HISTOLOGICAL AND BIOCHEMICAL STUDIES ON THE EFFECT OF NICKEL IN BIOLOGICAL SYSTEMS

A THESIS

submitted in fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOSCIENCES AND BIOTECHNOLOGY

By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"HISTOLOGICAL AND BIOCHEMICAL STUDIES ON THE EFFECT OF NICKEL IN BIOLOGICAL SYSTEMS"** in fulfilment of the requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY** and submitted in the Department of Biosciences and Biotechnology of the University is an authentic record of my work carried out during a period from March, 1988 to June, 1992 under the supervision of **Prof. C.B. Sharma** and **Dr. Vinay Sharma**, Department of Biocsciences and Biotechnology, University of Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

Ritushree Garg
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This is to certify that the above statement made by the candidate is correct to the best of my (our) knowledge.

Date : 12.11.93 Signature of Supervisor 12.11.93 Signature of Supervisor

The Ph.D. Viva-Voce examination of Miss Ritushree Garg Research scholar, has been held on 9th Feb. 1994.

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Signature of External Examiner

WITH LOVE TO

MUMMY AND DADDY

ABSTRACT

The present study is in two parts. Part I deals with biochemical, histopathological and structural changes induced by intramuscular administration of cumulative doses of $NiSO_4.7H_2O$ in rat liver and kidney. Part II reports the biochemical effects of $NiSO_4.7H_2O$ on peanut seedlings with special reference to some key enzymes involved in mobilizing the stored food from the cotyledons to different anatomical parts of the plant during germination and early growth and development period and also some plasma membrane bound enzymes which may act as bioindicators for the stress conditions.

Part I: Intramuscular administration of cumulative dose of $NiSO_4.7H_2O$ (0.5 - 2mg/100g body weight) in rats for a period of 15 days produced a dose dependent loss in body weight. The nickel contents of liver and kidney increased from 15ppm to 166ppm in liver and from 16ppm to 268ppm in kidney in rats which were given $NiSO_4.7H_2O$ doses (2mg/100g body weight) indicating bioaccumulation of nickel in these tissues. Protein and carbohydrate contents of both liver and kidney were also significantly decreased, but in contrast the concentration of total lipids in these organs was markedly increased. In fact, a 3-4-fold increase in neutral and phospholipids was observed in both rat liver and kidney as a result of nickel treatment.

A detailed study of the changes induced by nickel in the composition of various components of neutral and glycerophosphatidyl lipids in rat liver and kidney by GC and HPLC showed that the increase in neutral lipids was largely due to increase in the level of triacylglycerol and esterified cholesterol while in the case of glycerophosphatidyl lipids, it was mainly due to phosphatidyl choline. These results indicated a selective action of nickel, apparently by enhancing the rate of synthesis of triacylglycerol, esterified cholesterol and glycerophosphatidyl choline in liver and kidney cells.

Nickel has also altered the percentage composition of various components of lipids of the subcellular membrane fractions. The

relative proportion of phosphatidyl choline, the major component of phospholipids in rat liver membranes, was significantly increased in all subcellular fractions with maximum increase in plasma membrane followed by Golgi apparatus, mitochondrial membranes and the endoplasmic reticulum. The proportion of phosphatidyl ethanolamine, the second most abundant glycerophosphatidyl lipid in rat liver, was decreased in all membrane fractions except nuclear membrane. Thus the *in vivo* effect of nickel on the composition of the component glycerophosphatidyl lipids in various membrane fraction was somewhat selective.

The histochemical analysis has shown that intramuscularly administered nickel induced the deposition of triacylglycerols and phospholipids at the specific sites in liver and kidney of rats. The triacylglycerol deposition was particularly pronounced in the central zone of the liver, in the pyramidal region and the proximal convoluted tubules of kidney. The phospholipids were heavily deposited at the perilobular zone in the liver and at the cortex region of kidney. These results indicate the morphological toxicity of rats by nickel.

The light microscopic examination of tissues has shown that intramuscular administration of $NiSO_4.7H_2O$ damaged both liver and kidney affecting the hepatic parenchymal cells and distrupting the renal organisation respectively. The former showed focal, centrolobular and perilobular necrosis, nuclear morphological changes and hydropic degeneration, whereas kidney showed glomerulonephritis, increased cellularity of glomeruli and degenerative changes in the proximal and distal convoluted tubules. Irregularly arranged small but thick reticulin fibres and excessive synthesis and deposition of collagen was also observed which favour progression of tissue injury.

Both biochemical and histochemical analysis clearly indicated that the intramuscular administration of nickel in rats greatly reduced the level of the activity of alkaline phosphatase, acid phosphatase, glucose- 6- phosphatase and lipase in liver and kidney. In addition, characteristic differences were observed in the anatomic localization

of these enzymes.

Nickel treatment causes drastic changes in blood and serum constitutents. Haemoglobin percentage and total RBC number were significantly decreased, whereas a slight elevation was noticed in the number of TLCs. After exposure of nickel, the activities of alkaline phosphatase, glutamic-oxaloacetic transminase, glutamic-pryuvic transaminase and the levels of reducing sugars, cholesterol, urea, total bilirubin and creatinine markedly increased, but the total serum protein exhibited a marked decrease.

PART-II : When peanut seeds were germinated in the presence of varying concentrations of nickel in distilled-deionized water culture, it was found that the nickel in excess of 15ppm (approx. 0.25mM) interfered with the germination process and adversely affected the growth and development of embryonic axis and formation of secondary roots, at 60ppm nickel concentration in the culture medium seeds failed to germinate. The development of radicle was relatively more sensitive to nickel than that of plumule. The decrease in growth of embryonic axis of germinating peanut seedlings led to decrease in dry weight. The nickel uptake by the seedlings also increased many-fold. As for example, in seedlings grown in liquid culture containing 60ppm nickel, nickel content increased from approximately 9ppm to 273ppm, a net increase in Ni-uptake of about 30-fold.

The retardation of seedling growth appears to be direct manifestation of the inhibition of some key enzymes e.g. phytase, amylase, lipase and acid phosphatase that are responsible for mobilization of stored food. Interestingly, the activity of the 5'-nucleotidase in plasma membrane of the nickel-treated cotyledons was found completely inhibited. Remarkably, nickel ions cause the highest stimulation in the activity of mannosyl transferase. Besides nickel also causes significant changes in the protein and lipids of membrane.

Nickel-treated germinating peanut cotyledons show greater proportion of callose (1,3-β-glucan) synthase II which has low Km for UDP-glucose and requires a primer whereas untreated cotyledons have high proportion of CS-I, a self priming enzyme with higher Km value. This is thought to be of physiological significance as the CS is a wound repair enzyme and gets activated under stress conditions. CS-I and CS-II isoenzymes have been purified using selective solubilization of plasma membrane bound enzyme by digitonin followed by sucrose desity gradient centrifugation and hydroxylapatite column chromatography and their properties were compared.

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Ritushree Garg

(RITUSHREE GARG)

CONTENTS

LIST OF FIGURES

CHAPTER-VI

CHAPTER-VII

8.0 PUBLICATION

Kamat, U.,Garg, R.and Sharma, C.B.: Purification to homogeneity and characterization of a 1,3-β-glucan (callose) synthase from germinating *Arachis hypogaea* cotyledons. Vol.298,No.2,pp.731-739,1992.

LIST OF TABLES

ABBREVIATIONS USED

AMP	-	Adenosine 5'-monophosphate
AMPase	-	Adenosine 5'- monophosphatase
^{o}C	-	Centigrade
cm	-	Centimeter
conc	-	Concentration
cpm	-	Counts per minute
Ci/mol	-	Curie/mole
CS	-	Callose synthase
EDTA	-	Ethylene diamine tetraacetic acid
ER	-	Endoplasmic reticulum
Fig.	-	Figure
g	-	Gram
GDP	-	Guanosine disphosphate
h	-	Hour
kg	-	kilogram
Km	-	Michaelis-Menten constant
L	-	Litre
μg	-	Microgram
μM	-	Micromolar
mg	-	Milligram
ml	-	Millilitre
mM	-	Millimolar
min	-	Minute
N	-	Normality
Ni	-	Nickel
pNPP	-	Para nitrophenyl phosphate
ppm	-	Parts per million
PM	-	Plasma membrane
TEMED	-	N-N-N'-N'- Tetramethylethylene diamine
UDP	-	Uridine 5'- diphosphate
v/v/v	-	Volume/Volume/Volume
Vmax	-	Maximum velocity attained by a enzyme catalyzed reaction
w/w	-	Weight/Weight

CHAPTER-I

1.0 INTRODUCTION

In recent years rapid industrialization, in both developed and developing countries, has resulted in man made redistribution of trace metals in environment. As a result entire population of living organisms in the biosphere has been brought more and more in contact with relatively rare metals without having any realization what kind of health hazard may be associated with long term and chronic exposures to these metals. This problem of heavy metal contamination of environment becomes even more significant in view of the fact that living organisms have failed to develop a homeostatic defense for metals which are unlikely to play essential biological role in body and whose excessive bioaccumulation is most likely to disturb various metabolic processes.

Nickel is one of such metals which has been considered to constitute serious hazard to the environment due to its wide spread environmental occurrence and use of its chemical compounds in various industries (Sunderman *et al.*, 1988). Nickel has been associated with a number of pathological conditions in man . It is a toxic, mutagenic and carcinogenic metal of great occupational and environmental concern (Higinbotham *et al.*, 1992; Chou, 1989; Furst and Fan, 1993). Among the well known toxic activities associated with nickel and its salts are acute pneumonitis, dermatitis, asthma, central nervous system disorders and cancer of the nasal cavity and lungs (Fisher, 1985). Administration of nickel salts through inhalation or intramuscular, intravenous and subcutaneous injections to laboratory animals results in various toxic systems including lipid peroxidation (Sunderman,1986), immunotoxicity (Smialowicz *et al.*, 1985), hypometabolism and hypothermia (Gorden and Stead, 1986).

The toxic effects of nickel in plants are equally severe and damaging to the health of plant. It include stunted growth of roots and shoots, deformation of various plant parts (Haselholf, 1893), inhibition of mineralization of organic phosphate, etc. The latter

effect is of serious natureandmay ultimately be related to stunted growth of roots and shoots and deformation of plant parts, as the mobilization of stored phosphate to other parts of the growing plant during the initial stages of seedling growth and development from the cotyledons will be inhibited and if the effect is severe enough due to high nickel contamination the germination process will not initiate.

Thus, despite the fact that nickel exposure may induce serious metabolic disorders and abnormalities in animals, human beings and plants, our understanding of the mechanism(s) of action of nickel toxicity in both plants and animals at the subcellular, molecular or even cellular levels is extremely poor and inadequate. Even the biochemical basis of the nickel toxicity is not clearly understood, although, in recent years some important studies regarding nickel toxicity in animals have been made (Chou, 1989; Higinbotham, 1992; Cheng *et al*., 1993; Furst and Fan, 1993; Herrero *et al*.,1993; Shainkin-Keslenbaum *et al*., 1991; Chorvatovicova and Kovacikova, 1992; Conway and Costa, 1989; Baleux *et al*., 1993).

From these reports it is evident that in animals nickel exposure induces alterations in lipid metabolism and results in tissue injury. Thus in order to understand the mechanism of nickel toxicity in animals data on the extent of damage to internal organs and metabolic disorders induced by nickel exposure are absolutely necessary. It was, therefore, considered worthwhile, as a part of the long term project, to investigate structural and functional changes induced by chronic exposure of laboratory animals to low as well as high doses of nickel sulfate, a commonly used salt. Since, lipid metabolism seems to be largely affected by nickel (Sunderman, 1986) biochemical investigation about the specific changes in the component lipids in target organs (liver and kidney) at cellular, and subcellular levels may lead to a better understanding of the biochemical basis of toxicity in animals.

As pointed out above most obvious and serious nickel toxicity in plants is related to the seed germination and initial growth and

development of plants, presumably due to imprairment of mobilization of stored food to the growing plant parts from the cotyledons. In other words *de novo* synthesis of enzymes involved in the mobilization of stored food may be inhibited. For example, in order to mobilize the myoinositol hexaphosphate (phytate), the major form of stored phosphate in seeds, *de novo* systhesis of phytase occurs only 24-36h after inhibition of seeds in water. If that is the case, the *de novo* synthesis of phytase will be inhibited. Similarly, there are other enzymes which are likely to be affected by nickel treatment. Some of these enzymes occur in multiple forms indicating polymorphism. It would be interesting to study relative effect of nickel on their *de novo* synthesis. At what molecular level nickel would effect is entirely unknown. We, therefore, thought it is worthwhile to study the effect of nickel on various hydrolases involved in mobilization of essential energy materials during seedling growth. The stunted growth of roots and shoots may be due to the damage in the cell wall formation, especially the cellulose synthesis. Nickel treatment is a kind of chemical injury inflicted to the plant cell. Often under such conditions cellulose synthesis stops and callose (1,3-β-glucan) synthesis occurs which is a wound repair. Defense mechanism in plants is entirely different from animals. Thus, effect of nickel on the glucan synthase activity is of interest and may throw significant light on the possible mechanism(s) of stunted growth of roots and deformation of other plant parts induced by nickel.

Thus in the present study we have used two entirely unrelated organisms i.e, rat and peanut to obtain relevant data on the nickel toxicity in animals and plants. Hence, the thesis is in two parts, part I dealing with nickel effects in rat liver and kidney and part II reporting the results of nickel treatment on peanut seedlings.This study in no way, is done for making a comparison between animals and plants, but instead to gain an insight into the bioprocesses affected by nickel in animals and plants,since the survival of the former species is dependent on the health and bioacceptability of the latter as food and both have to coexist, in any case.

CHAPTER-II

2.0 HISTORICAL RESUME

Nickel is a silver white malleable metal found primarily in sulfide and oxide ores. It is used primarily in steel production, in electroplating in the form of nickel sulfate and nickel hydroxide and is also used in nickel cadmium batteries (Stokinger, 1981). Forchhammer in 1855 was the first to find nickel in oak wood. Again McHargue (1925) demonstrated the presence of nickel, copper, manganese and cobalt in plant, animal and soil samples. Later studies have shown that nickel is necessary for functioning of certain enzyme systems (Bertrand, 1974; Dixon *et al.*, 1975; Polacoo, 1977) and in trace levels it is also beneficial to plant growth (Mishra and Kar, 1974). Dixon *et al.* (1975) noted that urease is a nickel metalloenzyme and reported 2g atoms of nickel per 105,000g of purified urease. Hutchinson and Kuja (1979) reported stimulation of metal-tolerant *Deschampsia cespitosa* clones in nutrient culture at 1.0ppm Ni. Bertrand (1974) found nickel to be an essential element for the microbiological fixation of nitrogen in soil and this could be a key role for nickel in ecosystem.

The toxic properties of excessive nickel in soil for plant life was first pointed out by Haselhoff (1893). As early as in 1913 Wolfe found that 8ppm Ni in solution rapidly killed barley. Later Cotton (1930) found that 0.5ppm nickel produced chlorosis in buck wheat while Brenchley (1938) found 2ppm Ni to be toxic to bean and barley. The symptoms of nickel toxicity appear to be a combination of induced iron deficiency, chlorosis and foliar necrosis. The nickel concentrations in plants growing on soils are usually low i.e. <10ppm. The root sap of peanut contained from 1 to 34.0ppm Ni. However if the nickel concentration in soil exceeds beyond 500ppm then the level of nickel uptake by plant increases resulting in increased level of nickel accummulation in plant parts and decreased growth. Among the major symptoms of nickel toxicity in plants are stunted growth of roots and shoots, deformation of various plant parts and unusual spottings on

leaves and stems. Juma and Tabatabai (1977) found that nickel inhibited the mineralization of organic phosphate and it has a variety effects on enzymes, because it can substitute more or less ably, for other divalent ions, specially Zn. The toxicity of nickel on microorganism such as bacteria (Giashuddin and Cornfield, 1979; Babich and Stotzky, 1983), algae (Fezy *et al.*, 1979; Babich and Stotzky, 1983) and mold (Babich and Stotzky, 1983) is well known.

The major source of nickel uptake by the average person is through food. It is likely to be present in the meat, fish, dairy products, some fruits and even in the cigarettes (Veien and Andersen, 1986). Snyder *et al.* (1975) estimated 0.16mg nickel in whole blood of 70kg reference man. The main excretion route for absorbed nickel is urine with normal values of 2 to 4μg/L and some nickel also appears in sweat and hair (USEPA, 1980). Absorbed nickel is transported by a plasma protein called "nickeloplasmin". Disc gel immuncelectrophoresis showed that purified nickeloplasmin migrates as a single protein band in the α_2- globulin region and sedimentation equilibrium studies indicated that nickeloplasmin is a α_2 - macroglobulin, with estimated molecular mass of 7.0×10^5 (Nomoto *et al.*, 1971).

Nickel is a toxic metal of environmental concern that has been found to be carcinogenic in man and animals (Haugen *et al.*, 1989). Nickel toxicity has been studied by many workers in the different animals (Foulkes and Blanck, 1984; Ciccarelli and Wetterhahn, 1984). Peligero *et al.* (1985) reported the hyperglycemia in the pregnant rats due to nickel poisoning. The effect of nickel on the primates has been noted by Haley *et al.* (1987). Due to nickel exposure, the immune function is affected in the rats (Smialowicz *et al.*, 1987). Benson *et al.* (1987, 1988) and Blackburn and Highsmith (1990) noted the alteration due to nickel poisoning. Novelli and Rodgrigues (1987) reported the effect of nickel on streptozotocin induced diabetes in rats. Many workers have reported the alterations in blood constituent in rat after various heavy metal treatment. Nickel sulfide induced chromosomal change in cell lines from mouse tumors (Christie *et al.*, 1988). Toxicity and carcinogenecity of nickel compounds was noted by

Sunderman (1984). Benson *et al*. (1987) observed the toxicity of nickel on the lungs of the rat and mice. Miura *et al*. (1989) reported the induction of cytotoxicity and morphological transformation in mouse embryo fibroblasts by soluble and insoluble carcinogenic nickel compounds. Recently Baleux *et al*. (1993) found the increased sister chromatid exchanges and tumor markers in workers exposed nickel containing dust.

Boysen *et al*. (1984) observed that nickel is accumulated in human lungs. Whanger (1973) found that feeding 500μg nickel acetate/g diet of weanling rats for 6 week significantly depressed growth, while high dietary levels of nickel acetate (500-100μg/g) significantly depressed haemoglobin concentration, PCV plasma and alkaline phosphatase activity. Waalkes *et al*. (1985) observed that high proportion (60%) of the animals receiving nickel alone died during exposure, whereas zinc pretreated animals showed a higher rate of survival (80%) during the same period.

It has been suggested that nickel penetrates the cell nucleus *in vivo* and may bind tightly to DNA with consequent inhibition of DNA-dependent RNA synthesis (Lee *et al*., 1982). It is observed that nickel carbonyl intervene at the level of messenger RNA, inhibiting DNA-dependent RNA polymerase in liver nuclei (Lau *et al*., 1972; Witschi, 1972). Biochemical studies also show that nickel (II) salts react easily with DNA and impair the fidelity of DNA synthesis (Sirover and Loeb, 1976; Miyaki *et al*., 1977). Nickel ions produce selective damage in heterochromatic regions of chromosomes. This deletion of a heterochromatic chromosomal regions may be an important feature of the nickel-induced carcinogenic process (Conway and Costa, 1989). Considerable evidence suggests that nickel can act indirectly upon DNA by inducing the formation of oxidized purines or pyrimidines that constitute promutagenic lesions (Higinbotham *et al*., 1992). Recently, Cheng *et al*. (1993) reported that Ni (III) can promote DNA cleavage with ambient dioxygen. Schroeder *et al*. (1964) suggested that nickel can cross the fetamaternal barrier and enter the fetus during late gestation. It can upset the hormonal balance of the mother, and

thus affect pregnancy. LaBella *et al.* (1973 a,b) showed that release of prolactin from the rat pituitary *in vivo* and *in vitro* is inhibited by nickel ion and might affect the interactions between hypothalamus and pituitary gland needed to maintain pregnancy.

Jasmin and Riopelle (1976) demonstrated marked erythrocytosis following intrarenal administration of nickel sulfide. Hopfer *et al.* (1979) have investigated the association between erythrocytosis and renal cancers in rat. Templeton (1987) investigated the binding of nickel to glomerular basement membrane of the kidney. Nickel was found to bind anionic glycosaminoglycan sites of the membrane. Ionic blocking of these sites leads to the loss of selectivity in the filtration of albumin (Assel *et al.*, 1984), which explains the proteinuria. Gitlitz *et al.* (1975) reported aminoaciduria and proteinuria in rats after intraperitoneal injection of 2 to 5mg Ni/kg. Kurokawa *et al.* (1985) showed that nickel chloride exerts a strong promoting effect on renal tumorigenesis in rats. Administration of nickel carbonate to rats induces DNA-protein cross-links and DNA strand breaks in kidney (Ciccarelli and Wetterhahn, 1984). Nickel is known to trigger coronary vasoconstriction and inhibit cardiac contractivity (Rubanyi and Kovach, 1980). Poisoning of dogs and cats by nickel nitrate was reported to produce acute renal injury with proteinuria and hyaline casts (Azary, 1879).

There are reports that alveolar macrophages are cellular target for nickel toxicity (Medinsky *et al.*, 1987). Pulmonary lesions were described by Boysen *et al.* (1984) and found hyperplastic/polyploid rhinistis. The effects of inhalation exposure to metal aerosol derived from nickel refinery waste were studied on the frequency of chromosome alterations in alveolar macrophages in Wistar rats (Chorvatovicova and Kovacikova, 1992) and effects on pulmonary surfactant and ascorbic acid was also observed by Kovacikova and Chorvatovicova (1992).

Various epidemiological studies showed that occupational exposure to certain nickel compounds is followed by an increased lung cancer incidence (Akslen *et al.*, 1990).Intraperitoneal administration of

nickel chloride enhanced hepatic lipid peroxidation (HLP) in 6-ωk-old and 8-12-ωk-old mice and depleted hepatic GHS in 8-12-ωk-old mice but not in the younger age group (Andersen and Andersen, 1989). Sbinson *et al*. (1992) observed that lipid peroxidation is not causally retaled to genetic damage. Nickel chloride-induced DNA strand breakage may be caused by the induction of Fenton reaction, generating hydroxyl radicals.The administration of nickel to mice resulted in an inhibition in the activity of free radical reductase, and enhanced lipid peroxidation and the activity of glutathione-S-transferase in a close dependent manner (Srivastava *et al*., 1990). Andersen and Andersen (1989) studied the effect of nickel chloride on hepatic lipid peroxidation (HLP) and glutathione concentration in mice and demonstrated age dependency and a protective effect of enhanced GSH synthesis in nickel chloride stimulated HLP. Rodriguez *et al*. (1991) studied nickel-induced lipid peroxidation in the liver of different strains of mice and its relation to nickel effects on antioxidant systems.

Nickel has also been reported to be related to various physiological conditions (Sunderman *et al*., 1987). At 125-750μmol/kg level of nickel the activity of serum alkaline phosphatase was depressed and the activity of serum aspartate aminotransferase (AST) and alanine aminotransferase was increased (Donskoy *et al*., 1986). Athar *et al*. (1987) observed that administration of nickel to rats resulted in increase in the activities of glutathione reductase and glutathione -S- transferase with concomitant decrease in the activities of glutathione peroxidase and γ - glutamyl transpeptidase. The increase in glutathione -S- transferase suggests the ability of the tissue to cope against toxic insult of nickel by increasing its detoxifying capacity (Athar *et al*., 1987). Sunderman (1977) observed that the toxic action of nickel carbonyl may be due to inhibition of ATpase activity or RNA polymerase activity in the target tissues and nickel inhibited induction of various enzyme systems.

Srivastava *et al*. (1988) demonstrated that nickel is concentrated in a number of critical target tissues such as kidney, lung, heart and

liver. The sulfhydryl content in liver, kidney and lung of the animals exposed to nickel were estimated as these groups may effect the cellular function (Peers *et al.*, 1983; Whanger, 1973; Marzouk and Sunderman, 1985). The level of sulfhydryl groups might also serve as an indication of the biological disturbances. Jasmin and Riopelle (1976) noted that kidney is highly susceptible to nickel.

Nickel was shown to be related to a wide variety of pathophysiological conditions (Gitlitz *et al.*, 1975; Horak and Sunderman, 1975). Sunderman *et al.* (1984) found renal tubular hyperplasia and arteriosclerosis in rats that developed erythrocytosis. Recently Knauf and Thiel (1993) studied gastric lymphoma after exposure to chromium and nickel. Nickel compounds affect the erythrocyte membrane lipid bilayer, as well as membrane proteins to various extents, depending on the type of compounds used which suggest that nickel compounds decrease water permeability across erythrocyte membranes and decrease erythrocyte thermostability, deformability, and the rate of oxygen release by erythrocytes (Tkeshelashvili *et al.*, 1989). The nickel exposure also leads to disturbance of the electrolyte, reticulocytic and erythrocytic metabolism (Vodichenska, 1991).

The kidneys of rats that received intrarenal injections of nickel subsulfide showed, active proliferation of fibroblastic cells, regenerative hyperplastic tubules near the needle tracks and arteriosclerotic changes in renal arterioles (McCully *et al.*, 1982). Electron microscopy revealed fusion of the foot processes of epithelial cells in renal glomeruli at 48h after injection of nickel (Gitlitz *et al.*, 1975). Foulkes and Blank (1984) observed tubular lesions after intraperitoneal injections of nickel. Nath and Kumar (1990) studied the gonadial histopathology in giant gaurami *Colisa fasciatus* after nickel treatment. Immortalization of normal human kidney epithelial cells due to nickel was observed by Tveito *et al.* (1989). Primary human kidney cells were immortalized or rescued from senescence after exposure to nickel sulphate (Haugen *et al.* 1989).

High level of nickel sulfate (25mg/kg body weight daily) orally administered to male rats for 120 days resulted in marked dystrophic histopathological changes in the testis. Sano *et al.* (1988) using histopathological and immunohistochemical studies distinguished immature myogenic tumors induced by nickel from other primitive tumors and malignant fibrous histiocytoma from other plemorphic mesenchymal tumors. Recently Alvarez *et al.* (1993) studied that α_2-adrenergic blockade prevents hyperglycemia and hepatic glutathione depletion in nickel injected rats. There are reports that inhalation exposure of rabbits at low levels (0.1-1mg/m^3) of nickel in metallic or soluble form affects alveolar epithelial type II cells and increases the content of phospholipids in the lung (Wiernik *et al.*, 1981). Misra *et al.* (1991) observed lipid peroxidation and active oxygen-detoxifying enzymes like catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), as well as glutathione (GSH) and some related enzymes in kidney of mice after intraperitoneal injection of nickel acetate and emphasize the importance of GSH and GSH-Px for preventing nickel-induced oxidative cell damage.

Misra *et al.* (1990) studied lipid peroxidation (LPO) and alterations in cellular systems protecting against oxidative damage in the liver, kidney and skeleton muscles of male rats and observed that LPO was highest in the kidney and lowest in the muscles, which concurs with the corresponding ranking of nickel uptake by these tissues.It has also been speculated that nickel readily binds to hepatic proteins which are ultimately transported to various sites and retained especially in the kidney (Behari *et al.*, 1984; Peers *et al.*, 1983) and bind to low molecular weight metallothionein like proteins (Behari *et al.*, 1984). The combination of excessive oxygen free radical production and inhibition of their elimination by inhibition of SOD activity may contribute to the nickel toxicity that has been reported industrial accidents, as well as to the high incidence of cancer occurring in nickel workers (Shainkin-Kestenbaum *et al.*, 1991).

From the above review of literature, it is evident that some of the toxicological aspects of nickel in animals have been studied but

very little data are available in plants. In addition, the mechanism of nickel toxicity at subcellular and molecular levels, in both animals and plants, is far from clear. The present study describes the results of our investigations on *in vivo* toxicity of nickel in rats and plants (ground nuts) based on biochemical and histological studies.

CHAPTER-III

3.0 EXPERIMENTAL PROCEDURES

3.1 Materials

All chemicals used in this study were of analytical reagent grade of the highest quality available and were purchased from the reliable firms. UDP-[^{14}C]glucose (296Ci/mol), GDP-[^{14}C]mannose (228Ci/mol) and UDP-N-[^{14}C]acetylglucosamine were purchased from the Radiochemical centre, (Amersham, UK). Unlabeled UDP-glucose, various nucleotides, sugar nucleotides, adenosine, glucose-6-phosphate, bovine serum albumin (BSA), authentic lipid standards, p-nitrophenyl phosphate, Tris, sodium dodecyl sulfate (SDS), cellobiose, acrylamide, N,N'-methylene-bis-acrylamide and TEMED and other substrates for enzyme assays were obtained from Serva (Germany). Hydroxylapatite gel material for column chromatography and the molecular weight standards were obtained from Bio-Rad (Richmond, CA). All other chemicals were reagent grade from standard commercial firms.

Adult male albino rats of Wistar Strain (100±10g) were obtained from Haryana Agricultural University, Hissar (India). Peanut seeds (large variety) were procured from the local seed store.

3.2 PART-I: STUDIES WITH ANIMALS

3.2.1 Maintenance of animals

Colony bred adult male albino rats originally derived from the Wistar strain were maintained in a well ventillated animal room with 12h light and 12h dark schedule. They were fed a standard pelleted diet (Lipton, India) and water was made available *ed libitum*. Essential cleanliness and sterile conditions were maintained.

3.2.2 Nickel treatment of rats

For nickel treatment forty, 60-day old male albino rats (*Rattus rattus albino*), weighing 100±10g were randomly selected from the laboratory stock and placed into 4 groups (I, II, III and IV group) of

10 rats each. The rats were housed individually in plastic cages with galvanized iron wire bar tops and were provided pellet diet (Lipton, India) and tap water *ad libitum*. Rats in groups I, II and III, in addition to receiving pellet diet, were intramuscularly administered $NiSO_4.7H_2O$ (0.5, 1.0 and 2.0mg/100g body weight, a sublethal dose), dissolved in 0.5ml of physiological saline (0.9% NaCl solution) on alternate days for a period of 15 days, respectively. Rats in group IV, which served as control, were given 0.5ml saline in an identical manner for the same. After the scheduled treatment rats were starved for 24h before collection of blood and other tissues of interest for haematological or biochemical studies.

3.2.3 Haematological analysis

For haematological analysis blood samples from rats of each group were collected directly from the heart of the animal with the help of a 5ml presterilised plastic disposable syringe in two vials, one containing EDTA (1.5mg/ml blood) and the other without EDTA. No haemolysis was observed during blood collection. The blood sample in the former vial (with EDTA) was analyzed for total red blood cells (RBC), leucocyte counts (LC) and haemoglobin (Dacie and Lewis, 1977). RBC and LC were counted by Neubauer double haemocytometer. The diluting fluid for RBC contained 0.6% NaCl, 1g sodium citrate and 1g formalin in 100ml double distilled water. For LC the composition of the diluting fluid was as follows: glacial acetic acid (1.5ml), gentian violet (1mg) and distilled water (98.5ml).

The blood sample collected without EDTA was first allowed to clot at room temperature for 60min followed by 6h incubation at 0-4°C. The serum was separated by centrifugation at 7000xg for 15min in a refrigerated centrifuge. The clear serum was anslyzed for reducing sugars (Somogyi, 1945), cholesterol (Zak *et al.*, 1954), total bilirubin (Malloy and Evelyn, 1937) creatinine (Bonsnes and Taussky, 1945) and urea (Levine *et al.*, 1961) using standard analytical procedures, which are briefly described below:

3.2.3.1 Determination of reducing sugars

One ml serum was deproteinized by adding 9.5ml of $Ba(OH)_2$ solution and equal volume of $ZnSO_4$ solution. After mixing, the precipitate was separated by centrifugation. To the clear filtrate (0.25ml) was added 0.5ml alkaline copper reagent. The reaction mixture was heated in a boiling water bath for 20min, cooled to room temperature. 2.5ml of arsenomolybdate colour reagent (120mg of $Na_2HASO_4.7H_2O$ added to 100ml of 5% ammonium molybdate solution in 0.6N H_2SO_4) was added and absorbance measured at 540nm against blank. Reducing sugars standard curve was prepared simultaneously in the same way

3.2.3.2 Determination of cholesterol

To 0.1ml serum was added 5ml of 0.1% $FeCl_3$ in acetic acid. After 10min incubation at room temperature the sample was centrifuged. H_2SO_4 (3ml) was then added dropwise to the supernatant and mixed on a vortex. After 20min absorbance was measured at 560nm against ferric chloride-acetic acid reagent blank. Standard curve was prepared in a similar manner with known amounts of cholesterol.

3.2.3.3 Determination of bilirubin

The serum was diluted 10 x with 0.9% NaCl solution. To 2.5ml of the diluted serum sample were added 0.5ml of diazo reagent (10ml of 0.5% sulphanilic acid in 0.7N HCl + 0.5ml of 20% aqueous solution of $NaNO_2$) and 3ml methanol. The mixture was mixed on cyclomixer and incubated at room temperature for 30min. The absorbance was measured at 540nm against a blank. Standard curve was prepared in the same manner, using standard solution of bilirubin.

3.2.3.4 Determination of creatinine

One ml serum sample was added dropwise to 2ml of acid-iodine reagent (1 vol of 0.05 N I_2 reagent, 3 vol H_2O and 4 vol of 0.7N H_2SO_4) in a test tube. After 5min at room temperature 2ml of 6.6% aqueous solution of sodium tungstate was added and further incubated

for 10min. The supernatant was extracted with 5ml of chloroform and the aqueous phase was transferred to a 15ml bottle containing granulated zinc (500 mg). After hydrogen gas evolution had ceased, 1ml of the clear supernatant was taken in a glass stoppered 15ml tube and 0.4ml of 0.033N picric acid was added. The mixture was mixed carefully on a cyclomixer followed by extraction with 3ml ether, lower phase (aqueous) was collected by Pasteur pipette. To 2ml of the aqueous phase 0.3ml of NaOH-EDTA reagent (0.75N NaOH containing 5% EDTA) was added. After 20 min incubation at room temperature the absorbance was measured at 520nm against a blank prepared simultaneously in which equal volume of water was used in place of serum. Standard plot was also prepared concurrently using pure creatinine sample.

3.2.4 Determination of enzyme activities

The activities of glutamic - oxaloacetate transaminase (GOT), glutamic-pyruvate transaminase (GPT), acid and alkaline phosphatases, glucose-6-phosphatase, and lipase in serum and in liver and kidney were assayed by standard procedures as described by Bergmeyer (1974). When tissues (liver and kidney) were used as source of enzyme, the crude enzyme extract was prepared as follows: Fresh tissue (10g, wet weight) was homogenized in 100mM citrate buffer, pH 6.0, containing 250mM sucrose in a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20min at 5,000xg. The post-nuclear supernatant was dialyzed against 50mM citrate buffer, pH 6.5, and used as source of enzyme. All operations, unless stated otherwise, were performed at 0-4^{o}C. The enzyme assays used are briefly described below:

3.2.4.1 Phosphatases

For assaying alkaline and acid phosphatase activities the method of Morton (1955) was adopted. The following incubation mixture was used in a total volume of 1ml. Sodium β-glycerophosphate (16mM) adjusted to pH 9.3 (or pH 5.0 for acid phsphatase) and 0.1ml enzyme extract(approximately 1mg protein). After incubation for 60min at 37^{o}C, the enzyme reaction was terminated by the addition of 1ml of 10%

trichloroacetic acid and then centrifuged for 20min at 10,000 xg to remove denatured protein. The released inorganic phosphorus was determined by Fiske and Subbarow method (1925). For controls, enzyme was added to the substrate after terminating the enzyme reaction with trichloroacetic acid.

3.2.4.2 Glucose-6-phosphatase

The activity of glucose-6-phosphatase was essentially measured by the method of Swanson (1965). Enzyme sample (0.1ml, 1mg protein) was incubated in 50mM citrate buffer, pH 6.5, containing 25mM glucose-6-phosphate (sodium salt), for 30min at 37°C. Rest of the procedure was the same as described for alkaline and acid phosphatases in section 3.2.4.1.

3.2.4.3 Lipase

The lipase activity was measured by the method of Bier (1955).

3.2.4.4 Glutamic-oxaloacetate transaminase (GOT)

The activity of GOT was assayed by the method of Karmen *et al.* (1955). The sample of serum or enzyme extract (0.2ml) was added to 1.7ml of 50mM potassium phosphate buffer, pH 7.4, containing 0.5ml L-aspartate (5mM, pH 7.4), 0.3ml $NADH_2$ (100μg/ml) and 0.1ml malic dehydrogenase (100 units). Simultaneously a blank was prepared without $NADH_2$. Incubation was carried out for 15min at 37°C in a quartz cuvette and 0.2ml of 5mM α-ketoglutarate (pH 7.4) was added and optical density was measured at 340nm at one min intervals. Decrease in optical density in 5min x 1000 = units of GOT activity/ml serum.

3.2.4.5 Glutamic-pyruvate transaminase (GPT)

The activity of GPT was assayed by the method of Wroblewski and La Due (1956). The incubation mixture contained the following components in a total volume of 2.8ml. Serum (0.2 ml), 1.7ml potassium phosphate buffer (50mM), pH 7.4, 0.5ml L-alanine (5mM, pH 7.4), 0.3ml $NADH_2$ (100μg/ml) and 0.1ml LDH (800 units).

Simultaneously a blank was prepared without $NADH_2$. Incubation was carried out in quartz cuvette for 15min at 37°C followed by the addition of 0.2ml of 5mM α-ketoglutarate. Optical density was measured at 340nm at one min intervals. Decrease in optical density in 5min x 1000 = units of GPT activity/ml serum.

3.2.5 Histopathological methods

For the histopathological studies rats were dissected. Liver and kidney were immediately separated from the adjoining tissues. Small pieces of these tissues procured at random were fixed in 10% neutral formalin. After dehydrating in graded alcoholic series and clearing in xylene the tissues were embedded in paraffin wax (m.p. 52-54°C). Serial sections of 3.4μm thickness were cut and stained with haematoxylin and counter stained with eosin. In addition, silver impregnation technique (Gordon and Sweet, 1936) and picric acid fuchsin were employed for reticulin and collagen fibres, respectively. The histopathological changes were noted by comparing the structure with that of the control tissues.

3.2.5.1 Histochemistry of lipids

Paraffin sections of tissues, fixed in 10% neutral formalin, were processed for the histochemical examination of neutral lipids (Pearse, 1961) and phospholipids (Pearse, 1961). For neutral lipids sections were stained in Sudan black-B (0.3%, w/v, in 70% ethanol) for 10min, rinsed in 70% ethanol for 5-10s, with gentle agitation, followed by washing with double distilled water. In some cases counter stain was also applied. Neutral lipids (triacylglycerols) appear in shades of very dense blue and black. For phspholipids sections of tissues, mounted on slides, were immersed in dichromate - calcium solution (5g of $K_2Cr_2O_7$ and 1.5g $CaCl_2$ in 100ml water) for 60min at 60°C. Sections were then repeatedly washed with water until excess dye was removed and then treated with borax - ferricyanide differentiator (0.25g of $K_3Fe(CN)_6$ and 0.25g of $Na_2B_4O_7 \cdot 10H_2O$ dissolved in 100ml distilled water) for 18h at 80°C. After that the sections were washed with water and mounted in an aqueous medium.

3.2.5.2 Histochemical localization of enzymes

For the histochemical localization of enzymes the fresh tissues were cut into pieces and fixed in two changes of chilled absolute acetone for 8h. After clearing with benzene, the tissue pieces embedded in paraffin wax and sections (2-3 μm) were cut and processed routinely for the enzyme localization as follows.

Alkaline phosphatase was localized by the following method of Gomori (1952) using sodium β-glycerophosphate as substrate at pH 9.3. Briefly, the sections were deparaffinized and placed in water for washing. The washed sections were then incubated in a reaction mixture consisting of 25mM sodium β-glycerophosphate (pH adjusted to 9.3), 0.4% sodium veronal. 100mM $CaCl_2$ and 2.5mM $MgCl_2$, for 2h at 37^{o}C. After incubation the sections were quickly washed with distilled water and treated with 2% (w/v) cobalt nitrate for 2min, followed by washing with water to remove excess of cobalt salt, and exposed to 10% ammonium sulfide for 1min. Finally, the sections were washed with water, dehydrated through a series of graded alcohols, cleared with xylene and mounted for microscopy.

Glucose-6-phosphatase was localized histochemically by the method of Wachstein and Meisel (1956) incubating the fresh sections for 15min at 32^{o}C in an incubation mixture containing 1.5mM potassium glucose-6-phosphate, 50mM Tris-HCl buffer (pH 6.7), and 1% (w/v) lead nitrate. After incubation, sections were washed in distilled water and developed in dilute yellow ammonium sulfide, followed by washing with water. The washed sections were then fixed in 6% neutral formalin and mounted in glycerol for microscopy.

3.2.6 Subcellular fractionation from rat liver

Various subcellular membrane fractions from rat liver were prepared essentially as described by Fleischer and Kervina (1974). Control and nickel-treated male Wistar rats were starved for 14-16h before sacrificing to reduce liver glycogen content. The rats were sacrificed, liver was excised and immediately processed for subcellular fractionation. All steps were carried out at 0-4 C unless stated otherwise.

Buffers used were :

(i) Homogenization buffer (Buffer H)

0.01M HEPES (pH 7.5) + 0.25M sucrose

(ii) Suspension buffer for nuclei pellet (Buffer N)

0.01M HEPES (pH 7.5) + 0.25M sucrose + 1mM $MgCl_2$

(iii) Suspension buffer for plasma membranes (Buffer P)

0.01M HEPES (pH 7.5) + 0.25M sucrose + 1mM EDTA

Fresh liver (10g) was cut into small pieces and homogenized in Potter-Elvehjem homogenizer in 50ml buffer H. The homogenate was filtered through four layers of cheese cloth and centrifuged at 960xg for 10min. The pellet and supernatant fractions were processed for various subcellular membrane fractionation following the procedure outlined in Fig.1.

3.2.7 Determination of lipid peroxidation

The tissues (1g fresh weight) were homogenized in a glass/teflon homogenizer in 10ml ice-cold 1.15% KCl. Triplicate aliquots (0.5ml) of the homogenate were used for the determination of lipid peroxidation as described by Sunderman *et al.* (1985). The results are expressed as nmol of malondialdehyde (MDA) per g of fresh weight of tissue.

3.2.8 Determination of neutral and phospholipids

3.2.8.1 Extraction of total lipids

Total lipids from fresh tissues (10g) were extracted according to the procedure of Folch *et al.* (1957). The tissue was homogenized in 10 volumes of chloroform-methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as an antioxidant. The homogenate was centrifuged at 7,000xg for 20 min and supernatant fraction was separated from the pellet by decantation. The extraction procedure was repeated thrice and the resulting supernantants were pooled and evaporated to dryness on a thin film rotatory evaporator at 40°C under a stream of nitrogen gas.

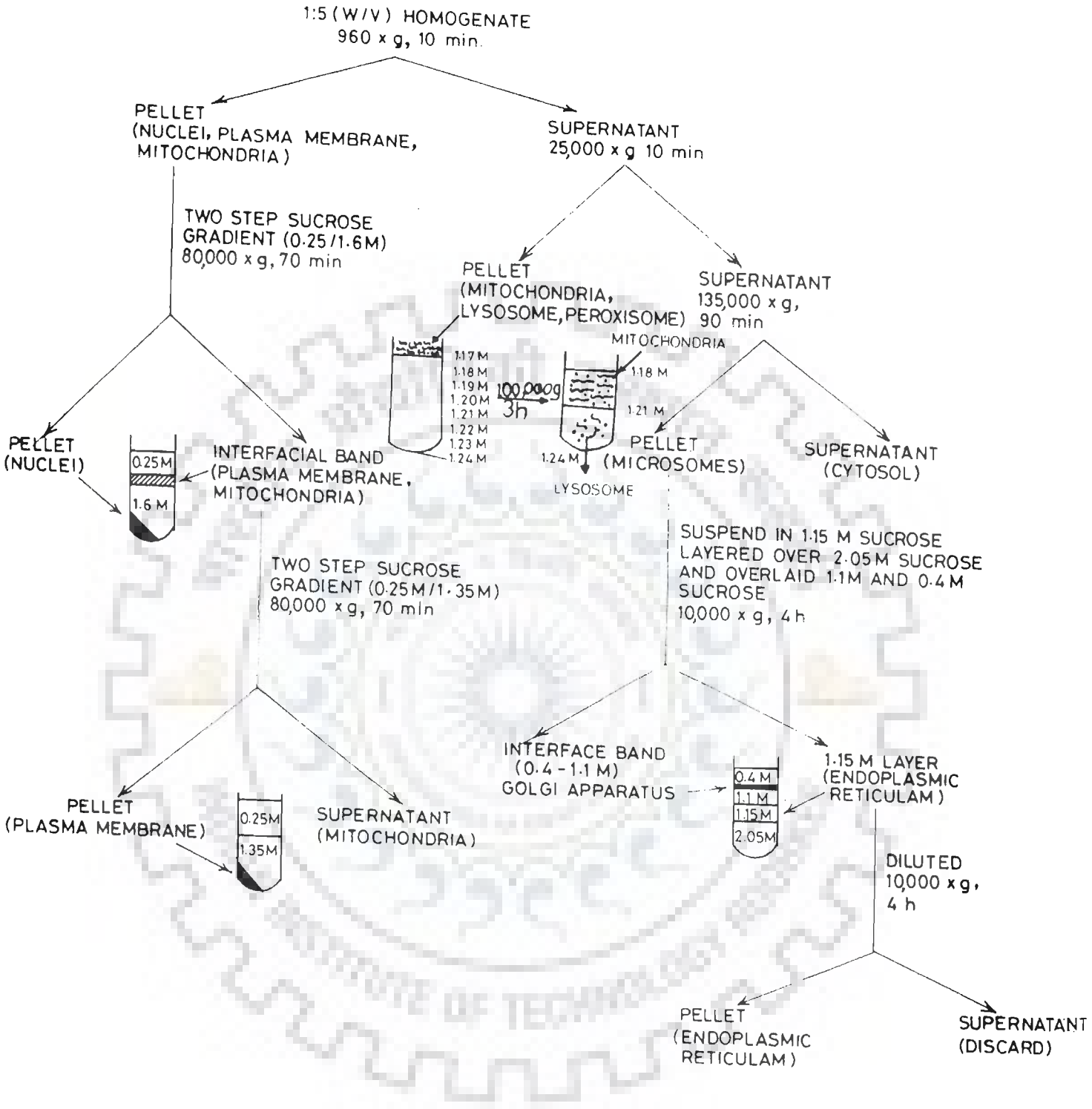


FIG. 1 SUBCELLULAR FRACTIONATION FROM RAT LIVER.

The residue was further suspended in chloroform- methanol-water (16:8:1, v/v/v) containing 0.01% BHT and then dried under nitrogen at 45°C. This process was repeated thrice to ensure breaking of proteolipid complex. Finally, the residue was extracted in 10ml of Chloroform - methanol (2:1) and partitioned into aqueous and organic phases by the addition of 1ml of 0.9% NaCl. The mixture is centrifuged at 5,000xg for 15min. The upper phase (aqueous methanol) contains glycolipids.

The lower phase which contained neutral and phospholipids was dried under a stream of nitrogen gas, redissolved in 1ml $CHCl_3$ and analyzed for neutral and phospholipids by thin layer chromatography (TLC), gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

3.2.8.2 TLC of neutral lipids

TLC was used for the separation of neutral lipids as described by Mangold (1965). Silica gel-G coated (0.25mM) glass plates (20x20 cm) were routinely used. Prior to use plates were developed in methanol to remove contaminants and activated by heating in an oven at 110°C for 60 min. The plates were cooled to room temperature and lipid samples were applied on the plates with the help of micropipettes. The chromatoplates were then developed sequentially in the following solvents:

Solvent I : n-hexane-diethylether-glacial acetic acid (60:40:1)

Solvent II : n-hexane-diethylether-glacial acetic acid (90:10:1)

Solvent III : n-hexane-diethylether-glacial acetic acid (30:70:1)

In solvent I the plates were developed upto a height of 7cm. The plates were dried in air and further developed in solvent II upto a height of 15cm. The plates were air dried once again and placed in an iodine chamber to identify the diacylglycerol fraction. After this solvent III was used and the solvent was allowed to run just below the diacylglycerol band. After air drying the plates were exposed to iodine vapours to locate various neutral lipids (monoacylglycerols, diacylglycerols, free cholesterol, triacylglycerol, and esterified

cholesterol) on the plate. Authentic neutral lipid standards were chromatographed simultaneously for identification of various classes of neutral lipids by matching the R_F values.

3.2.8.3 TLC of phospholipids

Phospholipids were analyzed by TLC as described by Abramson and Blecher (1969) using chloroform-methanol - 7N NH_3 (115:45:7.5) as the solvent system. The lipid spots were located by exposing the plate to iodine vapours. The individual phospholipids were identified by comparing the R_F values with that of authentic phospholipid standards (phosphatidyl inositol, PI; phosphatidyl serine, PS; sphingomyelin, SM; phosphatidyl choline, PC; phosphatidyl ethanolamine, PE; and phosphatidic acid, PA).

3.2.8.4 Quantitative estimation of triacylglycerol by GLC

The amount triacylglycerol in the neutral lipid fraction was quantitatively estimated by GLC according to Mares and Husek (1985). Analysis was carried out on the Hewlett-Packard-1 (methyl silicon gum) gas chromatograph equipped with flame ionization detector and linear temperature programme. Capillary columns (5m x 0.55mm x 2.65mm) were used. Prior to use, columns were stabilized overnight at 300°C with a helium gas flow rate of 2.6ml/min. Stock solution (1mg/ml) of standard triacylglycerol (tripalmitin) was prepared in chloroform. All reagents used were reagent grade quality.

3.2.8.5 Phospholipid analysis by HPLC

Phospholipids were analyzed, both qualitatively and quantitatively, by HPLC, according to Curstedt *et al.* (1983) on a Shimadzu LC-4A equipped with a SPD-2AS variable wavelength UV-spectrophotometric detector and a chromatopac C-RZA integrator. The separation of phospholipids was achieved with a Nucleosil-5 C_{18} reverse phase column (25cm x 4.6mm I.D) using methanol-water-acetonitrile (7:2:1, V/V/V) at a flow-rate of 1.5ml/min isocratically. The phospholipid peaks were detected at 214nm. The

column was calibrated using authentic phospholipid standards.

3.3 PART-II : STUDIES WITH PLANTS

3.3.1 Germination of seeds

Healthy peanut (*Arachis hypogaea L.*) seeds were surface sterilized by treatment with 0.1% NaOCl solution for 5 min, followed by thorough washing with sterilized deionized water. These seeds were then soaked in deionized water without or with specified amount of $NiSO_4.7H_2O$ for 24h and spread singly in plastic trays lined with 2 layers of cheesecloth or Whatman No. 3 filter paper which was premoistened with the same solution. The trays were then kept in BOD incubator at 30°C for germination under asceptic conditions. Seedlings at various stages of germination were picked up at random for analysis. On an average about 100 seedlings were used per experiment, and a set of three experiments were performed for each treatment.

3.3.2 Preparation of plasma membrane fraction

Unless stated otherwise all preparations were carried out at 0-4°C. The plasma membrane fraction was prepared from peanut cotyledons as described by Sharma *et al.* (1986). Briefly, 200 cotyledons (80-100g wet wt) from peanut seedlings that had been grown for 7 days in the dark at 35°C were suspended in 400ml (50mM) Tris-HCl, pH 7.4, containing 0.25M sucrose, 3mM EDTA, 1mM phenylmethylsulfonyl fluoride (protease inhibitor), and 0.04% β-mercaptoethanol (buffer A) and homogenized by 30-s bursts in a Waring blender. The homogenate was squeezed through four layers of cheese cloth and the filtrate was successively centrifuged at 12,000g for 20min and 150,000g for 60min. The resulting, 12,000-130,000g pellet (crude microsomes) was suspended in 4ml of buffer B (25mM Tris-HCl, pH 7.4, containing 20% (w/w) sucrose, and 1mM β-mercaptoethanol), layered carefully over 5ml 34% (w/w) sucrose in the same buffer, and centrifuged at 80,000g for 90min. The pellet was resuspended in 2ml of buffer B, layered over a discontinuous sucrose gradient prepared in 38ml tubes by layering 4ml 45% (w/w) sucrose and 6.4ml each of 38,34,30,25 and 20% sucrose in the same buffer and immediately centrifuged at 95,000g for 2h in an SW-27

rotor. Visible bands of membranes were removed with a Pasteur pipette. The plasma membrane fraction (the 45-38% interface band) was identified by the presence of marker enzyme glucan synthase II (Hall, 1983). The total protein in the plasma membrane fraction varied between 30 and 40mg per 100g (wet wt) cotyledons. The membrane fraction was suspended in buffer B (20mg/ml) and used in subsequent experiments.

3.3.3 Enzyme Assays

3.3.3.1 Assay of the callose synthase activity

The activity of callose synthase was measured as described by Ray (1977), using a high concentration of UDP-glucose in the absence of Mg^{2+} with a slight modification. The standard incubation mixture, unless stated otherwise, contained the following in a final volume of 100ml. Tris-HCl (50mM, pH 7.4), UDP-[^{14}C]-glucose (0.1μCi, 290 Ci/mol), 0.5mM unlabeled UDP-glucose, 5mM cellobiose, 2mM $CaCl_2$, 0.01% digitonin, and 0.02 to 0.1mg protein, depending upon the status of purification of the enzyme. After incubation for 20min at 25^{o}C the reaction was terminated by the addition of 1ml ethanol, 0.05ml 50mM $MgCl_2$, and 0.15ml boiled plasma membrane (0.1-1.5mg protein) as a carrier for the labeled products. The mixture was immediately boiled for 1min and after standing overnight at 4^{o}C the polymer was separated by centrifugation at 3000g for 10min. The pellet was washed four times with 70% (v/v) ethanol to remove all the unreacted radioactive substrates and ethanol soluble products. The washed precipitate was suspended in 5ml scintillation fluid (dioxan cocktail) and radioactivity measured in a Beckman L.S. 1801 liquid scintillation counter. Control assays were performed exactly the same way except that an equivalent amount of boiled enzyme was used in place of active enzyme preparations. Under the assay conditions the transfer of radioactive glucose from UDP-[^{14}C]glucose to the glucan polymer was linear up to 20min. One unit of activity is defined as that which catalyzes the incorporation of 1nmol of glucose/min into ethanol-insoluble glucan.

3.3.3.1.1 Solubilization of the callose synthase

Solubilization was done as described by Kamat *et al*. (1992). All steps were carried out at 0-4°C. To the plasma membrane fraction (300mg of protein) was added dropwise, with gentle stirring, 1% (w/v) digitonin in buffer B, so that the final detergent concentration was 0.5% and the protein: detergent ratio was 1:6. The enzyme was solubilized by three strokes in a Teflon homogenizer followed by 30min incubation in ice. The homogenate was then centrifuged at 105,000g for 60min to remove the insoluble material. The clear supernatant liquid, which contained the solubilized callose synthase, was carefully removed with the use of a Pasteur pipette. This enzyme preparation was fully active for up to 30 days when stored at -20°C and about 96h at 0°C.

3.3.3.1.2 Sucrose density gradient centrifugation

All steps were carried out as described in Kamat *et al*. (1992). Digitonin-solubilized enzyme (1.5ml) was layered onto a continuous sucrose density gradient (20-60%, w/v, dissolved in buffer B). Centrifugation was carried out for 8h at 200,000g using a TST 41.14 Swing bucket Centrikon rotor. Fractions of 0.5ml were collected by piercing the bottom of the tube and an aliquot (0.1ml) from each fraction was assayed for enzyme activity and protein.

3.3.3.1.3 Hydroxylapatite column chromatography

The hydroxylapatite gel material (20gm) was suspended in 100ml of 10mM sodium phosphate buffer, pH 6.8 by a gentle swirling motion and was allowed to swell for 60min at room temperature. The clear supernatant fluid along with fine particles of the phosphate gel was decanted off slowly. The fully generated gel was packed in a glass column (1cm x 10cm) and equilibrated with buffer C (10mM phosphate buffer containing 1mM β-mercaptoethanol and 0.01% digitonin) till the pH of the eluent was 6.8. Routinely the column was equilibrated overnight at a flow-rate of 0.2ml/min in cold (0-4°C). The enzyme fractions from the density gradient centrifugation step was dialyzed

overnight against the equilibration buffer (buffer C) and loaded onto the hydroxylapatite column at the same flow-rate. After washing the column with 20ml of equilibration buffer, the adsorbed proteins were eluted by the linear gradient from 10 to 350mM sodium phosphate buffer, pH 6.8, using a single mixing containing 0.01% digitonin and 1mM β-mercaptoethanol. Fractions of 2ml each were collected at a flow-rate of 0.2ml per min. Aliquots of 0.1ml and 0.07ml from every alternate fraction were assayed for protein content and callose synthase activity respectively.

3.3.3.1.4 Product characterization.

Radioactive products formed during incubation of callose synthase under assay conditions were analyzed by testing the susceptibility of radioactive glucan to digestion by specific glucanohydrolases, exo-(1-3)-β-D-glucanase (EC 3.2.1.58) and amylases (EC 3.2.1. and EC 3.2.1.2.) as described by Orlean (1982) with slight modification. For 1,3-β-D-glucanase digestion radioactive product (30,000cpm) was incubated with enzyme (0.1mg/ml) in 0.1ml of 50mM sodium phosphate buffer, pH 7.5, containing 0.1mg enzyme. The controls contained radioactive product in 0.2ml Tris-HCL buffer, pH 7.5, without glucanohydrolases. In other controls amylose and cellulose were used as substrates to check the action of amylases and 1,4-β-glucanase, respectively. After incubation for the indicated period, the reactions were stopped by adding 0.02ml glacial acetic acid. The whole digest was then chromatographed on Whatman No. 1 paper by descending chromatography using n-butanol:ethylacetate: acetic acid: water (40:30:25:40,v/v) solvent for 37h. Unlabeled glucose was used as reference. The radioactive bands on the chromatogram were located by scanning with the help of a TLC linear analyzer, LB 282 (Berthold) fitted with data acquisition systems LB 500. The unlabeled sugar bands were detected by alkaline silver nitrate reagent. Alternatively, glucanase digestion of the radioactive ethanol insoluble glucan was stopped by the addition of 1ml 70% ethanol. The remaining insoluble glucan was separated by centrifugation and washed four times with 70% ethanol and the radioactivity was determined.

3.3.3.2 5'-Nucleotidase (AMPase) assay

5'-Nucleotidase activity was measured with 5'-AMP as substrate as described by Riemer and Widnell (1975) with slight modifications. The standard reaction mixture, unless stated otherwise, contained (1 to 2.5mM AMP), 50mM sodium acetate buffer (pH 5.0) and 0.1ml of the enzyme preparation (8-70μg protein depending upon the form of enzyme assayed) which was added in the end, in a total volume of 1.0ml; control incubations contained no substrate. Incubation was carried out at 30^{o}C for 15min and the enzyme activity was terminated by adding 0.5ml cold 20% trichloroacetic acid. Protein was removed by centrifugation and Pi was determined in the supernatant fluid by the procedure of Fiske and Subbarow (1925). The specific activity of AMPase corresponds to the μ moles of Pi liberated by dephosphorylation of 5'-AMP per min per mg protein under assay conditions. Control and treated sample reactions were run parallel.

3.3.3.3 Acid Phosphatase assay

Acid phosphatase activity was measured by a slightly modified method as described by Odds and Hierholzer (1973) using p-nitrophenyl phosphate as substrate. The incubation mixture (1ml), contained 50-100μg enzyme protein, 2.5mM p-nitrophenyl phosphate, 50mM acetate buffer, pH 5.0. Reaction was terminated after 15 minutes incubation at 30^{o}C by addition of 1.5ml of 4% Na_2CO_3. The absorbance of yellow colour of p-nitrophenol released was measured at 420nm against the control, to which enzyme had been added after terminating the reaction. Specific activity was expressed as μmol p-nitrophenol produced per min, per mg protein. Control and treated sample reactions are run side by side.

3.3.3.4 Phytase assay

Phytase activity was assayed by measuring the amount of inorganic phosphorous liberated from phytic acid substrate. The reaction mixture contained the following in the final volume of 2ml:0.50mM of phytic acid, 50mM sodium acetate buffer (pH 5.0) and 5-15 units of

phytase enzyme (0.05mg - 0.20mg protein). The enzyme action was started by adding the buffered substrate solution to the reaction mixture and then incubated at 40°C for 30-60min. Enzyme activity was terminated by adding cold 20% trichloroacetic acid. The denatureed protein was removed by centrifugation and the amount of Pi was measured by the method of Fiske and Subbarow (1925). The intensity of the blue colour developed was measured at 690nm by a colorimeter. Controls both without substrate or without enzyme were incubated and processed concurrently with samples. One unit of enzyme is the amount of enzyme in mg which liberates one μmole of inorganic phosphorous (Pi) per minute under assay conditions.

3.3.3.5 Mannosyl transferase assay

The method of Lehle *et al.* (1976) was used to measure the mannosyl transfer from GDP-[^{14}C]mannose to endogeneous as well as exogeneous lipid acceptor (dolichol phosphate). The reaction mixture contained 50mM Tris-HCL (pH 7.5), 10mM $MnCl_2$, 5mM $MgCl_2$ and 0.1μCi GDP-[^{14}C]mannose. 100-200μg protein (particulate membrane fraction) was incubated with the reaction mixture in a total volume of 70μl. For the incorporation of mannose to the exogenous lipid acceptor, 10μl of dolichol monophosphate was mixed with 10μl of 0.1M Mg-EDTA and dried under nitrogen. The dried lipid was dispersed with 10μl of 5% nonidet. This lipid was then incubated with the reaction mixture as described above. The reaction was stopped after 30min by the addition of 2ml of chloroform/methanol (3:2 v/v). The precipitated protein was separated by centrifugation and the soluble portion was partitioned with 0.4ml of 4mM $MgCl_2$ solution. After thorough mixing the phases were separated by centrifugation. Upper aqueous phase was discarded and the lipid was dried in a vial and the radioactivity was measured after suspending the dried material in scintillation fluid (dioxane cocktail). Blank was prepared under identical conditions and the enzyme protein was added after termination of the reaction.

3.4 Other chemical and biochemical methods

Total lipids were estimated by the method of Colowick and Kaplan

(1963). Total carbohydrate and protein were determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956) and Folin-phenol method (Lowry *et al.*, 1951) using glucose and bovine serum albumin as standards, respectively. Nickel was determined by atomic absorption spectroscopy.Observations were recorded using a graphite kiln (Andersen *et al.*,1986).

3.5 Statistical analysis

The data were subjected to statistical analysis and expressed as Mean ± S.E.M. (Standard error of mean). The S.E.M. was calculated by the following formula :

$$S.E.M. = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}}$$

where x = individual observations

n = number of observations

Students 't' test was used to calculate the degree of significance by the formula given below:

$$t = \frac{X_1 - X_2}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where $$S = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}}{n_1 + n_2 - 2}}$$

n_1 and n_2 denote the number of observations in the two classes being compared (Ostle, 1954).

Based on the degree of freedom, value of probability was obtained from the standard table given by Fischer and Yates (1948). If the calculated value was more than the table value, it is significant at that probability level.

The following levels of significance were used: $P<0.001$ to $p<0.05$ for significant data and $p>0.05$ for nonsignificant data.

CHAPTER-IV

4.0 RESULTS

4.1 PART-I : STUDIES ON THE *IN VIVO* EFFECTS OF NICKEL SULPHATE IN RATS

4.1.1 Effects of $NiSO_4.7H_2O$ on animal growth

The effect of the intramuscularly administered $NiSO_4.7H_2O$ on the overall growth of male albino rats was measured in terms of the changes in the whole body weight. In a typical experiment four groups of rats, each containing 10 animals, were intramuscularly administered 0, 0.5, 1.0 and 2.0mg $NiSO_4.7H_2O$ per 100g body weight, respectively, on alternate days for a period of 15 days. The body weight of the animals was taken on a top loading electronic balance before and after the scheduled treatment. Results shown in Table-I indicate that barring the control group, animals in other groups receiving the nickel treatment showed a decrease in the weight and this decrease in the weight was increased with increasing dose of $NiSO_4.7H_2O$.

4.1.2 Effect on liver and kidney composition induced by $NiSO_4.7H_2O$ in rats

The alterations in the dry matter, water content, protein, carbohydrate, lipids and minerals of liver and kidney induced by $NiSO_4.7H_2O$ in rats are summarized in Table-II and Table-III. It can be seen that the total dry weight and water contents of liver and kidney were not affected by administering cumulative doses of $NiSO_4.7H_2O$ in rats. However, protein and total carbohydrate contents of liver and kidney were significantly reduced, particularly in those animals which had received higher doses (2mg/100g body weight) of $NiSO_4.7H_2O$, the decrease was insignificant in rats which received low dose (0.5 or 1.0mg/100g body weight). In contrast, however, lipid concentration increased quite substantially. For example, in the case of liver, lipid content increased from 11 to about 16% in 15 days in rats which were given a dose of 2mg/100g body weight on alternate days for 15 days. Similarly, in kidney the concentration of lipid was nearly

TABLE - I

Changes induced by $NiSO_4.7H_2O$ in whole body weight of rats

$NiSO_4.7H_2O$ dose[a] (mg/100g body weight)	Whole body weight [b]		
	Initial (g)	Final (g)	Change (%)
None (control)	100 ± 10	130 ± 10	+ 30
0.5	100 ± 10	90 ± 10*	- 10
1.0	100 ± 10	90 ± 10*	- 10
2.0	90 ± 10	75 ± 10**	- 24.2

a. Indicated amount of $NiSO_4.7H_2O$ in physiological saline (0.9% NaCl) was intramuscularly injected in rats on alternate days for a period of 15 days. To rats in control group only saline was injected. Each group consisted of 10 rats of approximately same age and body weight.

b. Results are mean ±SE; values are significant at * = $P<0.05$; ** = $P<0.001$ (Fischer's "t" test).

TABLE - II

Changes induced by $NiSO_4.7H_2O$ in the composition of liver of rats

Component[1]	$NiSo_4.7H_2O$ dose administered (mg/100g body weight)			
	Liver			
	0.0	0.5	1.0	2.0
Dry matter (%)	24.7 ± 1.0	25.4 ± 1.0	25.5 ± 2.0	25.6 ± 3.0
Water content (%)	75.3 ± 4.1	74.6 ± 4.0	74.5 ± 0.4	74.4 ± 3.1
Protein (%)	9.8 ± 0.6	9.4 ± 0.4	9.1 ± 0.4	7.8 ± 0.5**
Total carbohydrate (%)	3.1 ± 0.32	3.1 ± 0.3	2.7 ± 0.3	1.9 ± 0.1
Total lipid (%)	10.9 ± 0.5	11.2 ± 0.5	12.7 ± 0.3*	15.8 ± 0.2***
Ash (%)	0.64 ± 0.2	0.82 ± 0.2	0.68 ± 0.18	0.63 ± 0.2
Nickel (µg/g fresh weight)	15.09 ± 0.6	24.39 ± 0.4	36.12 ± 0.4	165.98 ± 1.5

1. All values are mean ± SE (three observations) and based on fresh weight of the organ. Values are significant at : * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Fischer's 't' test)

TABLE - III

Changes induced by $NiSO_4.7H_2O$ in the composition of kidney of rats

Component[1]	$NiSo_4.7H_2O$ dose administered (mg/100g body weight)			
	kidney			
	0.0	0.5	1.0	2.0
Dry matter (%)	25.9 ± 1.0	24.6 ± 2.0	25.4 ± 1.7	25.9 ± 1.2
Water content (%)	74.5 ± 3.0	74.5 ± 3.0	74.5 ± 2.0	74.3 ± 2.4
Protein (%)	12.9 ± 0.6	12.0 ± 0.4	11.5 ± 0.5	9.2 ± 0.6**
Total carbohydrate (%)	4.0 ± 0.6	3.3 ± 0.2	2.9 ± 0.16	1.9 ± 0.16
Total lipid (%)	6.9 ± 0.4	7.2 ± 0.47	8.1 ± 0.2*	14.1 ± 0.2***
Ash (%)	1.0 ± 0.2	0.80 ± 0.15	0.9 ± 0.2	1.1 ± 0.18
Nickel (µg/g fresh weight)	16.11 ± 0.60	33.87 ± 0.40	55.73 ± 0.40	268.29 ± 1.50

1. All values are mean ± SE (three observations) and based on fresh weight of the organ. Values are significant at : * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Fischer's 't' test)

doubled as compared to that of controls. The nickel content of liver and kidney in rats which received nickel treatment was more than those of controls without nickel treatment. In fact, in both liver and kidney the level of nickel accumulation showed an exponential increase indicating the inability of the animal to maintain the nickel beyond a certain level. In addition, increased level of nickel seems to be responsible for alterations in the intermediary metabolism in rats leading to lipid accumulation in liver and kidney.

4.1.3 Specificity in accumulation of different lipids in liver and kidney induced by $NiSO_4.7H_2O$ in rats

Since intramuscular administration of cumulative doses of $NiSO_4.7H_2O$ induced accumulation of lipids, the specificity of nickel action in different classes of lipids was investigated by separating various types of lipids by solvent-solvent extraction followed by chromatographic analysis. It was found (Table-IV) that the accumulation of both neutral lipids and phospholipids in liver and kidney was markedly increased by nickel. The effect on neutral lipids appeared to be more dose dependent than phospholipids as accumulation of neutral lipids was highly pronounced in rats which had been administered a cumulative dose of $NiSO_4.7H_2O$ (2mg/100g body weight) on alternate days for a period of 15 days. While the total phospholipids concentration in both liver and kidney reached a maximum level with cumulative dose of 0.5mg $NiSO_4.7H_2O$/100g body weight and further increase in $NiSO_4.7H_2O$ dose did not increase the phospholipid level in these tissues. These results suggest that the phospholipid metabolism is more sensitive to nickel salt concentrations in blood than probably the metabolism of neutral lipids. This trend is clearly reflected in alteration in ratios of phospholipids/neutral lipids, as $NiSO_4.7H_2O$ dose beyond 0.5mg/100g body weight favoured the formation (or accumulation) of neutral lipids more than the phospholipids, resulting in a decrease in phospholipid-to-neutral lipid ratio.

4.1.3.1 Effect of $NiSO_4.7H_2O$ on the abundance of different neutral lipids in rat liver and kidney

The total neutral lipid fraction was further analysed by TLC and

TABLE - IV

Alteration induced by $NiSO_4.7H_2O$ in neutral and phospholipid contents in rat liver and kidney

Experimental conditions were those given in Table-I except that three levels of $NiSO_4.7H_2O$ doses (0.5, 1.0 and 2.0mg/100g body weight) were used. Values are mean ± SE (3 experiments)

Dose of $NiSO_4.7H_2O$ (mg/100g body weight)	Concentration of lipids (mg/g fresh tissue)					
	Liver			Kidney		
	NL[1]	PL[2]	Ratio	NL	PL	Ratio
0.0 (control)	18.3±1.0	80.8±1.4	4.41	14.9±1.2	40.2±1.2	2.70
0.5	34.2±1.2	215.6±1.5	6.30	30.3±1.3	92.2±1.5	3.00
1.0	36.6±2.0	217.5±1.5	5.94	42.2±1.1	94.2±1.2	2.20
2.0	63.9±3.1	220.2±1.2	3.44	60.2±2.1	118.9±1.5	1.97

NL[1] = Neutral lipids (total) measured as triacylglycerol

PL[2] = Phospholipids (total)

GLC with a view to examine the effect and specificity of action of $NiSO_4.7H_2O$ on the abundance of various types of components of neutral lipids present in the total neutral lipid fraction. Fig. 2 shows a TLC profile of the neutral lipids in liver and kidney. It can be seen that the neutral lipid fraction was resolved into five distinct lipids corresponding to monoacylglycerol, diacylglycerol, cholesterol, triacylglyerol and cholesterol ester according to the increasing R_F values (Lane A). Similar results were obtained by GLC (Figs.3, 4 and 5). The relative concentration and percentage of abundance (Table-V) clearly indicate that in both liver and kidney the maximum effect in the accumulation of different types of neutral lipids induced by the intramuscularly administered $NiSO_4.7H_2O$ was on triacylglycerol followed by cholesterol ester. For example, in nickel-treated rats the level of triacylglycerol in liver and kidney was increased to about 3- and 4-fold, respectively. Similarly the cholesterol ester level was increased about 4-fold. Other neutral lipid components (diacylglycerol, monoacylglycerol and free cholesterol) were not significantly affected. It is interesting to note that the increase in the relative percentage of abundance in triacylglycerol and esterified cholesterol is at the cost of di-and monoacylglycerol, and free cholesterol, respectively. In addition, it may be noted that unlike other component lipids, percentage abundance of esterified cholesterol in both control and nickel-treated animals is somewhat comparable.

4.1.3.2 Effect of nickel on different types of phospholipids of liver and kidney in rats

The effect of the intramuscularly administered $NiSO_4.7H_2O$ on different types of component phospholipids of rat liver and kidney was analyzed by HPLC. The phospholipid fraction separated from the neutral lipids by solvent extraction was analyzed on a reverse phase C_{18} HPLC column. The elution of component phospholipids was calibrated with the help of authentic phospholipid standards from Sigma. Fig. 6 shows the separation of a standard mixture of phospholipids, consisting of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl glycerol (PG) and phosphatidyl

Figure 2 : Analysis of neutral lipids by TLC.

Lane A, spots of standard neutral lipids. From bottom to top: monoacylglycerol, diacylglycerol, free cholesterol, triglycerides and esterified cholesterol. Lane B, neutral lipids obtained from the liver of control rats. Lane C, neutral lipids obtained from the kidney of control rats. Lane D, neutral lipids obtained from the liver of nickel-treated (2mg $NiSO_4.7H_2O$/100g body weight) rats. Lane E, neutral lipids obtained from the kidney of nickel-treated (2mg $NiSO_4.7H_2O$/100g body weight) rats.

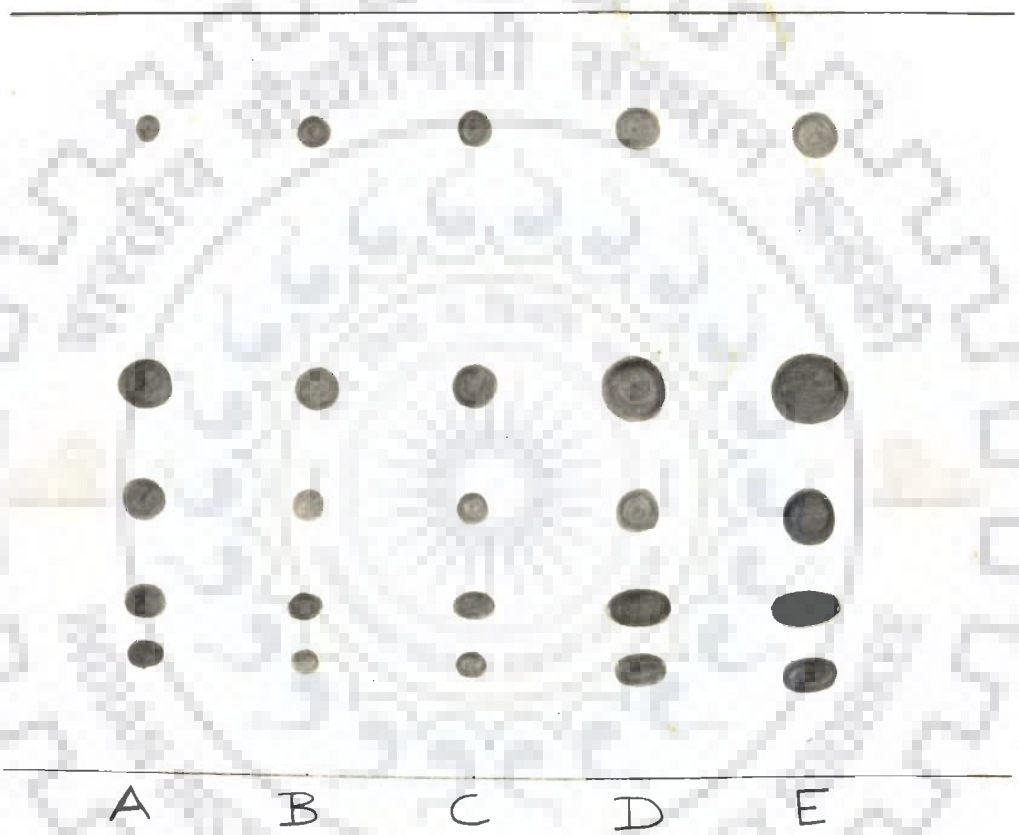

A
B
C
D
E

Figure 3 : Gas chromatogram of standard neutral lipids.

Analyses were carried out on the Hewlett - Pakard-1 (methyl silicon gum) gas chromatograph using capillary column (5m x 0.55mm x 2.6mm, fused-silica column) and helium gas flow-rate of 2.6ml/min.

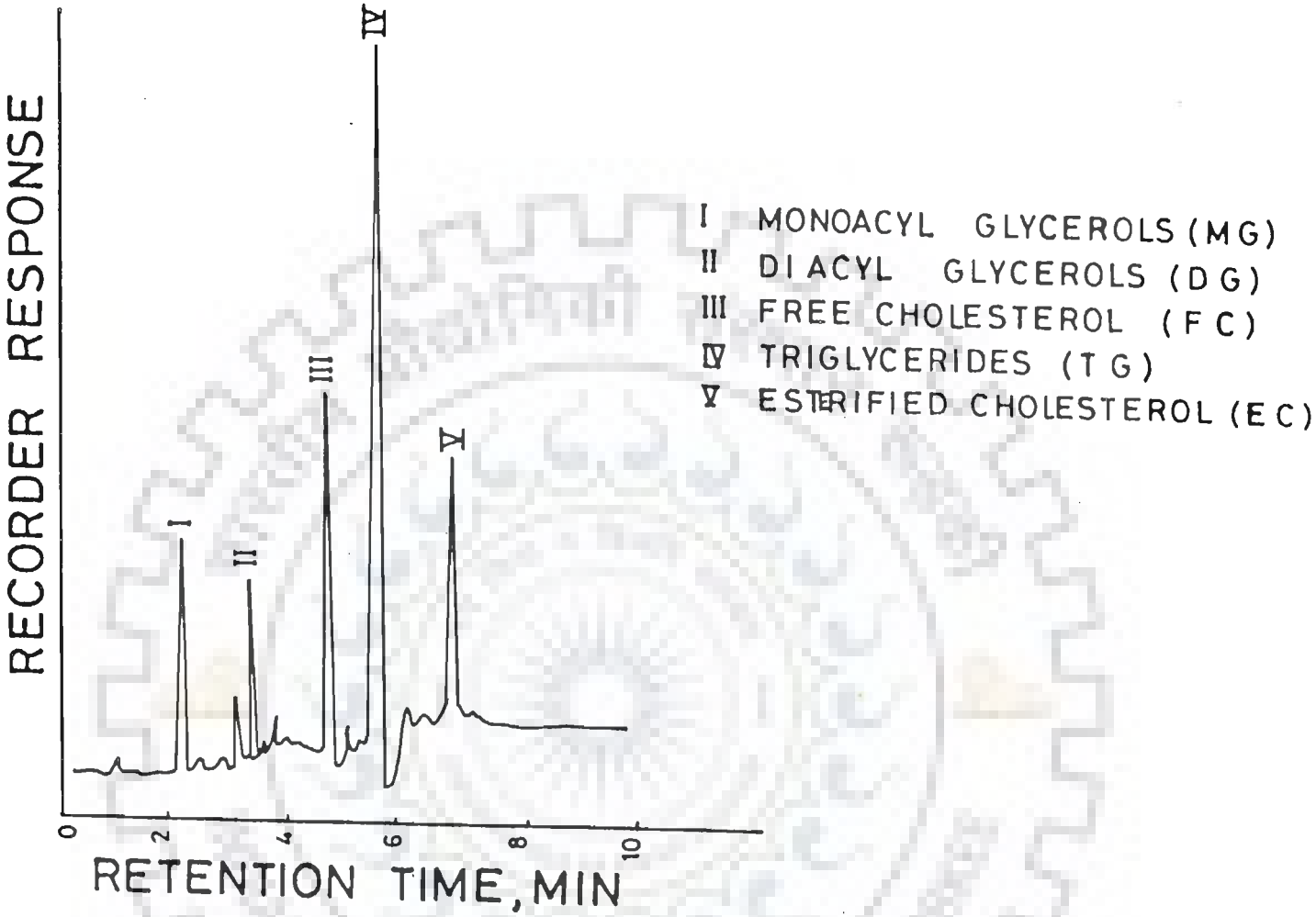

RECORDER RESPONSE
I
II
III
IV
V
0
2
4
6
8
10
RETENTION TIME, MIN
I MONOACYL GLYCEROLS (MG)
II DIACYL GLYCEROLS (DG)
III FREE CHOLESTEROL (FC)
IV TRIGLYCERIDES (TG)
V ESTERIFIED CHOLESTEROL (EC)

Figure 4 : Analysis of neutral lipids by GLC.

(a) and (b) are gas chromatograms of neutral lipids obtained from the liver and kidney of control rats, respectively. MG, Monoacylglycerol; DG, Diacylglycerol; FC, Free cholesterol; TG, Triglyceride; EC, Esterified cholesterol.

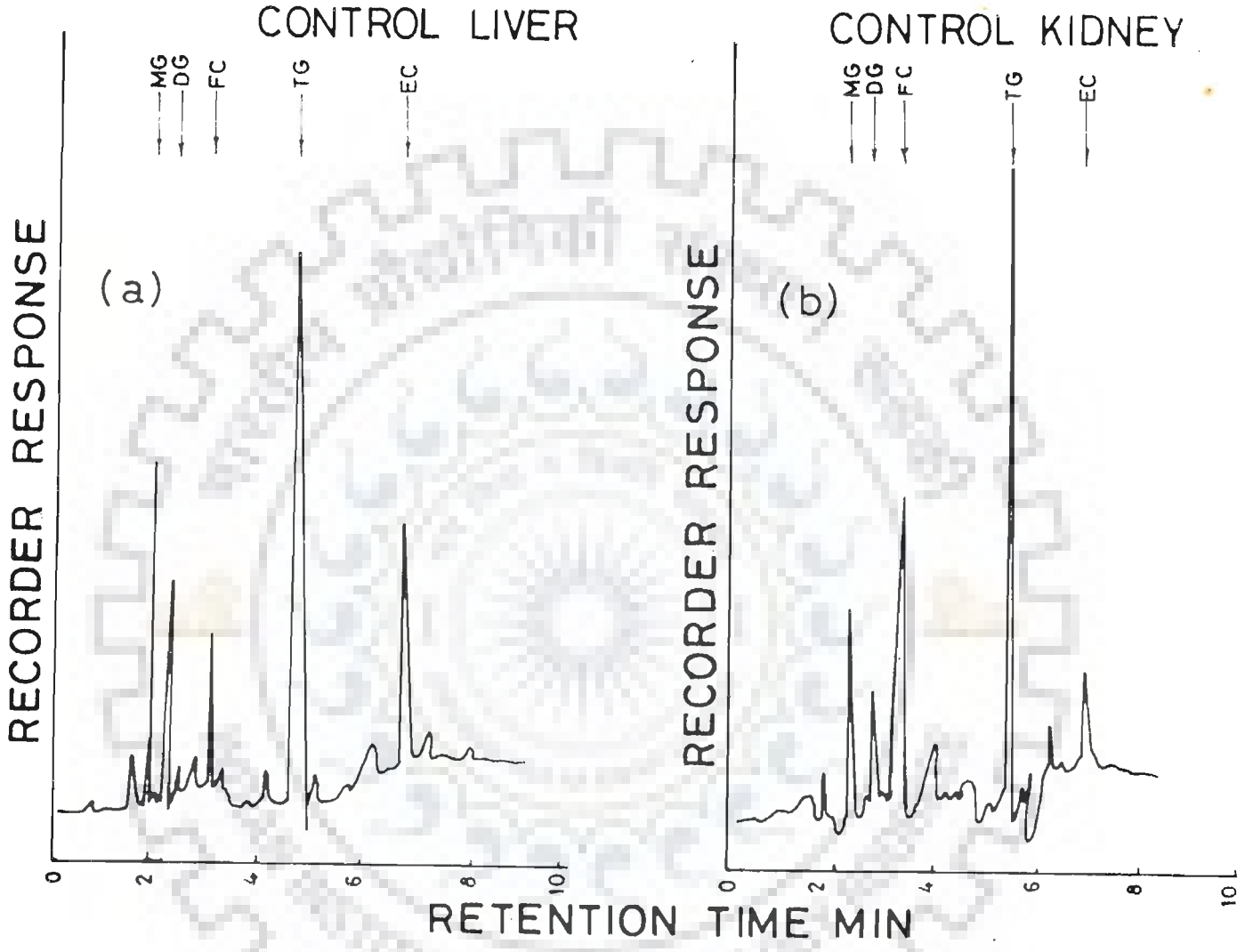

CONTROL LIVER
CONTROL KIDNEY
MG
DG
FC
TG
EC
(a)
(b)
RECORDER RESPONSE
RECORDER RESPONSE
0
2
4
6
8
10
RETENTION TIME MIN

Figure 5 : GLC analysis of neutral lipids obtained from nickel-treated rats.

(a) and (b) are gas chromatograms of neutral lipids obtained from the liver and kidney of nickel-treated (2mg $NiSO_4.7H_2O$/100g body weight) rats, respectively. MG, Monoacylglycerol; DG, Diacylglycerol; FC, Free cholesterol; TG, Triglyceride; EC, Esterified cholesterol.

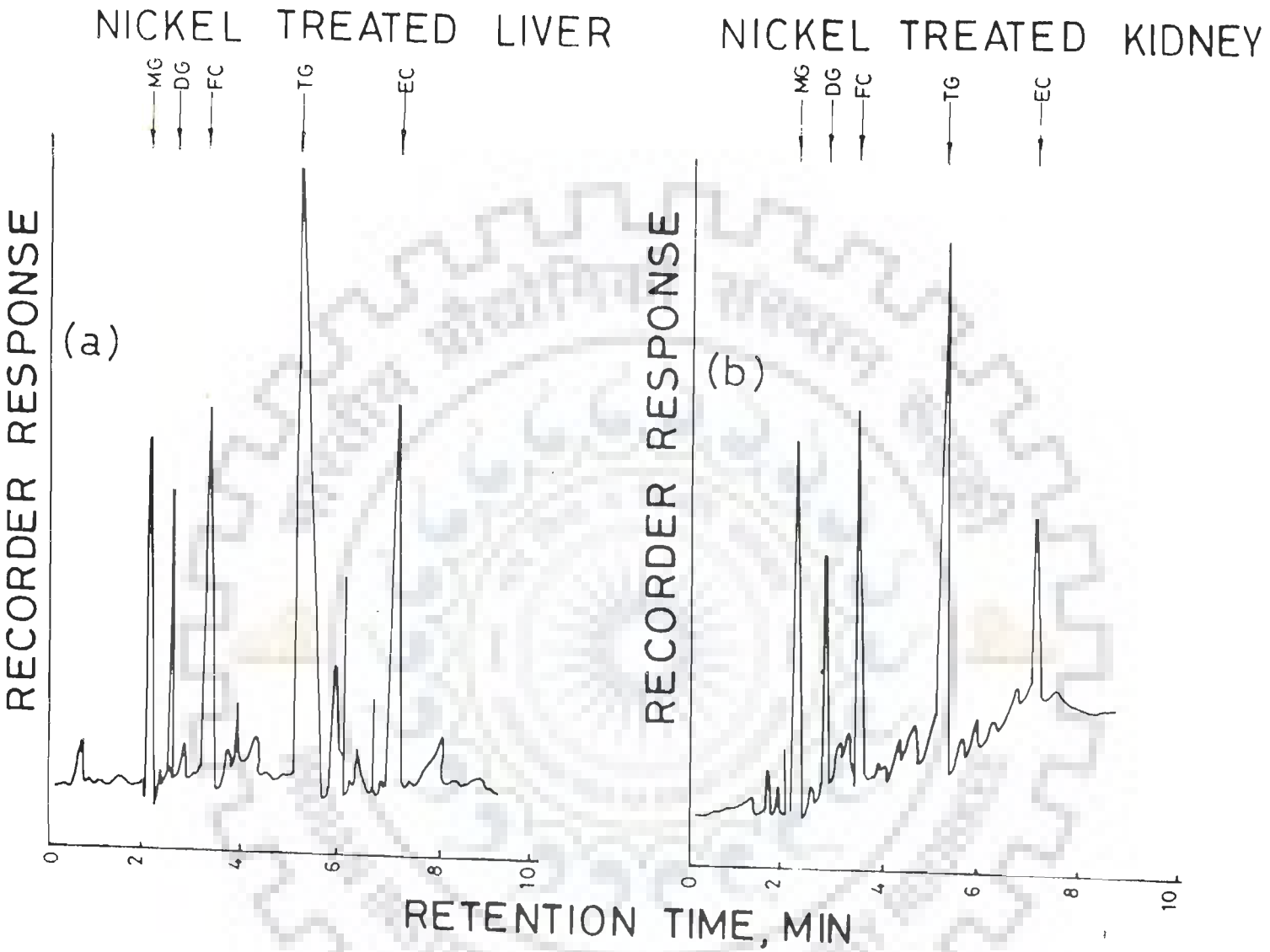

NICKEL TREATED LIVER
NICKEL TREATED KIDNEY
MG
DG
FC
TG
EC
RECORDER RESPONSE
(a)
(b)
0
2
4
6
8
10
RETENTION TIME, MIN

TABLE - V

Changes induced by $NiSO_4.7H_2O$ in the composition of various components of neutral lipids of liver and kidney in rats

A dose of $NiSO_4.7H_2O$ (2mg/100g body weight) was intramuscularly administered in rats on alternate days for a period of 15 days. Control rats were without nickel treatment. After the scheduled treatment liver and kidney were removed and processed for analysis of neutral lipids by G.L.C. as described under "Experimental Procedures". Ten rats per treatment were used and values are mean±SE of four treatments. Values in parenthesis are percent of total neutral lipids by weight.

Lipid	Lipid concentration (mg/g fresh tissue)			
	Liver		Kidney	
	Control	Ni-treated	Control	Ni-treated
Triacylglycerol	19.1 ± 1.1 (74.0)	63.9 ± 11.5 (82.4)	15.3 ± 1.1 (77.8)	60.4 ± 2.4 (86.5)
Diacylglycerol	1.3 ± 0.01 (5.0)	1.6 ± 0.01 (2.0)	1.0 ± 0.01 (5.3)	1.1 ± 0.01 (1.6)
Monoacylglycerol	1.5 ± 0.01 (5.8)	2.2 ± 0.01 (2.8)	1.1 ± 0.01 (5.9)	1.9 ± 0.60 (2.7)
Cholesterol ester	1.8 ± 0.01 (7.0)	6.8 ± 1.70 (8.8)	1.2 ± 0.01 (6.4)	4.3 ± 1.20 (6.2)
Free cholesterol	2.1 ± 0.01 (8.0)	3.0 ± 0.02 (3.1)	1.1 ± 0.01 (5.9)	2.1 ± 1.00 (3.0)

serine (PS). As can be seen the standard phospholipids were clearly separated from each other. Figs. 7 and 8 show the phospholipid profiles of liver and kidney as influenced by different doses of $NiSO_4.7H_2O$ administered into rats, respectively. Both liver and kidney were found to contain PC, PS, PE and PG plus some unidentified phospholipids with relatively higher retention time which was prominent in rats that received high dose (2mg/100g body weight) of $NiSO_4.7H_2O$. The concentration and percentage of abundance of various components of phospholipids in liver and kidney of control and nickel-treated rats are shown in Table-VI and Table-VII. It was found that of the indicated component phospholipids, the highest concentration was of PC followed by PS. Other phospholipids, including the unidentified lipids constituted less than 10%, while the percentage of abundance of PC and PS was about 57 and 35, respectively, accounting at least 90% of the total phospholipids in liver and kidney in untreated rats. In nickel-treated rats concentration of PC (mg/g fresh tissue) as well as the percentage of abundance increased significantly. For example, in liver of rats which had received $NiSO_4.7H_2O$ dose (2mg/100g body weight) on alternate days for a period of 15 days the concentration of PC was increased from 44.6mg to 79mg/g fresh tissue, while the percentage abundance increased from 57 to 70. Identical pattern was observed in kidney. In control whereas the level of PS in liver and kidney was almost not affected by nickel, its percentage abundance decreased significantly as a result of nickel treatment. Other phospholipids were not affected by nickel. From these results, it appears that metabolism of PC is preferentially affected by nickel both in liver and kidney of rats, which may be of physiological significance.

4.1.3.3 Nickel induced lipid peroxidation in liver and kidney in rats

The effect of nickel on lipid peroxidation in liver and kidney of rats is presented in Table-VIII. These results show that intramuscular administration of $NiSO_4.7H_2O$ increases lipid peroxidation in liver and kidney. Compared to controls there was about four-fold and three-fold increase in lipids in liver and kidney of rats which were intramuscularly administered nickel (2mg/100g body

Figure 6 : Analysis of standard phospholipids by HPLC.

Phospholipids were analysed in a Shimadzu LC-4A equipped with a SPD-2AS variable wavelength UV-Spectrophotometric detector and a chromatopac C-RZA integrator. The separation of phospholipids was achieved with a Nucleosil-5 C_{18} reverse phase column (25cm x 4.6m I.P) using methanol-water-acetonitrile (7:2:1, v:v:v) at a flow-rate of 1.5ml/min.PE, Phosphatidyl ethanolamine; PC, Phosphatidyl choline; PG, Phosphatidyl glycerol; PS, Phosphatidyl serine.

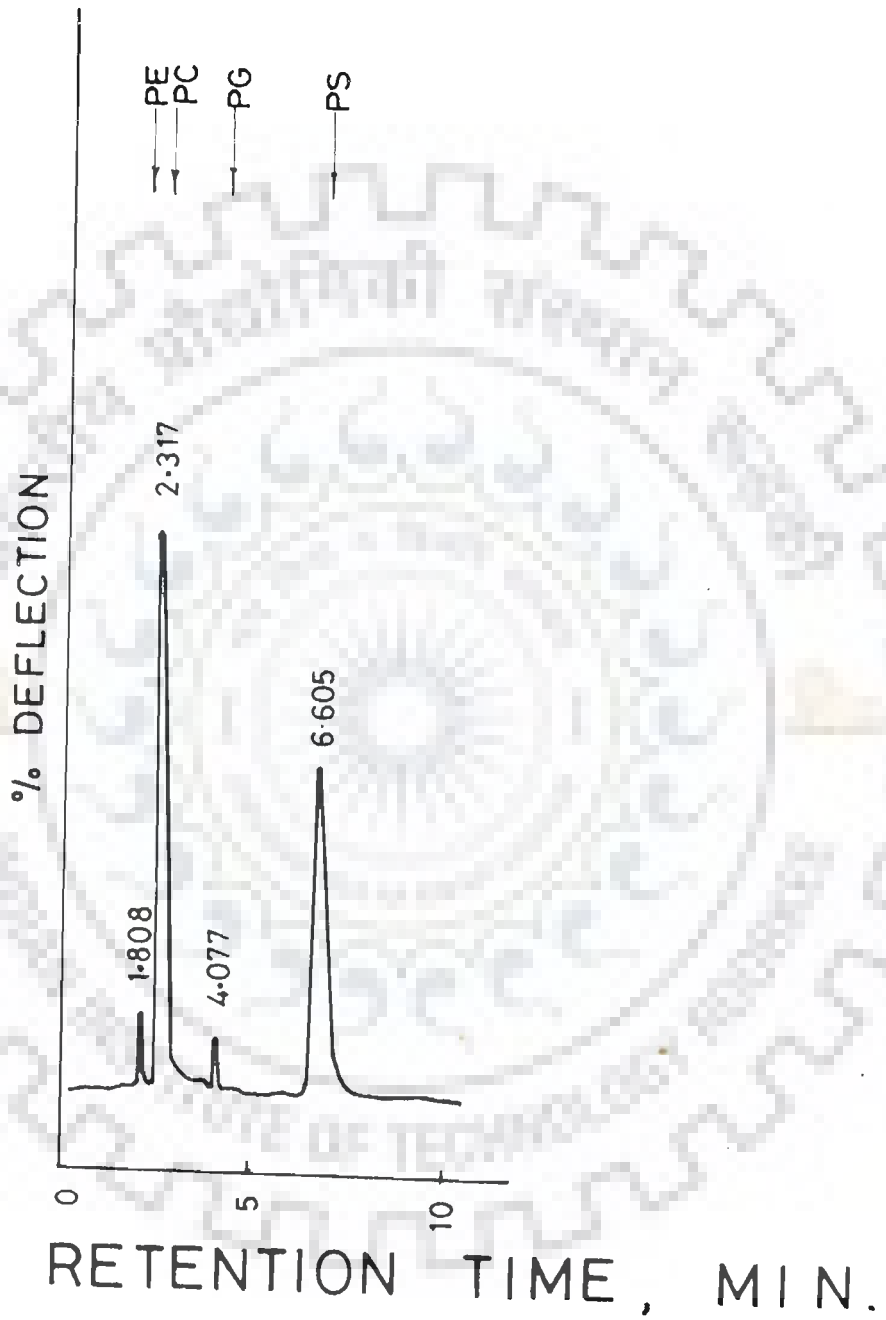

PE
PC
PG
PS
% DEFLECTION
2·317
6·605
1·808
4·077
0
5
10
RETENTION TIME, MIN.

Figure 7 : HPLC chromatograms of phospholipids obtained from liver of control rat and livers of rats treated with the increasing dose of $NiSO_4.7H_2O$.

(a) 0.0mg/100g body weight (control), (b) 0.5mg/100g body weight. (c) 1mg/100g body weight, (d) 2mg/100g body weight. PE, Phosphatidyl ethanolamine; PC, Phosphatidyl choline; PG, Phosphatidyl glycerol; PS, Phosphatidyl serine.

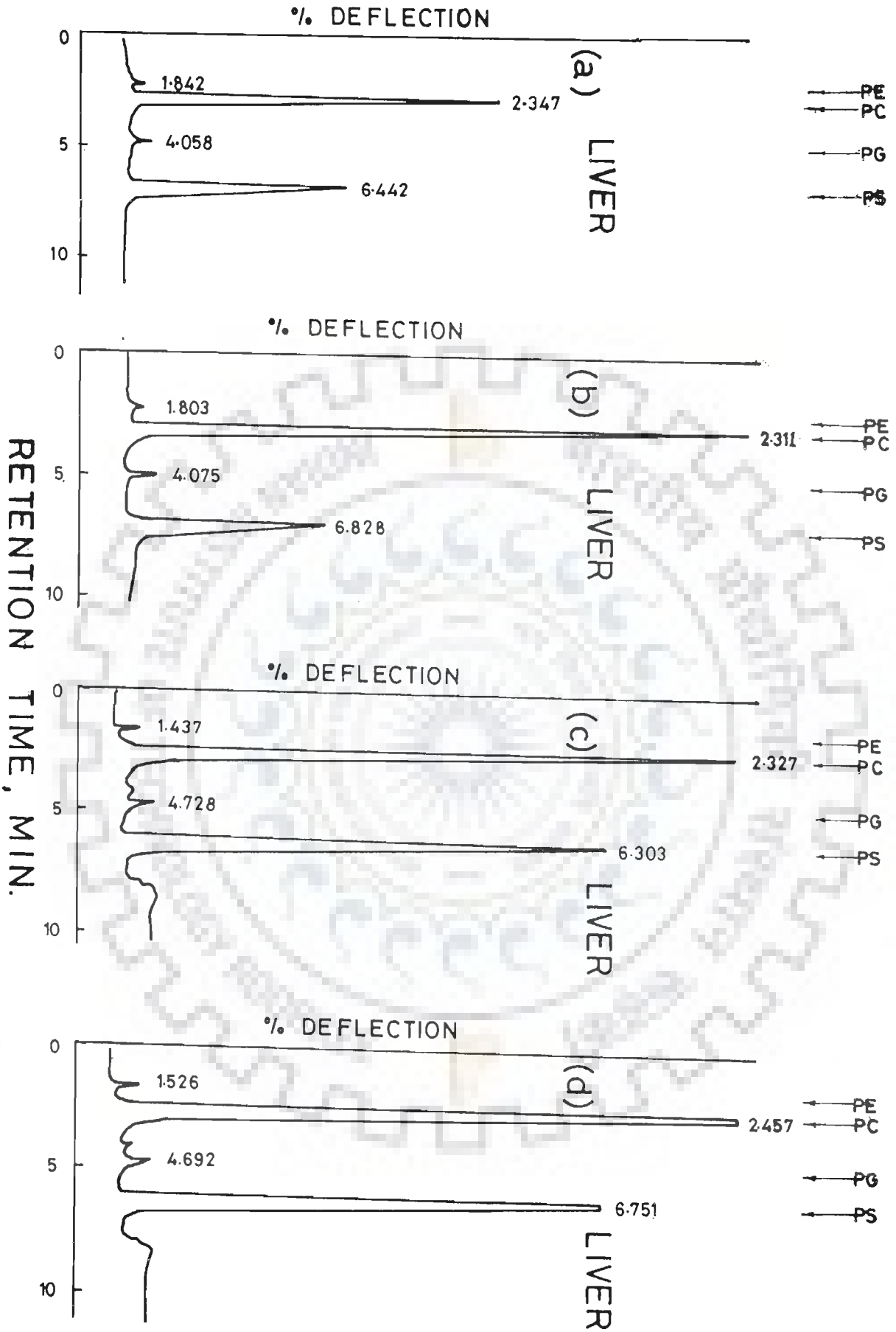

% DEFLECTION
0
5
10
1·842
2·347
4·058
6·442
(a) LIVER
PE
PC
PG
PS
% DEFLECTION
1·803
2·311
4·075
6·828
(b) LIVER
PE
PC
PG
PS
% DEFLECTION
1·437
2·327
4·728
6·303
(c) LIVER
PE
PC
PG
PS
% DEFLECTION
1·526
2·457
4·692
6·751
(d) LIVER
PE
PC
PG
PS
RETENTION TIME, MIN.

Figure 8 : HPLC chromatograms of phospholipids obtained from kidney of control rat and kidneys of rats treated with the increasing dose of $NiSO_4.7H_2O$.

(a) 0.0mg/100g body weight (control),(b) 0.5mg/100g body weight, (c) 1mg/100g body weight (d) 2mg/100g body weight. PE, Phosphatidyl ethanolamine; PC, Phosphatidyl choline; PG, Phosphatidyl glycerol; PS, Phosphatidyl serine.

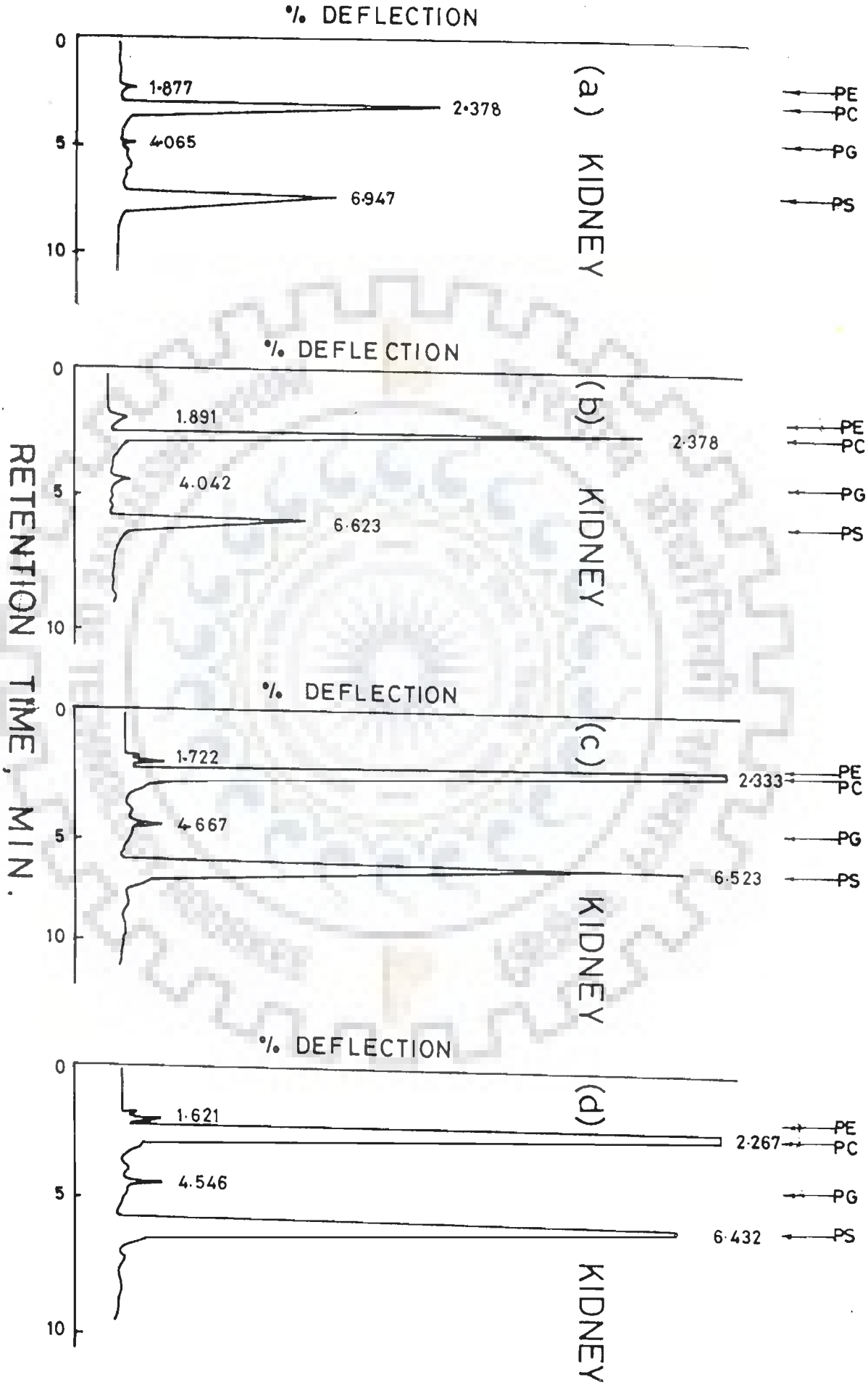

% DEFLECTION
0
5
10
1·877
2·378
4·065
6·947
(a) KIDNEY
PE
PC
PG
PS
% DEFLECTION
1.891
2·378
4.042
6·623
(b) KIDNEY
% DEFLECTION
1·722
2·333
4·667
6·523
(c) KIDNEY
% DEFLECTION
1·621
2·267
4.546
6·432
(d) KIDNEY
RETENTION TIME, MIN.

TABLE-VI

Changes induced by $NiSO_4.7H_2O$ in the composition of various components of phospholipids in rat liver

Indicated doses of nickel sulphate solution in physiological saline was intramuscularly administered in rats on alternate days for a period of 15 days. Liver was then excised and processed for analysis of various phospholipids by HPLC as described under "Experimental Procedures". Control rats received physiological saline injections without nickel sulphate. Ten rats were used for each treatment. Values are mean ± SE. Values in parentheses are percent of total phospholipids by weight.

Phospholipid	Concentration of phospholipids (mg/g fresh tissue)			
	Dose of $NiSO_4.7H_2O$ (mg/100g body weight)			
	0.0	0.5	1.0	2.0
Phosphatidyl ethanolamine (PE)	1.6 ± 0.1 (2.05)	0.4 ± 0.08 (0.45)	0.7 ± 0.01 (0.66)	0.8 ± 0.03 (0.70)
Phosphatidyl choline (PC)	44.6 ± 3.1 (57.03)	66.0 ± 13 (69.25)	72.5 ± 4.1 (68.85)	79.0 ± 3.1 (69.9)
Phosphatidyl glycerol (PG)	2.1 ± 0.1 (2.7)	1.6 ± 0.20 (1.68)	1.8 ± 0.09 (1.71)	1.9 ± 0.2 (1.66)
Phosphatidyl serine (PS)	27.4 ± 2.6 (35.04)	20.6 ± 0.20 (24.30)	23.2 ± .2 (24.7)	26 ± 1.8 (24.5)
Uni-dentified phospholipids	2.5 ± 0.3 (3.2)	4.1 ± 0.10 (4.3)	4.3 ± 0.1 (4.08)	4.5 ± 0.13 (3.9)

TABLE VII

Changes induced by $NiSO_4.7H_2O$ in the composition of various components of phospholipids in rat kidney

Indicated doses of nickel sulphate solution in physiological saline was intramuscularly administered in rats on alternate days for a period of 15 days. kidney was then excised and processed for analysis of various phospholipids by HPLC as described under "Experimental Procedures". Control rats received physiological saline injections without nickel sulphate. Ten rats were used for each treatment. Values are mean ± SE. Values in parentheses are percent of total phospholipids by weight.

Phospholipid	Concentration of phospholipids (mg/g fresh tissue)			
	Dose of $NiSO_4.7H_2O$ (mg/100g body weight)			
	0.0	0.5	1.0	2.0
Phoshatidyl ethanolamine (PE)	0.9 ± 0.01 (0.51)	0.6 ± 0.03 (0.78)	1.0 ± 0.03 (1.17)	1.1 ± 0.04 (1.15)
Phosphatidyl choline (PC)	34.8 ± 2.9 (58.3)	50.0 ± 2.1 (65.02)	56.5 ± 4.0 (66.08)	62.0 ± 5.1 (64.65)
Phosphatidyl glycerol (PG)	1.8 ± 0.12 (3.01)	2.0 ± 0.1 (2.6)	2.1 ± 0.1 (2.5)	2.4 ± 0.5 (2.5)
Phosphatidyl serine (PS)	20.6 ± 1.9 (34.51)	22.6 ± 1.9 (29.4)	24.0 ± 1.0 (28.7)	28.3 ± 0.1 (29.51)
Uni-dentified phospholipids	1.6 ± 0.5 (2.68)	1.7 ± 0.9 (2.2)	1.9 ± 1.1 (2.2)	2.1 ± 0.8 (2.2)

TABLE - VIII

Effect of $NiSO_4.7H_2O$ on lipid peroxidation in liver and kidney of rats

$NiSO_4.7H_2O$ dose (mg/100g body weight	Lipid peroxidation	
	Liver (nmole MDA/g fresh weight)	Kidney (nmole MDA/g fresh weight)
None (control)	130.7 ± 9	165.7 ± 10
0.05	174.2 ± 10	204.1 ± 13
1.0	309.7 ± 10	366.3 ± 14
2.0	513.0 ± 13	534.3 ± 17

Values are mean ± SE of 10 animals and three determinations. MDA = malondialdehyde

Figure 9 : Effect induced by nickel sulphate in the composition of major lipid components of subcellular membranes from rat liver.

Rats were intramuscularly injected nickel sulphate (2mg/100g body weight) on alternate days for 15 days period. After treatment livers were excised and various subcellular membrane fractions were isolated and lipid components were determined as described in Experimental Procedures. - Ni = control without nickel treatment ; + Ni = Nickel-treated rat liver membranes.

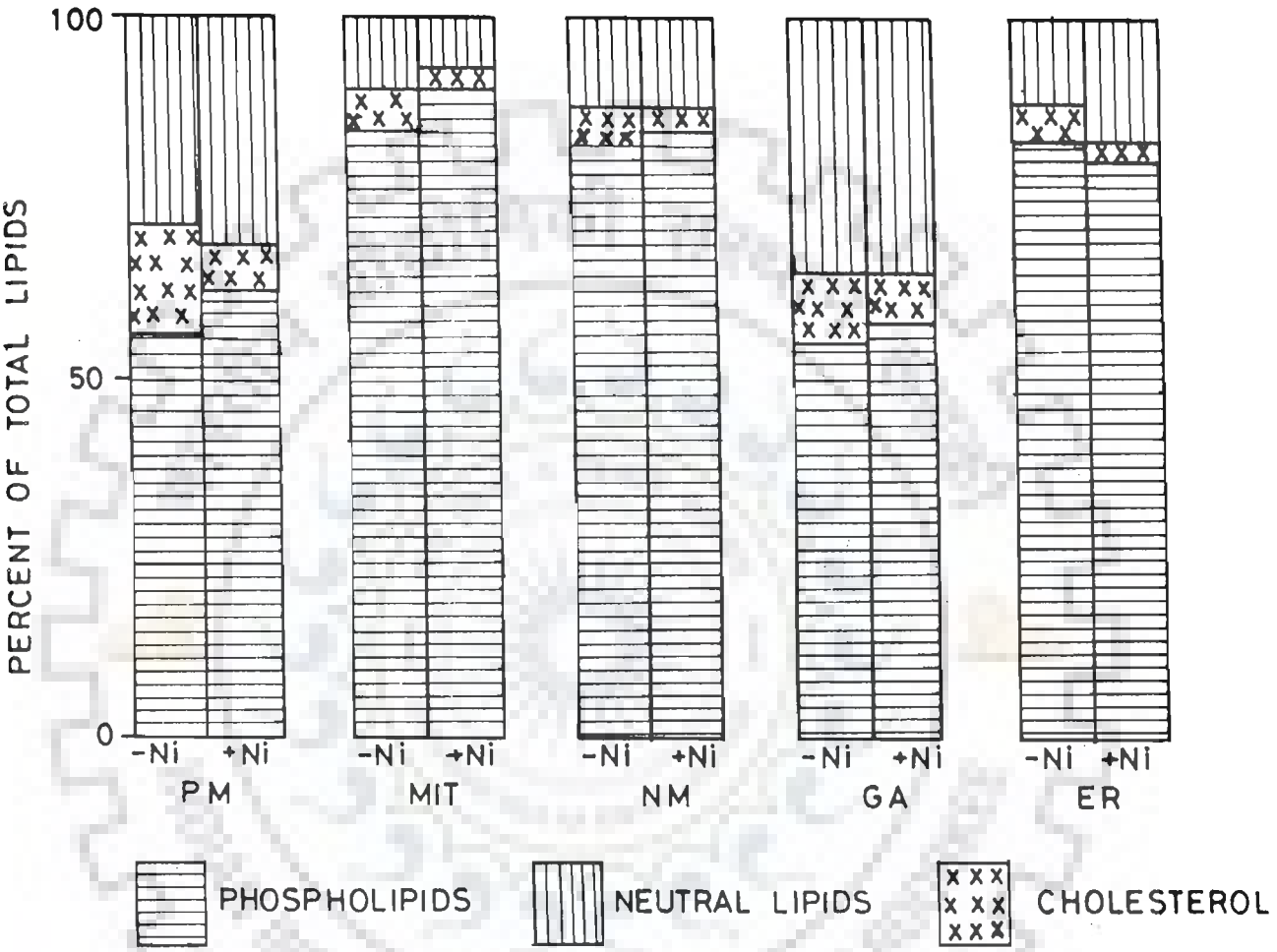
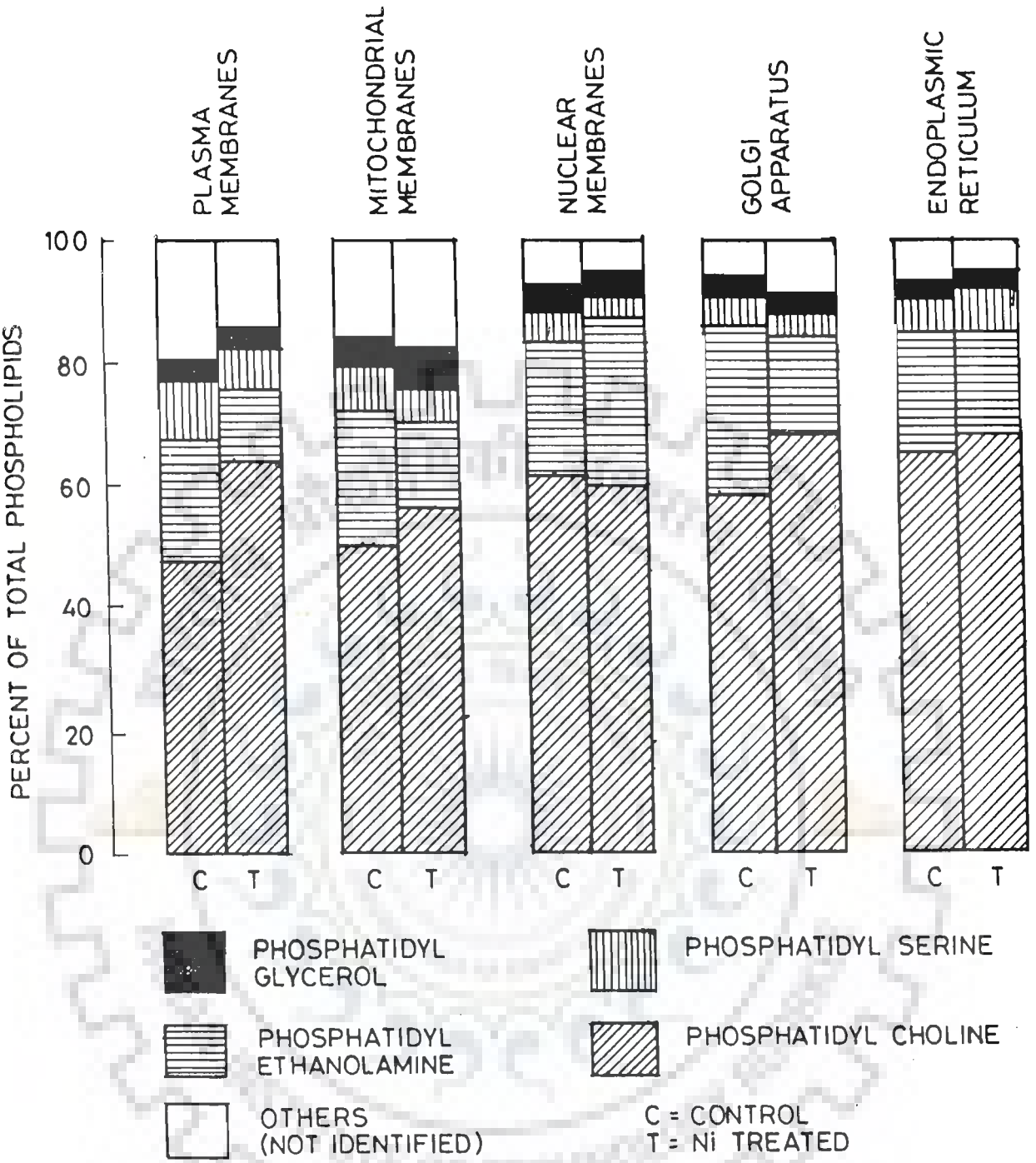

100
50
0
PERCENT OF TOTAL LIPIDS
-Ni +Ni
PM
MIT
NM
GA
ER
PHOSPHOLIPIDS
NEUTRAL LIPIDS
CHOLESTEROL

Figure 10 : A comparison of phospholipid composition of various subcellular membrane fractions isolated from livers of normal and nickel-treated rats.

Nickel sulphate (2mg/100g body weight) was intramuscularly injected in rats on alternate days for a period of 15 days. After treatment livers were excised and various subcellular membrane fractions were isolated and phospholipid composition was determined as described in Experimental Procedures.


PLASMA MEMBRANES
MITOCHONDRIAL MEMBRANES
NUCLEAR MEMBRANES
GOLGI APPARATUS
ENDOPLASMIC RETICULUM
PERCENT OF TOTAL PHOSPHOLIPIDS
100
80
60
40
20
0
C T
PHOSPHATIDYL GLYCEROL
PHOSPHATIDYL ETHANOLAMINE
OTHERS (NOT IDENTIFIED)
PHOSPHATIDYL SERINE
PHOSPHATIDYL CHOLINE
C = CONTROL
T = Ni TREATED

weight) on alternate days for a period of 15 days, respectively. The lipid peroxidation was also found to increase with nickel dose.

4.1.3.4 Changes in the relative proportion of various classes of lipids in subcellular fractions of rat liver

Results presented above clearly demonstrated that both simple (neutral lipids) and complex (phospholipids) lipids were greatly influenced by nickel treatment. Since lipids constitute an important component of cellular membranes, the changes in the composition of various classes of lipids, namely phospholipids, acylglycerols and cholesterol were determined in various subcellular membrane fractions of rat liver. The membrane fractions were prepared, from rat liver following the procedure outlined in Fig. 1. The total lipids were extracted and analysed by a combination of TLC and GC, or TLC and HPLC. The results (Fig.9) show that the relative proportion of the phospholipid components in plasma membrane and mitochondrial (inner and outer) membranes was significantly increased by nickel, administered intramuscularly in the form of $NiSO_4.7H_2O$. The effect on phospholipid concentration in nuclear membrane , Golgi apparatus and endoplasmic reticulum fractions was relatively smaller compared to that on plasma membrane and mitochondrial membranes. These results suggest that the rat liver plasma membrane and mitochondrial membranes are affected most by the *in vivo* nickel treatment.

Relative proportion of acylglycerols in plasma membrane increased more than in the mitochondrial membrane fraction as a result of nickel treatment. In fact in mitochondria the mole percentages of neutral lipids (acylglycerols) and cholesterol were reduced. In nuclear membranes the proportion of acylglycerols remain unchanged but the relative concentration of cholesterol was decreased. On the contrary, like plasma membrane, in the endoplasmic reticulum the relative proportion of acylglycerol was increased and concomitantly the proportion of cholesterol decreased. Of the neutral lipids and phospholipids the proportion of phospholipids in all the subcellular membrane fractions was much higher than the total neutral lipids.

4.1.3.5 *In vivo* effect of $NiSO_4.7H_2O$ on the percentage composition of various phospholipids in rat liver subcellular membrane fractions

Fig.10 shows the changes induced by nickel sulfate in the percentage composition of various components of phospholipids (glycerophosphatides) in various subcellular membrane fractions from rat liver. It was found that the relative proportion of the phosphatidyl choline, the major component of phospholipid, was significantly increased in the plasma membrane, mitochondrial (inner and outer) membrane, Golgi apparatus and the endoplasmic reticulum fractions obtained from livers of rats which were given $NiSO_4.7H_2O$ injections ; the maximum increase in phosphatidyl choline was observed in plasma membrane followed by Golgi apparatus, mitochondrial membrane and endoplasmic reticulum.

Significant change in the proportion of phosphatidyl choline in nuclear membrane was observed. The proportion of phosphatidyl ethanolamine the second most abundant glycerophosphatide in rat liver was reduced as a result of nickel treatment in all the subcellular membrane fractions except in nuclear membrane. Similarly, the proportion of phosphatidyl serine was decreased by nickel treatment in rat liver plasma membrane, mitochondrial membranes, nuclear membrane and Golgi apparatus. In the endoplasmic reticulum fraction, however, the proportion of phosphatidyl serine appeared to be only marginally increased. The plasma membrane and mitochondrial membrane fractions contained significant amount of unidentified lipids, probably phosphatidyl inositol and sphingomyelin. The proportion of these unidentified lipids in the plasma membrane was decreased but it was increased in the mitochondrial fraction as a result of nickel treatment. In all other fractions the change in the proportion of unidentified lipids was not significantly affected by the nickel treatment. Phosphatidyl glycerol was found to be present in all subcellular membrane fractions. Except in the mitochondrial membrane fraction nickel treatment apparently did not produce any change in the relative proportion of this lipid. In the mitochondrial membrane

fraction its proportion was doubled in the nickel-treated rat liver compared to control animals. Since in mitochondrial membrane fraction, except phosphatidyl ethanolamine, all other components namely phosphatidyl choline, phosphatidyl glycerol, phosphatidyl serine and unidentified lipids, were increased, it seems that the Ni-induced increase in phosphatidyl choline, phosphatidyl glycerol, phosphatidyl serine and unidentified phospholipids was probably at the cost of phosphatidyl ethanolamine. In totality, most changes in the composition of various phospholipids due to nickel treatment appeared to be in the liver plasma membrane, mitochondrial membranes and Golgi apparatus, and the nuclear membrane, and endoplasmic reticulum fraction were much less affected. Thus, the *in vivo* effect of nickel on the phospholipid composition in various membrane fraction is somewhat selective.

4.1.4 Effect of $NiSO_4.7H_2O$ on haematological parameters in rat

Alterations in the haematological parameters induced by nickel in rats are shown in Table-IX. It can be seen that at low doses of $NiSO_4.7H_2O$ (0.5mg/100g body weight) no significant change, except in reducing sugars, was induced in the concentration of different blood components. However, in contrast to this animals treated with higher $NiSO_4.7H_2O$ doses (2mg/100g body weight) showed a markedly prominent change in the levels of different blood components, including some enzymes. For example, while the level of total RBC, haemoglobin and total protein in blood decreased by about 45, 21 and 60%, the concentration of total leucocytes, reducing sugars, cholesterol, urea bilirubin and creatinine increased by about 50, 31, 37, 98, 103 and 150%, respectively. In addition, the activity of acid and alkaline phosphatases, GOT and GPT blood serum were greatly increased by about 96, 45, 220 and 527%, respectively. All these results clearly point that $NiSO_4.7H_2O$ doses, in excess of 0.5mg/100g body weight are detrimental to the health of the animal, perhaps by damaging liver and other vital organs of the body.

4.1.5 HISTOCHEMICAL ANALYSIS OF LIPID DISTRIBUTION IN LIVER AND KIDNEY

TABLE -IX

Alteration in haematological parameters induced by nickel in rats

Indicated amounts of nickel sulphate doses were intramuscularly administered in rats on alternate days for a period of 15 days. Blood samples from the control and nickel-treated animals were analysesd for different parameters as described under "Experimental Procedures". Results are mean ± SE of five observations. Rats in control, I, II and III groups (10 rats in each group) received 0,0.5, 1.0 and 2.0mg $NiSO_4.7H_2O$/100g body weight. Control values were used to calculate the percentage alteration in different components. (-) and (+) indicate inhibition and stimulation, respectively. IU = International unit.

Blood component	Control value	Experimental groups of animals					
		I		II		III	
		Value	alteration %	value	alteration %	value	alteration %
RBC (10^6/mm^3)	5.56 ± 0.4	5.15 ± 0.58	(-)7.4	4.9 ± 0.68	(-)12.0	3.0 ± 0.4	(-) 45.5
Haemoglobin (g/dl)	11.8 ± 0.2	11.6 ± 0.40	(-)2.0	11.0 ± 0.2	(-) 6.8	9.3 ± 0.2	(-) 21.35
Total Leucocytes (10^6/mm^3)	8.6 ± 0.9	8.6 ± 0.80	0.0	8.8 ± 0.6	(+) 2.3	12.9 ± 0.7	(+) 50.0
Protein (g/dl)	7.2 ± 0.8	5.7 ± 1.00	(-)20.1	4.2 ± 0.8	(-)41.7	2.8 ± 0.8	(-) 61.1
reducing sugar (mg/dl)	92.0 ± 2.0	94.0 ± 1.20	(+) 2.2	108.0 ± 2.0	(+)17.4	121.7 ± 2.0	(+) 31.5
Cholesterol (mg/dl)	210.0 ± 6.3	213.0 ± 6.00	(+) 1.8	223.0 ± 6.3	(+) 6.2	267.0 ± 6.1	(+) 27.1
Urea (mg/dl)	20.2 ± 2.7	20.9 ± 2.40	(+) 3.6	28.0 ± 2.0	(+)38.6	40.0 ± 2.6	(+) 98.0
Bilirubin (mg/dl)	0.33 ± 0.1	0.34 ± 0.18	(+) 3.0	0.41± 0.12	(+)24.2	0.67± 0.1	(+)103.0
Creatinine (mg/dl)	1.0 ± 0.3	1.0 ± 0.30	0.0	1.2 ± 0.36	(+)20.0	2.5 ± 0.3	(+)150.0
Acid Phosphatase (I.U)	25.0 ± 2.8	27.2 ± 2.30	(+) 8.8	29.0 ± 2.2	(+)16.0	49.2 ± 2.7	(+) 96.0
Alkaline Phosphatase (I.U)	47.0 ± 2.3	47.3 ± 0.73	0.0	49.0 ± 4.2	(+) 4.2	68.3 ± 2.5	(+) 45.2
GOT (I.U)	33.0 ± 1.5	40.0 ± 2.40	(+)21.2	49.0 ± 2.8	(+)48.5	106.0 ± 2.7	(+)221.2
GPT (I.U)	11.0 ± 1.5	17.0 ± 1.80	(+)54.5	29.0 ± 1.3	(+)163.1	69.0 ± 2.2	(+)527.0

4.1.5.1 Liver triacylglycerols

In control rats, a uniform distribution of triacylglycerols was observed in the parenchymal cells throughout the liver lobule from centrolobular zone to portal zone (Fig.11 a). Increased accumulation of triacylglycerols was particularly noticed at the central zone in the liver of rats after injected intramuscularly with 2.0mg/100g body weight of $NiSO_4.7H_2O$ (Fig.11 b).

4.1.5.2 Kidney triacylglycerols

Tubular epithelium showed a positive reaction for triacylglycerols in kidney of control rats (Fig. 11 c). An intense reaction for triacylglycerols was found in the proximal convoluted tubules of cortex zone in kidney of rats injected intramuscularly with 2.0mg/100g body weight of $NiSO_4.7H_2O$ (Fig.11 d).

4.1.5.3 Liver phospholipids

Phospholipids are the chief constituents of healthy hepatic cells. For instance a moderate and uniform reaction was noticed by the parenchymal cells of centrolobular zone and portal zone in the liver of control rats (Fig. 12 a). A strong deposition of phospholipid was particularly noticed at the perilobular zone in the liver of rats injected intramuscularly with 2.0mg/100g body weight of nickel (Fig.12 b).

4.1.5.4 Kidney phospholipids

Control rat kidney showed phospholipids in the epithelium of proximal convoluted tubules in the cortex zone (Fig. 12 c). Intertubular deposition of phospholipids along with tubular necrosis was noticed in the cortex zone of the kidney of rats after injected intramuscularly with 1mg/100g body weight of nickel (Fig. 12 d).

4.1.6 Histological changes induced by nickel

EXPLANATION OF THE FIGURES

Figure 11 : Photomicrographs showing deposition of triacylglycerols in liver and kidney of *Rattus rattus albino*.

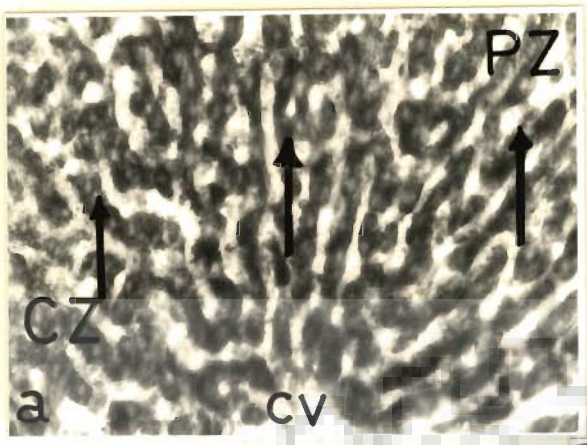
(a) Uniform deposition of triacylglycerols was exhibited by the hepatic parenchymal cells of centrolobular zone (CZ) and portal zone (PZ) of control liver. x 500

(b) High stimulation of triacylglycerolswas observed particularly at the centrolobular zone (CZ) in the liver of rats after injected intramuscularly with 2.0mg/100g body weight of nickel. Necrosis (N) and balloon cells (BC) fill up most part of the liver. x 500

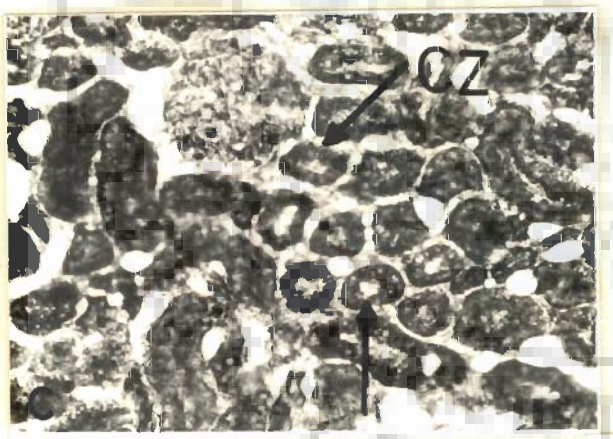
(c) In the kidney of control rats, tubular epithelium of cortex zone (CZ) showed a positive reaction for triacylglycerols. x 500.

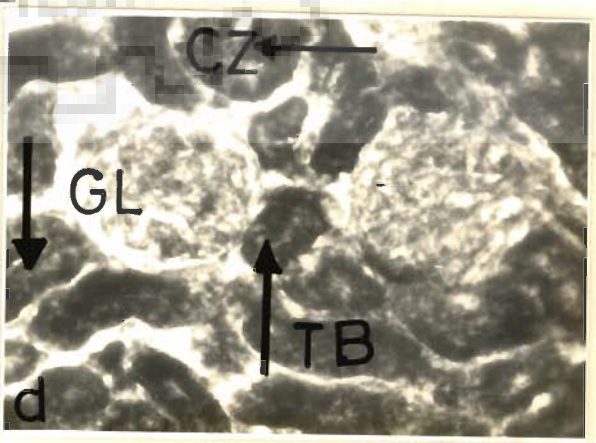
(d) High deposition of triacylglycerols was showed by the renal tubules (TB) of cortex zone (CZ) in the kidney of rats after injected intramuscularly with 2.0mg/100g body weight of nickel. x 500

CV, Central vein ; GL, Glomerulonephritis.


PZ
CZ
a
CV


BC
N
b


CZ
c


CZ
GL
TB
d

EXPLANATION OF THE FIGURES

Figure 12 : Photomicrographs showing deposition of phospholipids in liver and kidney of *Rattus rattus albino*.

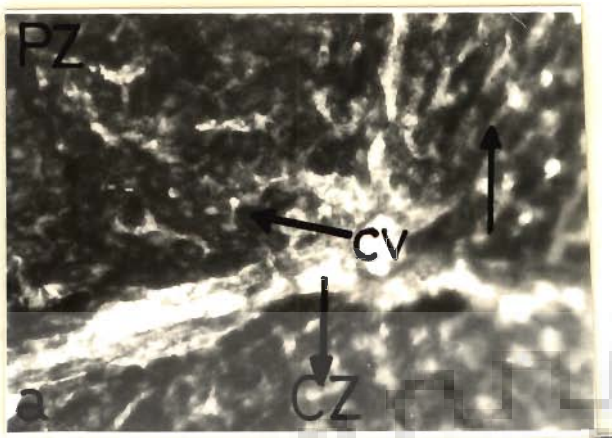
(a) In control liver, a uniform and moderate deposition of phospholipids was observed by the parenchymal cells of centrolobular zone (CZ) and portal zone (PZ). x 500

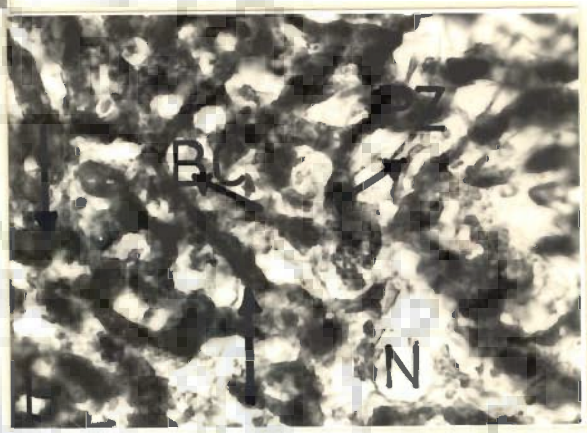
(b) Phospholipids were found more accumulated particularly at the portal zone (PZ) in the liver of rats after injected intramuscularly with 2.0mg/100g body weight of nickel. Necrosis (N) and balloon cells (BC) fill up most part of the liver. x 500

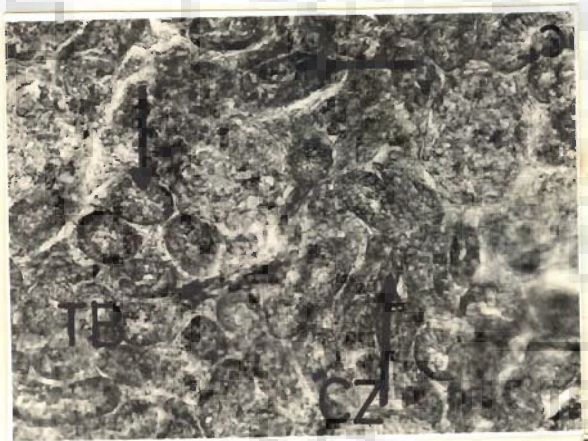
(c) Epithelium of renal tubules (TB) of cortex zone (CZ) of control kidney showed a positive reaction for phospholipids. x 500

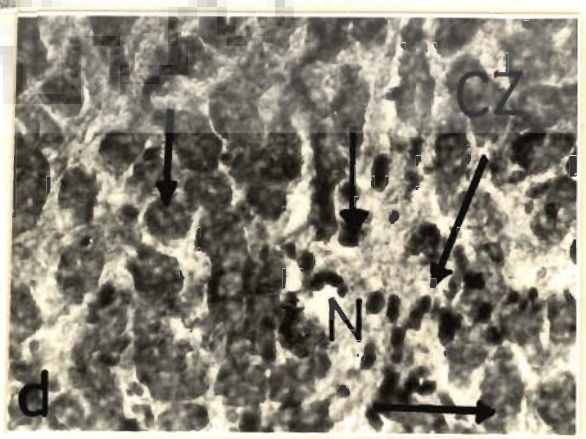
(d) Intertubular deposition of phospholipids along with tubular necrosis (N) was noticed in the cortex zone (CZ) of the kidney of rats after injected intramuscularly with 1.0mg/100g body weight of nickel. x 500

CV, Central vein ; GL, Glomerulonephritis.


PZ
CV
CZ
a


BL
N


TB
CZ


CZ
N
d

4.1.6.1 Histopathological evaluation of liver in nickel-treated rats

The histopathological changes that were induced by nickel treatment in rat liver (the main detoxifying organ and which is likely to be affected most) were examined by comparing the results with that of the liver of control animals which were treated in an identical manner but, without the nickel treatment. The results are presented in fig. 13 a. The photomicrograph of the transverse section of normal rat liver (without nickel treatment) consists of polyclonal glandular hepatic cells arranged in groups and enclosing the bile passage. Each hepatic cells is composed of a spherical central nucleus and strongly stained cytoplasm. However in the nickel-treated animals the changes in the structure of the liver are clearly observed (Fig. 13 b). Rats which were intramuscularly administered 0.5mg $NiSO_4.7H_2O$/100g body weight on alternate days for a period of 15 days showed nuclear pycnosis and nuclear clumping of cells surrounding the central vein. The cytoplasm of hepatocytes was vacuolated and characteristic polygonal shape of hepatocytes was lost due to excessive vacuolation and they were of different shape and size (Fig. 13 b). In addition, necrosis, and widened intercellular spaces were observed.

4.1.6.2 Effect on reticulin and collagen fibres in liver

Some of the characteristic types of fibres found in connective tissue are reticulin and collagen. Accumulation of these fibres has been implicated in various pathological lesions which can be demonstrated by selective staining. It can be seen (Fig.13 c) that the normal rat liver possess a network of fine branching of reticulin fibres that provides supporting frame work to the tissues. Regular distribution of reticulin fibres bound to sinusoids is also observed in the section of liver. As expected these normal structures were greatly damaged by the treatment of nickel given to the animal intramuscularly (Fig.13 d). Rats which were intramusculary administered 2.0mg/100g body weight showed the loss of reticular fibres, accumulation of the branching fibres at certain places of centrolobular region. Thick reticular fibres were also observed. In addition fibro-proliferative activity was noticed in the transverse section of the liver (Fig.13 d).

Fig.13 f represent the effect of nickel on the liver collagen. Changes can be seen that compared to the untreated rat liver (Fig. 13 e). The thickness of the collagen fibres is markedly increased in the central vein of liver in the nickel treated rats. Besides thick collagen fibres covering the central vein of the liver lobule the fibro-proliferative activity is also clearly evident (Fig. 13 f).

4.1.6.3 Histopathological evaluation of kidney in nickel-treated rat

The kidney of untreated rats (Fig. 14 a) consists of a large number of uriniferous tubules or nephrons surrounded by interstitial haematopoietic tissue and some endocrine glands, particularly the adrenal gland. The exposure of rat to any pollutant is, therefore, likely to disturb the normal functioning of kidney and its associated structure. The loosely coiled nephrons converge to form a collecting duct system which drain it to the nepheric duct.

Each nepheron is made up of the renal capsule, a short neck, proximal convoluted tubule, distal convoluted tubule and collecting duct. Renal capsule is an oval or rounded structure formed of two layers of squamous epithelium. Renal corpuscle consists of highly coiled thin walled capillaries while Bowman's capsule is an oval or round structure formed of two layers of squamous epithelium. The inner or visceral layer covers the glomerulus whereas the parietal layer is continuous with tubule. Blood is supplied to glomerulus by afferent arteriole and is collected by an efferent arteriole and is collected by an efferent arteriole. Well developed brush border towards the tubular lumen is present in the neck segment as well as in proximal tubule. The neck segment is a thin and long tubular structure which connects the Bowman's capsule with the proximal segment. It is lined with columnar epithelial cells having centrally packed nuclei and faintly eosinophilic cytoplasm. The cells are provided with cilia that project into the lumen of the segment.

The proximal tubule is highly convoluted presenting various shapes in the section. The proximal segment can be distinguished into two parts on the basis of brush border, which is more prominent in the

first part than the second. The cells in this part are cuboidal with basophilic nuclei and columnar epithelial cells with a well developed brush border lysosome system and numerous mitochondria.

The second part of proximal segment is predominant segment. In this region the brush border is less well developed than in first segment. The cells are tall columnar. This segment is of variable length and is composed of cuboidal cells more or less similar to those in neck and have clumps extending from the cell.

The distal tubule is composed of columnar epithelial cells which are arranged perpendicular to the basement membrane. The diameter is usually lesser than that of proximal tubules. The cells lining the segment are without brush border. The nuclei are large and centrally placed. The distal tubule open into the collecting tubule and ducts are constituted by simple epithelial cells with no brush border, few mircovilli and apical mucous glands also characterised this segment. They are still lesser in diameter.

Photomicrograph (Fig. 14 b) of kidney from nickel treated rats shows the structural changes and damages that are induced by nickel treatment. Conspicuous degenerative changes are clearly evident in kidney. A moderate loss of tubular epithelium border and loss of interstitial cells was noticed. The enlargement of tubules and loss of brush border was observed. In addition to extended and necrotic tubules, glomerulonephritis was also evident in the cortex zone of kidney of rat after injecting in intramuscularly with 2.0mg/100g body weight of nickel.

4.1.6.4 Effect on reticulin and collagen fibres in kidney

A transverse section of control rat kidney showed thin and regular reticulin fibres encircling the renal tubules (Fig. 14 c). As expected these normal structure were greatly damaged by the treatment of nickel given to the rat intramuscularly (Fig. 14 d). In general the kidney of rats which were intramuscularly administered 2.0mg/100g body weight showed irregular branching and proliferation of intertubular reticular fibres. Comparatively thick reticular fibres

EXPLANATION OF THE FIGURES

Figure 13 : Photomicrographs showing histological features, arrangement of reticular fibres and arrangement of collagen fibres in liver of *Rattus rattus albino*.

(a) A transverse section of the liver from a control rat showed normal mononucleated intact parenchyma from centrolobular zone (CZ) to portal zone (PZ). x 500

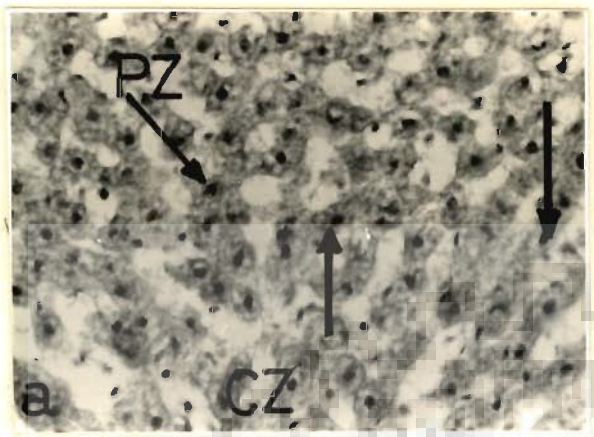
(b) Nuclear pycnosis, nuclear clumping in the cells surrounding the central vein (CV) and necrosis (N) is observed by the liver of rats after injected intramuscularly with 0.5mg/100g body weight of nickel. x 500

(c) Uniform and fine network of reticular fibres firmly bound to the hepatic cells was seen throughout the liver lobule in control rats. x 500

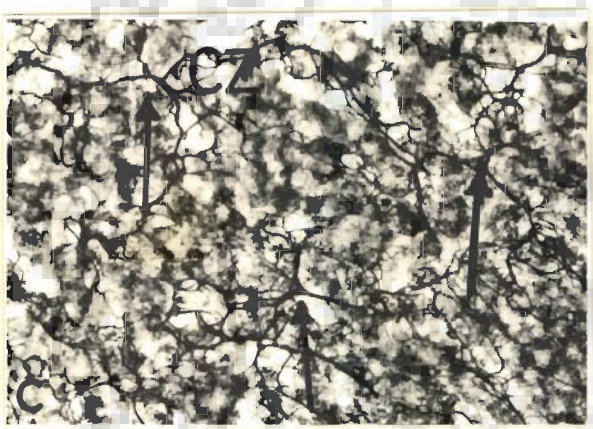
(d) Thick reticular fibres and marked fibro-proliferative (FP) activity was seen throughout the liver lobule in rats after injected intramuscularly with 2.0mg/100g body weight of nickel. x 500

(e) Thin collagen fibres covering the central vein (CV) was seen in the liver of control rat. x 500

(f) Thick collagen fibres covering the central vein (CV) and fibro-proliferative (FP) activity was seen in the liver of rat after injected intramuscularly with 1.0mg/100g body weight of nickel. x 500


PZ
CZ
a


CZ
PZ
N
V
b


CZ
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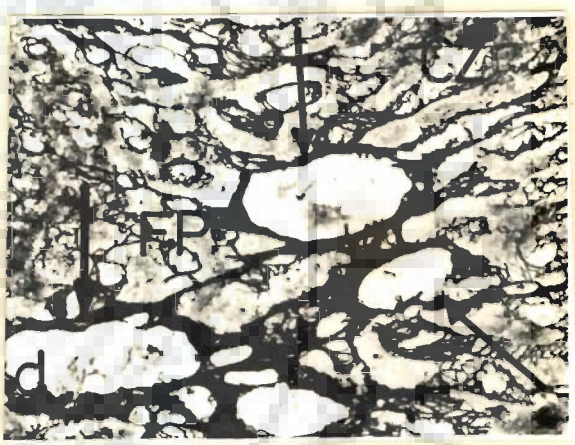
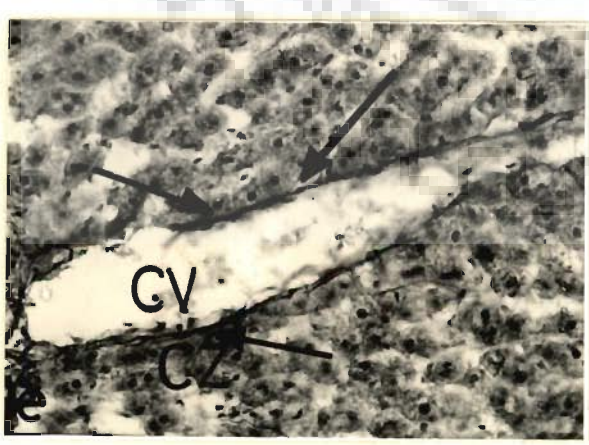
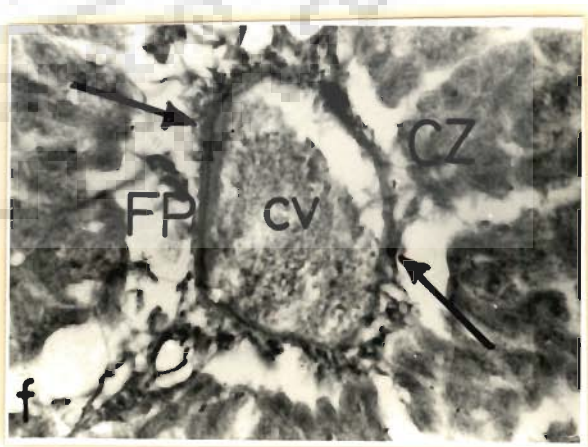

PZ
CZ
FP
d


CV
CZ
e


FP
CV
CZ
f

EXPLANATION OF THE FIGURES

Figure 14 : Photomicrographs showing histological features, arrangement of reticular fibres and arrangement of collagen fibres in kidney of *Rattus rattus albino*.

(a) A transverse section of the kidney from control rat showed intact renal tubules and round nuclei of cortex zone (CZ). x 500

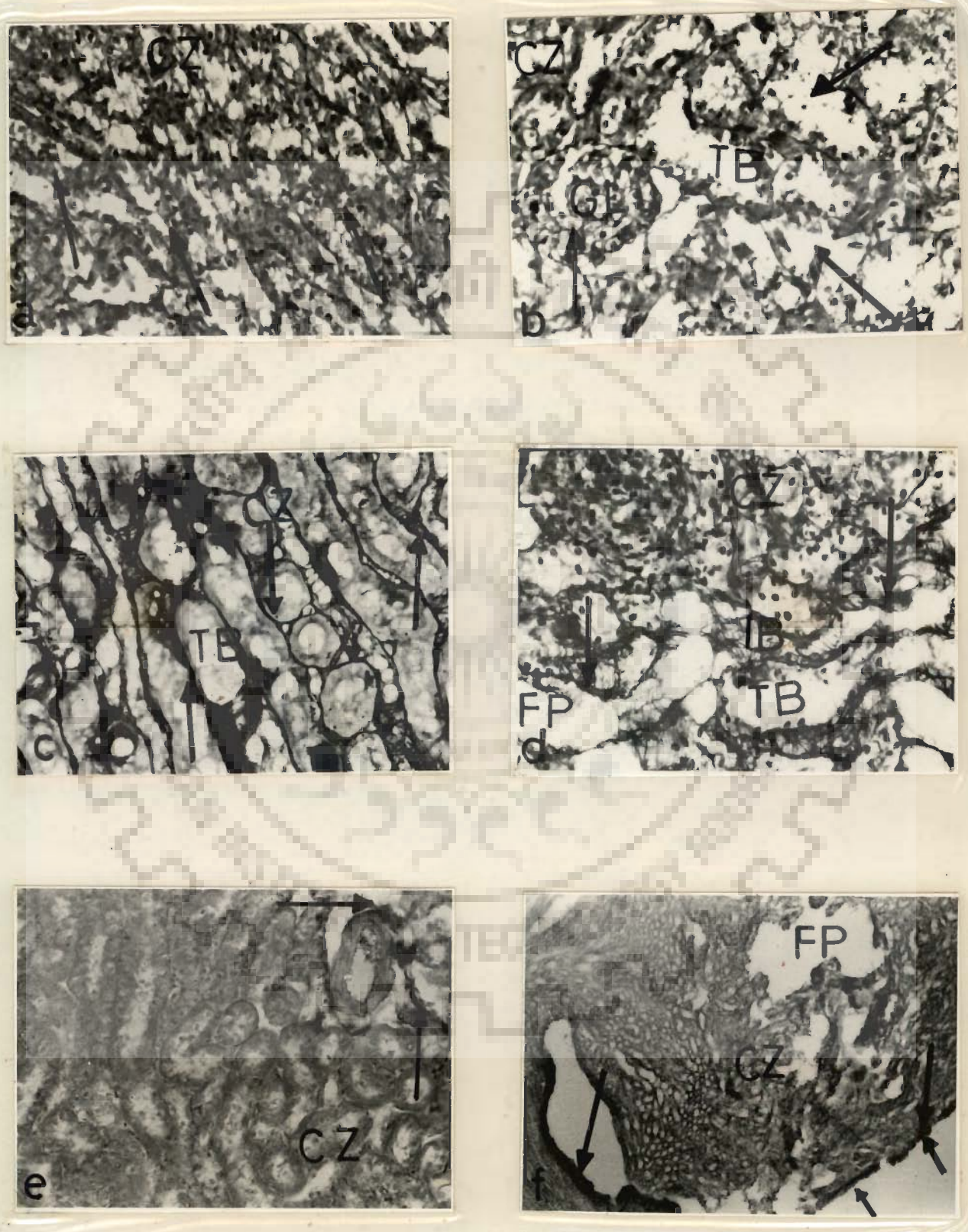
(b) Extended and necrotic tubules (TB), loss of tubular epithelium, brush border, pycnotic nuclei and glomerulonephritis (GL) was found in the cortex zone (CZ) of kidney of rat after injected intramuscularly with 2.0mg/100g body weight of nickel. x 500

(c) In the kidney of control rat, reticular fibres encircled the renal tubules (TB) of cortex zone (CZ). x 500

(d) Comparatively thick reticular fibres encircling the renal tubules (TB), intratubular branching (IB) and fibro-proliferative (FP) activity was exhibited by the cortex zone (CZ) of the kidney of rat after injected intramuscularly with 2.0mg/100g body weight of nickel. x 500

(e) Thin collagen fibres were noticed around the renal tubules in the cortex zone (CZ) of kidney of control rat. x 500

(f) Thick collagen fibres showed increased amounts of collagen and high fibro-proliferative (FP) activity was seen in the cortex zone (CZ) of kidney of rat after injected intramuscularly with 1.0mg/100g body weight of nickel. x 500


CZ
a
CZ
TB
b
TB
c
CZ
TB
FP
TB
d
CZ
e
FP
CZ
f

were also observed encircling the renal tubules (Fig. 14 d).

In control rat thin collagen fibres were observed around the renal tubules of the kidney and also the blood vessels (Fig. 14 e). Changes can be seen that compared to the untreated rat kidney, the thickness and elevated amount of the collagen fibres is markedly increased in the kidney of rats injected intramuscularly with 2.0mg/100g body weight of nickel (Fig. 14 f).

4.1.7 Effect of nickel on some hydrolases in rat liver and kidney

Table-X shows the results of the effect of $NiSO_4.7H_2O$ injected intramuscularly into the rats, on the activities of some major hydrolases (alkaline phosphatase, acid phosphatase, glucose-6-phosphatase and lipase) in liver and kidney of rats. As expected the influence on the enzyme activities was dose dependent and in animals of III group which received cumulative doses of 2.0mg $NiSO_4.7H_2O$/100g body weight, the activity of alkaline phosphatase, acid phosphatase, glucose-6-phosphatase and lipase in both liver and kidney was inhibited from 20 to 44%. At lower doses of $NiSO_4.7H_2O$, no significant reduction in the activities of these enzymes was noticed. Thus, it seems reasonable to assume that lower doses of $NiSO_4.7H_2O$ were relatively less toxic than the higher doses (2.0mg/100g body weight) to liver and kidney. These results follow the same pattern as was observed in case of other biochemical parameters.

4.1.8 Histochemical analysis of nickel effect on enzymes in liver and kidney of rats

4.1.8.1 Alkaline phosphatase

Photomicrograph (Fig. 15 b) show the effect of nickel on alkaline phosphatase activity in a fresh liver section. The normal liver (Fig. 15 a) shows alkaline phosphatase rich parenchymal cell. The overall distribution of enzyme activity is evident from the large number of black granules scattered in the cytoplasm. Relatively less activity is present in the cell membranes. In nucleolus the enzyme activity is noticed around the nucleolar membrane and in the nucleolus as well.

TABLE - X

Effect of nickel on the activity of some hydrolases in liver and kidney in rats

Enzyme	Enzyme activity[1]							
		Ni-treated[2]						
	Control units	I		II		III		
		Units[3]	(%)[4]	Units	(%)	Units	(%)	
Liver								
Alkaline phosphatase	24.2±1.0	23.1±1.0	(-) 4.5	22.0±1.0	(-) 8.9	16.6±1.0	(-)31.4	
Acid Phosphatase	16.1±1.0	14.0±1.0	(-)13.2	14.8±1.0	(-) 8.3	10.7±1.0	(-)33.7	
Glucose-6-phosphatase	36.6±1.1	32.0±1.8	(-)11.8	30.7±1.5	(-)16.2	20.4±1.0	(-)44.1	
Lipase	14.0±1.0	13.0±1.0	(-) 7.1	9.0±1.0	(-)35.7	8.0±1.0	(-)42.8	
Kidney								
Alkaline phosphatase	28.0±1.0	26.9±1.0	(-) 3.9	26.4±1.0	(-) 5.7	21.5±1.0	(-)23.2	
Acid Phosphatase	17.7±1.2	17.2±1.0	(-) 2.8	18.3±1.0	(-) 6.4	10.8±1.0	(-)37.5	
Glucose-6-phosphatase	32.3±1.6	31.2±1.0	(-) 3.4	31.2±1.0	(-) 3.4	24.2±1.0	(-)25.0	
Lipase	16.0±1.0	14.0±1.0	(-)14.2	10.5±1.0	(-)21.9	9.0±1.0	(-)43.7	

1. Results are mean ±SE (three experiments).

2. In groups control, I, II and III animals were intramuscularly given 0.5, 1.0 and 2.0mg nickel sulphate/100g body weight doses, respectively. Control were treated without nickel sulphate. Each group contained 10 rats.

3. Unit of alkaline/acid and glucose-6-phosphatase is that which liberates one μmole Pi per min under assay conditions. Unit of lipase was that which liberates free acid equivalent to 1ml of 0.2mM of NaOH.

4. Percentage change was calculated using control as 100%; (-) and (+) indicates inhibition or stimulation.

In connective tissues the alkaline phosphatase activity is only mild. Liver of rat after injected intramuscularly with 2.0mg/100g body weight of nickel showed diffused patches of alkaline phosphatase in the entire hepatic parenchyma of centrolobular zone (Fig 15 b).

The photomicrograph (Fig. 15 d) show the effect of nickel on alkaline phosphatase activity level in kidney as a function of nickel dose. Kidney from control rat (Fig.15 c) exhibit very strong alkaline phosphatase reaction in the brush border of proximal and distal tubules. Intense activity is found in the cytoplasm, nuclei and in the luminal border of these cells. Mild activity was observed in the glomeruli while the RBC's trapped in the glomeruli gave strong alkaline phosphatase activity. Interstitial hematopoietic tissue also showed mild activity of this enzyme. A poor reaction for alkaline phosphatase was observed in the tubules of the kidney of rat treated with 2.0mg $NiSO_4.7H_2O$/100g body weight of nickel. Decreased activity was also found in the cytoplasm, nuclei and glomeruli (Fig. 15 d).

4.1.8.2 Glucose-6-phosphatase

Photomicrograph (Fig. 16 b) show the effect of nickel on the activity of glucose-6-phosphatase as a function of nickel dose. Liver shows powerful glucose-6-phosphatase activity. The highest activity of this enzyme is found in the cytoplasm of the hepatocytes which are present in the centrolobular portion and surrounding the bile canaliculi while it is weak in the perilobular area. The cell membrane show moderate enzyme activity. Unlike alkaline phosphatase, connective tissue, endothelial lining of blood vessels and nuclei give negative results for this enzyme (Fig. 16 a). In the nickel-treated rat liver the level of alkaline phosphatase in the parenchymal cells, cytoplasm and around the nucleolar membrane was found to decrease with 2.0mg/100g body weight of nickel administered intramuscularly into the animal (Fig.16 b).

Photomicrograph (Fig. 16 d) show the effect of nickel on glucose-6-phosphatase activity in kidney of rats. It can be seen that a strong positive reaction for glucose-6-phosphatase was recorded in

the epithelium of renal tubules of control kidney. However, the activity was comparatively less to that of alkaline phosphatase. Intense activity was observed in the luminal border of the tubules. The cytoplasm of the tubular cells also show good activity. Mild activity of the enzyme was localized in the red blood cells. The interstitial hematopoietic tissue and glomerulus did not show any enzyme activity (Fig. 16 c). A strong inhibition for glucose-6-phosphatase was observed in the epithelium of renal tubules in cortex zone of kidney of rat after injected intramuscularly with 2.0mg/100g body weight of nickel. The interstitial hematopoietic tissue and glomeruli give no reaction (Fig. 16 d).

EXPLANATION OF THE FIGURES

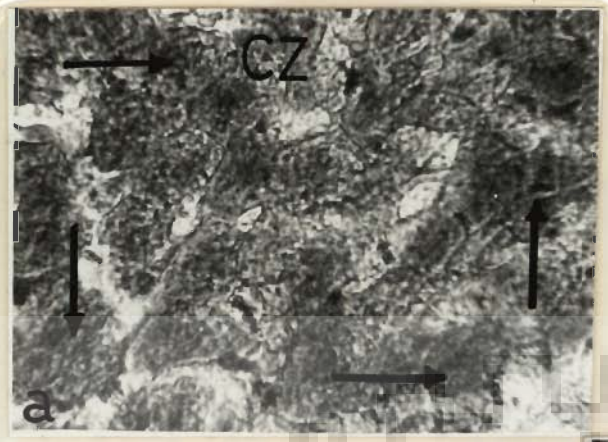
Figure 15 : Photomicrographs showing histochemical localization of alkaline phosphatase in liver and kidney of *Rattus rattus albino*.

(a) Nucleus and cytoplasm of hepatic parenchymal cells of centrolobular zone (CZ) show a strong positive reaction for glucose-6-phosphatase in control rat liver. x 600

(b) Liver of rat after injected intramuscularly with 2.0mg/100gm body weight of nickel showed diffused patches of alkaline phosphatase in the entire hepatic parenchyma of centrolobular zone (CZ). x 600

(c) Kidney from control rat exhibited very strong alkaline phosphatase reaction in the brush border (BR) of proximal and distal tubules (TB) of cortex zone (CZ). x 600

(d) A poor reaction for alkaline phosphatase was observed in the tubules (TB) of cortex zone (CZ) of the kidney of rat after injected intramuscularly with 2.0mg/100g body weight of nickel. x 600


CZ
a


CZ


CZ
TB
c


CZ
TB
d

EXPLANATION OF THE FIGURES

Figure 16 : Photomicrographs showing histochemical localization of glucose-6-phosphatase in liver and kidney of *Rattus rattus albino*.

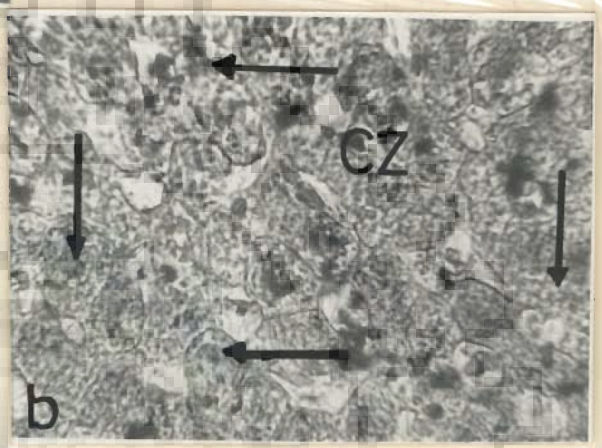
(a) Liver of control rat showed strong positive reaction for glucose-6-phosphatase by the hepatic parenchyma of centrolobular zone (CZ). x 600

(b) Inhibition in the activity of glucose-6-phosphatase was observed in the hepatic parenchymal cells of centrolobular zone (CZ) of liver in rat after injected intramuscularly with 2.0mg/100g body weight of nickel. x 600

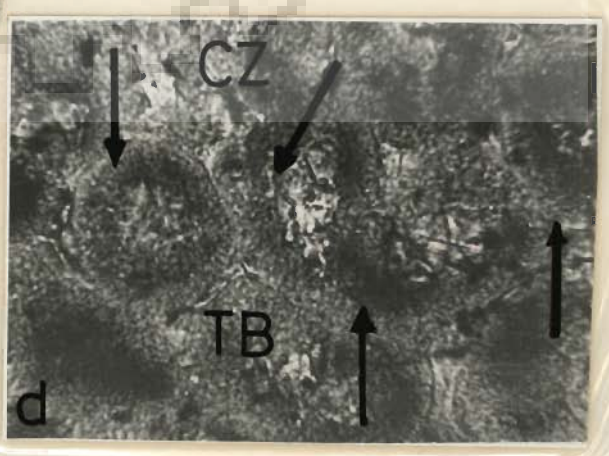
(c) A strong positive reaction for glucose-6-phosphatase was recorded in the epithelium of renal tubules (TB) of cortex zone (CZ) of control kidney. x 600

(d) A strong inhibition for glucose-6-phosphatase was observed in the epithelium of renal tubules (TB) in cortex zone (CZ) of kidney of rat after injected intramuscularly with 2.0mg/100g body weight of nickel. x 600


CZ
a


CZ
b


CZ
TB
c


CZ
TB
d

4.2 PART-II : BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON THE EFFECTS OF NICKEL SULPHATE ON PEANUT SEEDLINGS

4.2.1 Uptake of nickel by peanut seedlings

In order to understand the *in vivo* effects of nickel uptake by peanut seedlings from the growth medium (3 mm filter paper soaked in $NiSO_4.7H_2O$ solution) was investigated. In a typical experiment healthy peanut seeds were inbibed at 35°C for 24h in a $NiSO_4.7H_2O$ solution of desired concentration followed by transfer of control (without $NiSO_4.7H_2O$) and nickel-treated peanut seeds in plastic trays lined with 2 layers of 3mm Whatman filter paper completely moistened with deionized water containing 0-60ppm of nickel as $NiSO_4.7H_2O$. For controls only deionized water was used. Seeds were allowed to germinate for four days in dark at 35° C. Seedlings were picked up at randomly, thoroughly washed with deionized distilled water until washings tested negative for nickel, and contents were determined as described in Experimental Procedures. At least three estimations using three different samples were made, results of these experiments were summarized in Table-XI. These results clearly show that uptake of nickel in peanut seedlings increases with increasing concentration of nickel salt in the culture medium . For example, when seedlings are grown in medium containing 15, 30, 60ppm of nickel, the nickel contents of whole peanut seedlings taken as unit was increased from approximately 9.2ppm in the control (grown in medium without $NiSO_4.7H_2O$) to about 31.5, 102, and 273ppm giving an increase of nickel concentration by 3.4, 11, and 29.7-fold respectively. Thus it would be expected that this abnormal high accumulation of nickel in peanut seedlings may adversely affect the cellular metabolism.

4.2.2 Effect of nickel on peanut seedlings

The effect of nickel on the growth and development of peanut seedlings was investigated by growing the seedlings in water culture in the presence of varying concentrations of $NiSO_4.7H_2O$ for four days in dark at 35°C. The results of this experiment are presented in Fig. 17. It can be seen that nickel has an extremely pronounced deleterious effects on the development of both hypocotyl and radicle

TABLE XI

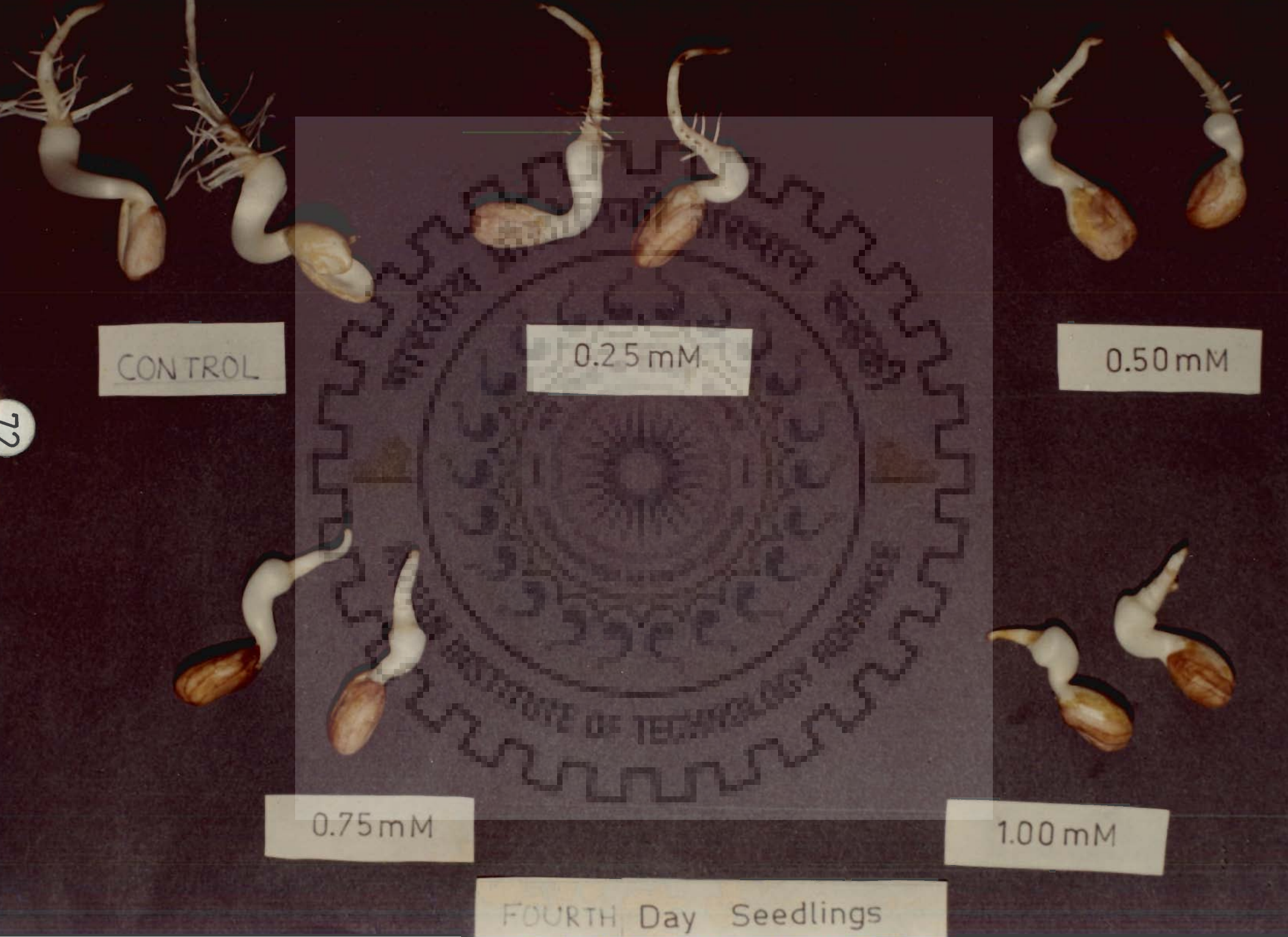
Uptake of nickel by peanut seedlings as a function of $NiSO_4.7H_2O$ concentration in growth medium

Peanut seedlings were grown in the presence of varying amount of $NiSO_4.7H_2O$ (0-66 ppm of nickel) in dark at 35^oC for four days. Seedlings were picked up at randomly, washed with running tap water followed by washing with deionized distilled water until the washings tested negative for nickel. Nickel contents were determined by the atomic absorption spectrophotometer as described in Experimental Procedures. Results are mean of three experiments.

Ni concentrations in culture medium (ppm)	Fresh weight of seedling (g)	Nickel content of seedling fresh weight (ppm)	Increases in Ni uptake (fold)
0	3.92 ± 0.5	9.2 ± 1.5	-
15	2.75 ± 0.4	31.5 ± 4	3.42
30	2.20 ± 0.5	102.5 ± 15	11.14
60	1.68 ± 0.4	273.3 ± 30	29.7

Figure 17 : Four-day old peanut seedlings showing the effect of different concentration of nickel on growth and development of hypocotyl, radicle and secondary roots.

Mature and healthy peanut seeds of equal size were allowed to germinate in aqueous culture containing indicated amounts of $NiSO_4.7H_2O$ in dark at 35^oC under asceptic conditions. Note the retardation of seedling growth, necrosis of root tips, loss of secondary roots formation and inhibition of germination at 1mM $NiSO_4.7H_2O$ concentration.


CONTROL
0.25 mM
0.50 mM
0.75 mM
1.00 mM
FOURTH Day Seedlings

and also on the number of secondary roots. As compared to the control the length of hypocotyl and radicle was greatly reduced and the secondary root formation was completely inhibited. At 0.75mM $NiSO_4.7H_2O$ concentration the nickel toxicity is clearly visible. In fact, degeneration of radicle and root tips is observed even at 0.25mM (ca. 15 ppm) $NiSO_4.7H_2O$. The quantitative data on the length as affected by different concentration of nickel in four -day old seedlings are given in Table-XII. It was found that at 1mM concentration of nickel the length of hypocotyl and radicle was reduced to about one-half and one-fourth, respectively.

The nickel effect on seedling growth was also analysed in terms of dry weight (Table-XIII). It was found that treatment with 1mM $NiSO_4.7H_2O$ greatly reduced the dry weight of hypocotyl and radicle. For example, the hypocotyl dry weight of seven -day old seedlings was reduced to 0.13g/20 seedlings from 2.2g/20 seedlings. At 0.1mM $NiSO_4.7H_2O$ concentration, however, a significant increase in the dry weight of hypocotyl was observed. In contrast, the dry weight of radicle was reduced to 0.34 $\pm$ 0.06 from 0.58 $\pm$ 0.07g/20 seedlings. Relatively there was little change in the dry weight of cotyledons, but the trend of decreasing dry weight with increasing dose of nickel treatment is evident both in cotyledons and the whole peanut seedlings.

4.2.3 Effect of nickel on the development of radicle and secondary roots

The effect of nickel on the development of radicle and secondary roots as a function of seedling age (germination period) is presented in Table-XIV. The results show that at 1mM concentration of $NiSO_4.7H_2O$ in the medium the development of radicle upto fourth day of germination is comparable with the control. In fact, upto third-day there seems to be some stimulation in the elongation of radicle by nickel, but as the age of seedling increases a drastic reduction in the elongation of radicle is observed and degeneration starts from the tip of the radicle from the seventh-day onwards of germination. Similarly, the toxicity of nickel is also indicated by the total inhibition of secondary root formation. For the purpose of experimentation five to six-day old seedlings were used as at this

TABLE- XII

Effect of nickel on growth and development of peanut seedlings

Peanut seedlings were grown in water culture containing indicated amounts of $NiSO_4.7H_2O$ in dark for four days at 35^oC. Twenty seedlings were randomly picked up for measurements. Results are mean ±SE of minimum three experiments. Controls were without nickel treatment

$NiSO_4.7H_2O$ concentration (mM)	Seedling parts		
	Hypocotyl length (mm)	Radicle length (mm)	Secondary roots
0.0(control)	25.0 ± 1.0	42.5 ± 2	Normal
0.25	16.1 ± 1.2	29.0 ± 2	none
0.50	14.5 ± 1.2	18.5 ± 2	none
0.75	14.0 ± 1.2	14.0 ± 2	none
1.0	12.5 ±1.4	10.5 ± 1.5	none

TABLE -XIII

Effect of $NiSO_4.7H_2O$ on growth and development of peanut seedlings

Peanut seedlings were grown in aqueous culture containing indicated amount of $NiSO_4.7H_2O$ for seven days in dark at 37°C. Cotyledon, hypocotyl and radicle parts of the seedlings were separated and lyophilized. Twenty seedlings were used for each determination and values are mean ± SE of three experiments.

$NiSO_4.7H_2O$ concentration (mM)	Dry weight (g/20 seedlings)			
	Cotyledon	Hypocotyl	Radicle	Total seedling
0.0	15.75 ± 1.5	2.21 ± 0.2	0.58 ± 0.07	18.54 ± 1.8
0.10	13.19 ± 1.4	3.03 ± 0.3	0.34 ± 0.06	16.56 ± 1.8
1.0	11.81 ± 1.4	0.13 ± 0.03	0.02 ± 0.01	11.96 ± 1.5

TABLE-XIV

Effect of nickel on the development of radicle and secondary roots in peanut seedlings

Peanut seedlings were grown in water culture medium containing 1mM $NiSO_4.7H_2O$ in dark for indicated days at 35°C. Twenty seedlings were randomly picked up at each stage of development and length of radicle and number of secondary roots were measured. Controls were without nickel treatment. Results are mean ± SE of three different experiments carried out under identical conditions.

Age of seedling (days)	Length of radicle(mm)		Development of secondary roots (number)	
	Control	Ni-treated	Control	Ni-treated
2	17.0 ± 8.2	21.0 ± 5.1	none	none
3	32.8 ± 1.3	36.4 ± 2.5	none	none
4	48.7 ± 2.9	43.8 ± 5.7	none	none
5	84.9 ± 4.9	55.6 ± 9.8	13 ± 2	none
6	103.2 ± 5.1	53.4 ± 14.6	22 ± 2	none
7	132.5 ± 8.7	degenerated[a]	34 ± 4	none
8	156.7 ± 8.8	degenerated[a]	47 ± 4	none

a. Degeneration starts from root tips

stage apparent degeneration of root tips was observed.

4.2.4 Effect of nickel on total lipids in peanut seedlings

The total lipid contents of radicle and hypocotyl of seven -day old peanut seedlings grown without and with nickel in water culture are summarised in Table-XV. The lipid concentration in both radicle and hypocotyl were greatly reduced by nickel. The maximum effect, however, was observed in radicles in which at 0.2mM concentration of nickel the lipid contents were reduced to 0.07mg from 0.85mg per g dry weight of the tissue and at 1mM nickel concentration the total lipid concentration in radicle was further reduced to 0.03mg/g dry weight. Somewhat identical pattern was observed in hypocotyls, although the lowering of lipid concentration by nickel in hypocotyl was much lower (from 0.86mg to 0.23mg/g dry weight of the tissue) compared to that of the radicle. Since, the radicle and hypocotyl are dividing tissues and cotyledons are the storage tissues which provide food for the growing seedlings during the early stage of germination and growth, the effect of nickel on the growth and development of peanut seedlings could be physiologically important.

4.2.5 Effect of nickel on some major enzymes involved in mobilizing the stored food from cotyledons to the growing parts of the seedling

It is well established that during the early part of germination the nutritional requirement of the growing plant is satisfied by mobilizing the food stores from the cotyledons. Among the major food stores that are mobilized, are carbohydrates (starch), lipids (fats), proteins, phosphate (phytate) and minerals. It is, therefore, expected that the level of enzymes which are involved in the digestion of the materials is greatly enhanced during germination process. Similarly, if the germination process and early growth of seedling is inhibited by an inhibitor, the likely major effect of the inhibitor would be on the enzyme activities mentioned above. In view of the above, the effect of nickel on the levels of amylases, lipases, proteases, phytases and non-specific acid phosphatases and nucleotidases has been studied in the germinating peanut cotyledons.

TABLE -XV

Effect of nickel on the lipid contents of radicle and hypocotyls of seven -day old germinating seedlings

Nickel conc (mM)	Lipid content of seedling (mg/g dry weight	
	Radicle	hypocotyl
0(control)	0.85 ± 0.1	0.86 ± 0.01
0.1	0.26 ± 0.05	0.58 ± 0.08
0.2	0.07 ± 0.05	0.25 ± 0.06
0.4	0.03 ± 0.03	0.20 ± 0.06
1.0	-	0.23 ± 0.06

4.2.5.1 Effect on α-amylase activity

Mature peanut seeds were germinated with and without nickel treatment for days, under aseptic conditions, in dark at 35° C. The activity of α-amylase was determined in germinating cotyledons, picked-up randomly at different stages of germination. Results are presented in Fig. 18.

It can be seen that the α-amylase activity in peanut cotyledons without nickel treatment is much higher than that of the nickel-treated cotyledons. As, for example, in six -day old nickel-treated germinating cotyledons the enzyme activity was about two-third of the untreated cotyledons. These results clearly indicate that the mobilization of stored carbohydrate was adversely affected by nickel that in turn would contribute to the poor growth of the seedling.

4.2.5.2 Effect of nickel on protease activity

Fig. 19 shows the effect of nickel on protease activity in peanut cotyledons at different stages of germination. The protease activity in nickel treated peanut cotyledons was found to be about 50% higher than the protease activity in the untreated cotyledons at each stage of germination, except in eight -day old plants. This shows that nickel induced higher rate of degradation of storage proteins during germination, which is unlike the α-amylase activity which was found to be inhibited. At the moment significance of this observation is unclear.

4.2.5.3 Effect on lipase

Lipase which catalyzes the hydrolysis of lipids into glycerol and fatty acids is important from the point of lipid utilization in growing plants. When germination of peanut seeds was carried out in the presence of 1mM $NiSO_4.7H_2O$, no lipase activity was detected in two -day old germinating cotyledons, but under identical germination conditions the lipase activity was highly predominant (Fig. 20). However, the enzyme activity in nickel-treated peanut cotyledons was rapidly increased between germination period of two and four days.

Figure 18 : Effects of nickel on α-amylase activity in peanut cotyledons

Peanut seeds were germinated in dark at 35°C and 100% humidity for the indicated periods. Cotyledons were excised, washed and α-amylase activity was assayed in cytosolic fractions (12,000-105,000xg).

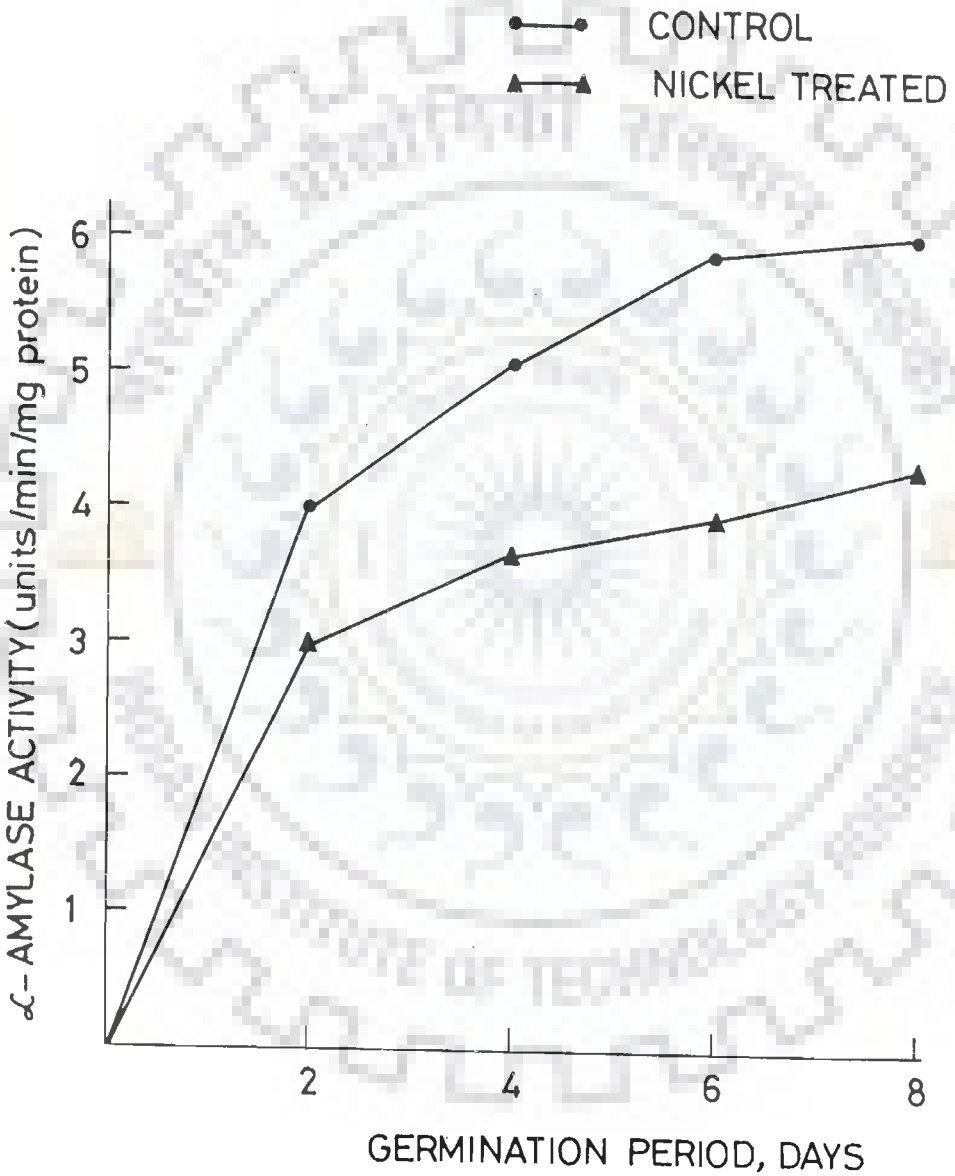

CONTROL
NICKEL TREATED
α-AMYLASE ACTIVITY (units/min/mg protein)
1
2
3
4
5
6
2
4
6
8
GERMINATION PERIOD, DAYS

Figure 19 : Effects of nickel on protease activity in peanut cotyledons

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and protease activity was assayed in cytosolic fractions (12,000-105,000xg).

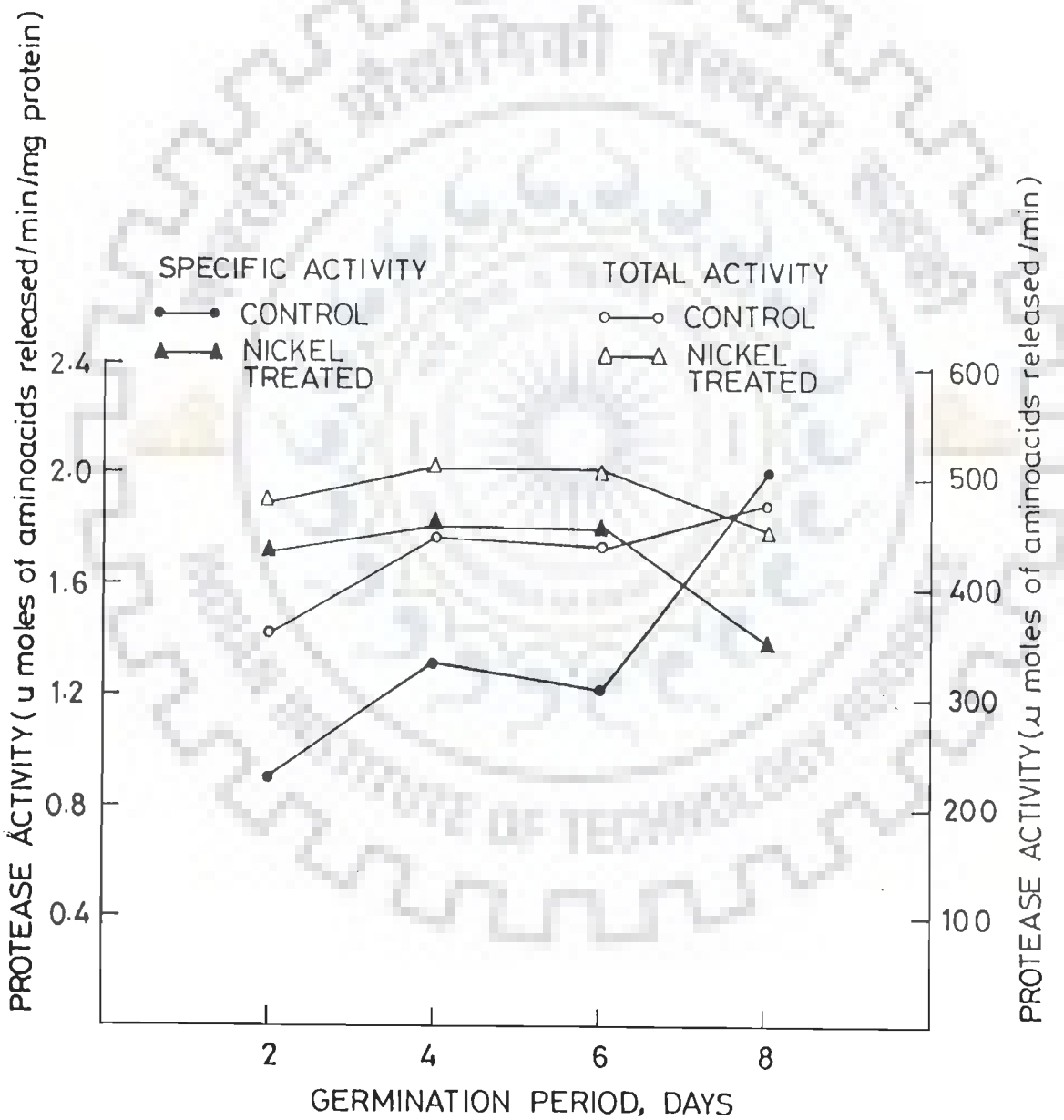
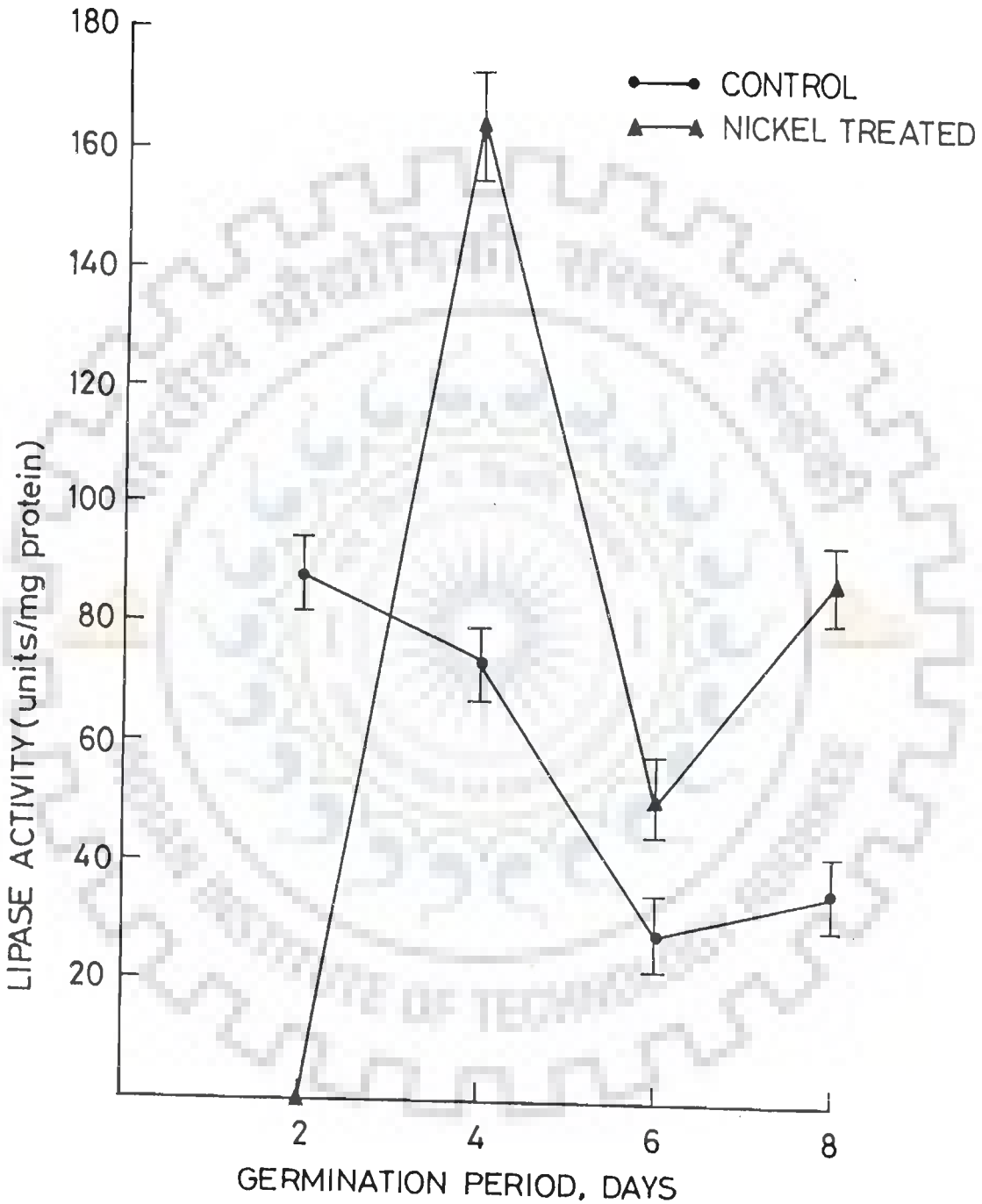

SPECIFIC ACTIVITY
CONTROL
NICKEL TREATED
TOTAL ACTIVITY
CONTROL
NICKEL TREATED
PROTEASE ACTIVITY (u moles of aminoacids released/min/mg protein)
PROTEASE ACTIVITY (u moles of aminoacids released /min)
2.4
2.0
1.6
1.2
0.8
0.4
600
500
400
300
200
100
2
4
6
8
GERMINATION PERIOD, DAYS

Figure 20 : Effects of nickel on lipase activity in peanut cotyledons

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and lipase activity was assayed in cytosolic fractions (12,000-105,000xg).


180
160
140
120
100
80
60
40
20
LIPASE ACTIVITY (units/mg protein)
CONTROL
NICKEL TREATED
2
4
6
8
GERMINATION PERIOD, DAYS

Following this period the lipase activity declined rapidly and in six -day old cotyledons the enzyme activity was reduced to one-third of the maximum activity observed on fourth-day of germination. The lipase activity in the untreated plants showed a continuous decrease.

4.2.5.4 Effect of nickel on phytase activity

Phytase catalyzes the hydrolysis of phytate (myoinositol hexa phosphate) into myoinositol and Pi. The released phosphorus is utilized by the growing seedling in its early stage of growth and development. Fig. 21 shows the effect of phytase activity during germination period. It was observed that the phytase activity in the nickel-treated cotyledons remains more or less constant and its level is lower than that of the untreated cotyledons at each stage of germination tested. The effect is more clearly noticed when data are presented as total phytase activity on 25 cotyledon basis. These results demostrate that nickel impairs the mobilization of stored phosphorus from cotyledon during germination.

4.2.5.5 Effect on 5'-nucleotidase

Fig. 22 shows the effect of nickel on 5'-nucleotidase activity in germinating peanut cotyledons. Like phytase the 5'-nucleotidase activity in the nickel-treated cotyledons during later stage of germination (after 4-days of germination) was significantly lower than enzyme activity in the untreated cotyledons. In fact, on eighth-day of germination 5'-nucleotidase activity in the untreated cotyledons was found to be about 3-fold higher than that of the nickel-treated cotyledons. The effect of nickel during early stages of germination (upto fourth-day) was not significant. These results also indicate that inhibitory effect of nickel on seedling growth is most likely, at least in part, due to the reduction in the level of 5'- nucleotidase activity.

4.2.5.6 Effect on non-specific phosphatases

So far we have seen that nickel had resulted in lowering the level of phytase and 5'-nucleotidase which are specific phosphatases. Results in Fig. 23 shows that the non-specific acid phosphatases are

Figure 21 : Effects of nickel on phytase activity in peanut cotyledons

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and phytase activity was assayed in cytosolic fractions (12,000-105,000xg).

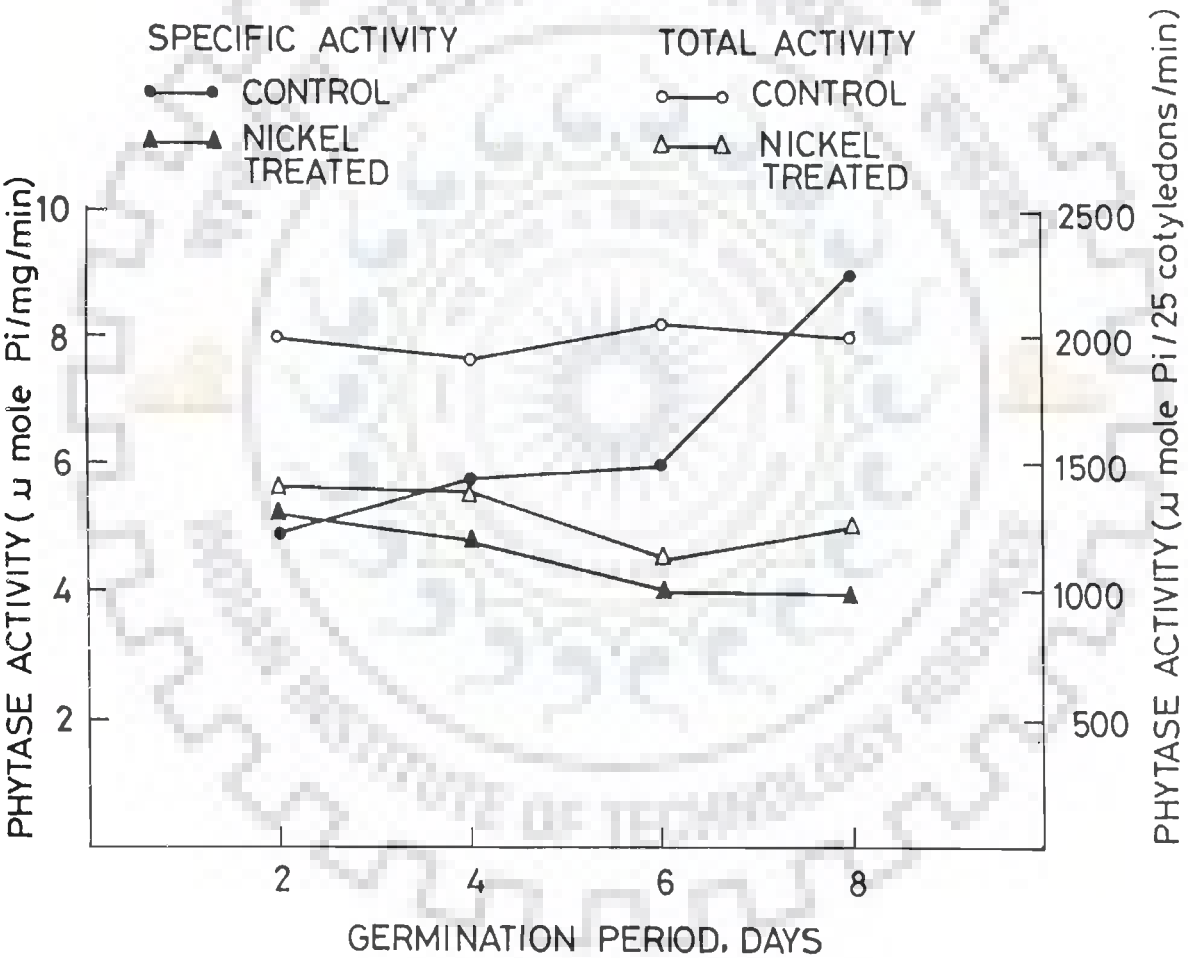

SPECIFIC ACTIVITY
CONTROL
NICKEL TREATED
TOTAL ACTIVITY
CONTROL
NICKEL TREATED
PHYTASE ACTIVITY (μ mole Pi/mg/min)
PHYTASE ACTIVITY (μ mole Pi / 25 cotyledons /min)
10
8
6
4
2
2500
2000
1500
1000
500
2
4
6
8
GERMINATION PERIOD, DAYS

Figure 22 : Effects of nickel on 5'-nucleotidase activity in peanut cotyledons

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and 5'-nucleotidase activity was assayed in cytosolic fractions (12,000-105,000xg).

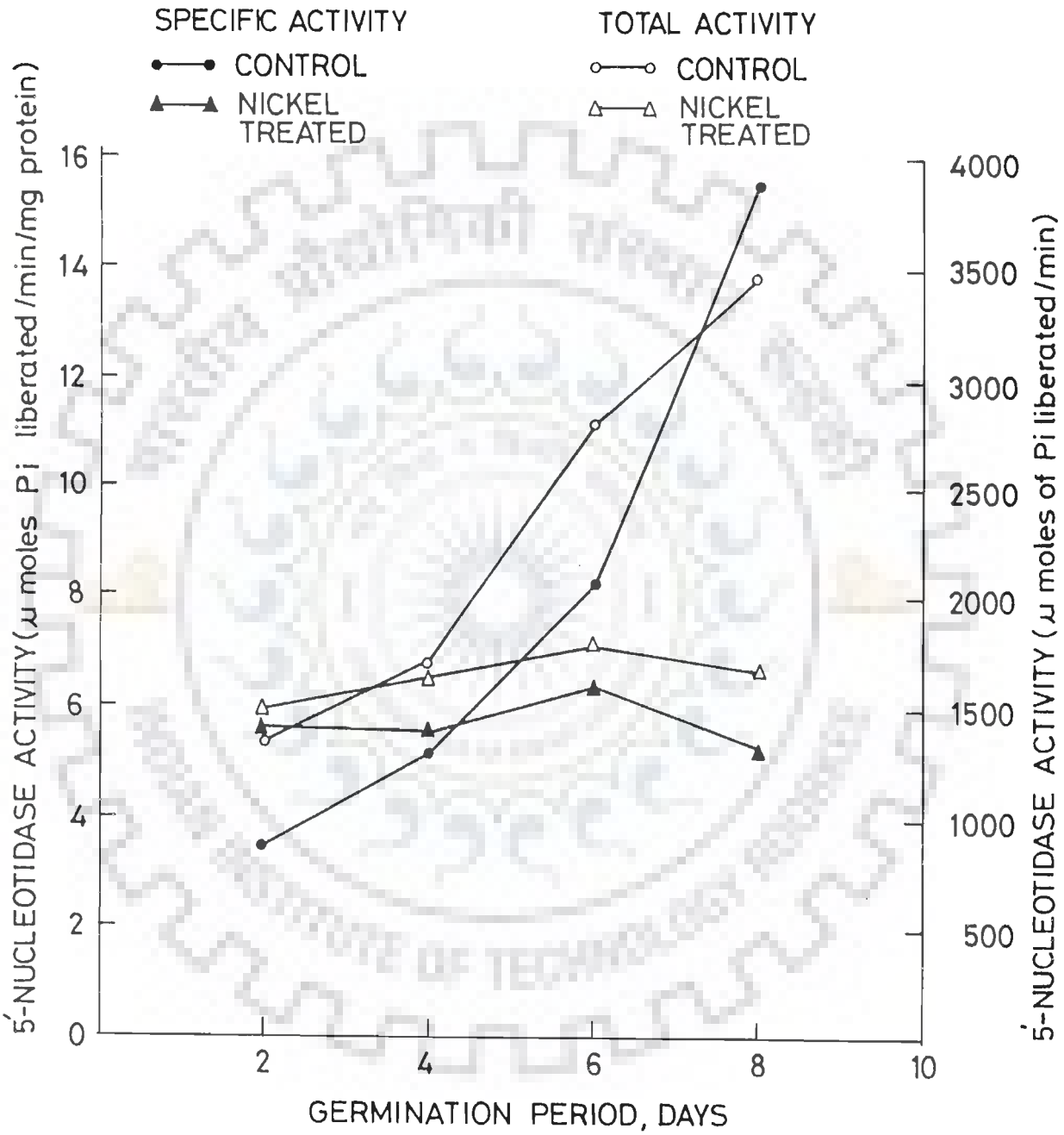
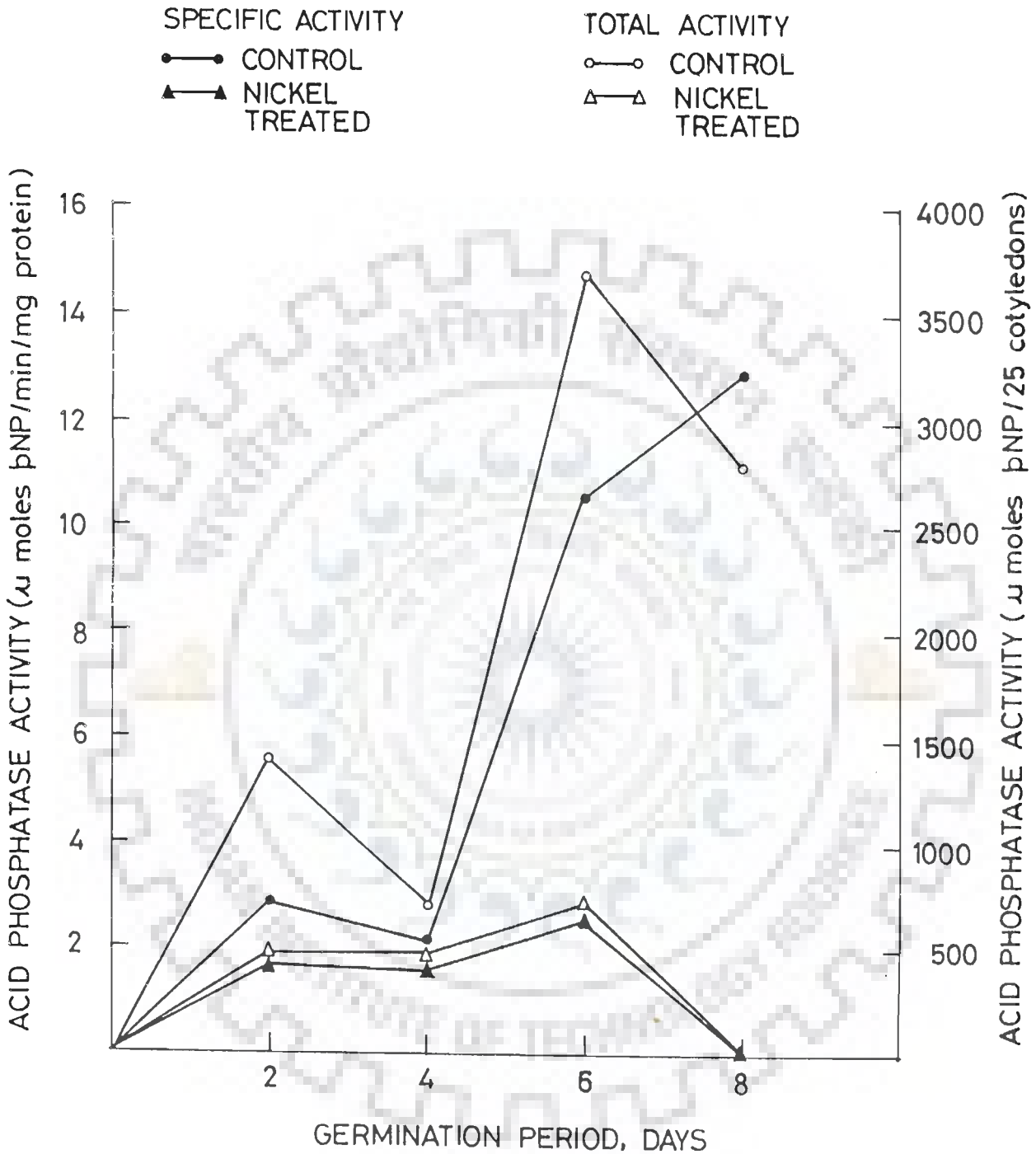

SPECIFIC ACTIVITY
CONTROL
NICKEL TREATED
TOTAL ACTIVITY
CONTROL
NICKEL TREATED
5'-NUCLEOTIDASE ACTIVITY(μ moles Pi liberated/min/mg protein)
5'-NUCLEOTIDASE ACTIVITY (μ moles of Pi liberated/min)
GERMINATION PERIOD, DAYS
0
2
4
6
8
10
12
14
16
500
1000
1500
2000
2500
3000
3500
4000

Figure 23 : Effects of nickel on non-specific phosphatases in peanut cotyledons

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and acid phosphatase activity was assayed in cytosolic fractions (12,000-105,000xg).


SPECIFIC ACTIVITY
CONTROL
NICKEL TREATED
TOTAL ACTIVITY
CONTROL
NICKEL TREATED
ACID PHOSPHATASE ACTIVITY (µ moles pNP/min/mg protein)
ACID PHOSPHATASE ACTIVITY (µ moles pNP/25 cotyledons)
16
14
12
10
8
6
4
2
4000
3500
3000
2500
2000
1500
1000
500
2
4
6
8
GERMINATION PERIOD, DAYS

also greatly inhibited by nickel treatment, especially during the later period (after fourth-day) of germination. It was found that while untreated eight -day old cotyledons showed very high phosphatase activity (13 μmol pNP/min/mg protein), no acid phosphatase activity was observed in the nickel-treated cotyledons of the same age. These results once again demonstrate that nickel strongly interferes with phosphorus metabolism in germinating cotyledons of peanut, which is probably responsible for growth of radicle and hypocotyl and secondary root formation during germination of seeds.

4.2.6 Effect of nickel on microsomal membrane fraction in germinating peanut cotyledons

In order to study the effect of nickel on various properties of the microsomal membrane fraction, peanut sedlings were grown without and with nickel sulphate in the aqueous medium. Cotyledons were harvested, the microsomes were prepared and analysed for various components.

4.2.6.1 Effect on membrane protein

Fig. 24 a shows the effect of varying concentrations of nickel on membrane proteins. The results represent the protein content of the total microsomal pellet obtained from 25 cotyledons detached from six -day old germinating seedlings. It was found that the concentration of protein in the microsomal membrane fraction increased with nickel concentration in the medium and compared to control microsomes from cotyledons treated with 0.25 mM $NiSO_4.7H_2O$ contained twice the amount of protein. However, at 1mM $NiSO_4.7H_2O$ concentration change in protein content of the membrane fraction was only marginally higher than the control. This observation was further confirmed by the results shown in Fig. 24 b in which the seedlings were grown in water containing 1mM $NiSO_4.7H_2O$ and protein content of microsomal membrane, prepared from cotyledons of different stages of seedling development, were determined and compared with the control. It was found that throughout the germination period the content of membrane protein in nickel-treated cotyledons were higher than the untreated cotyledons. In addition the membrane protein content in both nickel-treated and control progressively decreased with germination

Figure 24a: Effects of varying concentrations of nickel on membrane protein of peanut cotyledons.

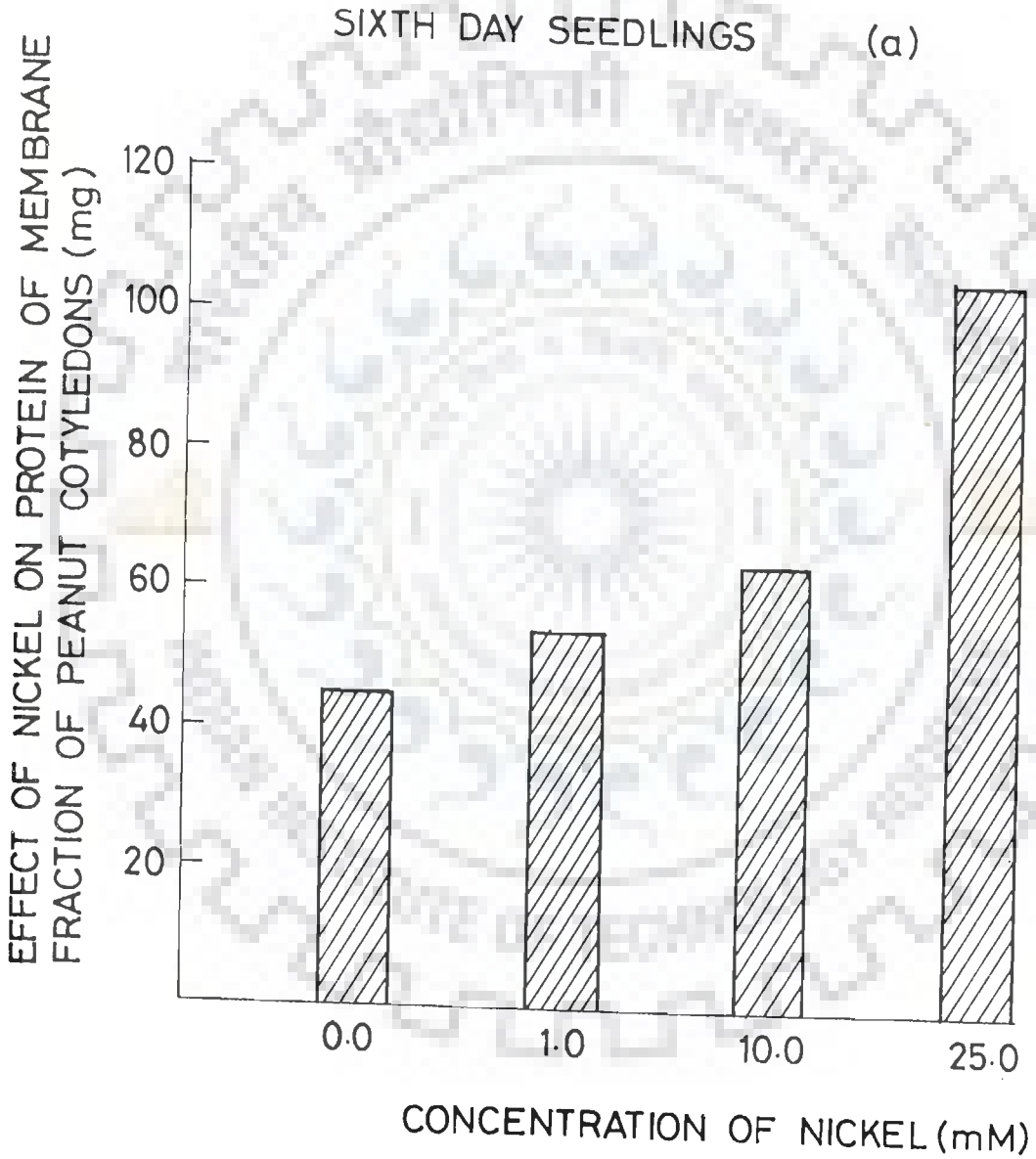
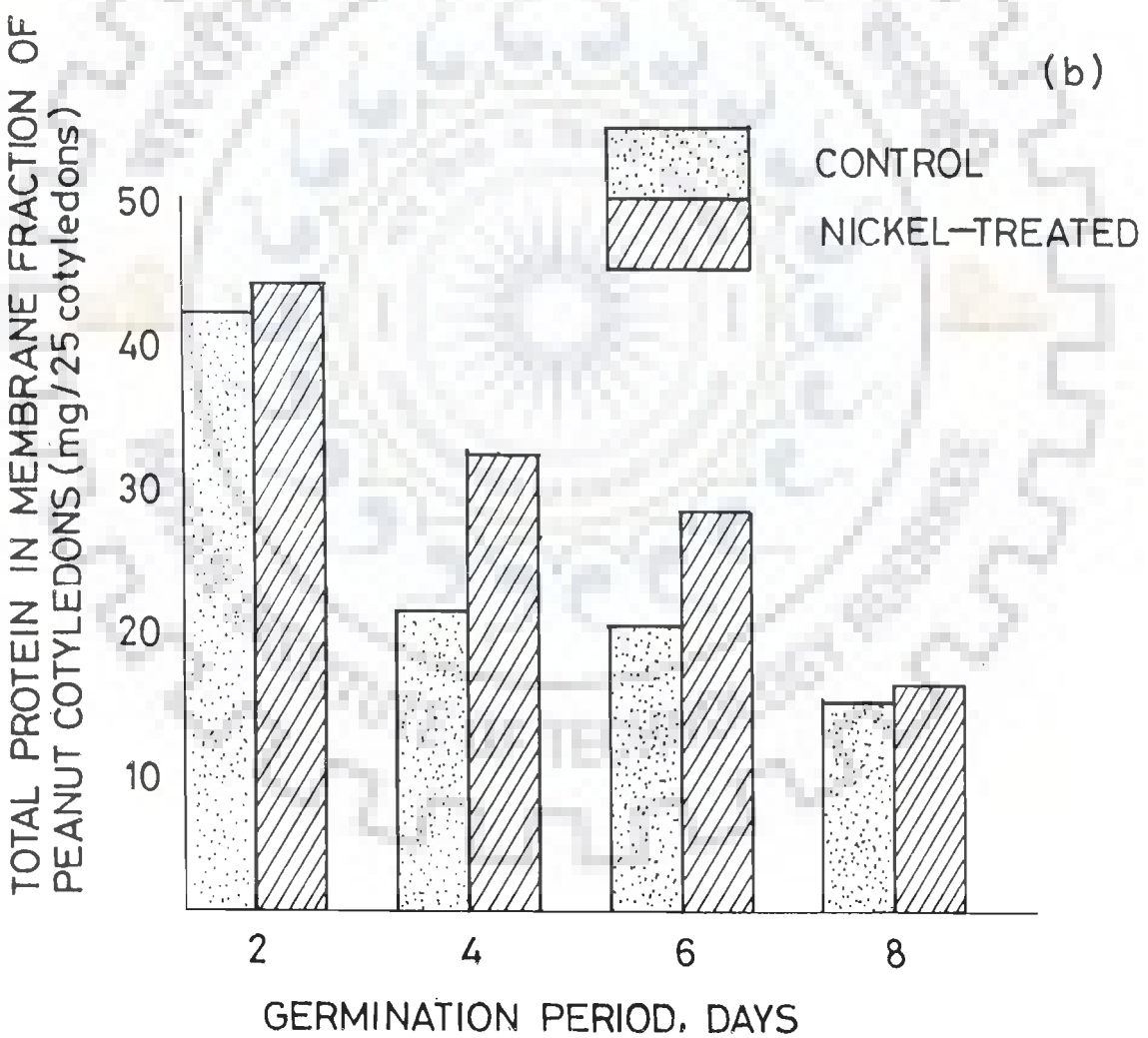

SIXTH DAY SEEDLINGS (a)
EFFECT OF NICKEL ON PROTEIN OF MEMBRANE FRACTION OF PEANUT COTYLEDONS (mg)
120
100
80
60
40
20
0.0
1.0
10.0
25.0
CONCENTRATION OF NICKEL (mM)

Figure 24b: Effects of nickel on protein content of microsomal membranes, prepared from cotyledons at different stages of seedling development.


(b)
CONTROL
NICKEL–TREATED
TOTAL PROTEIN IN MEMBRANE FRACTION OF PEANUT COTYLEDONS (mg/25 cotyledons)
50
40
30
20
10
2
4
6
8
GERMINATION PERIOD, DAYS

period, but the decrease in both was comparable.

4.2.6.2 Effect on membrane carbohydrates and phospholipids

The effect of the fixed concentration (1mM) of nickel on membrane carbohydrate and phospholipid contents of cotyledons are summarized in Table-XVI. At all stages of germination the carbohydrate as well as the phospholipid contents of microsomal membrane fraction of the nickel-treated cotyledons were found to be significantly lower than that of the untreated peanut cotyledons. These results point towards the alterations in membrane properties due to the nickel treatment. The age of seedling is also found to have profound effect on the membrane carbohydrate make up. In controls the concentration of membrane carbohydrates rapidly decreased with germination period. In contrast, however, nickel reversed this process and there was continuous increase in membrane carbohydrate concentration with seedling age. The same pattern i.e.,increasing concentration with germination period is evident in case of membrane phospholipids in nickel-treated cotyledons.

4.2.6.3 Membrane-bound 5'-nucleotidase and non-specific acid phosphatase

Results presented in previous sections, showed that in general cytosolic acid phosphatases were greatly inhibited by nickel treatment. Since 5'-nucleotidase is a marker enzyme for the plasma membranes, effect of nickel on the activity of the membrane bound enzyme was studied. Surprisingly, very little or no 5'-nucleotidase activity could be detected in the plasma membrane fraction obtained from nickel-treated cotyledons (Fig. 25), but the plasma membrane fraction from the untreated germinating cotyledons exhibited high 5'-nucleotidase activity. For instance in eight -day old germinating cotyledons, in controls, the enzyme activity was about 25 μmole Pi/min/mg protein whereas in the nickel-treated cotyledons no activity of 5' nucleotidase was found. Similarly, the activity of membrane-bound nonspecific acid phosphatase was greatly suppressed by nickel (Fig. 26). From these results it is quite apparent that in the presence of nickel the phosphorus metabolism is strongly inhibited in the germinating seedlings, causing retardation in germination as well

TABLE-XVI

Effect of nickel on the carbohydrate and phospholipid content of the microsomes of germinating peanut cotyledons

Peanut seedlings were grown with and without 1mM $NiSO_4.7H_2O$ in water for indicated period in dark at 35^oC. Cotyledons (25) were randomly picked up at each stage of seedling growth, cotyledons were detached from the seedling and microsomal fraction was prepared as described in "Experimental Procedures". Result are mean ± SE of three experiments.

Age of seedling (days)	Carbohydrate (mg/g protein)		Phospholipids (µg/g protein)	
	Control	Ni-treated	Control	Ni-treated
2	2.69 ± 0.1	0.67 ± 0.08	46.5 ± 6	19.1 ± 4
4	2.09 ± 0.2	0.92 ± 0.05	62.5 ± 7	30.0 ± 5
6	1.5 ± 0.1	1.20 ± 0.05	52.4 ± 6	33.6 ± 5
8	0.27 ± 0.04	1.95 ± 0.06	97.3 ± 9	48.7 ± 7

Figure 25 : Effects of nickel on membrane-bound 5'-nucleotidase activity in the plasma membrane fraction.

Peanut seeds were germinated in dark at 35°C and 100% humidity for the indicated periods. Cotyledons were excised, washed and membrane-bound 5'-nucleotidase activity was assayed in membrane fraction (12,000-105,000xg).

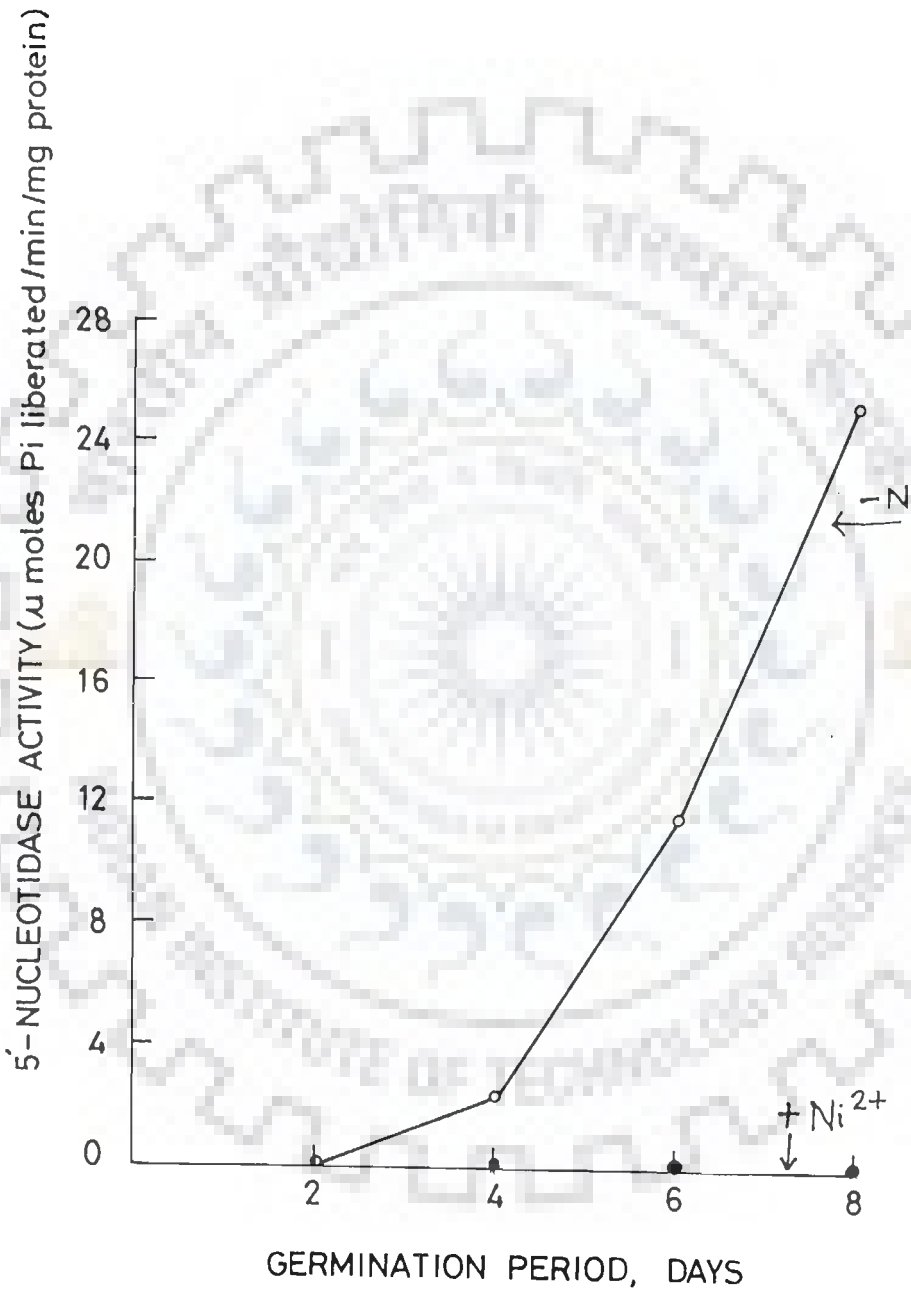

5'-NUCLEOTIDASE ACTIVITY (μ moles Pi liberated/min/mg protein)
28
24
20
16
12
8
4
0
2
4
6
8
GERMINATION PERIOD, DAYS
-Ni 2+
+Ni 2+

Figure 26 : Effects of nickel on membrane-bound non-specific acid phosphatase activity in the plasma membrane fraction

Peanut seeds were germinated in dark at 35^{o}C and 100% humidity for the indicated periods. Cotyledons were excised, washed and membrane-bound acid phosphatase activity was assayed in membrane fraction (12,000-105,000xg).

as in growth and development of seedling as a whole.

4.2.6.4 Effect of Ni^{2+} on the mannosyl transferase activity *in vitro*

Nickel is found to stimulate the activity of dolichyl-phosphomannose synthase (Table-XVII). It can be seen that when EDTA was present in the incubation mixture only about 2.5% and 0.5% of the radioactivity was incorporated into the lipid-saccharide fraction (chloroform-methanol, 3:2, v/v extractable lipid) and into the membrane glycoproteins. On the contrary in the presence of 1mM $NiSO_4.7H_2O$ the mannosyl incorporation in the lipid and glycoprotein fractions was increased 26 and 31-fold, respectively. Of all the metal ions tested stimulation of the mannosyl transferase activity by Ni^{2+} was the highest. These results also indicate the metal ion requirement of the enzyme.

The mannosylated lipid fraction as identified by TLC contained dolichyl-phosphomannose as the major lipid component (Fig. 27). Some slow moving lipid-saccharide fractions corresponding to Dol-PP-$GlcNAc_2$-Man_{5-9}, Dol-PP-$GlcNAc_2Man_3$, Dol-PP-$GlcNAc_2$-Man were also present. These results indicate that *in vitro* Ni^{2+} not only stimulated the dol-P-man synthase activity, but also could replace divalent metal ions (Mg^{2+} and Mn^{2+}) required by the enzyme. Since Dol-P-Man is the important donor substrate of the mannosyl residue of the last four mannose residues in the assembly of the large lipid oligosaccharide, Glc_3-Man_9-$GlcNAc_2$-PP-dol, it is expected to influence the N-glycosylation of protein as well. The results of radioactivity in the glycoprotein fraction supports this view. If this is also true *in vivo* remains to be established.

4.2.7 *In vivo* effect of $NiSO_4.7H_2O$ on callose (1,3-β-glucan) synthase activity

The *in vivo* effect of $NiSO_4.7H_2O$ on the activity of callose (1,3-β-glucan) synthase (CS) in the germinating peanut seedlings was investigated by measuring the radioactivity incorporated by the plasma membrane bound CS into the glucan polymer (ethanol insoluble product) from the donor substrate UDP-[^{14}C]-glucose (Kamat et al., 1992). The results summarized in Table - XVIII clearly indicate that the CS

TABLE XVII

Effects of $NiSO_4.7H_2O$ and some other common divalent metal salts on the activity of mannosyl transferase *in vitro*

Microsomal membrane fraction from sixth-day old germinating cotyledons was incubated with GDP-^{14}C-Mannose (50000, cpm) in the presence of metal ions. In the controls, 4mM EDTA (pH 7.4) was also added to remove endogenous metal ions present in the membrane fraction. After incubation the radioactive mannosylated lipids were extracted with chloroform-methanol (3:2, v/v). The membrane pellet glycoprotein fraction obtained after lipid extraction was washed 4x with theoretical upper phase and radioactivity was measured. CM=Chloroform-methanol ; GP=Glycoprotein.

Metal salts added	Radioactivity incorporated			
	CM(3:2)		GP	
	cpm	%	cpm	%
none + EDTA, 4mM	1285	2.57	245	0.49
1mM $NiSO_4$	33440	66.88	7713	15.42
1mM $ZnSO_4$	29070	58.14	125	0.25
1mM $CuSO_4$	17915	35.83	115	0.23
2mM $MnCl_2$	28660	57.32	1917	3.84
10mM $MgCl_2$	27685	43.37	4452	9.90

Figure 27 : TLC profile of th e [^{14}C]-mannose containing lipid-saccharides formed during the incubation of the microsomes from the 6-day old germinating peanut cotyledons with GDP-[^{14}C]-mannose in the presence of $NiSO_4.7H_2O$ as described in the "Experimental procedures ". (a), [^{14}C]-mannose-containing lipid-saccharides formed in the normal assay mixture containing Mg^{2+} and no Ni^{2+} and (b), [^{14}C]-mannose-containing lipid-saccharides formed in the presence of Ni^{2+}without Mg^{2+}. Arrows show the position of standard dolichyl-phosphomannose, Dol-P-man; dolichyl diphospho -N- acetylglycosamine (Dol-PP-GlcNAc); Dol$PP-GlcNAc_2$; Dol-PP-$GlcNAc_2$-Man; Dol-PP-$GlcNAcMan_3$ and Dol$PP-GlcNAc_2-Man_5$.

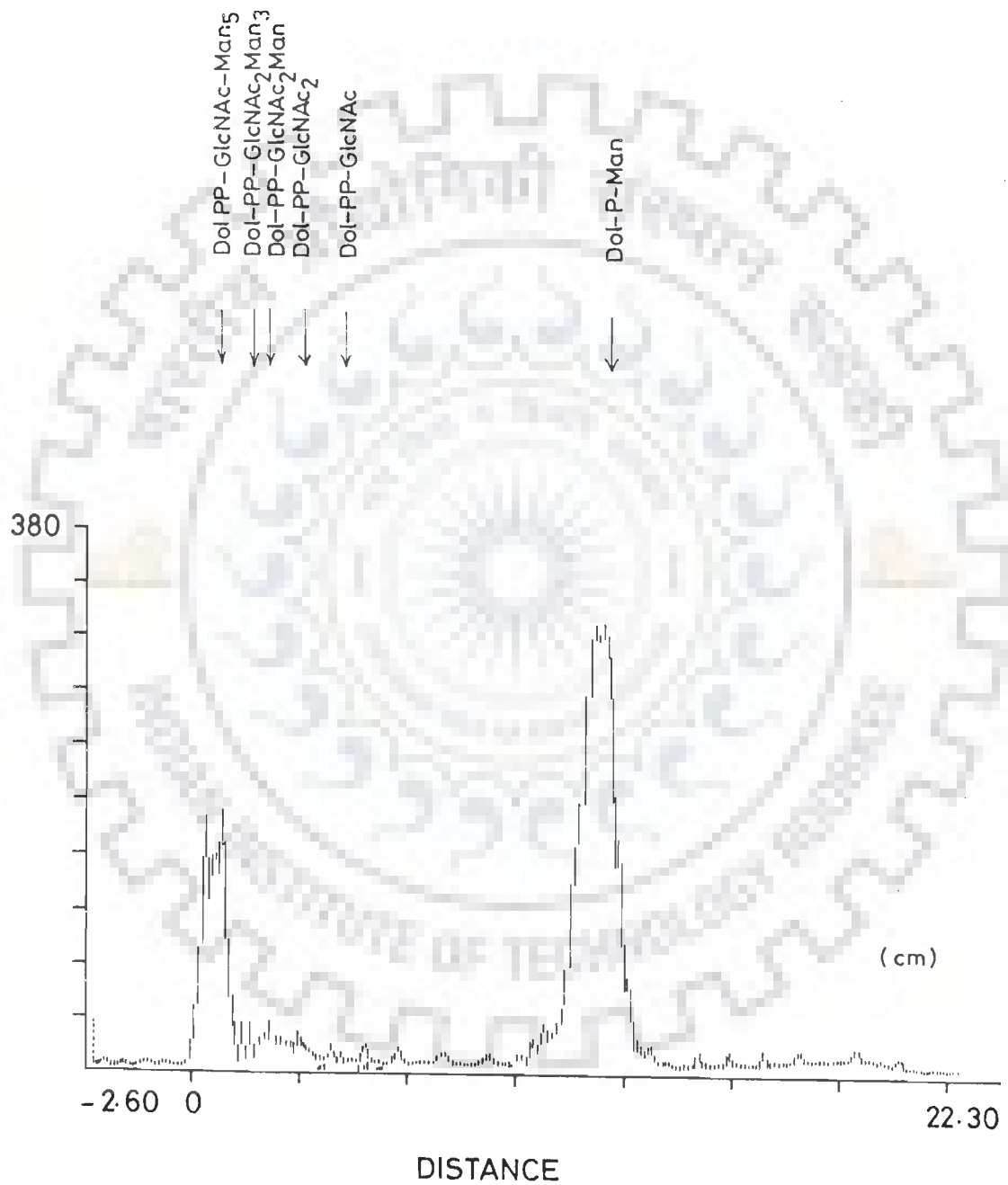

Dol PP-GlcNAc-Man5
Dol-PP-GlcNAc2Man3
Dol-PP-GlcNAc2Man
Dol-PP-GlcNAc2
Dol-PP-GlcNAc
Dol-P-Man
380
(cm)
-2·60
0
22·30
DISTANCE

TABLE-XVIII

In vivo effect of $NiSO_4.7H_2O$ on the callose (1,3-β-glucan) synthase activity in germinating peanut seedlings.

Crude microsomal fraction (12,000-105,000xg pellet) from six-days old germinating seedlings grown in without and with $NiSO_4.7H_2O$ (1mM) in culture was prepared and activity of callose synthase was assayed as described in Experimental Prcedures.

Microsome	Protein (mg)	Callose synthase activity	
		Total cpm x 10^{-3}	Sp. activity cpm/mg/min
Control seedlings	16.4	1523	92.8
Ni-treated seedlings	16.8	1725	102.6

activity in nickel-treated seedlings was somewhat higher than the enzyme activity in control. Since, CS is a wound repair enzyme in plants, it seems reasonable to assume that the nickel treatment has induced a kind of chemical stress in cells increasing the CS activity to counteract the stress caused by nickel in plant cells. Normally under such stress conditions the synthesis of cellulose is completely stopped and callose synthesis starts (Delmer, 1987). Cessation of cellulose formation would lead to defective cell wall formation, growth retardation and necrosis. We have previously reported that peanut seedlings contained two forms of CS viz CS-I and CS-II (Fig.28) with molecular masses of 48,000 and 57,000 daltons, respectively (kamat *et al.*, 1992) and CS-I being the dominant form.

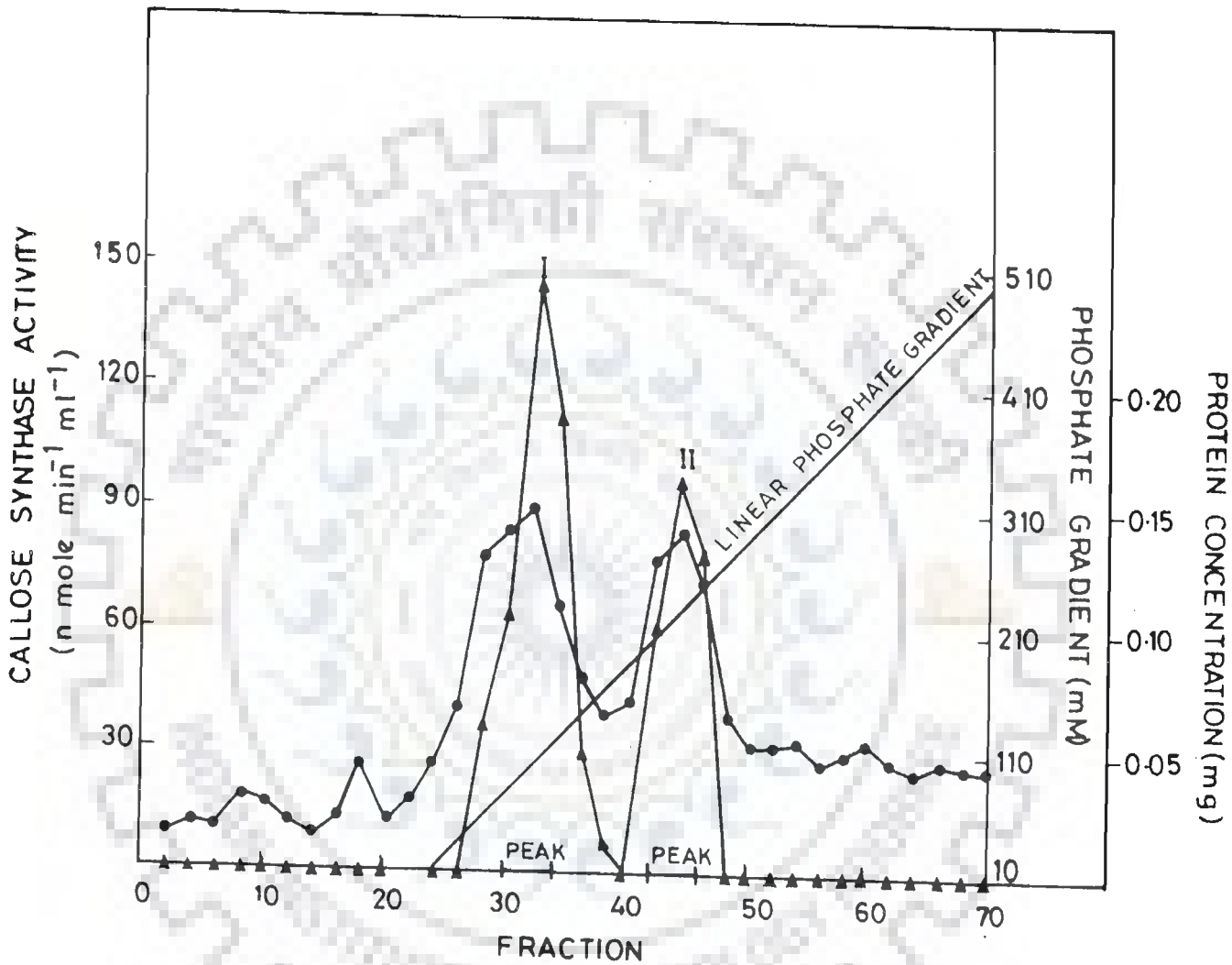
In order to find out the effect of $NiSO_4.7H_2O$ on the relative concentrations of CS-I and CS-II, the two molecular forms of the enzyme were separated by chromatography on hydroxylapatite column from the digitonin solubilized enzyme from the plasma membrane fraction from both untreated (control) and nickel-treated peanut cotyledons. Results are shown in Fig. 28 and Fig. 29. Surprisingly, it was found that whereas CS-I was dominant form of the enzyme in control germinating peanut cotyledons, it was the CS-II form which was dominant in the nickel-treated cotyledons. Thus differential effect of nickel on CS-I and CS-II synthesis in germinating cotyledons is evident, CS-II synthesis being more than that of CS-I.

4.2.8 Purification of CS-I and CS-II forms from nickel-treated peanut cotyledons

Table-XIX summarizes the stepwise purification data. The first step in the purification scheme was the preparation of high specific activity plasma membrane fraction from the germinating peanut cotyledons. The plasma membrane fraction prepared by the procedure outlined in Fig.30 and Fig.31 give a purification of about 12-fold based on the CS activity. The membrane bound enzyme was solublized by treatment with digitonin . The total solublized enzyme activity (450,000 cpm) was significantly higher than that of the activity present in the PM fraction (220,000 cpm) indicating at least a 2-fold stimulation of the enzyme activity by digitonin. At this stage the purification fold was about 85-fold. The digitonin solublized enzyme

Figure 28 : Elution profile of callose synthase from hydroxylapatite column from control germinating peanut cotyledons.

The elution profile of the dialyzed sucrose density peak fraction loaded on the hydroxylapatite column. Callose synthase activity (▲—▲) was assayed in alternate fractions. Callose synthase activity was eluted in two distinct peaks (peak I and peak II).


CALLOSE SYNTHASE ACTIVITY
(n mole min⁻¹ ml⁻¹)
150
120
90
60
30
0
10
20
30
40
50
60
70
FRACTION
PEAK
PEAK
II
LINEAR PHOSPHATE GRADIENT
PHOSPHATE GRADIENT (mM)
510
410
310
210
110
10
PROTEIN CONCENTRATION (mg)
0·20
0·15
0·10
0·05

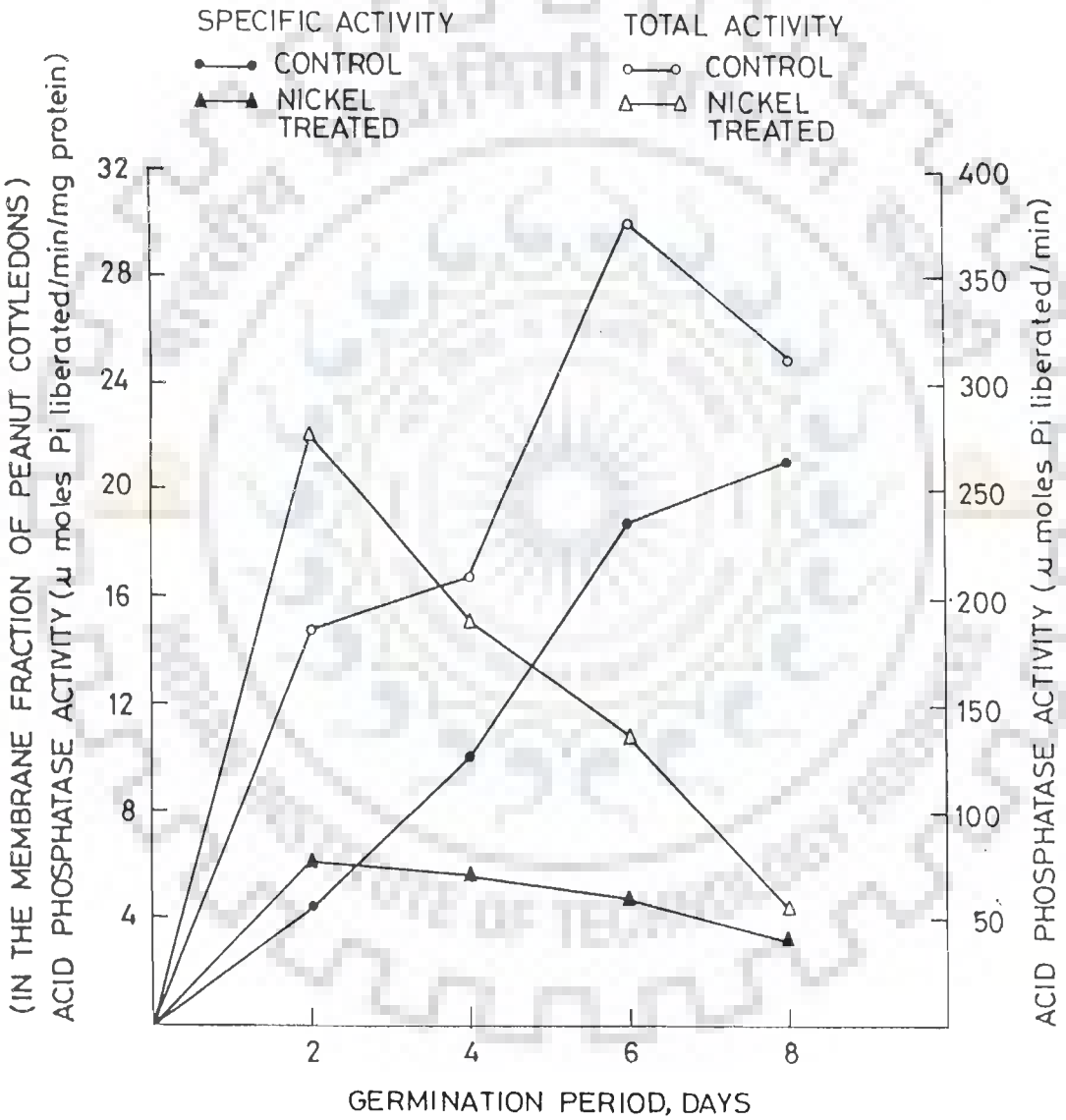

SPECIFIC ACTIVITY
CONTROL
NICKEL TREATED
TOTAL ACTIVITY
CONTROL
NICKEL TREATED
(IN THE MEMBRANE FRACTION OF PEANUT COTYLEDONS)
ACID PHOSPHATASE ACTIVITY (μ moles Pi liberated/min/mg protein)
ACID PHOSPHATASE ACTIVITY (μ moles Pi liberated/min)
32
28
24
20
16
12
8
4
400
350
300
250
200
150
100
50
2
4
6
8
GERMINATION PERIOD, DAYS

Figure 29 : Elution profile of callose synthase from hydroxylapatite column from nickel-treated germinating peanut cotyledons.

The elution profile of the dialyzed sucrose density peak fraction loaded on the hydroxylapatite column. Callose synthase activity (O—O) was assayed in alternate fractions. Callose synthase activity was eluted in two distinct peaks (peak I and peak II).

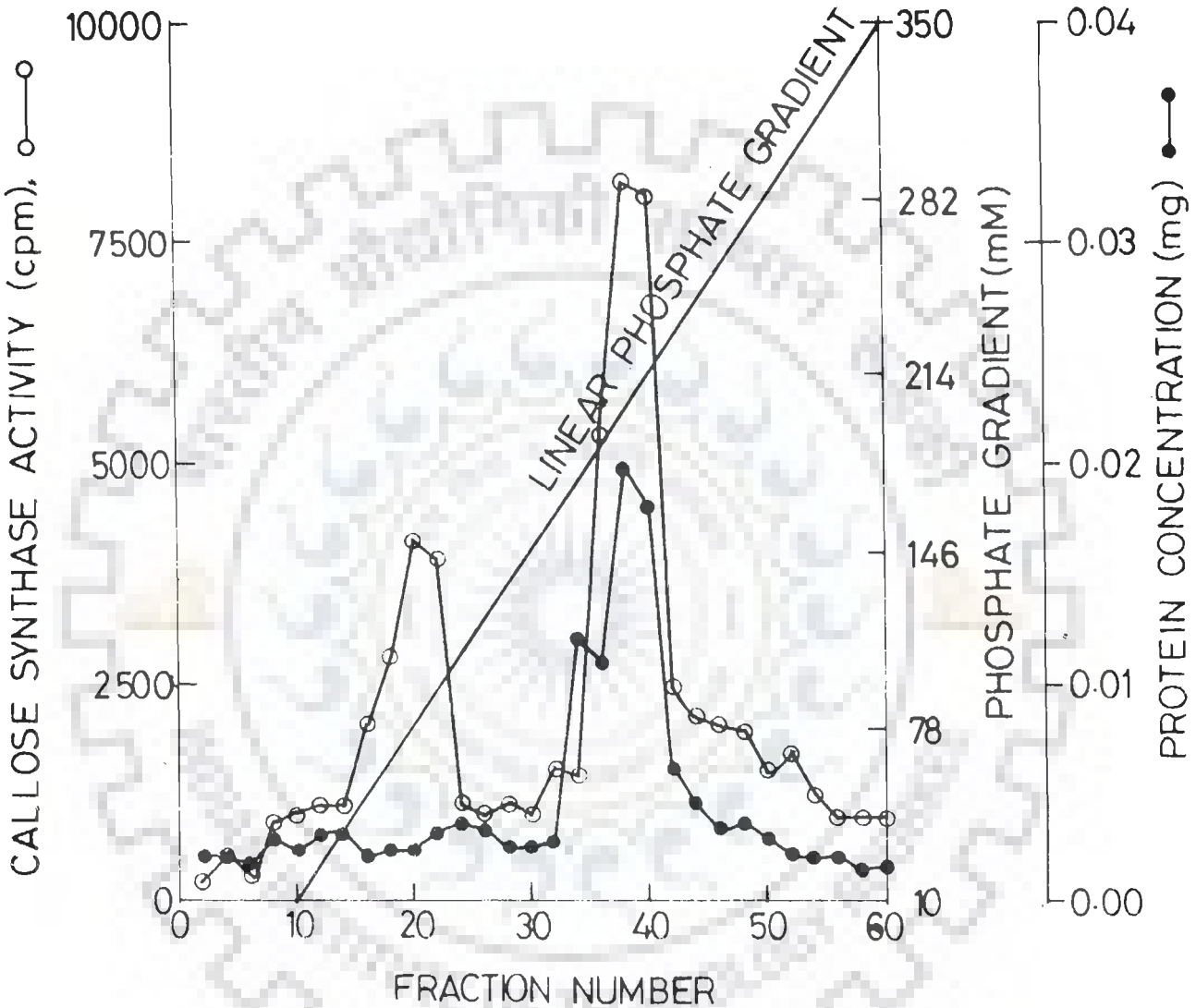

CALLOSE SYNTHASE ACTIVITY (cpm), o—o
10000
7500
5000
2500
0
0
10
20
30
40
50
60
FRACTION NUMBER
LINEAR PHOSPHATE GRADIENT
350
282
214
146
78
10
PHOSPHATE GRADIENT (mM)
0·04
0·03
0·02
0·01
0·00
PROTEIN CONCENTRATION (mg) •—•

Figure 30 : Flow diagram for the isolation and purification of different membrane fractions from *Arachis hypogaea* cotyledons.

Unless otherwise indicated, all steps were carried out at $4^{o}C$.

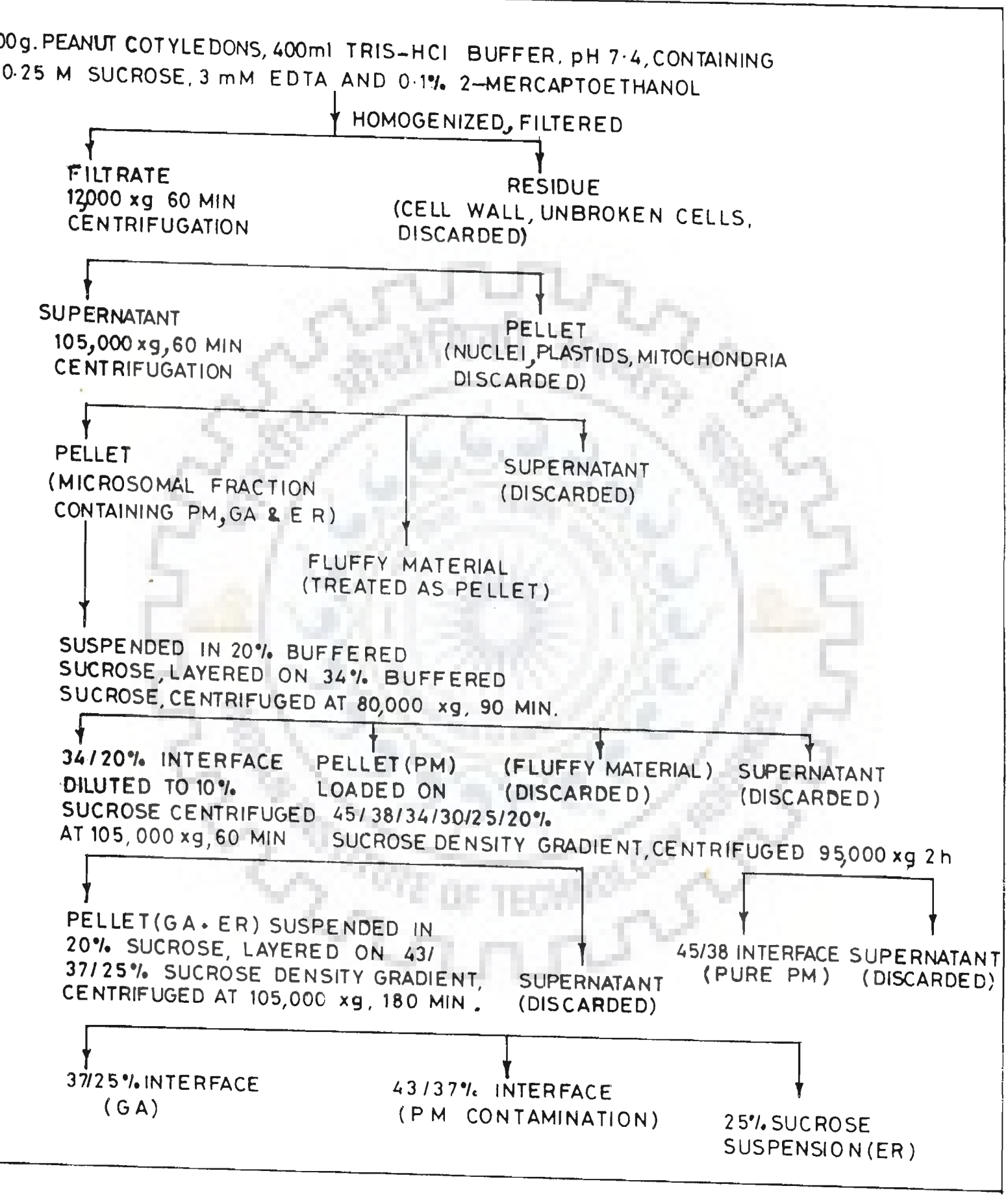

00g. PEANUT COTYLEDONS, 400ml TRIS-HCl BUFFER, pH 7·4, CONTAINING
0·25 M SUCROSE, 3 mM EDTA AND 0·1% 2-MERCAPTOETHANOL
HOMOGENIZED, FILTERED
FILTRATE
12,000 xg 60 MIN
CENTRIFUGATION
RESIDUE
(CELL WALL, UNBROKEN CELLS,
DISCARDED)
SUPERNATANT
105,000 xg, 60 MIN
CENTRIFUGATION
PELLET
(NUCLEI, PLASTIDS, MITOCHONDRIA
DISCARDED)
PELLET
(MICROSOMAL FRACTION
CONTAINING PM, GA & E R)
SUPERNATANT
(DISCARDED)
FLUFFY MATERIAL
(TREATED AS PELLET)
SUSPENDED IN 20% BUFFERED
SUCROSE, LAYERED ON 34% BUFFERED
SUCROSE, CENTRIFUGED AT 80,000 xg, 90 MIN.
34/20% INTERFACE
DILUTED TO 10%
SUCROSE CENTRIFUGED
AT 105, 000 xg, 60 MIN
PELLET (PM)
LOADED ON
45/38/34/30/25/20%
(FLUFFY MATERIAL)
(DISCARDED)
SUPERNATANT
(DISCARDED)
SUCROSE DENSITY GRADIENT, CENTRIFUGED 95,000 xg 2 h
PELLET (GA • ER) SUSPENDED IN
20% SUCROSE, LAYERED ON 43/
37/25% SUCROSE DENSITY GRADIENT,
CENTRIFUGED AT 105,000 xg, 180 MIN.
SUPERNATANT
(DISCARDED)
45/38 INTERFACE
(PURE PM)
SUPERNATANT
(DISCARDED)
37/25% INTERFACE
(GA)
43/37% INTERFACE
(PM CONTAMINATION)
25% SUCROSE
SUSPENSION (ER)

Figure 31 : Separation of various endomembrane fractions by discontinuous sucrose density gradient centrifugation.

Step I : Layering of crude microsomes in 20% sucrose on 34% sucrose buffered solution.

Step II : Separation of PM from GA and ER fractions after centrifugation at 80,000xg for 90min.

Step III : Layering of PM pellet from step II in 0.25M sucrose on discontinuous gradient of 45/38/34/30/25/20% sucrose.

Step IV : Purified plasma membrane after centrifugation on the above graident for 120min at 95,000xg.

Step V : Layering of 34/20% interface membrane fraction in 20% sucrose on 43/37/25% sucrose gradient system.

Step VI : Separation of Golgi apparatus fraction (37/25% interface) from the endoplasmic reticulum (suspension above the 37/25% interface) after centrifugation at 105,000xg for 180 min.

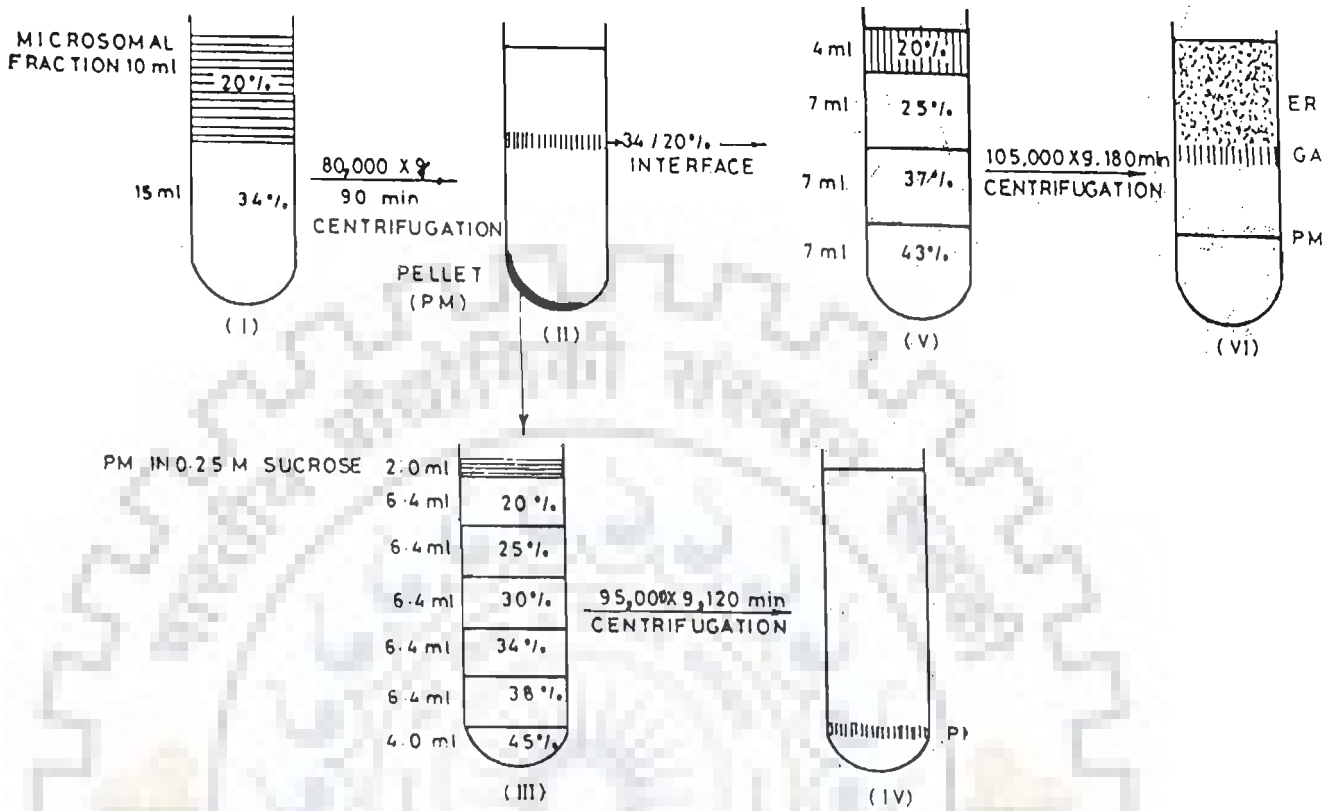

MICROSOMAL FRACTION 10 ml
20%
15 ml
34%
(I)
80,000 X g
90 min
CENTRIFUGATION
PELLET (PM)
(II)
34 / 20% INTERFACE
4 ml
20%
7 ml
25%
7 ml
37%
7 ml
43%
(V)
105,000 X g. 180 min
CENTRIFUGATION
ER
GA
PM
(VI)
PM IN 0.25 M SUCROSE
2.0 ml
6.4 ml
20%
6.4 ml
25%
6.4 ml
30%
6.4 ml
34%
6.4 ml
38%
4.0 ml
45%
(III)
95,000 X g 120 min
CENTRIFUGATION
P
(IV)

TABLE-XIX

Purification of CS-I and CS-II forms from nickel-treated peanut cotyledons

Fraction	Protein (mg)	Callose synthase			
		Total activity (cpm x 10^{-4})	Specific activity (cpm.mg^{-1})	yield (%)	Purification (-fold)
Crude microsomes	860	30.1	350	100	1
Plasma membrane	50	22	4,000	73.1	12.57
Digitonin-solublized enzymes	15	45	30,000	(149.5)[a]	85.72
Sucrose gradient	4	40	100,000	132.9	285.71
Hydroxylapatite					
Peak I	0.5	7	140,000	23.25	400
Peak II	0.4	12.1	302,500	40.19	864.28

a. Increase in yield is indicative of activation of enzyme by digitonin.

was then subjected to sucrose gradient centrifugation. The CS activity was concentrated in fractions 7,8 and 9 representing the sucrose gradient 44 to 40% (Fig.32). This step yielded a purification of about 3-fold over the digitonin solublized fraction . As can be seen the enzyme peak is not symmetrical and two peaks are heavily overlapped indicating the presence of at least two forms of CS. These peaks were pooled, dialyzed overnight against 200 ml of 10mM phosphate buffer, pH 6.8 containing 0.01% digitonin and 0.01% β-mercaptoethanol. The dialyzed fractions were the applied to hydroxylapatite column (1 x 10cm) previously equilibrated with the same buffer. The column was washed with 20ml of the dialyzing- cum-equilibration buffer and than eluted with a linear 100ml gradient from 10-350mM phosphate buffer, pH 6.8. Two ml fractions were collected at a flow-rate of 0.2ml/ per min and assayed for the enzyme activity and proteins. The CS activity was eluted in two distinct peaks (I and II) at approximately 85 and 195mM phosphates gradient (Fig.29). The CS forms in peak I and II were designated as CS-I and CS-II . Fractions containing CS activity in peak I (fractions 18-22) and in peak II (fractions 35-40) were pooled separately and dialyzed overnight against 25mM Tris-HCl, pH 7.2, containing 0.01% digitonin and 0.01% β-mercaptoethanol. The partially purified fractions were used for characterization of CS-I and CS-II forms. The purification of CS-I and CS-II was 400 and 860-fold, respectively.

4.2.9 Effect of cellobiose (primer) on CS-I and CS-II activity

CS-II activity increased by increase in the concentration of cellobiose which acts as a primer and a maximum activity was attained at a concentration of about 600mM cellobiose with a Km value of approximately 300mM (Fig.33). Thus, CS-II enzyme requires a primer and in the absence of a primer (cellobiose in the present case) no CS activity was observed. In contrast, activity of CS-I was not significantly affected by cellobiose, indicating that unlike CS-II, CS-I does not require a primer for its activity and is probably a self priming enzyme. Apparently nickel has , therefore, selectively increased the production of primer requiring callose synthase, (CS-II) while suppressing the synthesis of CS-I, a self-priming enzyme.

Figure 32 : Activity profile of callose synthase after sucrose density gradient centrifugation.

The digitonin solubilized fraction was layered onto a linear sucrose gradient of 20% (w/w) to 60% (w/w) sucrose and centrifuged at 200,000xg for 8 hours using the TST 41.14 Centrikon rotor as described in Experimental Procedures. Callose synthase activity (▲——▲) and protein (●——●) were determined in the eighteen fractions obtained after centrifugation according to the description in Methods. The activity profile in the figure shows callose synthase activity obtained was concentrated in the fraction numbers 7,8, and 9, corresponding to 44-40% sucrose gradient.

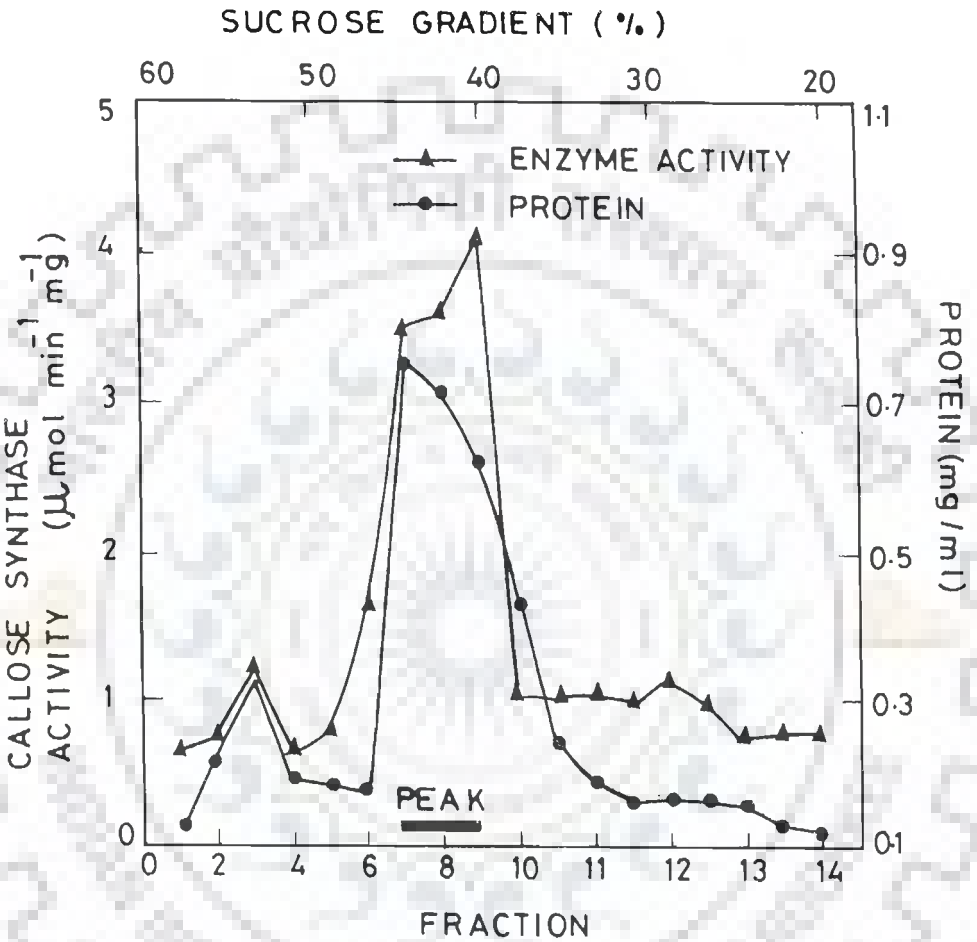
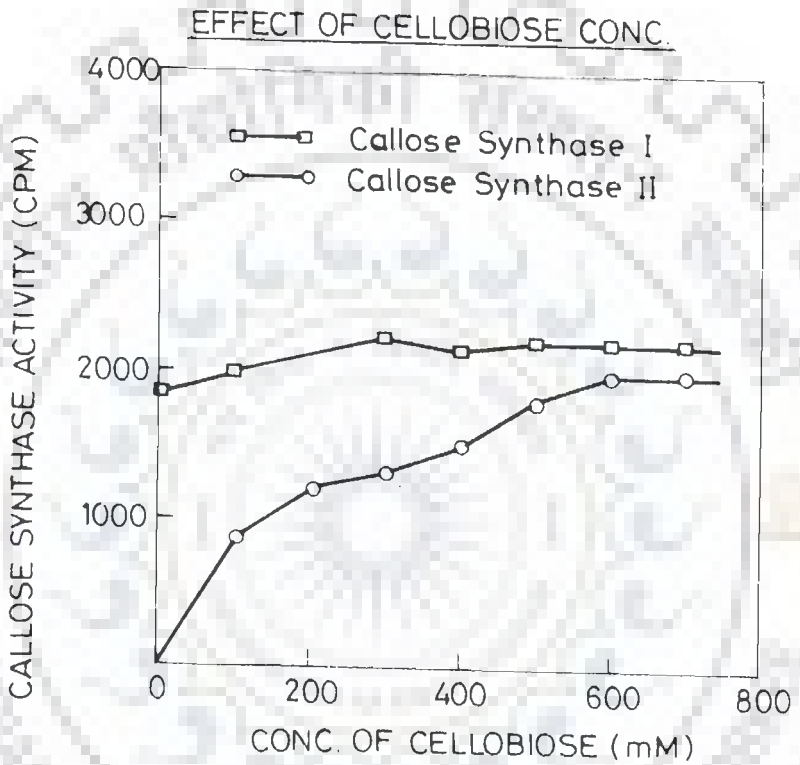

SUCROSE GRADIENT (%)
60
50
40
30
20
ENZYME ACTIVITY
PROTEIN
CALLOSE SYNTHASE ACTIVITY (μmol min⁻¹ mg⁻¹)
PROTEIN (mg/ml)
PEAK
FRACTION
0
1
2
3
4
5
0·1
0·3
0·5
0·7
0·9
1·1
0
2
4
6
8
10
11
12
13
14

Figure 33 : Effect of cellobiose on the activity of callose synthase-I and callose synthase-II.


EFFECT OF CELLOBIOSE CONC.
Callose Synthase I
Callose Synthase II
CALLOSE SYNTHASE ACTIVITY (CPM)
4000
3000
2000
1000
0
200
400
600
800
CONC. OF CELLOBIOSE (mM)

4.2.10 Effect of UDP-glucose.

The effect of UDP-glucose (donor substrate) concentration on the activity of CS-I and CS-II was measured using Lineweaver-Burk plot method (Fig.34). The Km values of enzymes CS-I and CS-II for UDP-glucose in the presence of a fixed amount of cellulose were found to be 2.0 and 1.0mM, respectively. In other words the affinity of CS-II was two times greater than that of CS-I. Thus , it seems likely that in order to meet the stress caused by nickel, the proportion of high affinity callose synthase (CS-II) was increased.

4.2.11 Product characterization

The radioactive reaction product formed by incubation of CS-II enzyme with UDP- [^{14}C]-glucose and cellobiose was characterized by using specific glycosidases and periodate oxidation. As can be seen (Table-XX), α and β amylases and periodate oxidation did not release glucose from the product indicating the absence of α-1,4 linkages. However , exo-(1,3)-β-glucanase released almost completely total radioactivity migrating as glucose on paper chromatography confirming formation of callose and no cellulose synthesis *in vitro* by the partially purified enzyme CS-II from nickel-treated germinating peanut cotyledons. Thus nickel treatment enhanced the level of activity of primer requiring callose synthase.

Figure 34 : Lineweaver-Burk plots showing the effect of UDP-glucose concentration on the activity of callose synthase-I and callose synthase-II.
Enzyme assays, with varying amounts of UDP-glucose, were carried out as described under Experimental Procedures.

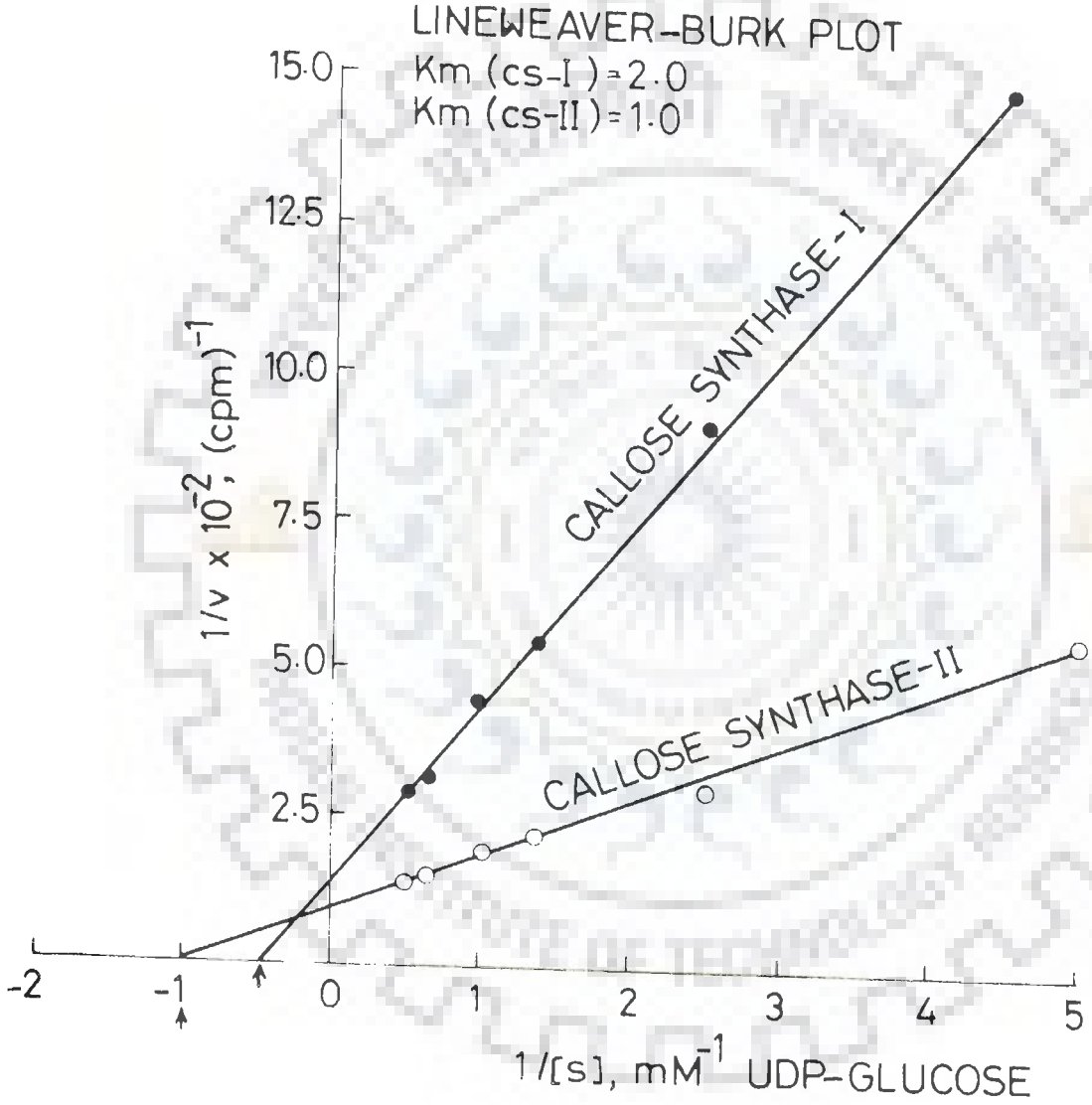

LINEWEAVER-BURK PLOT
Km (cs-I) = 2.0
Km (cs-II) = 1.0
CALLOSE SYNTHASE-I
CALLOSE SYNTHASE-II
15.0
12.5
10.0
7.5
5.0
2.5
1/v x 10^-2; (cpm)^-1
-2
-1
0
1
2
3
4
5
1/[s], mM^-1 UDP-GLUCOSE

TABLE-XX

Characterization of product

Treatment[a]	Radioactivity distribution on chromatogram	
	Origin (cpm)	Migrated as glucose (cpm)
Control	21,090 ± 450	105 ± 25
α-amylase	19,720 ± 450	156 ± 40
β-amylase	20,017 ± 450	123 ± 40
Exo-(1,3)-β-D-glucanase	699 ± 150	17,979 ± 315
Periodate Oxidation	19,542 ± 400	

a. Radioactive product obtained by the purified callose synthase-II was subjected to various treatments and analysed by paper chromatography as described under Experimental Procedures.

CHAPTER-V

5.0 DISCUSSION

5.1 PART-I : STUDIES ON THE *IN VIVO* EFFECTS OF NICKEL SULPHATE IN RATS

In recent years the widespread contamination of the environment with toxic chemicals, including the heavy metals has become an important health hazard mainly due to the disposals of the industrial wastes (Adelman *et al.*, 1976). Metals present in industrial wastes find their way into the body of living organisms and get accumulated over the time due to long and continuous exposure. As a result biochemical defects and structural disorders arise which differ from species to species depending upon the bioavailability and toxicity of the element(s) and its presistance in the system. In general, the biological activity of a metal ion is governed by the general principle that only soluble metal ions are absorbed, then systemically distributed and eliminated.

In the present study the *in vivo* effects of $NiSO_4.7H_2O$ in rats have been investigated in some detail. The general approach used was to administer cumulative dose of $NiSO_4.7H_2O$ in rats for 15 days, period which was sufficient to produce detectable changes in the structure and function of different organs. After the scheduled nickel treatment animals were sacrificed, organs of interest were excised and biochemical and histopathological as well as haematological changes induced by nickel were examined. $NiSO_4.7H_2O$ was used in the present studies since Benson *et al.* (1986) reported that the biochemical, cytological and morphological toxicity of $NiSO_4.7H_2O$ in rat lungs was comparable with Ni_3S_2 and $NiCl_2$ but was much greater than NiO. Like Ni_3S and $NiCl_2$, $NiSO_4$ treatment increased the activity of lactic dehydrogenase, β-glucuronidase, glutathione reductase, and glutathione peroxidase. In addition, the total protein content , ascorbic acid, pulmonary surfactants, sialic acid and total nucleated cells in lavage fluid, which are indicators of pulmonary inflammation were also increased (Coogan *et al.*, 1989 ;Benson *et al.*, 1989). The effect of $NiSO_4.7H_2O$ administration in liver and kidney in rats was

however not reported. As will be discussed later nickel induced marked changes in these organs, as well.

The effects of a metal ion are largely dependent on its availability and concentration, duration of exposure and its uptake toxicokinetics, including detoxification. The biological activity of inorganic nickel ion, Ni^{2+}, in the body is characterized by the general principle that only the soluble nickel is absorbed, distributed in the system and eliminated (Coogan *et al.*, 1989 ; Dunnick *et al* ., 1989). Results presented here show that following the intramuscular administration of nickel the concentration of nickel in different tissues increased many-fold in liver and kidney (Table-II and III). It was expected, since nickel is known to accumulate predominantly in soft tissues like liver, kidney, brain, lungs and blood (Parker and Sunderman, 1974) as these soft tissues provide particularly highly receptive sites (Coogan *et al.*, 1989). Retention of nickel ion in tissues is known to cause various disorders and the most commonly noticed ones in rats are loss in body weight, decrease in renal and hepatic proteins and carbohydrates and elevated lipids and nickel contents in addition to the histopathological lesions (Coogan *et al.*, 1989). Results of the present study are in agreement with these findings (Table-I,II and III).

The present study shows that nickel enhances the rate of fat accumulation in both liver and kidney. Although a fatty liver can be induced by a multiplicity of agents, in most cases the mechanism responsible is not understood. Moreover, since a common response to hepatic parenchymal cells to damage is the intracellular accumulation of lipid, the general mechanism that could lead to accumulation of triglycerides in the hepatic cell has received considerable attention compared to kidney. The rate of 1-α- glycerophosphate formation could be a factor in producing fatty liver (Lombardi, 1966). Under most circumstances many of the free fatty acids (FFA) that are made available to the liver are converted to triglycerides. Therefore, the input of free fatty acids seems to be an important factor. It looks tempting to speculate that after nickel treatment the input of FFA causing triglyceride formation is greater than the output processes such as mitochondrial oxidation and secretion of lipoprotein by the

ER, resulting in a fatty liver. Activation of lipid peroxidation induced by nickel may be another factor especially at NADPH oxidation reaction (Athar *et al.*, 1987). Administration of nickel results in an inhibition in the activity of free radical reductase and enhanced lipid peroxidation and the activity of glutathione S-transferase. Ultimately the formation of free radicals initiates the peroxidation reaction. This mechanism of lipid peroxidation has been established in hepatic tissue (Pfeifer and McCay, 1971 ; Srivastava *et al.*, 1990). Our results lend further support to the lipid peroxidation mechanism. Also, Andersen and Andersen (1989) observed the effect of nickel chloride on hepatic lipid peroxidation in mice and lipid peroxidation induced by Ni(II) has been reported *in vitro* in primary epithelial cell cultures of rat kidney (Helmutz *et al.*, 1985). The overall mechanism remains associated with the injury of biomembranes. An injury in the ER affecting its ability to synthesize the protein moieties of lipoprotein seems to be the biochemical mechanism involved.

The effect of nickel on kidney included ultrastructural and functional changes and lipid peroxidation is implicated as a general mechanism of tissue damage in the light of present studies. Whether the significant rise in malondialdehyde concentration in nickel treated rat liver and kidney (Table-VIII) may be related to the possibility of Ni (II)/Ni(III) redox couple generate oxygen free radicals by one electron transfer reaction or not remains to be investigated.

Changes in different classes of neutral lipids induced by nickel did not follow the same pattern and some kind of preferential action was evident (Table-V). Accumulation of triacylglycerol was induced by nickel in both liver and kidney, but there was little or no change in the concentrations of diacylglycerol, monoacylglycerol or free cholesterol. It seems that nickel treatment induces the change (s) in such a way that intermediates do not accumulate. Interestingly however, relative percent abundance of diacylglycerol in nickel treated liver and kidney was much lower than the untreated tissues. The latter may be of greater physiological importance than the absolute concentration of a component neutral lipid.

Aggregation of lipids induced by nickel at a specific localization observed in liver and kidney showed morphological toxicity. Accumulation of phospholipids indicates a blockade in the process of oxidative phosphorylation. Inhibited oxidative phosphorylation is known to raise the accumulation of lipids (Hartmann, 1960) Furthermore the enhanced accumulation of phospholipids is possibly due to their slow rate of breakdown, reduced rate of their transport from these tissues and progressive loss of esterases as reported in the case of chromium (Cr) by Kumar and Rana (1982).

Effect of nickel on phospholipid concentration in laboratory animals have been investigated by a number of workers (Jobe *et al.*, 1978, Johansson *et al.*, 1983; Curstedt *et al.*, 1983, 1984 and Casarett-Bruce *et al.*, 1981). In all cases, regardless of the tissue or the animal the phospholipid content in tissues increased by nickel treatment. Thus, it seems that as far as the effect of nickel on phospholipids is concerned there is no tissue specificity and action seems to be universal. Furthermore, we found that of various phospholipids, the concentration of phosphatidyl choline (PC) was greatly increased in both liver and kidney in nickel-treated rats (Table-VI and Table VII). These results are in good agreement with those reported by other workers (Johansson *et al.*, 1983, Curstedt *et al.*, 1983, 1984; Caserett Bruce *et al.*, 1981, and Jobe *et al.*, 1978). The selective accumulation of PC in liver and kidney cells may be of physiological significance. Casarett-Bruce *et al.* (1981) found a close correlation between disaturated PC and volume density of type II cells of rabbit. Biochemically, elevation in PC level might affect the activity of glycosyltransferases involved in the biosynthesis of glycoproteins, since O-seryl mannosyl transferase (Sharma *et al.*, 1991) and oligosaccharyl transferase (Chalifour and Spiro, 1988) are highly activated by PC. In addition, nickel has also been found to stimulate the activity of dolichyl-phosphomannose synthase *in vitro* and also glycoprotein biosynthesis (Table-XVII) In fact, *in vitro* nickel can replace the Mg^{2+} and/or Mn^{2+} requirement of the mannosyl transferase. There is also some evidence which shows that in presence of nickel, lipid-saccharide intermediates of dolichol pathway are also accumulated. But these results would require deeper study in order to

establish the correlation between nickel and glycoprotein/glycolipid biosynthesis and also to define the actual physiological significance of this observation. Nickel effect is of interest since high proportion of ether lipids is observed in many tumors (Curstedt *et al* ., 1984).

The composition of various components of phospholipids in various subcellular membrane fractions of liver was found to be quite significantly altered by $NiSO_4.7H_2O$. The sharpest changes were observed in plasma membrane, mitochondria and Golgi apparatus. Similarly neutral lipid proportions were altered in the subcellular membrane fractions as a result of $NiSO_4.7H_2O$ treatment. These results were interpreted to mean that the functional properties of the membranes forming the cytoskeleton structure in eukaryotic cells is altered by $NiSO_4.7H_2O$ leading to various metabolic , structural and morphological abnormalities.

Histology of different organs before and after the exposure of the amimal to the toxicants gives us useful information regarding the external manifestation of abnormalities and can serve as an indicator of the extent of toxicity. The major lesions caused by nickel involve the liver and kidneys, being the centres for resorption, detoxication and excretion. Liver is the main detoxifying organ in the body, it is susceptible to a number of toxic and metabolic disturbances. Liver after nickel intoxication showed certain lesions viz, necrosis, nuclear pycnosis, hydropic degeneration, hypertrophical cells with vacuolated cytoplasm. Swelling of cells narrowed the sinusoids that ultimately reduced blood flow and oxygen supply resulting in mitochondrial damage and necrosis.

Histopathological manifestations of nickel toxicity in liver include microvesicular steatosis, necrotic hepatocytes and nuclear clumping and in kidney it includes severe tubular necrosis, glomerular degeneration and nuclear clumping and karyolysis. The leakage of glomerular filtrate out of nephron into the interstitium may result into necrosis. However, Knight *et al.* (1991) showed microvesicular steatosis, and necrotic hepatocytes were scattered throughout the

lobules as supported by present histological observation of liver and Kumar *et al.* (1987) reported proximal tubule damage and alteration in brush border during the course of copper nephropathy as supported by present histological observation of kidney.

A connective tissue usually consist of cellular portion in an enveloping framework of a non-cellular substance which is made up of basic proteins, the reticular fibre possesses reticulin and disarrangement of these fibres is known to produce varying types of lesions in the liver and kidney. Nickel treatment induce several lesions in hepato-renal tissues which showed that increased accumulation of reticular fibres are in agreement with other findings (Rana *et al.*, 1982). A normal staining for collagen as compared with controls supports their pharmacological importance by controlling fibro-proliferative inflammation (Rana, 1977).

Metals inactivate the intracellular proteins and this is one of the major mechanism of toxicity (Passow *et al.*, 1961 ; Novelli *et al.*, 1990). In the present investigation, the decrease in the protein content in all treated rats was noted. It may be due to one or more of the following factors (Kadiiska *et al.*, 1985) (i) Inhibition of amino acids incorporation and/or short supply of amino acids available for protein synthesis, (ii) break down of the protein synthesis, (iii) diffusion of proteins out of the cell, (iv) blockage of protein by direct chemical union between it and toxin and (v) inactivation of sulphydryl function. The defect of protein synthesis was due to an altered relationship between the ribosomes and the membranes of the ER (Smuckler *et al.*, 1962). The change in lipid composition of the ER (Fig.9) supports this view.

The plasma membrane and membranes of the various organelles contain a large number of important enzymes and enzyme sequences that are readily disrupted by primary toxic agents like inorganic metals (Slater, 1968), their covalent binding with thiol groups perturb the thiol dependent enzymes that control vital membrane functions such as active transport. Under these conditions the brush border of the renal tubular cells is especially sensitive to heavy metal salts

(Rana, 1974; Rana and Kumar, 1980). Serious ionic imbalance thus results in necrosis of the proximal tubules and other renal disorders. Although nickel is known to damage liver and kidney of experimental animals (Berndt, 1976; Mackenzie *et al.*, 1958), its effects on the membranous enzymes are not known for comparison. The present observations indicate that it significantly inhibited alkaline phosphatase, acid phosphatase, glucose-6-phosphatase and lipase. The general mechanism of inhibition might involve (i) removal of essential metal ion leaving apo-enzyme alone, and/or (ii) replacement of some of the protein groups, resulting in a mixed enzyme-metal inhibitor complex. The results obtained on lipase further indicate the interference of nickel with phospholipid metabolism (Kumar and Rana, 1982).

The biochemical processes affected by chemical modification of these enzymes might include phosphorylation, adenylation, ADP-ribosylation and oxidation of thiol groups as well as the respective reverse reaction (Holzer and Duntze, 1971). Detailed study is further required to identify the level of enzyme protein and lipid with consequent involvement of the cellular organelles, the highly dynamic structures.

The possibilities of this inhibition seem to be via

(i) The removal of the essential metal ion and formation of apoenzyme.

(ii) Replacement of some of the protein groups giving a mixed enzyme inhibitor metal complex.

Inhibition of acid phosphatase noted in the present study may be due to the disintegration of the affected cells as direct binding of metal ions with enzyme protein. Loss of acid phosphatase indicates lysosomal damage. Some inclusion bodies formed cause cell injury by altering lysosomal structure and functions as evidenced by reduced acid phosphatase activity. Inhibition is considered either due to cytotoxic action of the metals or due to the possible leakage of the enzymes from inflammed tissue to the blood (Chennoweth and Ellman,

1957; Rees and Sinha, 1960). Rapid loss of lysosomes from atrophying cells may be a reason for the diminished activity of acid phosphatase.The lower activity of this enzyme might be the consequence of unbalanced catabolism of enzyme protein of disintegrated cells. The present investigations showed that the alkaline phosphatase is distributed in both liver and kidney of the rats. Alkaline phosphatase is brush border enzyme involved in the transphosphorylation reaction and mediates membrane transport. Alkaline phosphatase being a key enzyme of metabolic pathway responds to a number of different controlling factors and their regulation will therefore, require a multitude of different controlling sites, in addition to the state of plasma membrane (Holzer and Duntze, 1971). Like other inorganic salts, nickel interact with plasma membrane causing damage to its mucopolysaccharides and ultimately pinocytosis and endocytosis.

The cause of inhibition of acid and alkaline phosphatase may be due to the uncoupling of oxidative phosphorylation by the metals. The uncoupling of oxidative phosphorylation was also suggested by Gallagher and Rees (1960) after carbon tetrachloride treatment. The other probable cause of inhibition of the alkaline phoshatase activity may be due to the alteration in lysosomes in response to toxic damage of metals.

Inhibition of glucose-6-phosphatase due to toxins greatly alter the ER accompanied by depletion in cellular glycogen. Glucose-6-phosphatase of liver serves for the release of free glucose into the blood stream by dephosphorylation of glucose-6-phosphate. So the release of glucose from the intercellular to extracellular space is totally dependent upon the availability of glucose-6-phosphatase, an enzyme found abundantly in hepatic tissue (Verma *et al.*, 1981).

The haematological tests have been an important diagnostic tool in medical sciences. These parameters are valuable indicators of disease or environmental stress in living organisms (Larsson *et al.*, 1976). We have investigated different blood parameters and serum enzymes in rats which were given intramuscular injections of nickel sulphate for

a period of 15 days. As haematology fluctuates much with species, ecological conditions, age, sex, length, weight, maturity and seasonal variations, for the present study the experimental animals were taken from identical categories. Here, significant decrease in red blood corpuscles count and haemoglobin percentage has been observed which reflects the anaemic state of nickel treated rats. Anaemia associated with decreased RBC, Hb% was recorded to occur in various organisms in response to various pollutants by many workers (Garg *et al.*, 1989 a; Garg *et al.*, 1989b; Rana and Kumar, 1980 ; Vodichenska, 1991). The acceleration of erythrocyte aging was observed by some nikel compounds in young erythrocytes (Tkeshelashvili, 1989).

Nickel treatment of rats induced an increase in leucocyte count which is similar to that of copper toxicity (Rana and Kumar, 1980). The leucocytosis has been considered to be of adaptive value to meet the stressful condition of animals and is consequence of direct stimulation of immunological defence due to tissue damage.

Elevation in the activity of alkaline and acid phosphatases is observed in serum of rats treated with nickel. The elevation in serum alkaline and acid phosphatases may be due to (i) consequence of tissue damage under toxic stress. (ii) the enzymes from the damaged tissue leak and elevate the serum phosphatase level . (iii) toxicant release the immature osteoblasts having high amount of alkaline and acid phosphatases in blood. (iv) toxicants might induce proliferation of smooth endoplasmic reticulum in hepatic parenchymatous cells that results in release of microsomal enzymes causing increase in the level of alkaline and acid phosphatases in serum. (v) degeneration and necrosis, induced in hepatic parenchymatous cells by various chemicals also increased the level of these enzymes. However, the diminished serum alkaline phosphatase activity has also been reported by Donskoy *et al.* (1986) in rats treated with nickel chloride. Thus, the effects of $NiSO_4.7H_2O$ appear to be different from that reported for $NiCl_2$.

The enzymes glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), generally found in liver were observed to be elevated in rats given $NiSO_4.7H_2O$ doses intramuscularly.

Transaminases allow an interplay between carbohydrate, fat and protein metabolism, an activity which can serve the changing demand of the body. The elevation of transaminases in serum might be due to (i) toxic material damage to the cells, in principle entails two type of injuries in cell membrane or suborganular membrane dispermeability and the more severe lesions to cell degeneration with necrosis that lead to the release of enzyme in blood, (ii) injury to cell resulted in an outflow of intracellular enzymes which come in the blood from extracellular spaces, (iii) as membrane integrity is critically linked with intracellular metabolic status, any disturbance in the latter expectedly results in submicroscopic membrane lesions with consequent enhanced enzyme leakage. Changes induced by $NiSO_4.7H_2O$ in the percentage composition of various components of phospholipids in various subcellular membrane fraction of rat liver (Fig.10) lend further support to the membrane integrity was damaged leading to enhanced enzyme leakage because of altered membrane permeability.

The protein content of blood serum have been found to be depleted as a consequence of nickel intoxication. Protein spectrum of blood serum is a sensitive index of the physiological state of animals. The reduced level of protein has been correlated to either its excessive loss due to nephrosis or to reduced protein synthesis due to liver cirrhosis (Lynch *et al*., 1969). Smuckler *et al*. (1962) have suggested that the defect in protein synthesis was due to altered relationship between ribosomes and membranes of ER under toxic stress. The decrease in the protein level might be a result of renal disorder (albunaria) or impaired protein synthesis, due to liver disorder.

The increased reducing sugar level in nickel-treated rats is a clear indication of disturbed carbohydrate metabolism which might be due to the enhanced breakdown of liver glycogen mediated by reduced insulin secretory activity by the influence of nickel on adrenocortical hormones. Many workers have observed hyperglycemia in serum of various animals (Cartana and Arola, 1992 ; Alcon *et al* ., 1991). At this moment the exact cause is uncertain.

Increased blood cholesterol was observed in nickel-treated rats. Since liver is the main site for the cholesterol synthesis, it might have been mobilized from necrosed liver cells into the blood. Nickel induced elevation in serum bilirubin , urea and creatinine level. Increased bilirubin level was a sign of malffunctioning of the liver (conjugation of bilirubin) or of haemolytic anaemia. While elevated levels of urea and creatinine are indcative of malfuctioning of kidney (Oser, 1965).

Humans are usually exposed to low level doses for prolonged period of time. Hence, discrepancies in dose, duration and route of exposure drastically complicate extrapolation of laboratory data to man. However, overall observations possess significant value if extrapolated to man. Any exposure which might have such effects should be unacceptable for public health. Much investigation on exposed human subjects still have to be performed to evaluate nickel hazards in terms of health and disease, like asthma, anaemia, dermatites, etc.

5.2 PARTS-II : BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON THE EFFECTS OF NICKEL SULPHATE ON PEANUT SEEDLINGS

The peanut seedlings were selected for studying the effect on plants, since peanut seeds are rich in protein, carbohydrate (starch), lipids and minerals. In addition, peanut is an important legume crop in India and is extensively used by both human and animal subjects. Further, development of peanut seeds takes place underground and it is likely that the effect of surface and water contamination on this crop will be more pronounced than those in which the seed development takes place above the ground.

The essentiality of nickel requirement in plants has never been established beyond doubt, although there are reports to show that trace amounts of nickel help in plant development and increased yield (Mishra and kar, 1974) and it is necessary for the functioning of certain plant enzyme systems (Polacco, 1977; Dixon *et al.*, 1975) like jack bean urease, which is involved in the utilization of urea. In fact, nickel was shown to be specifically essential for the conversion

of urea into ammonia. However, there are equally strong evidences of nickel toxicity when present in excess. Among the symptoms of nickel toxicity in plants were induced iron deficiency, chlorosis and foliar necrosis, stunted growth of roots and shoots, deformation of various plant parts and unusual spottings on leaves and stems (Misra and Kar, 1974). The early effects of nickel toxicity in bush bean were decreased dry matter production, abnormal vertical orientation of leaves, an abnormal starch accumulation and an accumulation of apolar soluble phenolics (Rauser, 1978). The results described here largely confirm the nickel toxicity in seed germination and early growth and development of the seedling. The decrease in growth of embryonic axis, hypocotyl and radicle of germinating peanut seedling lead to decrease in dry matter which suggests that nickel suppresses mobilization and utilization as well as translocation of reserve materials from the cotyledons of germinating seeds. In this study the toxic effect of excess of nickel concentration in water culture medium is clearly evident on seedling growth (Fig. 22). There was almost complete inhibition of radicle formation at 1mM concentration of $NiSO_4.7H_2O$ in the water culture medium and the secondary roots formation was also inhibited at 0.5mM concentration even though the radicle was formed like in untreated seedlings. Seeds failed to germinate when nickel concentration was 1mM or more. Since the germination of seeds and seedling growth and development was performed in deionized water without any nutrient, the early growth and development of seedling was sustained by utilizing the storage food from the cotyledons. Therefore, the experimental set-up presented ideal physiological conditions to study the effect of nickel on the mobilization and utilization of storage proteins, carbohydrates, fats, minerals and phosphate.

Amylases, phytases, nucleotidases, lipases, and proteases are some of the major hydrolases that are either induced or activated by germination and which are known to play key roles in the mobilization and utilization of stored food (starch, lipid, protein and organic phosphorus) from the cotyledons to growing seedling parts. Inhibition of germination and seedling growth by nickel would be reflected by the inhibition of these hydrolases in nickel-treated seedlings. This is

exactly what has been observed, indicating a clear direct correlation between the inhibition of germination and seedling growth by nickel with the enzyme activities.

A special mention of phytase has to be made. This enzyme is involved in the mobilization of phytate phosphorus which is the primary storage form of phosphate and is generally localized in the aleurone grains (Sharma and Dieckert, 1975). During germination these aleurone grains are digested by proteases and phytases (Bagley *et al.*, 1963). The released phosphate then enters into various reactions in developing axis (Hall and Hodges, 1966).

Extremely low level of phytase in nickel treated cotyledons is indicative of the suppression of the mobilization of reserve phosphate due to which the growth and development of seedlings and dry matter production were almost completely stopped. In this context it is worth mentioning that nickel also inhibited the activity of both acid and alkaline phosphatases, the key enzymes in the mineralization of organic phosphate to plant available form (Juma and Tabatabai, 1977).

The plasma membrane localized 5'- nucleotidase was another key enzyme which was greatly suppressed. This enzyme has been implicated in a number of important cellular phenomenon including cell proliferation (Sun *et al.*, 1982), transmembrane nucleotide transport (Fleit *et al.*, 1975) and nucleotide pool size regulation (Itoh, 1981; Carter and Tipton, 1986). Since, 5'-nucleotidase activity is very strongly inhibited by nickel it is tempting to suggest that the important cellular phenomenon mentioned above are suppressed by nickel leading to retarded in seedling growth. Further studies in this direction would be interesting from the point of mechanism of nickel action in retarding the seedling growth. It may also be pointed out that nickel ions did not inhibit 5'- nucleotidase activity *in vitro*, meaning there by the effect of nickel *in vivo* was perhaps due to inhibition either at transcriptional or translational levels.

One of the most interesting *in vivo* effects of nickel on plant enzymes was observed on the callose (1,3-β-glucan) synthase (CS), a plasma membrane localized enzyme which becomes activated only under stress conditions and is thought to be involved in wound repair process and several other specialized developmental processes in plants like sieve pore formation and gravitropism, etc (Delmer, 1987).

In a recent communication (Kamat *et al.*, 1992) we have reported the existence of two dominant forms of CS designated as CS-I and CS-II in normal untreated germinating peanut cotyledons (Fig. 28). Of these, the CS-I was highly dominant accounting for about 60% of the total CS activity. In nickel-treated peanut cotyledons of same germinating age however, the proportion of CS-II was greater than CS-I (Fig. 29) representing a completely reversed position in regard to the proportions of CS-I and CS-II forms found in untreated peanut cotyledons. This observation is considered of physiological importance, since CS-I was found to be self-priming enzyme (Kamat *et al.*,1992) requiring no primer (like cellobiose), whereas CS-II requires a primer for its activity (Fig.33). Furthermore the Km value of CS-I is two times higher than the CS-II indicating much higher affinity of CS-II for the donor substrate (Fig.34). From these results it seems that the chemical stress induced by nickel on germinating seedlings is related to the increase in the proportion of CS-II activity. In other words system seems to repair the damage caused by nickel by enhancing the activity of CS-II which requires a primer and has much greater affinity for the UDP-glucose, the donor substrate. It may also be pointed out here that CS-I and CS-II are immunologically distinct enzymes (Kamat *et al.*, 1992). In addition, it is hypothesised that callose synthase and cellulose synthase belong to same enzyme complex (Jacob and Northcote, 1985) which under normal undamaged conditions catalyze cellulose (1,4-β-glucan) synthesis while under perturbed conditions the cellulose synthesis is switched off and callose synthesis starts immediately. Nickel treatment seems to favour the callose synthesis.

5.3.5 Some limitations

The present study though establishes the toxicity of nickel *per se* in animals and plants, there are number of limitations which need consideration. Since, in any environment nickel exists along with a wide range of other elements, interactions of a synergistic or antagonistic nature need to be investigated, in order to obtain correct toxicity parameters, especially in areas where nickel from smelters pollutes the environment.

CHAPTER-VI

6.0 SUMMARY AND CONCLUSIONS

Although nickel is one of the heavy metals involved in environmental pollution, very little is known about its toxicological effects in mammals and higher plants. The present study is in two parts. Part I deals with biochemical, histopathological and structural changes induced by intramuscular administration of cumulative doses of $NiSO_4.7H_2O$ in rat liver and kidney. Part II reports the biochemical effects of $NiSO_4.7H_2O$ on peanut seedlings with special reference to some key enzymes involved in mobilizing the stored food from the cotyledons to different anatomical parts of the plant during germination and early growth and development period and also some plasma membrane bound enzymes which may act as bioindicators for the stress conditions.

6.1 Part I: STUDIES ON THE *IN VIVO* EFFECTS OF NICKEL SULPHATE IN RATS

Physical examination of nickel-treated rats (*Rattus rattus albino*) revealed a change in the colour of paws from pink to white and retardation of growth, indicating adverse effects of nickel on the health of the animal. Intramuscular administration of cumulative dose of $NiSO_4.7H_2O$ (0.5 - 2mg/100g body weight) in rats for a period of 15 days produced a dose dependent loss in body weight. The nickel contents of liver and kidney increased from 15ppm to 166ppm in liver and from 16ppm to 268ppm in kidney in rats which were given $NiSO_4.7H_2O$ doses (2mg/100g body weight) indicating bioaccumulation of nickel in these tissues. Protein and carbohydrate contents of both liver and kidney were also significantly decreased, but in contrast the concentration of total lipids in these organs was markedly increased. In fact, a 3-4-fold increase in neutral and phospholipids was observed in both rat liver and kidney as a result of nickel treatment.

A detailed study of the changes induced by nickel in the composition of various components of neutral and glycerophosphatidyl lipids in rat liver and kidney by GC and HPLC showed that the increase

in neutral lipids was largely due to increase in the level of triacylglycerol and esterified cholesterol while in the case of glycerophosphatidyl lipids, it was mainly due to phosphatidyl choline. These results indicated a selective action of nickel, apparently by enhancing the rate of synthesis of triacylglycerol, esterified cholesterol and glycerophosphatidyl choline in liver and kidney cells.

Nickel has also altered the percentage composition of various components of lipids of the subcellular membrane fractions. The relative proportion of phosphatidyl choline, the major components of phospholipids in rat liver membranes, was significantly increased in all subcellular fractions with maximum increase in plasma membrane followed by Golgi apparatus, mitochondrial membranes and the endoplasmic reticulum. The proportion of phosphatidyl ethanolamine, the second most abundant glycerophosphatidyl lipid in rat liver, was decreased in all membrane fractions except nuclear membrane. Thus the *in vivo* effect of nickel on the composition of the component glycerophosphatidyl lipids in various membrane fraction was somewhat selective.

The histochemical analysis has shown that intramuscularly administered nickel induced the deposition of triacylglycerols and phospholipids at the specific sites in liver and kidney of rats. The triacylglycerol deposition was particularly pronounced in the central zone of the liver, in the pyramidal region and the proximal convoluted tubules of kidney. The phospholipids were heavily deposited at the perilobular zone in the liver and at the cortex region of kidney. These results indicate the morphological toxicity of rats by nickel.

The light microscopic examination of tissues has shown that intramuscular administration of $NiSO_4.7H_2O$ damaged both liver and kidney affecting the hepatic parenchymal cells and distrupting the renal organisation respectively. The former showed focal, centrolobular and perilobular necrosis, nuclear morphological changes and hydropic degeneration, whereas kidney showed glomerulonephritis, increased cellularity of glomeruli and degenerative changes in the proximal and distal convoluted tubules. Irregularly arranged small but

thick reticulin fibres and excessive synthesis and deposition of collagen was also observed which favour progression of tissue injury.

Both biochemical and histochemical analysis clearly indicated that the intramuscular administration of nickel in rats greatly reduced the level of the activity of alkaline phosphatase, acid phosphatase, glucose- 6- phosphatase and lipase in liver and kidney. In addition, characteristic differences were observed in the anatomic localization of these enzymes.

Haemoglobin percentage and total RBC number were significantly decreased, whereas a slight elevation was noticed in the number of TLCs. After exposure of nickel, the activities of alkaline phosphatase, acid phosphatase, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and the levels of reducing sugars, cholesterol,urea, total bilirubin and creatinine markedly increased, but the total serum protein exhibited a marked decrease.

6.2 PART-II BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON THE EFFECTS OF NICKEL SULPHATE ON PEANUT SEEDLINGS

When peanut seeds were germinated in the presence of varying concentrations of nickel in distilled-deionized water culture, it was found that the nickel in excess of 15ppm (approx. 0.25mM) interfered with the germination process and adversely affected the growth and development of embryonic axis and formation of secondary roots, at 60ppm nickel concentration in the culture medium seeds failed to germinate. The development of radicle was relatively more sensitive to nickel than that of plumule. The decrease in growth of embryonic axis of germinating peanut seedlings led to decrease in dry weight. The nickel uptake by the seedlings also increased many-fold. As for example, in seedlings grown in liquid culture containing 60ppm nickel, nickel content increased from approximately 9ppm to 273ppm, a net increase in Ni-uptake of about 30-fold. These results point that increased level of nickel in cells suppresses mobilization and and utilization of the reserve food which the growing plant utilizes during early period of growth. The reduced levels of key enzymes such as phytase, amylase, lipase and phosphatases which are involved in the

mobilization and utilization of stored food in cotyledons, also supports this view.

The phospholipid content of microsomal membrane fraction prepared from peanut seedlings grown in presence of $NiSO_4.7H_2O$ were reduced to almost half indicating damage in the permeability functions of membranes. The activity of the 5'-nucleotidase in plasma membrane of the nickel-treated cotyledons was found completely inhibited, indicating the impairment of nucleotides transport across the membrane which will adversely affect the process of cell divisions. It is of interest to note that Ni^{2+} ions do not inhibit the PM-5'-nucleotidase activity *in vitro*, whereas *in vivo* the nickel was found to completely inhibit the 5'-nucleotidase activity. This means that *in vivo* excess of nickel probably suppresses the expression of the 5'-nucleotidase gene(s).

The callose (1,3-β-glucan) synthase (CS) is another PM-localized enzyme which is activated only under stress conditions. Normal peanut cotyledon cells contain two molecular forms, CS-I and CS-II. The former is dominant, requires no primer and has higher Km than CS-II. On the other hand CS-II requires a primer like cellobiose and has lower Km for UDP-glucose. In nickel-treated seedlings, the level of CS-II was found to be much higher (almost double) than CS-I, indicating a selective effect of nickel on the CS-II production when cells are under stress and thus reversing the relative proportions of CS-I and CS-II in nickel-treated seedlings. CS-I and CS-II isoenzymes were purified 400 and 864-fold respectively, using selective solubilization of plasma membrane bound enzyme with digitonin followed by sucrose density gradient centrifugation and chromatography on hydroxylapatite column. The latter step separated the CS-I and CS-II isoenzymes.

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Purification to Homogeneity and Characterization of a 1,3-β-Glucan (Callose) Synthase from Germinating *Arachis hypogaea* Cotyledons

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A 1,3-β-D-glucan (callose) synthase (CS) from a plasma membrane fraction of germinating peanut (*Arachis hypogaea* L.) cotyledons has been purified to apparent homogeneity as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), amino-terminal analysis, and the Western blots pattern. The purification protocol involved preparation of a high specific activity plasma membrane fraction, selective solubilization of the enzyme from the membrane with 0.5% digitonin at a protein-to-detergent ratio of 1:6, sucrose gradient centrifugation, and chromatography on hydroxylapatite and DEAE-Sephadex A-50. The purified CS shows a molecular mass of approximately 48,000 by SDS–PAGE, pH optimum of 7.4, leucine as the amino-terminal residue, K_m for UDP-glucose of 0.67 mM, and V_{max} of 6.25 μmol/min/mg protein. The enzyme is specific for UDP-glucose as the glucosyl donor and required Ca^{2+}, at an optimum concentration of 2–5 mM, for activity. The enzyme activity was inhibited by nucleotides (ATP, GTP, CTP, UTP, UDP, and UMP). The enzyme activity was also inhibited by the addition of EDTA or EGTA to the enzyme, but this inhibition was fully reversible by the addition of Ca^{2+}. The reaction product formed during incubation of UDP-[^{14}C]glucose and cellobiose with purified enzymes was susceptible to digestion by exo-(1,3)-β-glucanase, but was resistant to α- and β-amylases and to periodate oxidation, indicating that the polymer formed was 1,3-β-glucan, and β-1,4 and β-1,6 linkages were absent.

1,3-β-D-Glucan (callose) synthase (UDP-glucose: 1,3-β-D-glucan 3-β-D-glucosyltransferase, EC 2.4.2.34) is a plasma membrane-localized enzyme which catalyzes the synthesis of callose from UDP-glucose in higher plants (1–4). In intact and undamaged cells the enzyme is fully latent and is activated only under perturbed conditions, such as mechanical injury or attack by pathogens, inducing the production of callose, which is then rapidly deposited *in vivo* (1, 5). These observations point toward the possible involvement of callose synthase (CS) in the wound-healing process and in defense against pathogens (6). In addition, the enzyme seems to play a vital role in a number of specialized developmental processes in plants, namely pollen maturation and sieve pore formation (7) and gravitropism (8), as callose formation is associated with these processes.

During the last several years a great deal of effort has been made to understand the properties and functions of CS using partially purified enzyme preparations only; all attempts to purify CS to homogeneity have so far been unsuccessful (1, 4, 5, 9–15). This paper, for the first time, describes the purification to apparent homogeneity of a CS from the germinating peanut cotyledons.

EXPERIMENTAL PROCEDURES

Materials. UDP-[^{14}C]glucose (296 Ci/mol), GDP-[^{14}C]mannose, and UDP-*N*-[^{14}C]acetylglucosamine were purchased from the Radiochemical Centre, (Amersham, UK). Unlabeled UDP-glucose, various nucleotides, adenosine, glucose 6-phosphate, Tris, *n*-octyl-β-D-glucoside, Triton X-100, sodium dodecyl sulfate (SDS),[2] Chaps 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate), Nonidet P-40, cellobiose, bovine serum albumin, DEAE-Sephadex, EGTA [ethylene glycol bis (β-aminoethylether) *N*, *N*′-tetraacetate],DEAE-cellulose, amino acid standards, and Freund's adjuvants were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, *N*,*N*′-methylene-bis-acrylamide, and TEMED were obtained from Serva (Germany). Bacterial α-amylase, β-amylase, digitonin, β-mercaptoethanol, and ammonium persulfate were procured from E. Merck (India). Hydroxylapatite gel material for column

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[2] Abbreviations used: CS, callose synthase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Chaps, 3-[(cholamidopropyl)dimethylammonio]propanesulfonate; EGTA, ethylene glycol bis(β-aminoethylether)*N*,*N*′-tetraacetate; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; NC, nitrocellulose; PTH, phenylthiohydantin; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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chromatography and the molecular weight standards were obtained from Bio-Rad (Richmond, CA). BAPTA [1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetate] was from Calbiochem. Insoluble Protein-A from *Staphylococcus aureus* was obtained from Fluka, (Buchs, Switzerland). Blotting detection kit (RPN 23) for rabbit antibodies was purchased from Amersham. The purified exo-(1,3)-β-D-glucanase from *Sporotricum dimorphosphorum* (Basidiomycetes QM 806) and endo-(1-3)-β glucanase from Rhizopus QM 6789 were a generous gift from Dr. E. T. Rees, U.S. Army Laboratories (Natick, MA). All other chemicals were reagent grade from standard commercial firms. Peanut (*Arachis hypogaea* L.) seeds (large variety) were obtained locally.

Preparation of plasma membrane fraction. Unless stated otherwise all preparations were carried out at 0–4°C. The plasma membrane fraction was prepared from peanut cotyledons as described previously (16). Briefly, 200 cotyledons (80–100 g wet wt) from peanut seedlings that had been grown for 7 days in the dark at 35°C were suspended in 400 ml (50 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose, 3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (protease inhibitor), and 0.04% β-mercaptoethanol (buffer A) and homogenized by 30-s bursts in a Waring blender. The homogenate was squeezed through four layers of cheesecloth and the filtrate was successively centrifuged at 12,000*g* for 20 min and 130,000*g* for 60 min. The resulting 12,000–130,000*g* pellet (crude microsomes) was suspended in 4 ml of buffer B (25 mM Tris–HCl, pH 7.4, containing 20% (w/w) sucrose, and 1 mM β-mercaptoethanol), layered carefully over 5 ml 34% (w/w) sucrose in the same buffer, and centrifuged at 80,000*g* for 90 min. The pellet was resuspended in 2 ml of buffer B, layered over a discontinuous sucrose gradient prepared in 38-ml tubes by layering 4 ml 45% (w/w) sucrose and 6.4 ml each of 38, 34, 30, 25, and 20% sucrose in the same buffer and immediately centrifuged at 95,000*g* for 2 h in an SW-27 rotor. Visible bands of membranes were removed with a Pasteur pipette. The plasma membrane fraction (the 45–38% interface band) was identified by the presence of marker enzyme glucan synthase II (17). The total protein in the plasma membrane fraction varied between 30 and 40 mg per 100 g (wet wt) cotyledons. The membrane fraction was suspended in buffer B (20 mg/ml) and used in subsequent experiments.

Solubilization of the callose synthase. All steps were carried out at 0–4°C. To the plasma membrane fraction (300 mg of protein) was added dropwise, with gentle stirring, 1% (w/v) digitonin in buffer B, so that the final detergent concentration was 0.5% and the protein:detergent ratio was 1:6. The enzyme was solubilized by three strokes in a Teflon homogenizer followed by 30 min incubation in ice. The homogenate was then centrifuged at 105,000*g* for 60 min to remove the insoluble material. The clear supernatant liquid, which contained the solubilized callose synthase, was carefully removed with the use of a Pasteur pipette. This enzyme preparation was fully active for up to 30 days when stored at −20°C and about 96 h at 0°C.

Assay of the callose synthase activity. The activity of CS was measured as described by Ray (18), using a high concentration of UDP-glucose in the absence of Mg^{2+} with a slight modification. The standard incubation mixture, unless stated otherwise, contained the following in a final volume of 100 μl. Tris–HCl (50 mM, pH 7.4), UDP-[^{14}C]glucose (0.1 μCi, 290 Ci/mol), 0.5 mM unlabeled UDP-glucose, 5 mM cellobiose, 2 mM $CaCl_2$, 0.01% digitonin, and 0.02 to 0.1 mg protein, depending upon the status of purification of the enzyme. After incubation for 20 min at 25°C the reaction was terminated by the addition of 1 ml ethanol, 0.05 ml 50 mM $MgCl_2$, and 0.15 ml boiled plasma membrane (0.1–1.5 mg protein) as a carrier for the labeled products. The mixture was immediately boiled for 1 min and after standing overnight at 4°C the polymer was separated by centrifugation at 3000*g* for 10 min. The pellet was washed four times with 70% (v/v) ethanol to remove all the unreacted radioactive substrate and ethanol soluble products. The washed precipitate was suspended in 5 ml scintillation fluid (dioxan cocktail) and radioactivity measured in a Beckman L.S. 1801 liquid scintillation counter. Control assays were performed exactly the same way except that an equivalent amount of boiled enzyme was used in place of active enzyme preparations. Under the assay conditions the transfer of radioactive glucose from UDP-[^{14}C]glucose to the glucan polymer was linear up to 20 min. One unit of activity is defined as that which catalyzes the incorporation of 1 nmol of glucose/min into ethanol-insoluble glucan.

Product characterization. Radioactive products formed during incubation of callose synthase under assay conditions were analyzed by testing the susceptibility of radioactive glucan to digestion by specific glucanohydrolases, exo-(1-3)-β-D-glucanase (EC 3.2.1.58) and amylases (EC 3.2.1.1 and EC 3.2.1.2) as described by Orlean (19) with slight modification. For 1,3-β-D-glucanase digestion radioactive product (30,000 cpm) was incubated with enzyme (0.1 mg/ml) in 0.1 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 0.1 mg enzyme. The controls contained radioactive product in 0.1 ml Tris–HCl buffer, pH 7.5, without glucanohydrolases. In other controls amylose and cellulose were used as substrates to check the action of amylases and 1,4-β-glucanase, respectively. After incubation for the indicated period, the reactions were stopped by adding 0.02 ml glacial acetic acid. The whole digest was then chromatographed on Whatman No. 1 paper by descending chromatography using *n*-butanol:ethylacetate:acetic acid:water (40:30:25:40, v/v) solvent for 37 h. Unlabeled glucose was used as a reference. The radioactive bands on the chromatogram were located by scanning with the help of a TLC linear analyzer, LB 282 (Berthold) fitted with data acquisition system LB 500. The unlabeled sugar bands were detected by alkaline silver nitrate reagent. Alternatively, glucanase digestion of the radioactive ethanol insoluble glucan was stopped by the addition of 1 ml 70% ethanol. The remaining insoluble glucan was separated by centrifugation and washed four times with 70% ethanol and the radioactivity was determined.

SDS–polyacrylamide gel electrophoresis. This was carried out under reducing conditions as described by Laemmli (20), using gels containing 10% acrylamide and calibrated with phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Proteins were stained in 0.5% Coomassie brilliant blue R-250 in methanol:acetic acid:water (25:10:65, v/v/v) and destained with methanol: acetic acid:water (25:7:68, v/v/v). Protein bands were also visualized by silver staining (21).

Sucrose density gradient centrifugation. Digitonin-solubilized enzyme (1.5 ml) was layered onto a continuous sucrose density gradient (20–60%, w/v, dissolved in buffer B). Centrifugation was carried out for 8 h at 200,000*g* using a TST 41.14 swing bucket Centrikon rotor. Fractions of 0.5 ml were collected by piercing the bottom of the tube and an aliquot (0.1 ml) from each fraction was assayed for enzyme activity and protein.

Production of antibodies. Polyclonal monospecific antibodies were made against the purified CS in New Zealand white female rabbits following the standard procedures. A 200-μg sample of the purified enzyme in 1 ml of a 1:1 mixture of Freund's complete adjuvant and phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate buffer, pH 7.2) was injected into multiple sites subcutaneously on the back of the animal. Booster injections of about 100 μg of enzyme in Freund's incomplete adjuvant were given 21 days after the first injection. The third injection was administered 10 days after the booster dose. Blood was collected 7 days after the last final booster injection. The antisera obtained after clotting of the blood were used directly for immunoinhibition of the enzyme activity.

For immunoblotting studies IgG fraction was partially purified from the immune serum by precipitation with ammonium sulfate (45% saturation) followed by column chromatography on DEAE-cellulose as described by Mayer and Walker (22). The preimmune serum was subjected to the same procedure.

Immunoprecipitation. The antiserum to callose synthase was serially diluted with phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and incubated for 4 h at 4°C with a constant amount of the enzyme preparation in a 1.5-ml Eppendorf tube. At the end of incubation, 200 μl of protein-A–Sepharose was added to each incubation and mixed end-over-end on a rotor for 3 h at 4°C. The mixture was centrifuged at 15,000*g* for 15 min and callose synthase activity was assayed in the

TABLE I

Purification of Callose Synthase from Peanut Cotyledons

Fraction	Protein (mg)	Callose synthase			
		Total activity (units)[a]	Specific activity (units · mg^{-1})	Yield (%)	Purification (-fold)
Cell-free homogenate (5000*g*)	82,030	41,330	0.50	100	1.0
Crude microsomes	5,130	26,676	5.2	64.5	10.4
Plasma membrane-rich fraction	300	16,413	54.7	39.7	109.4
Digitonin-solubilized enzyme	166	25,200	151.8	60.9	303.6
Sucrose gradient	52	16,112	309.8	39.0	619.6
Hydroxylapatite (I)					
Peak I	6.7	6,405	955.9	15.5	1911.8
Peak II	6.5	4,251	654.0	10.3	1308.0
Hydroxylapatite (II)					
Peak I	2.8	4,221	1507.5	10.2	3015.0
Peak II	3.0	1,206	402.0	2.9	804.0
DEAE-Sephadex A-50					
Peak I	0.88	2,118	2406.8	5.2	4813.6

Note. Results are shown for one preparation of enzyme from 1000 g (fresh wt) of 7-day-old germinating peanut cotyledons. The enzyme activity was assayed using standard assay procedure as described under Experimental Procedures.

[a] One unit of enzyme activity is the amount required to incorporate 1 nmol of glucose into the product in 1 min under standard assay conditions.

supernatant. Control experiments with preimmune serum were performed concurrently under identical conditions.

Western blotting. Purified peanut cotyledon plasma membrane CS-I, digitonin-solubilized enzyme, and hydroxylapatite CS-II enzyme fraction were subjected to SDS–PAGE on 10% gels and then electrophoretically transferred onto nitrocellulose (NC) sheets (23). Immunodetection of the blotted protein on NC sheet was done using polyclonal antibodies directed against purified CS-I (1:50) dilution followed by incubation with donkey anti-rabbit IgG conjugated to alkaline phosphatase according to the instructions supplied along with the blotting kit (Amersham).

Analytical and other methods. Protein was determined by the method of Lowry *et al.* (24) with bovine serum albumin as standard. Amino-terminal analysis was carried out as described by Fraenkel-conrat and Harris (25). The phenylthiohydantin (PTH) derivative of the NH_2-terminal amino acid residue released from the polypeptide was identified by thin layer chromatography using benzene:ethylacetate (15:3, v/v) as solvent system and authentic PTH-amino acids as reference standards. Dolichyl phosphomannose (dol-P-man), dol-P-glucose, dol-PP-GlcNAc, and dol-PP-$GlcNAc_2$ were prepared as described by Sharma *et al.* (26).

RESULTS

Purification of Callose Synthase

The results of the purification procedure for callose synthase are summarized in Table I. The initial enzyme preparation was a microsomal fraction that sedimented between 12,000*g* and 130,000*g*. This fraction showed a 10.4-fold increase in the specific activity of the enzyme as compared to that of the cell-free homogenate (5000*g* supernate). The next step in the purification scheme involved the preparation of a high specific activity plasma membrane fraction by sucrose gradient centrifugation as described under Experimental Procedures. A membrane fraction that separated at the interface of the 45 and 38% sucrose layers on the gradient showed a further increase of 10.5-fold in the specific activity of CS, giving an overall purification of 109.4-fold. Hence, separation of the plasma membrane fraction from other endomembranes was an important purification step.

The next step involved the solubilization of the PM-bound enzyme with detergents in stable and active form. The effect of five different nonionic detergents, namely *n*-octyl-β-glucoside, Triton X-100, Nonidet P-40, digitonin, and Chaps on the activity of the membrane-bound enzymes, was examined. It was found that octylglucoside, Triton X-100, and Nonidet P-40 strongly inhibited the CS activity and at 0.5% detergent concentration nearly three-fourths of the enzyme activity was lost (Table II). However, under identical conditions digitonin and Chaps

TABLE II

Effect of Various Detergents on the Activity of Plasma Membrane-Bound 1,3-β-D-Glucan Synthase

Detergent	Concentration (%)	CMC[a] (%)	1,3-β-Glucan synthase activity (nmol · min^{-1} · mg^{-1})	Percentage of control (%)
None	—	—	106.0	100.0
β-Octylglucoside	0.5	0.73	26.5	25.0
Triton X-100	0.5	0.02	23.3	21.9
Nonidet P-40	0.5	0.02	25.4	23.9
Digitonin	0.5	0.02	261.6	246.7
Chaps[b]	0.5	0.60	174.4	161.7

[a] CMC, critical micelle concentration.

[b] Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonate).

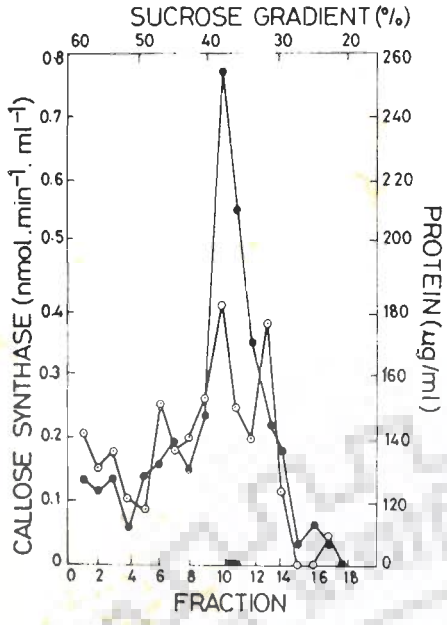

FIG. 1. Distribution of digitonin-solubilized callose synthase activity and membrane proteins from peanut cotyledon cells in sucrose density gradient. The digitonin-solubilized enzyme was layered on a linear 20–60% (w/w) sucrose in buffer B and centrifuged for 8 h at 200,000*g*. Fractions of 0.5 ml were collected from the bottom of the tube and assayed for the enzyme activity (●) and protein (○). Fractions indicated by bar were pooled.

stimulated the CS activity by 2.4- and 1.6-fold, respectively (Table II). These results clearly indicated that digitonin or Chaps would be suitable for the solubilization of PM-bound CS activity. But because of the higher stimulation of CS activity by digitonin than by Chaps the enzyme activity in the plasma membrane fraction was solubilized with 0.5% digitonin in the presence of 20% sucrose and at a protein:detergent ratio of 1:6. The solubilized enzyme thus obtained showed 1.5 times higher total activity than the total activity of the membrane-bound enzyme initially present before solubilization. These results indicate that the CS activity was activated by digitonin treatment. In addition, there was a 2.7-fold increase in the specific activity of the solubilized enzyme compared to that of the plasma membrane fraction, giving an overall purification and yield of 303.6-fold and 60.9%, respectively. At this stage the solubilized enzyme was stable for at least 1 month at −20°C, losing less than 15% of the initial activity. The digitonin-solubilized enzyme was then subjected to sucrose gradient centrifugation as shown in Fig. 1. It was found that about two-thirds of the enzyme activity was concentrated in fractions 10 and 11, representing the sucrose gradient from 38 to 36%. By this step the enzyme activity was further purified by 2-fold (Table I). These fractions were pooled, dialyzed overnight against 100 vol of 10 mM phosphate buffer, pH 6.8, containing 0.1% digitonin and 0.01% β-mercaptoethanol (buffer C). The dialyzed fraction was then applied to a hydroxylapatite column (1.5 × 8 cm) previously equilibrated with the same buffer. The column was washed with 50 ml of buffer C and then eluted with a linear 100-ml gradient from 10 to 500 mM phosphate buffer, pH 6.8, containing 0.1% digitonin and 0.01% β-mercaptoethanol. Two-milliliter fractions were collected at a flow rate of 0.2 ml/min and assayed for the enzyme activity and protein. The CS activity was eluted in two distinct peaks (peaks I and II) at approximately 95 and 220 mM phosphate buffer (Fig. 2). The callose synthase forms in peak I and peak II were designated CS-I and CS-II. As judged from the peak area, about 60 and 40% of the total CS activity recovered from the column were separated as CS-I and CS-II. Since peak I and peak II are the major protein peaks as well and together account for about one-fourth of the protein applied on the hydroxylapatite column, it appears that a large portion of protein was not eluted from the column under the elution conditions used, resulting in substantial purification of CS-I and CS-II enzymes. Fractions containing CS activity in peak I (fraction 30–35) and in peak II (fractions 42–46) were pooled separately and concentrated to 1 ml by ultrafiltration on an Amicon YM-30 membrane. This chromatographic step was repeated to remove the overlapping proteins and obtain homogeneous enzyme preparations. Only the top three peak fractions with the highest specific activity were pooled and concentrated. After the second column of hydroxylapatite the purification of CS-I was increased 1.5 times, but there was a substantial decrease in the specific activity as well as in the total activity of CS-II (Table I), indicating that the latter enzyme was unstable. All attempts to stabilize this CS-II enzyme fraction with glycerol or with phospholipids were unsuccessful and, there-

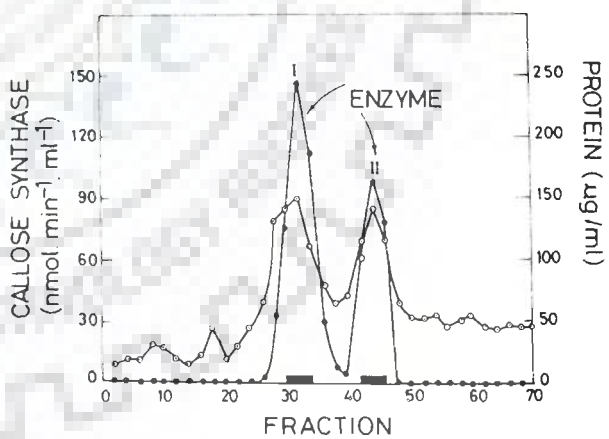

FIG. 2. Purification of callose synthase by chromatography on hydroxylapatite. Active fractions 10 and 11 (Fig. 1) from sucrose density gradient were pooled, dialyzed against buffer C, and applied to a 1.5 × 8-cm column of hydroxylapatite. The column was washed with 50 ml of the same buffer and then eluted with a 100-ml linear gradient of 10–500 mM sodium phosphate in the same buffer. Fractions of 2 ml were collected and assayed for callose synthase activity (●) and protein (○). Fractions indicated by bar were pooled.

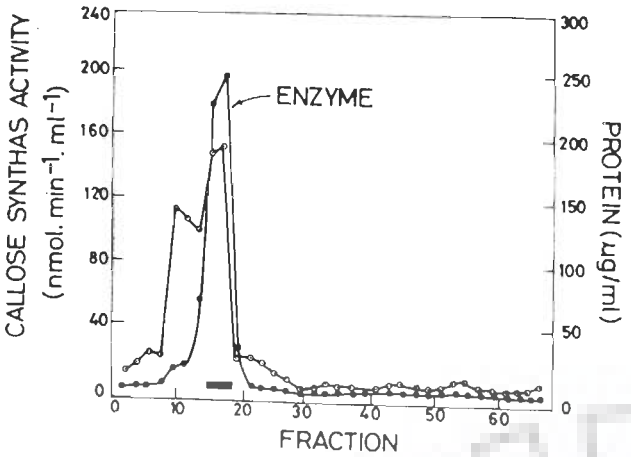

FIG. 3. Purification of callose synthase by chromatography on DEAE-Sephadex A-50. Active peak I fractions from the hydroxylapatite column (Fig. 2) were pooled, dialyzed against buffer D, concentrated to about 1 ml, and applied to a column (1 × 10 cm) of DEAE-Sephadex A-50. The column was washed with 20 ml of the same buffer and then eluted with a linear gradient of 10–350 mM NaCl. One-milliliter fractions were collected and assayed for the enzyme activity (●) and protein (○). Fractions shown by bar were pooled.

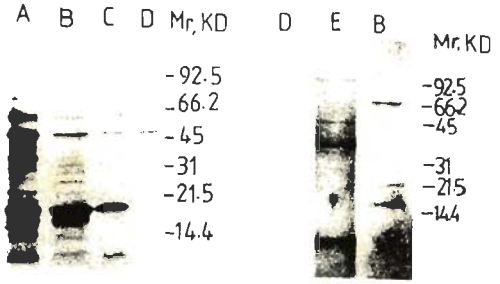

FIG. 4. Analysis by SDS gel electrophoresis of callose synthase fractions on a 10% gel. Lane A, plasma membrane; lane B, digitonin-solubilized enzyme fraction; lane C, sucrose density gradient fraction; lane D, purified callose synthase from DEAE-Sephadex A-50; lane E, peak II from hydroxylapatite column. Standard protein markers are shown at the right as follows: 92.5-kDa protein, phosphorylase b; 66.2-kDa protein, bovine serum albumin; 45-kDa protein, ovalbumin; 31-kDa protein, carbonic anhydrase; 21.5-kDa protein, soybean trypsin inhibitor; 14.4-kDa protein, lysozyme. The gels on the left and right sides were stained with Coomassie brilliant blue and silver, respectively, and represent two different enzyme preparations from two different batches of peanut cotyledons.

fore, they could not be purified further. On the other hand CS-I was quite stable and was purified further as described below.

Subsequent to hydroxylapatite, DEAE-Sephadex A-50 column (1 × 10 cm) chromatography, as indicated in Fig. 3, was used as the final step of purification for the CS-I fraction of the enzyme. The column was equilibrated with 10 mM Tris–HCl, pH 7.4, containing 0.1% digitonin and 0.01% β-mercaptoethanol (buffer D) and loaded with CS-I fraction. The column was washed with 20 ml of buffer D and then eluted with a linear gradient of 10–350 mM NaCl in buffer D. One-milliliter fractions were collected at a flow rate of 0.2 ml/min. It can be seen that essentially all of the enzyme activity emerged in a peak toward the end of the washing of the column in fractions 16–18, while a considerable amount of inactive proteins was separated from the enzyme in the early flow-through fractions as well as in the fractions that followed the enzyme peak (Fig. 3). Peak fractions 16–18 showing constant specific activity were pooled, concentrated, and stored at −20°C in 50 mM Tris–HCl, pH 7.4, containing 0.1% digitonin and 0.01% β-mercaptoethanol and 20% sucrose or glycerol. Under these conditions the purified enzyme was stable for at least 1 week, losing less than 10% of the initial activity. The purification procedure was quite reproducible. The overall purification was about 4813-fold, and the yield of the active enzyme was about 5%.

Homogeneity and Molecular Mass of CS-I

As seen in Fig. 4 (left), when the enzyme fraction, CS-I, from the DEAE-Sephadex A-50 column was subjected to SDS-gel electrophoresis carried out under fully reduced conditions, it showed only one protein band (lane D) with a molecular mass of 48,000 even when concentrated CS-I sample (10 µg protein) was applied on the gel. Also shown in this figure for comparison are SDS-gel profiles of the plasma membrane fraction proteins (lane A), digitonin-solubilized enzyme fraction (lane B), and the preparation from the sucrose gradient (lane C). Figure 4 (right) shows the silver-stained SDS-gel profiles of proteins of the purified CS-I fraction (lane D), hydroxylapatite peak II fraction, CS-II enzyme (Lane E), and the digitonin-solubilized enzyme fraction (lane B). These results indicate that the enzyme preparation from the DEAE-Sephadex A-50 was homogeneous but the CS-II fraction contained several protein bands.

The homogeneity of CS-I enzyme was further confirmed by Western blotting (Fig. 5) performed with polyclonal

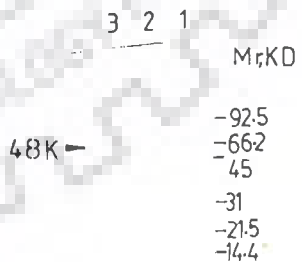

FIG. 5. Western blots of digitonin-solubilized enzyme (lane 1), purified CS-I (lane 2), and CS-II enzyme fraction (lane 3). Immunodetection of the blotted protein on NC sheet was done using polyclonal antibodies directed against the purified CS-I followed by incubation with donkey anti-rabbit IgG conjugated to alkaline phosphatase as described under Experimental Procedures. Note the homogeneity and the immunological specificity of CS-I enzyme.

TABLE III

Susceptibility of the Callose Synthase Assay Product to Digestion by α-Amylase, Exo-(1,3)-β-D-Glucanase, and Periodate Oxidation

Treatment[a]	Radioactivity distribution on chromatogram (cpm)	
	Origin	Migrated as glucose
Control	23,974 ± 350	79 ± 15
α-Amylase	22,693 ± 325	108 ± 20
β-Amylase	23,002 ± 360	90 ± 20
Exo-(1,3)-β-D-glucanase	201 ± 50	20,162 ± 280
Periodate oxidation	22,732 ± 350	

[a] Radioactive callose synthase assay product was subjected to various treatments and analyzed by paper chromatography as described under Experimental Procedures.

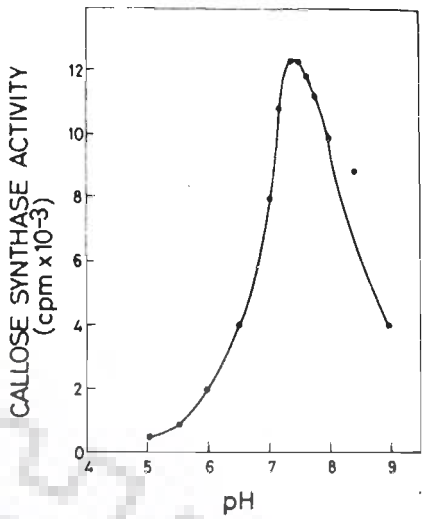

FIG. 6. Effect of pH on the activity of the purified callose synthase. Assay conditions were as described under Experimental Procedures.

monospecific antibody made against this enzyme fraction. It can be seen that only a single band corresponding to CS-I was obtained with purified CS-I enzyme (lane 2) and with the digitonin-solubilized enzyme preparations (lane 1) while no band was observed with the CS-II enzyme fraction from the hydroxylapatite column (lane 3). These results clearly show that the antibodies are specific for CS-I and do not cross-react with CS-II.

Amino-Terminal Amino Acid

The amino-terminal analysis by generation of PTH amino acid derivatives showed that leucine occupied the amino-terminal position of CS-I; the PTH–leucine derivative was detected with no accompanying traces of other amino acid derivatives (data not shown). These results further demonstrate the homogeneity of the CS-I preparation. In addition, these results together with those of SDS–PAGE suggest that CS-I consists of a single polypeptide chain, although the possibility of having more than one molecule of the same polypeptide chain per molecule of the enzyme can not be ruled out.

Reaction Product

The radioactive product formed during the incubation of CS-I under standard assay conditions was found resistant to α- and β-amylase digestion and the total amount of the radioactivity was completely immobile on paper chromatography; no peaks corresponding to glucose, maltose, or lower oligosaccharides were detected (Table III), indicating the absence of glycogen. However, after the digestion of the radioactive product with exo- and endo-1,3-β-glucanase, the total radioactivity traveled on the paper chromatogram with the glucose peak. The radioactive product was also resistant to periodate oxidation (Table III). These results clearly indicate that the reaction product formed by CS-I was predominantly β-1,3-linked glucan, and if any other linkages were formed these were undetectable. The exo- and endo-1,3-β-glucanases used were pure and did not act on cellobiose or amylase (data not shown).

Kinetic Properties

The purified peanut cotyledon microsomal CS-I had a pH optimum of 7.4 (Fig. 6). The K_m and V_{max} values of CS-I for UDP-glucose, as determined by Lineweaver–Burk plot (Fig. 7) were found to be 0.67 mM and 6.25 μmol glucose incorporated/min/mg protein, respectively.

Substrate Specificity

CS-I was found to be highly specific for UDP-glucose as the donor of the D-glucosyl residue in the *in vitro* for-

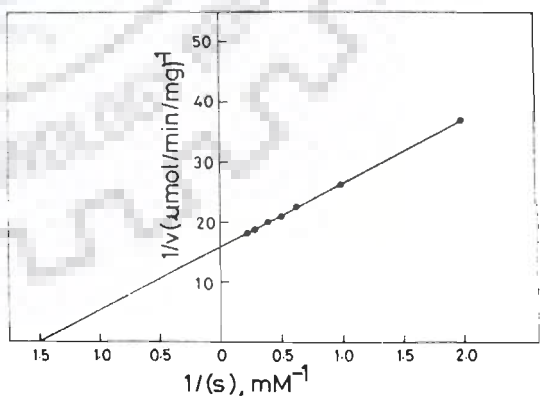

FIG. 7. Lineweaver–Burk plots showing the effect of UDP-glucose concentration on the purified callose synthase activity. Enzyme assays, with varying amounts of UDP-glucose, were carried out as described under Experimental Procedures.

TABLE IV

Donor Substrate Specificity

Substrate	Concentration (cpm)	Activity of CS (cpm)
UDP-[^{14}C]glucose	50,000	12,140
GDP-[^{14}C]mannose	50,000	993
UDP-[^{14}C]GlcNAc	50,000	701
Dol-P-[^{14}C]mannose	10,000	175
Dol-P-[^{14}C]glucose	10,000	180
Dol-PP-[^{14}C]GlcNAc	10,000	198
Dol-P-[^{14}C]GlcNAc$_2$	10,000	208

Note. Enzyme assays were performed using standard incubation mixture except the substrate. When lipid saccharides were used components of incubation mixture were added after drying the lipids under a stream of nitrogen gas.

mation of 1,3-β-D-glucan (callose) polymer and very little activity was observed when other sugar nucleotides, namely GDP-[^{14}C]mannose and UDP-β-*N*-[^{14}C]acetylglucosamine (UDP[^{14}C]GlcNAc), or the lipid saccharides (dol-P-mannose, dol-P-glucose, dol-PP-GlcNAc, and dol-PP-GlcNAc$_2$) were used as the donor substrates (Table IV). These results rule out the possibility, at least *in vitro*, of the involvement of the lipid–saccharide intermediates in the biosynthesis of callose. Five to eight percent of the radioactivity from GDP-[^{14}C]mannose or UDP-[^{14}C]-GlcNAc was incorporated into ethanol-insoluble material by the purified enzyme. At the moment we are not certain if these sugars are covalently bound to β-1,3-glucan polymer.

Metal Ion Requirement

The enzyme activity was inhibited 61, 80, and 91% by 2 mM EDTA, EGTA, and BAPTA, respectively. Since both EGTA and BAPTA are highly selective for Ca^{2+}, these results suggested that the enzyme had a requirement for a specific divalent cofactor. The inhibition of callose synthase by the chelating agents was almost fully (about 95%) reversed by the addition of an excess of Ca^{2+}, but not by other metal ions that were tested. For instance, the CS-I activity was assayed in the presence of 2 mM EGTA. Full activity was obtained by the addition of 5 mM $CaCl_2$, whereas the addition of 5 mM $Fe^{2+/3+}$, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+} was ineffective. In fact, Mn^{2+} and Zn^{2+} were found to be strong inhibitors, as at 1 mM concentration 50–60% of the enzyme activity was inhibited by these metals ions. Magnesium was not used since the enzyme is assayed in the absence of Mg^{2+} (18).

Effect of Substrate Analogues

The effect of different nucleotides and related compounds on the activity of the purified CS-I is shown in Table V. The enzyme activity was inhibited from 40 to 50% by UTP, ATP, GTP, CTP, UDP, and UMP at 5 mM concentration of the nucleotide. However, except UDP and UMP, the extent of inhibition by other nucleoside di- and monophosphates was only marginal, varying from 10 to 20%. Similarly, adenosine, adenine, D-ribose, glucose 6-phosphate, and glucose 1-phosphate had no significant effect on the activity of the enzyme.

TABLE V

Effect of Some Nucleotides and Related Compounds on the Activity of CS

Nucleotide added	Activity of CS (nmol · min^{-1})	Inhibition (%)
None	7.0	
UTP	3.0	57.2
ATP	3.6	48.6
GTP	4.2	40.0
CTP	3.8	45.8
ADP	5.9	15.8
GDP	5.6	20.0
UDP	3.9	44.3
AMP	6.0	14.3
GMP	6.2	11.5
UMP	4.3	38.6
Adenosine	6.8	3.0
Adenine	6.0	14.3
D-Ribose	5.9	15.8
Glucose-1-PO$_4$	5.8	17.2
Glucose-6-PO$_4$	5.7	8.6

Note. Enzyme activity was assayed in the presence of 5 mM nucleotide or related compounds as described under Experimental Procedures.

Effect of Cellobiose

The activity of the purified CS-I was not significantly affected by cellobiose (Table VI). It appears that the latter was not used as primer by the purified enzyme.

Immunological Studies

Incubation of the antiserum against the purified CS-I preparation with CS-I fraction resulted in immunopre-

TABLE VI

Effect of Cellobiose on the Activity of the Purified CS-I

Cellobiose concentration (mM)	CS-I activity	
	cpm/70 μl	nmol/min/mg
0	16,771	417.9
5	14,969	373.0
10	13,358	332.9
20	15,672	390.59

Note. 1,3-β-Glucan synthase activity was assayed in the presence of varying concentrations of cellobiose using standard incubation mixture as described under Experimental Procedures. CS-I, 1,3-β-glucan (callose) synthase I.

cipitation of the CS-I enzyme activity (Fig. 8). Similarly, the addition of protein-A–Sepharose to the enzyme/antiserum mixture removed the CS-I activity by adsorption. No inhibition or removal of enzyme activity was observed when nonimmune serum was substituted for the antiserum.

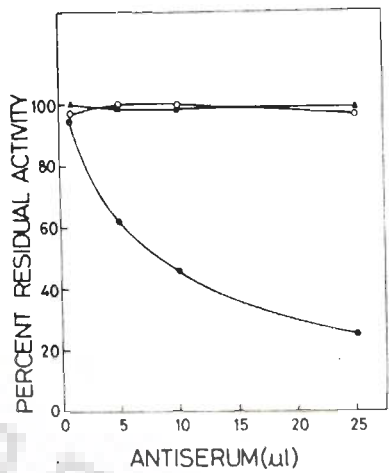

FIG. 8. Precipitation of enzyme activity with immune serum. Twenty microliters of the enzyme fraction from DEAE-Sephadex A-50 (20 μg protein) was incubated in a total volume of 100 μl containing 10 mM Tris/HCl, pH 7.4, 0.2% digitonin, and 0.01% β-mercaptoethanol with indicated amounts of serum followed by incubation with 50 μl Protein-A–Sepharose for 3 h at 4°C. The immune complexes were centrifuged and standard enzyme assays were performed with 50-μl aliquots from the supernatants. (●) Immune serum added to the purified enzyme, CS-I; (○) preimmune serum added; (▲) immune serum added to hydroxylapatite enzyme fraction II (CS-II).

DISCUSSION

The purification scheme for callose synthase described here is relatively simple and involved the use of conventional methods of purification of membrane proteins, namely, preparation of high specific activity PM fraction, selective solubilization of the membrane-bound enzyme with digitonin, linear density gradient fractionation, and chromatography on hydroxylapatite and DEAE-Sephadex A-50 columns. The enzyme preparation was homogeneous as judged by SDS–PAGE, immunological specificity, and H_2 N-terminal amino acid determinations.

As far as we know this enzyme has not yet been purified to homogeneity from any plant tissue, although a number of workers reported the partial purification of the enzyme from a few plant sources (4, 9, 12). Recently, Fink *et al.* (14) have purified the enzyme 12-fold from the suspension-cell culture of *Glycine max* and made polyclonal antibody against a 31K polypeptide band which was enriched during solubilization by digitonin followed by linear sucrose density gradients. This is an interesting piece of information, but direct evidence for the enzyme activity in the 31K protein is yet to be obtained.

The enzyme is tightly bound to the membrane and was best solubilized by 0.5% (w/v) digitonin. The digitonin-solubilized enzyme exhibited 1.5- to 2.8-fold increase in the total and specific activity compared to that of the membrane-bound enzyme, clearly indicating the activation and selective solubilization of the enzyme. The mechanism by which digitonin activates the CS activity is not known. However, Wasserman and McCarthy (3) believed that the digitonin-extracted enzyme contained a layer of attached boundary lipid, which was responsible for the enzyme activation. One of the widely used techniques used for purifying the membrane-bound enzymes is density gradient centrifugation (4, 14, 17). In the present purification scheme also this step was found very useful as a 2-fold purification was achieved. The hydroxylapatite column chromatography not only yielded a substantial purification, but also resolved two forms of CS, CS-I and CS-II, eluting at about 95 and 220 mM phosphate gradients. This was an unexpected observation. It is not clear whether CS-I is the degradation product of the CS-II enzyme or not. The antibodies made against CS-I did not cross-react with CS-II, however, suggesting that CS-I and CS-II are immunogenically different enzymes.

Since peanut CS-I enzyme was stable through the purification scheme, its purification could be achieved. In addition, the purified peanut enzyme does not seem to require a primer, or it has a glucan primer associated with it as the addition of cellobiose in the incubation mixture did not increase the incorporation of radioactive glucose into the ethanol-insoluble glucan (Table VI). These two factors appeared to have facilitated the purification of peanut enzyme. Moreover, it seems likely that digitonin, a steroidyl glycoside containing an O-linked pentasaccharide, xylose (glucose → galactose) → glucose → galactose, used for solubilizing the enzyme is also acting as a primer. This would also explain as to why cellobiose did not enhance the incorporation of [^{14}C] glucose into the ethanol-insoluble glucan.

The polysaccharide formed during the reaction by the purified glucosyl transferase was not attacked by α- or β-amylase, but was completely hydrolyzed by an apparently pure β-(1,3)-glucanase. These results were consistent with the characteristic observation that the isolated plant plasma membranes largely synthesize 1,3-β-D-glucan and their ability to synthesize 1,4-β-D-glucan polymer from the external UDP-glucose is lost (1). In contrast, the plasma membrane-located 1,3-β-D-glucan synthase becomes active on cell homogenization which otherwise was fully latent in intact cells (14). The possibility that a very small number of 1,4-β-linkages remained undetected cannot be excluded completely as the plant membranes do incorporate glucose from UDP-glucose into xyloglucan *in vitro* (29). Furthermore, it is hypothesized that both callose synthase and cellulose synthase (1,4-β-glucan synthase) belong to the same enzyme complex which may change its specificity on perturbation of PM (30).

The activity of the purified CS-I was greatly stimulated by Ca^{2+}. Kohle *et al.* (31) have proposed a direct and reversible action of Ca^{2+} ions without mediation of calmodulin. However, covalent modification of CS cannot be ruled out at the moment and would require further research in this direction. Surprisingly, the enzyme activity was inhibited by Mn^{2+} and Zn^{2+}. The physiological significance of the inhibition of these divalent metal ions is not understood. The enzyme activity was also inhibited by nucleotides (Table V), suggesting that nucleotides may have a role in the regulation of CS activity. In this respect the peanut enzyme resembles the *Saprolegnia* enzyme which is also inhibited by nucleotides (32).

The molecular mass of the purified CS-I polypeptide as determined by SDS–PAGE was found to be 48K. In the absence of homogeneous preparations of the enzyme from other plant sources, direct comparison of the molecular mass is not possible. However, recently, using the affinity probe or isoelectric focusing, the molecular masses of mung bean (9), red beet (13), and pea (15) enzymes have been predicted as 50,000, 57,000 and 55,000, respectively. Thus the molecular mass of the peanut CS is fairly comparable.

ACKNOWLEDGMENTS

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