legend to Fig 4A .

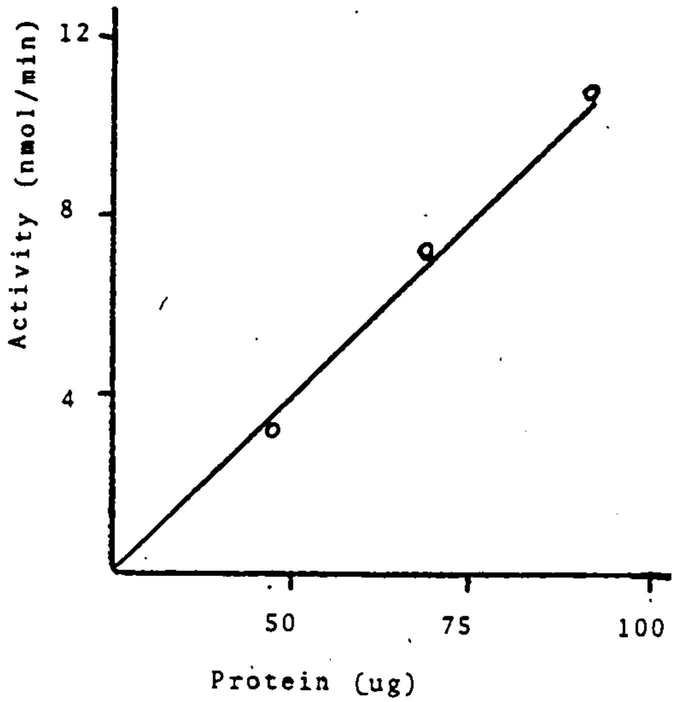

Fig. 6. The effect of enzyme concentration on testes adenosine deaminase activity

For details, refer to the Methods section and the legend to Fig 4A.

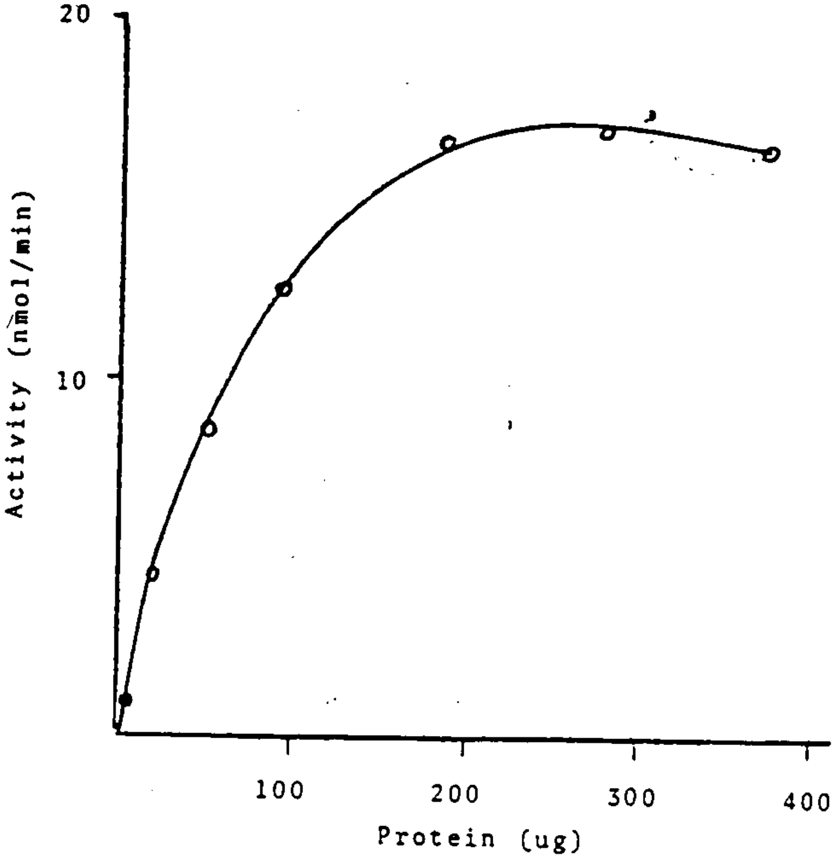

Table 10- Adenosine Deaminase Activity (nmol/ug/min)

Group	Tissue		
	Liver	Spleen	Testes
lean-control	.184±.008^{a}	1.08±.04	0.25±.006
lean-zinc suppl	.157±.003^{a}	1.16±.04	0.25±.007
ob/ob-control	.079±.003^{b}	1.06±.06	0.27±±.01
ob/ob-zinc suppl	.076±.006^{b}	1.13±.05	0.24±.007

Adenosine deaminase activity was assayed as described in Methods. $[^{14}C]$-adenosine was used in these experiments. Values are means ±SEM for six animals in each group. For further details see the Methods section.

DISCUSSION

1) Tissue levels of zinc

The results indicate some basic differences in the metabolism and physiology of zinc between lean and obese mice. An analysis of the steps from consumption to excretion of zinc can only offer some speculation as to the mechanisms whereby obese mice accumulate and utilize their zinc in a different manner than lean mice.

On a control diet, obese mice consume at least twice as much zinc as lean mice by virtue of their hyperphagia (See Table 1). They also eat continuously and do not demonstrate a diurnal pattern. In contrast, zinc deficient animals show greatly reduced food intake and demonstrate a very erratic food consumption from day to day (Huber et al, 1973, Wallwork et al, 1981). This suggests that from the point of view of appetite and timing of feeds the obese mouse is behaving in an opposite fashion to a zinc deficient mouse.

In the absence of absorption or balance studies, discussion on the amount of zinc absorbed by the ob/ob mouse is purely speculative. Some studies suggest that the obese mouse on a control diet absorbs at least an equal quantity of zinc as the lean mouse. 1) the gut of lean and obese mice is histologically similiar (Binder et al,1966) 2) absorption of various nutrients by the obese mouse has been found to be equal to or greater than that of the lean mouse (Mayer et al, 1956; Bihler et al, 1975). 3) Chemically diabetic rats (streptozotocin treated) absorbed three times the amount of zinc than control rats on similiar diets (Craft et al, 1983). These diabetic rats would be hypoinsulinemic as opposed to the obese mice who are hyperinsulinemic, however, no studies as yet have determined the effect of plasma insulin levels on zinc absorption from the intestine. 4) The plasma levels of zinc were found to be comparable in lean and obese mice (See Table 5). It is well established that plasma zinc is reduced in zinc deficient animals (Prasad, 1982).

Once absorbed, the distribution of zinc to other tissues could occur differently in lean and obese mice. The results of tissue analysis indicated lower zinc concentration in the bone of the obese mouse but higher zinc content in certain soft tissues. Although, of the tissues analyzed, a significantly higher zinc content was only found in liver and BAT of the ob/ob mouse, the muscle and testes did demonstate trends of higher zinc levels in the ob/ob mouse. The genetically diabetic mouse (db/db), has been reported to have depressed serum and femur zinc concentrations although kidney and liver levels were normal (Levine et al, 1983). In contrast, streptozotocin treated rats exhibited increased hepatic and renal zinc concentration (Failla, 1981, 1983) whereas similiarly treated mice did not exhibit any changes in tissue or serum zinc concentrations (Levine et al, 1983). These varied results indicated that the handling of zinc among the experimental animals is different.

The accumulation of zinc in the soft tissues of the ob/ob mouse may be a function of increased need in these metabolically active tissues. When ^{65}Zn is given to an animal it first goes to more metabolically active tissues such as liver and kidney and then slowly goes to the bone. In the ob/ob mouse there seems to be accumulation of zinc in certain soft tissues and low levels of zinc in the bone. This is the result, however, of certain long-term homeostatic mechanisms in the ob/ob mouse and not comparable to that which occurs after a single dose of zinc such as when ^{65}Zn is given to an animal.

The accumulation of zinc may also be the result of altered distribution of zinc due to increased storage in certain tissues. Abnormal metallothionein levels may be binding excessive amounts of zinc. In the liver, once zinc increases above basal levels, zinc binds principally to a hepatic metallothionein (Cousins, 1979). In contrast to the intestine, hepatic zinc metabolism is not only induced by fluctuations in dietary zinc supply but by stresses as well. As a result of exercise, heat, or infections plasma zinc is decreased and a concomitant increase in hepatic metallothionein occurs (Whanger et al, 1978).

High levels of glucocorticoids have a similiar effect (Squibb et al, 1979). Glucocorticoids may induce synthesis of metallothionein mRNA by a method independent of zinc accumulation (Karin et al, 1980). The ob/ob mouse has high levels of glucocorticoids in the plasma (Dubuc, 1975) and it could be visualized that this hyper-corticoid state could produce a situation whereby metallothionein synthesis is constantly induced in the ob/ob mouse. This, in turn, would lead to accumulation of zinc in the liver such as found in these studies.

One study demonstrated that high zinc levels in the diet can induce the synthesis of metallothioneins in a variety of tissues such as kidney and pancreas in addition to the liver (Whanger et al, 1981). If other tissues have this capacity for the synthesis of metallothionein, it is possible that in the obese mouse the target tissues for glucocorticoids would have permanently elevated metallothionein levels. Zinc would thus accumulate in these tissues and be sequestered there in a form which is unavailable for other purposes. Studies on metallothionein levels in a variety of tissues as well as the effect of adrenalectomy on the zinc status of the ob/ob mouse would be interesting.

The effect of the ob/ob genotype on skeletal zinc is more difficult to elucidate. Low bone zinc has classically been used to ascribe a state of zinc deficiency. Decreased zinc concentrations in bone in a zinc deficient animal represents a decreased rate of zinc deposition rather than increased resorption (Murray et al, 1981). One cannot directly correlate these findings with the ob/ob mouse who has adequate zinc in the diet and increased zinc in most tissues. It is possible the bone is low in zinc due to other tisues sequestering zinc in an form unavailable for bone. The plasma levels of zinc are, however, comparable in lean and obese mice indicating an adequate supply of zinc to bone tissue. Therefore, it is possible that the ob/ob mouse has a slow rate of deposition of zinc in the bone which could account for an overall decrease in femur zinc.

The brown adipose tissue (BAT) of the obese mouse has much higher quantities of zinc than that of the lean mouse. The BAT of the obese mouse is largely infiltrated with triglycerides and surrounded by white fat. White fat is believed to contain only negligible amounts of zinc (Underwood. 1977). The high zinc content of the brown adipose tissue can thus be attributed to the BAT cells per se. BAT is the main thermogenic organ in small mammals (Foster et al, 1978). In light of the fact that both zinc deficient animals (Topping et al, 1982) and obese mice (Bray and York, 1979) have reduced body temperature and impaired thermogenesis, it is possible that the high zinc content of the BAT in the obese mouse may be involved in this phenomenon. Zinc has been found to be an inhibitor of the electron transport chain in certain experimental conditions (Chvapil, 1973; Bettger et al, 1981).

2) Zinc supplementaton

It is difficult to estimate the proportion of zinc that was absorbed on a 1,000 ppm supplemented diet. Zinc has limited toxicity in large part due to the ability of animals to regulate the extent of absorption. Homeostatic mechanisms must exist to ensure an adequate but not deleterious supply of zinc to the body. Studies in ruminants (Miller et al, 1969) and rats (Richards et al, 1976) indicate that zinc deficient animals absorb more zinc while high levels of zinc in the diet do not increase zinc absorption proportionally.

Cousins (1979) has recently proposed a scheme to explain zinc absorption based on accumulated experiments to date. Zinc passes the intestinal mucosal brush border probably by an active process, and then enters a "zinc pool". In times of zinc demand, this zinc is then transferred immediately to the serosal boundary and onto a plasma carrier, thus increasing plasma levels. During a zinc adequate period, the zinc has a number of possible routes: 1) it induces synthesis and is bound to an intestinal metallothionein 2) it enters the intestinal cells own intracellular zinc pools 3) it is excreted back in the intestinal lumen, or 4) it enters into plasma.

The obese and lean mice on a zinc supplemented diet (1,000 ppm), did not exhibit any toxic symptoms or did not increase the zinc content of any tissues or plasma significantly. Thus, it appears the these mice use a variety of the mechanisms proposed by Cousins to regulate their zinc absorption. Other excretory pathways such as bile and urine could also be playing a role in maintaining zinc levels. Careful balance studies, absorption studies using ^{65}Zn, measurement of intestinal zinc and metallothionein content could confirm these postulates.

Previous studies have shown rodents to be more resistant to high zinc levels than most larger animals. In rats, only minor increases in tissue zinc occur after feeding high but nontoxic levels of zinc. 600 ppm of zinc in the diet of rats did not result in significant accumulation in any of the tissues studied (Ansari et al, 1975). At 1,000 ppm of zinc in the diet , there was a 20-30% increase in the zinc content of the liver (Chen et al, 1974; Chvapil et al, 1974) with other tissues being relatively insensitive to high zinc intakes. At 2,000 ppm there was significant accumulation in all tissues studied (Chen et al, 1974). The mice used in this study were supplemented with 1,000 ppm zinc and seemed even more resistant to accumulation of zinc in their tissues than rats. Although, the liver as well as other tissues showed trends of having higher levels of zinc, the differences were not significant.

In one study, rats consuming 1,000 ppm zinc increased their plasma zinc levels significantly (Chen et al, 1974). In contrast, the plasma zinc levels of the supplemented lean and obese mice did not show any differences. One possibility is that mice in general, or the particular strain of mice used here, may have more efficient homeostatic mechanisms at the level of the intestine i.e. they may be excreting more zinc via the gut and/or storing more zinc in intestinal metallothionein. The plasma levels of zinc in the obese and lean mice must, however, be interpreted with caution. Plasma zinc in rats demonstrate a circadian rhythmn in relation to timing of food intake (Hurley et al, 1982). There is a drop of 25% in plasma zinc concentration when food intake increases rapidly with return to basal levels a few hours later. Plasma zinc was measured at 9:00 h in the experiments described in this thesis. If this coincides with a prior period of high food intake by the lean mice, plasma zinc could be relatively lower than in the obese mice who do not exhibit a particular period of increased food consumption.

Zinc supplementation did not significantly alter the zinc content of the triceps muscle, a primarily white muscle, in either lean or obese mice. This was mainly due to a high variation of zinc content of the muscles. A 3-4 fold variation has previously been reported with porcine and bovine muscles (Underwood, 1977). In addition, the obese mouse muscle may be functionally different than the lean mouse muscle due to low locomotor activity and reduced protein deposition. Zinc supplementation did not increase the zinc content of the bone indicating that whatever processes are responsible for reduced zinc skeletal levels in the obese mouse are not ameliorated by increasing zinc in the diet.

3) Zinc and glucose homeostasis

It was shown that the effect of zinc supplementation on peripheral glucose handling by the ob/ob mouse was negligible. Some evidence has been presented for a post-secretory role for the zinc-insulin complex. Liver plasma membrames, from mice pretreated with zinc, or treated with zinc in vitro, have enhanced binding of insulin which correlates with slower degradation of the hormone (Arquilla et al, 1978). Zinc exerts a stimulatory effect on the rate of lipogenesis in rat adipocytes and the effect is additive with that of insulin when they were present together (Coulston et al, 1980).

Zinc deficient animals have impaired glucose tolerance . When repleted with zinc their glucose handling returns to normal (Huber et al, 1973). At the pancreatic level, previous studies in our laboratory demonstrated that zinc supplementation (1,000 ppm) of the ob/ob mouse increases the insulin content and the granulation of the pancreatic islets. The treatment also decreased the abnormally high insulin secretory response to glucose in vitro (Begin-Heick et al, 1982; Dalpe-Scott, 1983). Zinc has been implicated as a regulator of insulin secretion. Zinc inhibits the release of insulin from isolated islets in vitro (Figlewicz et al 1981; Ghafghazi et al, 1981).

Prior to zinc supplementation of the obese mouse, metabolic aberrations are already present. The ob/ob mouse has decreased numbers and responsiveness of insulin receptors in several tissues. In contrast to zinc deficient animals zinc supplementation does not improve their glucose handling. In the case of the zinc deficient animal, addition of zinc changes an abnormal metabolic state ie.zinc deficiency , to a normal one. In the case of the obese mice we were attempting to normalize an abnormal metabolic state. There is no basis to assume the two conditions could be compared.

Zinc could be acting on peripheral cells that respond to insulin by 1) a direct insulin-like stimulatory effect or 2) indirectly, by possible enhancement of binding of insulin to its receptor. Due to the fact that the obese mouse has abnormal numbers and affinity of insulin receptors in several tissues, a lack of peripheral response by the zinc-supplemented ob/ob mouse does not preclude such a post-secretory role for the insulin-zinc complex. This may lead to a defective response of these tissues in the obese mouse to the action of zinc.

4) Immunity

The obese mouse maintained on a control diet had a greatly enhanced response to Con A compared to the lean mice. Examination of the literature reveals that adequate zinc status is necessary for a normal respond of T-cells to mitogens. Zinc deficient animals have impaired lymphocyte proliferative response to in vitro exposure to mitogens and antigens (Gross et al, 1979; Good et al, 1980). In rats, lymphocyte transformation in response to T-cell mitogens phytohemagglutin (PHA) or Concanavalin A (Con A) is impaired by zinc deficiency (Pefarek et al, 1977; Gross et al, 1979). In contrast zinc supplementation of normal mice improves the mitogenic response to PHA and Con A (Gaworski et al, 1978) while in humans it exerts an immunoregulatory effect (Duchateau et al, 1981). On the basis of this test, therefore, the lymphocytes of obese mice may be said to be zinc sufficient. This conclusion cannot, however, be upheld when the results of other tests are examined.

Proliferative response in a mixed-lymphocyte reaction was reduced in the obese mouse. As was seen in the introduction there are varying responses of the ob/ob lymphocytes in immunological assays. This indicates that various subpopulations of thymus derived lymphocytes are reacting differently. Both the mixed lymphocyte reaction and the plaque cell assay were improved in animals receiving zinc supplemented diets. Zinc supplementation improved the response in the immune assays to an equal extent in both lean and obese mice therefore one cannot conclude from these assays that the obese mouse was preferentially more sensitive to a high zinc intake. The improved responses generated by zinc supplementation are another indication, along with changes at the level of the endocrine pancreas that the extra zinc was being utilized.

Most of the hypotheses advanced to explain the immune alterations in the ob/ob mouse reside on the hormonal/metabolic abnormalities present. In light of our findings of high zinc levels in the liver, immune alterations may be due to trace mineral interactions. Copper competes with zinc for binding to metallothionein during absorption and for storage in the liver (Sandstead et al, 1981). Rats fed a high zinc diet have low serum and liver copper levels despite adequate copper in the diet (Fischer et al, 1980). An impaired immune response was observed in severe and marginally copper deficient mice (Prohaska et al, 1981, 1983). Similarly, iron excess or deficiency can result in immune dysfunction in both humans and animals (Beisel, 1982). Interactions with other trace minerals can thus affect immune function.

The obese mice on a control diet consume 2-3 times more zinc, as well as other trace minerals such as copper and iron, than the lean mice. On the control diet the nutrients are consumed in adequate proportions relative to each other, however, nothing is known of the relative absorption of these nutrients in the obese mouse. Similiarly, the effect of zinc supplementation of the obese mice on absorption of other trace minerals is not known.

5) Adenosine deaminase

The activity of adenosine deaminase was normal in spleen and testes of the obese mouse but decreased activity was observed in the liver. At present, the significance of this finding is unclear. The liver of the obese mouse is fat infiltrated but the total protein content is similiar to that found in a lean mouse. The activity of the enzyme measured in the liver is thus not the result of a different reference amount of protein.

It does not seem likely that the lower activity in the liver is the result of a mutant gene producing this enzyme. All the cases in humans with genetic deficiency of this enzyme have reported barely detectable activity present in all tissues measured (Osborne et al, 1981). These patients also have severe derangement of both T and B-cell function. This does not resemble the ob/ob mouse in whom only liver adenosine deaminase activity was decreased and in whom the immune system is not severely compromised.

In the liver, 60% of the enzyme is present in the bound to a tissue specific binding protein, while only 10% is present in the form in the spleen. This binding protein has no known catalytic activity and is antigenically distinct from the small form of adenosine deaminase (Van der Wedden et al, 1976; Schrader et al, 1979). The binding protein does not seem to be necessary for catalytic activity but may have a role in stabilization of the enzyme (Fox et al, 1979). Assuming the adenosine deaminase is structurally sound and produced in adequate amounts , it is possible the tissue binding protein is abnormal in the liver.

The reduced activity of adenosine deaminase in the liver could lead to higher adenosine concentration present. Adenosine has many physiological functions; it is postulated to be a neurotransmitter in the CNS and in peripheral smooth muscle, it dilates certain blood vessels, and inhibits platelet aggregration (Schwabe, 1981; Snyder, 1981). Higher concentrations of adenosine in the liver of the obese mouse, if released to the circulation, may exert an influence on these functions. Adenosine is also a modulator of adenylate cylcase activity. Obese mice have been shown to have defective adenylate cyclase activity in several tissues including white fat and brown fat (Bégin-Heick, 1980, 1981, 1982). It is possible that altered metabolism of adenosine by the enzyme adenosine deaminase could be a contributing factor.

The major underlying difference between zinc metabolism of lean and obese mice may ultimately lie at the membrane level. Zinc is proposed to play an important role in membrane function and structure (Bettger et al, 1981). A brief examination of some of the purported functions of zinc at the membrane level reveals many areas in which abnormalities have previously been reported for the ob/ob mouse.

Zinc affects the activities of membrane bound enzymes such as adenylate cyclase and Na^+K^+ ATPase. Abnormalities in the activity of both these enzymes are reported in the ob/ob mouse (Bray and York, 1979). Zinc may modulate specific plasma membrane receptor, especially of lymphocytes. The ob/ob mouse has defective insulin receptors in many tissues including lymphocyte (Soll et al, 1974). Various experiments have demonstrated that zinc inhibits electron transport in the mitochondrial respiration chain. Defective thermogenesis in the ob/ob mouse (Foster et al, 1978) may be related to this phenomenon.

Finally, a role for zinc has been proposed in lipid and prostaglandin synthesis by affecting essential fatty acid metabolism. This idea arose initially due to the many clinical similiarities between zinc deficiency and essential fatty acid deficiency. Horrobin et al,(1980), have suggested that zinc is required for the activity of d-6-desaturase that converts linoleic acid to C18:3n-6 and for the mobilization of dihomo-γ-linoleic acid (C20:3n-6). This implies that zinc is needed for the generation of the one series of prostaglandins. The exact link between zinc and lipids is not yet well documented.

A recent report did not identify differences in prostaglandin levels in several tissues in zinc deficient rats (Meydani et al, 1982). An increase in the cholesterol/phospholipid ratio of liver microsomes was reported in zinc deficient rats (Clejan et al, 1981).

In the ob/ob mouse, there is an increased in the cholesterol/phospholipid ratio in the liver (Sena et al, 1982). Analysis of the fatty acid composition of the phosphatides and phospholipids of the liver revealed there was a general polyunsaturated deficiency and a higher content of monounsaturates (Rouer et al, 1980; Sena et al, 1982). These groups have postulated the ob/ob mouse may have a higher requirement for essential fatty acids than their lean littermates. Altered membrame function may possibly explain certain phemomena observed during my experiments on the zinc status of the ob/ob mouse.

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