IMMUNOPATHOLOGICAL RESPONSE OF THE DUCK *(Anas platyrrhyncos domesticus)* TO SUBLETHAL DOSE OF SELECTED AGRO-CHEMICALS

By
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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**IMMUNOPATHOLOGICAL RESPONSE OF THE DUCK (*Anas platyrrhyncos domesticus*) TO SUBLETHAL DOSE OF SELECTED AGRO-CHEMICALS**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy

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CERTIFICATE

Certified that the thesis entitled "**IMMUNOPATHOLOGICAL RESPONSE OF THE DUCK (*Anas platyrrhyncos domesticus*) TO SUBLETHAL DOSE OF SELECTED AGRO-CHEMICALS**" is a record of research work done independently by **Shri. Vijayan, N.,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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Introduction

INTRODUCTION

Asia is considered to be the homeland of domesticated ducks. In most of the watershed areas and coastal states in India, duck rearing is popularly practised. According to 1987 census, the duck population in India is 23.48 millions which is 8.53 per cent of the total poultry population in the country. By and large traditional duck farmers belong to marginal and landless labour category whose main source of income is from duck farming. The layer duck operation is of nomadic type. Farmers migrate from place to place in search of post-harvest paddy fields. Elaborate housing is not provided. The laying ducks are flocked in the evening after feeding in the harvested paddy fields in a circular enclosure made with chicken mesh or bamboo slates on the bank of the paddy field or river in the open.

The ducklings are handfed till about one month of age and thereafter they are flocked to harvested paddy fields and ponds. The paddy fields are likely to be contaminated with the various agrochemicals used in agricultural operations.

The Ministry of Agriculture and Co-operation, Government of India, has published the list of pesticides registered under the Insecticides Act, 1968 and has recommended carbofuran to be used for a variety of crops as a pesticide

and 2,4-D as a herbicide. On local enquiry, it was found that these two agro-chemicals are being used extensively in agricultural operations.

The farmers in their curiosity to protect their crop from pests, use agro-chemicals more than the recommended dosage and they are likely to remain as residues in the soil. The soil act as an environmental reservoir for these residues from which they move into the atmosphere, water or living organism (Edward, 1973). Water and the mud at the bottom of the rivers, streams, lakes and ponds are other major reservoirs for persistent pesticide residues. The pesticides reach the water either through aerial sprays or as residues which reach water as surface run off from treated soil. Insecticides may be discharged into rivers with factory and sewage effluents.

A report in the National daily `The Hindu' stated "The university, along with the State Agriculture Department, advocated intensive use of pesticides in paddy fields like that of Kuttanad. Its advice for preventive application of pesticides went even to the extent of telling the farmers to spray them even in the peripheral areas of paddy fields. It was no secret that scientists and agriculture officers often nodded their heads at conferences at which pesticide companies advised farmers to use pesticides at dosages two or three times higher than what is required".

The pollution control board detected letting out of highly toxic effluents into the periyar river which caused massive death of fish over a stretch of six kilometres by one factory in the Elur industrial belt (The Hindu dt. 13.6.98).

In Kerala, heavy mortality in ducks was reported in Alleppey district during 1976-77 (Nair, 1978; Rajan *et al.* 1980). Since then, Kerala state has become endemic for duck plague and regular reports are received from Alleppey, Kottayam, Pattanamthitta, Thrissur and other waterlogged areas (Punnose *et al.* 1993).

In an investigation during 1991, six outbreaks of duck plague with 33 per cent morbidity and 26 per cent mortality were reported from various parts of Kerala in spite of the vaccinations adopted (Kulkarni, 1993).

Immunomodulation by agrochemicals is gaining significance in toxicity evaluation as low level dietary intake through feed residues might cause breakdown of immunity following vaccination (Varshneya *et al.* 1988).

In contrast to the reports of acute toxicity, continuous presence of one or more major insecticides at low level in acquatic environments have been documented by several countries where agricultural pesticides are widely used.

The major infectious diseases with considerable economic loss in ducks in India are Duck Plague and Pasteurellosis. Outbreaks among the vaccinated flocks were very frequent and these were suspected as a result of breakdown of immunity. It was not known whether breakdown of immunity was due to the immunosuppressive effects of environmental pollutants like agrochemicals applied in the agricultural fields, since the ducks are mostly reared in the agricultural fields. However, this was suspected to be an important factor in causing immunosuppression and breakdown of immunity.

Against this background an investigation was undertaken to clarify and evaluate the role of certain agro-chemicals in causing immunosuppression in ducks when they are exposed to low levels of these agro-chemicals.

Review of Literature

REVIEW OF LITERATURE

2.1 The use of agro-chemicals and the presence of their residues in the environment

Residues of lindane was observed in chicken and eggs, after spraying of poultry house (Ivey *et al.* 1961) and after feeding caged laying hens at a dosage level of 0.10 to 10 ppm in their ration (Ware *et al.* 1961).

The Directorate of Plant Protection, quarantine and storage under the Ministry of Agriculture, Government of India has listed the various herbicides, plant growth regulators, fungicides and insecticides registered under the insecticide Act, 1968 for use in agricultural operations. 2,4-D has been recommended as a herbicide to destroy about 26 varieties of weed affecting about 11 crops. Carbofuran has been recommended for use against 51 pests affecting about 23 crops including paddy (Ministry of Agriculture, 1968).

The Miller Amendment Act of 1954 laid down that toxic residues on the crop surface should be kept at minimum, consistent with effective control of pest species. The magnitude of the residue problem has become significant in advanced countries with the extensive use of persistent pesticides in larger volumes. Hazards were therefore recognized in respect of residues, remaining on produce after

direct treatment or treatment of the substratum and also in respect of those occurring through drift of pesticides from a treated to an untreated area (Somasekhar, 1969).

Carbofuran left residues of 1.26 ppm after 21 days of application of the chemical at the rate of 1.12 kg/ha (Shaw *et al.* 1969).

In contrast to many short exposures to very high concentrations, many of the large rivers in countries where agricultural pesticides are widely used were now recording the almost continuous presence of one or other insecticide at low but continuous dosage (Muirhed and Thomson, 1971).

According to Edwards (1973) large amounts of pesticides reached the soil, either as direct application, as fall out from aerial spraying, in rain or dust or from plant or animal remains which become incorporated with the soil. According to him the soil is an environmental reservoir for these residues from which they move into the atmosphere, water or living organisms. Water and mud at the bottom of the rivers, streams, lakes, ponds or the sea bed are the major reservoirs of persistent pesticide residues (Edwards, 1973).

A few organocarbomate insecticides were reported to be absorbed by roots or leaves, metabolised and translocated in non-insecticidal amounts, to leave persistent and undersizable

residues at harvest. Forage and fodder carrying residues, even in parts per billion may represent a danger, when fed to livestock, in which chemicals reappear concentrated in body fats and other products (Finlayson and Mac Carthy, 1973).

The yolk of hens fed on feeds containing 5 mg dicofol per kilogram for 2 months contained residues of the chemical (Gladenko *et al.* 1988).

Poisoning by carbofuran was reported in dogs, cats, ducks and cattle (Smith and Lewis, 1988).

Aerial sprays of herbicides are used to induce changes in wetland emergent vegetations (Ling *et al.* 1996).

2.2 Carbofuran

Carbofuran was developed in Canada in 1960s and introduced by FMC corporation in 1965 as a systemic and contact insecticide. Carbofuran is available as a 75 per cent wettable powder, 2,3,5 and 10 per cent granules and as 48 per cent flowable paste. The metabolism of carbofuran was found to be essentially similar in mice, rats, cows, insects and plants, although a few compounds were formed by plants only (Metcalf *et al.* 1968). Carbofuran is used to control about 8 pests which included the leaf roller, stem borer, nematodes in paddy and various other crops (Ministry of Agriculture, 1968)

Single LD-50 for carbofuran as 98 per cent technical product was 25-39 mg/kg in chicken (Tobin, 1970).

2.3 2,4-Dichlorophenoxy acetic acid (2,4-D)

2,4-D was recommended as a herbicide to be used in about eight crops and also against acquatic weeds and in non-crop area at different dosage levels (Ministry of Agriculture, 1968).

2,4-D concentrations as high as 25 per cent was recommended for eradicative purposes and concentrations as low as 3 ppm to prevent premature dropping of fruit. In the body, the salts or esters of chlorophenoxy compound depends mainly on the acid involved. However, different derivatives of the same compound may have very different properties of herbicides. The solubility of 2,4-D in water was observed to be 620 ppm at 25°C. This compound was reported to be particularly effective against broad leaved weeds and often was used in combination with other herbicides (Hayes, 1982).

2.4 Embryotoxicity

2.4.1 Pesticides

Reproductive and teratological studies with carbofuran, did not show any evidence of teratogenesis in rats, rabbits or dogs (McCarthy *et al.* 1971).

Carbofuran at a dose rate of 2.5 mg/kg administered to rats by gavage on the 18th day of gestation produced cholinergic signs within 5 minutes and killed one of the dams within 30 minutes. A dosage of 0.05 mg/kg by gavage produced slight, brief inhibition of choline esterase in the dams, but not in the foetus (Cambon *et al.* 1979).

Unincubated chicken eggs immersed in different concentrations of an aqueous solution of a commercial formulation of maneb (Maneb-80 containing 80 per cent manganese ethylene bisdithiocarbonate and 20 per cent inert ingredients) proved to be teratogenic at all concentrations and produced mainly unilateral lower limb deformities (Maci and Arios, 1987).

Nair (1990) while studying the immune system in ducks, observed that ducklings hatched from eggs dipped in 1% testosterone recorded significant decrease in the mean body weight between the control and the two treatment groups. He also observed that ducklings hatched out from testosterone treated eggs did not reveal any significant difference in bursal weight compared to the control group in contrast to the results obtained by earlier workers.

2.4.2 Herbicides

2,4-D did not cause a significant increase in tumour development following oral administration to two strains of mice at the maximal tolerated levels for 18 months (Innes *et al.* 1969).

No congenital malformations occurred in lambs whose mothers received 2000 mg of 2,4-D per day during the first 30, 60 or 90 days of gestation (Binns and Johnson, 1970).

The occurrence of terata in hamsters receiving 2,4-D was not dose related (Collins and Williams, 1971).

Compared to 2,4,5-T, labelled 2,4-D showed a slight tendency to accumulate in the visceral yolk sac and pass into the foetus (Lindquist and Velberg, 1971).

Aleksashina *et al.* (1973) reported that 2,4-D was not teratogenic, but a large single dose did reduce growth and survival and caused other toxic effects on the foetus. Daily administration of 0.5 mg/kg reduced the growth, but not survival, and 0.1 mg/kg was without any effect.

At a dosage rate of 110 mg/kg/day, 2,4-D was teratogenic and embryotoxic in mice (Baage *et al.* 1973).

Konstantinova *et al.* (1975) found that an oral dosage of 2,4-D as high as 80 mg/kg in rats did not increase embryonal mortality or malformations but it did increase the proportion of foetuses with haemorrhage of the internal organs.

An expert committee concluded that no evaluation of the carciniogenecity of 2,4-D and 2,4,5-T could be made (WHO, 1977).

The foetal toxicity of 2,4-D depends largely on the derivative when each compound was administered to rats as a single dose at half the LD-50 rate or daily throughout pregnancy at the highest level tolerated by the dam. The ammonium and sodium salts did not affect the development of the embryos, but the ammonium salt especially the butyl ester of 2,4-D caused a significant increase in post implantation mortality (Aleksashina *et al.* 1979).

Paraquat when injected into the yolk sac of fertile eggs on the 5th and 9th days of incubation, and when administered orally to 2 groups of pregnant rats on the 8th and 15th days of gestation proved to be more embryotoxic than teratogenic in both chick embryo and white rats (Ahmed *et al.* 1988).

A large scale multireplicated developmental toxicity study conducted in various strains of mice with the herbicide, 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) by gavage on

gestational days 6 through 14, developmental toxicity was observed at doses below those producing maternal toxicity. Reduced fetal weight and increased incidence of cleft palate and embryo lethality were the most significant prenatal effects of 2,4,5-T (Holson *et al.* 1992).

The herbicide 3,4-dichloropropionanilide (propanil) at doses of 100-200 mg/kg, induced significant thymic atrophy between 2 and 7 days post exposure in mice (Cuff *et al.* 1996).

2.5 Immunopathological response

2.5.1 Humoral immune response

2.5.1.1 Body weight and organ weight

2,4-D proved slightly less toxic and there was no significant effect on the growth and organ weights in rats when compared with dogs (Hansen *et al.* 1971).

Khera and McKinley (1972) did not find any difference in the normal weight gain in prenatal and post natal studies conducted in rats with 2,4 dichlorophenoxy acetic acid and 2,4,5 trichlorophenoxy acetic acid.

2,4-D at a dietary level of 5000 ppm inhibited the growth in chicks (Whitehead and Pettigrew, 1972).

When chicks were fed 2,4-D for 8 weeks at 100, 50 and 10 ppm caused significant reduction in food intake and growth. The reduction caused by 5 ppm was not statistically significant (Whitehead, 1973).

A routine toxicological study scheme envisaged 90 day toxicity study, at the termination of which, a gross examination of all the lymphoid organs, thymus, spleen, lymphnode and bursa of Fabricius was made and later on examined for histopathological abnormalities and organ to body weight ratios (Dean *et al.* 1979).

Weights of lymphoid organs (thymus, spleen and lymph nodes) have been used as one of the general tests employed for immunotoxicological evaluation of environmental chemicals (Sharma, 1982).

The immunotoxicological effects of sodium methyl dithiocarbamate (SMD) as oral administration to mice at 300 mg/kg/day for 3, 5, 10 or 14 days decreased thymus weight at all time points; increased spleen weight after 10 or 14 days of exposure (Pruett *et al.* 1992).

Sub-chronic toxicity studies in dogs conducted on three forms of 2,4-D at doses of 1.0, 3.75 and 7.5 mg/kg/day resulted in reduction in body weight gain and food consumption (Charles *et al.* 1996).

2.5.1.2 Haematological changes

Decreased serum immunoglobulin levels were reported in laboratory animals in studies on immunotoxicologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (Sharma *et al.* 1978).

Cockerels fed on a diet containing 0, 25, 50 and 150 µg/g of lindane for 90 days did not show any alteration in the values of serum protein, bilirubin, aminotransferase, cholinesterase and protein bound iodine (Varshneya *et al.* 1986).

Subtoxic doses of malathion administered to rabbits showed slight increased TLC in non-immunised rabbits whereas the same dose showed decreased TLC in the immunised rabbits (Veena, 1987).

Cockrels given fenthion at different dietary levels showed low blood values which indicated cellular damage in the haemopoietic organs of birds and development of haemolytic anaemia (Singh *et al.* 1989).

While studying the role of bursa of Fabricius in the production of immunoglobulins in the duck by surgical, chemical, hormonal and antibursal serum methods, Nair (1990) observed significantly higher lymphocyte count between the treated (immunosuppressed) and the control group at the 4th

and 8th week. He also observed significantly lowered heterophil count in the treatment groups compared to the control. Eosinophil counts in the bursectomised ducklings were higher than in the controls, while the basophil and monocyte counts in the control and treated groups were more or less the same.

Moregaonkar *et al.* (1993) observed that BUN level in birds treated with oncol (Benfurocarb) was slightly increased on the 11th day and at the termination of the experiment. The elevated BUN level indicated adverse effect of benfurocarb on the renal function of the birds.

Propanil has been shown to cause methaemoglobulinemia and anaemia through direct action on the erythrocyte. The result of the study suggested that propanil is myelotoxic to early haemapoietic stem cells (Blyler *et al.* 1994).

Butocarboxim, a carbamate insecticide at 500 ppm level did not alter the erythrocyte count upto 45 days of feeding, but thereafter it lowered the erythrocyte counts. At 1000 and 1500 ppm levels, there was a significant reduction in the circulating erythrocytes as compared to 0 ppm. This reduction was attributed to depression of erythropoieis. Feeding of butocarboxim at all three levels significantly lowered the haemoglobin level which was considered due to anaemic

condition as a result of depressed erythropoiesis (Moregaonkar *et al.* 1994).

The chicks in the butocarboxin fed group had significantly higher heterophils on the 75th day of post treatment as compared to the control group. They opined that the heterophilia observed in the chicks fed on 1500 ppm butocarboxim diet may be because of the toxic injury to the visceral organs due to the ingestion of pesticide. The lowered lymphocyte and monocyte counts observed in the chicks were considered as manifestation of immunosuppression. However, the mean eosinophil and basophil counts were similar (Moregaonkar *et al.* 1994).

Pande *et al.* (1995) observed that repeated oral exposure of the benfurocarb had no significant effect on blood glucose, alkaline phosphatase and serum protein levels of the birds at the two treatment intervals of study.

2.5.1.3 Antibody titre

Suppression of the humoral immune response was recorded following the administration of sub-toxic doses of DDT and sevin in rats (Wasserman *et al.* 1969). Wasserman and Wasserman (1972) observed a decreased anti-Salmonella titres and decreased anti-ovalbumin titres in rabbits dosed with DDT.

Ercegovich (1973) opined that certain organophosphorus chemicals have the potential to stimulate immunologic reactions through the production of heptenic determinants by protein binding.

Carbofuran was demonstrated to cause immunosuppression in rabbits (Street and Sharma, 1975).

Street and Sharma (1975) opined that the variation in the response might result from the type and route of antigen administration and duration of insecticide treatment.

2,3,7,8 tetrachlorodibenzo-p-dioxin was reported to cause immunosuppression (Sharma *et al.* 1978).

Sharma *et al.* (1978) studied the antibody response to tetanus toxoid and serum immunoglobulin level in experimental animals, to assess the immunotoxicological effects of 2,3,7,8 tetrachlorodibenzo-p-dioxin.

Wiltrout *et al.* (1978) noted conflicting reports on the effects of repeated dosing of pesticides prior to immunization. The humoral response to bacteriophage in DDT pretreated rabbits was seen to lag than that of the control. However, by the 3rd and 4th week, the antibody titres were similar. Fan *et al.* (1978) observed the host defense in mice

dosed with carbofuran in the diet for varying times to diminish with increasing duration of the pesticide treatment.

Immunostimulation was recorded following insectide exposure (Rozakoweski (1979).

Faith *et al.* (1980) reported that secondary antibody response to tetanus toxoid was depressed only at higher dose levels of TCDD.

The antibody titre was initially reduced by 300 mg/kg body weight and severely reduced by 400 mg/kg body weight in rabbits by malathion. The immunosuppression by malathion was more pronounced immediately after malathion administration and lasted for 24 h to 7 days. The sub-toxic doses of malathion administration in general, slightly stimulated immune response in non-immunized rabbits and suppressed significantly in immunized rabbits (Veena, 1987).

Cockerels receiving lindane at 50 and 100 ppm and endosulfan at 25, 50 and 100 ppm had significantly ($P<0.05$) lower haemagglutinin titres. Highest decline was observed in cockerels receiving lindane at 100 ppm. Endosulfan produced immunosuppressive action even at the lowest dietary concentration. Malathion however produced only a slight non-significant decrease ($P>0.05$) in haemagglutinin titres at 1600 ppm (Varshneya *et al.* 1988).

Vijayan *et al.* (1990) observed stimulation of humoral immune response in ducks administered aqueous solution of furadan at 0.25 mg/kg body weight on alternate days for two months.

Trust *et al.* (1990) observed that HI titres to SRBC were lower in lead poisoned mallard ducks than in the controls, suggesting that ingested lead may have immunosuppressive effect on mallards.

Propanil (3,4-chloropropionaniline) used extensively as a post emergence herbicide in rice and wheat has been shown to affect the antibody response at a high dose of 150 mg/kg in mice (Barnett *et al.* 1992).

Pruett *et al.* (1992) observed that sodium methyldithiocarbamate (SMD) on oral administration to mice at 300 mg/kg/day for 3, 5, 10 or 14 days, did not suppress the antibody production *in vivo* of splenocyte responses to mitogens or allogenic lymphocytes.

There was a stimulatory effect on total antibody titre, IgM and IgG levels to sheep RBC antigen when malathion was fed at the rate of 22.6 mg/kg or 45.2 mg/kg for 10-20 days. However, suppressive effect was noticed at a higher dose of 90.4 mg/kg for 10-20 days (Rishi and Garge, 1993).

Indications of immunotoxic or oncogenic response were not observed by Charles *et al.* (1996) in subchronic and chronic dietary toxicity studies of 2,4-D in dogs.

2.5.1.4 Electrophoretic studies

Glick (1968) observed the absence of stained area 4-8 mm below the origin which indicated reduced immunoglobulin levels in chemically bursectomized birds, when compared with normal birds.

Rats treated with lindane or dieldrin showed a reduced response of the 7S fraction of the serum gammaglobulin after the administration of *Salmonella typhi* (Wassermann *et al.* 1969).

Morgan and Glick (1972) employed PAGE to study the immunological status in bursectomised and irradiated chickens. Chickens administered DDT or mirex showed a reduction in the level of IgG, but IgM level was elevated (Glick, 1974).

Follow up study conducted in mice dosed with carbofuran revealed that IgG was markedly depressed but the IgA and IgM levels were not greatly affected. The dosed mice were also deficient in gamma globulin compared to the controls (Fan *et al.* 1978).

2.6 Cell-mediated immune response

2.6.1 DNCB

Eliber and Morton (1970) stated that DNCB test could be considered as one of the reliable tests to assess the cellular immune response in man. DNCB test was employed for the first time to assess the cell-mediated immunity in calves (Brummerstedt *et al.* 1973). The test was also performed by Jennings in 1979. Reddi *et al.* (1981) standardised the DNCB test in cattle. Subsequently a study was also undertaken to assess the efficacy of DNCB sensitization test in evaluating the cell-mediated immune response in goats (Rajan *et al.* 1981).

Valsala *et al.* (1981) described the DNCB test for assessing the cell-mediated immune response of ducks.

Rajan *et al.* (1982) used the DNCB test for evaluating the cell-mediated immunity in pigs.

Nair (1986) evaluated the cell-mediated immunity in experimental aflatoxicosis in pigs using DNCB test.

Yadav *et al.* (1986) used DNCB test to measure the cell-mediated immune response following vaccination with goat pox vaccine.

Chaudhary (1987) employed DNCB test to measure the cell-mediated immune response in Japanese quails in experimental aspergillosis.

Haynls and Chubb (1987) observed that Delayed Type Hypersensitivity (DTH) to DNCB could be induced by a single painting of the skin with 1 mg of DNCB/bird without adjuvant and could be elicited at a challenge site with 50/μg DNCB in chicken. The sensitization lasted for several weeks and was mediated by sensitized cells. Doses above 1 mg/bird induced some form of suppression.

Ashturkar *et al.* (1989) used the DNCB test to assess the cellular immunity in experimental diaphragmatic hernioplasty in buffalo calves.

CMI was assessed by DNFB skin sensitivity in cypermethrin pesticide toxicity in goats and found that CMI was lower compared with the control (Jha *et al.* 1989).

Sreeramalu and Krishnaswamy (1989) evaluated the cell-mediated immunity using DNCB in experimentally infected lambs with *Mycoplasma arginini* cell antigen.

Balakrishnan (1992) employed DNCB to assess the cell-mediated immune response in ducks fed on aflatoxin.

Farshid and Rajan (1996) reported significant reduction in CMI in quails fed on ochratoxin employing DNCB test.

Delayed type hypersensitivity was utilised to assess the immunosuppressive effect of the pesticide, fenitrothion in rats (Kunimatsu *et al.* 1996).

Remadevi *et al.* (1996) observed a cellular response consisting of oedema and infiltration of heterophils, lymphocytes, monocytes and macrophages in the DNCB hypersensitivity test in chicken fed with ochratoxin.

2.6.2 Skin sensitivity to PHA-M

Nowell (1960) observed the usefulness of phytohaemagglutinin (PHA), an extract of the red kidney bean *Phaseolus vulgaris*, for inducing lymphocyte transformation.

Janossy and Greaves (1971) recognized PHA as a T-cell specific mitogen.

2, 3, 7, 8 tetrachloro dibenzo-p-dioxin (TCDD) when added directly into the splenic lymphocytic culture, a dose related reduction in lymphocyte transformation to PHA was observed. These reports, therefore, indicated that mice and rats when exposed to TCDD pre-natally and post-natally suppressesed several cell-mediated immune functions including *in vitro* response of the thymic and splenic lymphocytes to T-cell

mitogen, (Faith and Moore, 1977). Zuckerman and LoBugli (1977) used PHA as a skin test for the evaluation of cellular immunocompetence in normal and cancer patients respectively.

Marchalonis (1978) employed PHA in evaluating the cell-mediated immunity in man since PHA caused a direct reaction without prior sensitization.

Sharma *et al.* (1978) reported decreased lympho-proliferative response to PHA as a result of immunotoxicologic effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Haggard *et al.* (1980) reported intradermal PHA response in experimental iodine toxicosis in young cattle.

Thein *et al.* (1981) employed both *in vitro* and *in vivo* PHA tests to assess the cell-mediated immunity in horses. They reported that the response to PHA was an indication of delayed type of hypersensitivity reaction.

Reddy and Rajan (1984) employed PHA intradermally to assess the cell-mediated immunity in cattle bearing carcinoma of the ethmoid mucosa.

Significant reduction in CMI in quails fed ochratoxin was reported based on the PHA response (Farshid and Rajan, 1996).

2.6.3 Leucocyte migration inhibition test (LMIT)

Tompkins *et al.* (1970) demonstrated the application of the macrophage migration inhibition test in assessing the cellular immunity induced by Fibroma virus.

Falecka (1979) assessed the cell mediated immunity using LMIT in guinea pigs vaccinated with measles virus.

Hussain and Mohanty (1979) assessed the cell-mediated immunity in bovine Rhinovirus type-1 infection in calves employing LMIT.

Kantoch *et al.* (1979) studied the cell-mediated immune response to measles and mumps viruses in monkey and guinea pigs using LMIT.

McCorkle and Simmons (1984) studied the ability of peripheral blood leukocytes from young poults to migrate *in vitro* using LMIT.

Haynls and Chubb (1987) opined that the migration inhibition test could be used as an *in vitro* method to measure the DTH response and they then correlated the results with DTH skin response.

Chandrasekhar *et al.* (1989) demonstrated the cell-mediated immune response to Ranikhet disease vaccine in chicks by employing LMIT.

LMIT was used in the assessment of immune response of guinea pigs during experimental leptospirosis (Ramakrishna *et al.* 1990).

Farshid and Rajan (1996) reported severe reduction in the cellular immunity in OA fed birds using LMIT.

2.6.4 Graft vs. Host Reaction (GVHR)

2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) when added directly into the splenic lymphocytic culture, a dose related reduction in lymphocyte transformation to PHA was observed indicating suppression of cell-mediated immune functions, including, delayed hypersensitivity reactions, graft vs. host reactivity of the spleen cells (Faith and Moore, 1977).

In mice even a low dose level of TCDD depressesed the graft Vs host reactivity (Faith and Moore, 1977). Cypermethrin at the rate of 30 mg/kg body weight caused suppression of the cell-mediated immunity which was assessed by GVHR (Jha *et al.* 1989).

Singh *et al.* (1990) evaluated the cell-mediated immunity in broiler chicks during chronic ochratoxicosis by using GVHR,

and found the test to be of significance in assessing the cell-mediated immunity.

Ghosh *et al.* (1991) employed GVHR as a parameter to assess the cell-mediated immune status of broiler chicks given aflatoxin B_1. They observed suppression of the cell-mediated immunity.

Farshid and Rajan (1996) employed GVHR to assess the cell-mediated immune functions in quails fed on ochratoxins.

2.7 Gross pathology

2.7.1 Herbicides

Dogs killed by 2,4-D showed bleeding gums and necrotic changes in the buccal mucosa (Drill and Heratzka, 1953).

Rats killed by 2,4-D showed irritation of the stomach, minor liver and kidney injury and sometimes congestion of the lungs (Rowe and Hymas, 1954).

In one case of fatal poisoning in man by 2,4-D autopsy findings were few and not diagnostic. Congestion of all the organs and severe degenerative changes of the brain ganglion cells were observed (Neelsen *et al.* 1965).

Degenerative changes of the kidney tubules and liver and severe congestion of the upper gastrointestinal tract were reported by Dudley and Thapar (1972) in fatal human ingestion of 2,4-D.

Konstantinova *et al.* (1975) found that an oral dosage of 2,4-D as high as 50 mg/kg did not increase embryonal mortality or malformation, but it did increase the proportion of foetuses with haemorrhage of the internal organs.

A routine toxicology study scheme envisaged 90 day toxicity study, at the termination of which a gross examination of all the lymphoid organs, thymus, spleen, lymph node and bursa of Fabricius is made and later on examined for histopathological abnormalities and organ to body weight ratio (Dean *et al.* 1979).

In a two year study in rats, a diet containing 2,4-5-trichlorophenoxyacetic acid (2,4,5-T) at a dosage of 30 mg kg/day reduced the growth without reducing the feed intake or survival. This dosage also increased kidney weight and mineralised deposits were seen in the renal pelvis. A dosage of 10 mg kg/day produced minimal effects, notably mineralised deposits in the renal pelvis (Kociba *et al.* 1979).

2,6-dichlorobenzonitrite (dichobenil) fed to broiler and White Leghorn chicks at a dietary concentration of 75, 150 and

225 mg/kg of feed for 2 months, did not affect feed consumption or growth, but it resulted in heavier kidneys and livers (Davison and Bakke, 1988).

The herbicide 3,4-dichloropropionanitide (propanil) at the dose rate of 100-200 mg/kg, induced significant thymic atrophy between 2 and 7 days post exposure in mice (Cuff *et al.* 1996).

2.7.2 Pesticides

Increased atrophy of the cortex of the thymus was noticed in rabbits treated with carbofuran (Street and Sharma, 1975).

Lymphoid follicles showed necrotic and hyperplastic changes and the liver showed degenerative changes. Adrenal cortex revealed regenerative lesions whereas thyroid did not show any gross changes in a toxicological evaluation of dietary lindane in cockerels (Varshneya *et al.* 1986).

The carcasses of 17 rams died of acute poisoning by maize seed which had been dressed with carbofuran were hyperaemic and there were degenerative changes in the internal organs (Topalski *et al.* 1987).

Sodium methyldithocarbamate (SMD) on oral administration to mice at 300 mg/kg/day for 3, 5, 10 or 14 days decreased the

thymus weight at all time points, increased spleen weight after 10 or 14 days of exposure (Pruett *et al.* 1992).

2.8 Histopathology

2.8.1 Herbicides

Degeneration of the convoluted tubules, protein in the glomerular spaces, and a little fatty degeneration of the kidneys were the only lesion found in the body of a man who survived after intentional drinking of 2,4-D (Curry, 1962). Severe degenerative changes of the brain ganglion cells, perhaps due to anoxia were found in one case of fatal poisoning in man by 2,4-D (Nielsen *et al.* 1965).

Degenerative changes of the kidney tubules and liver and severe congestion of the upper gastrointestinal tract were reported by Dudley and Thapar (1972). They observed especially in perivascular areas acute demyelination, occasionally with central petechiae confined almost entirely to the white matter. But the interpretation of the lesion was complicated by the fact that hypoxia and/or the atrophic state of the brain could not be excluded as causes.

Histopathological examination of the thymus revealed characteristic depletion of lymphocytes in the cortex while

thymic medullary region was unaffected in TCDD toxicity (Faith *et al.* 1980).

In an experimental study in dogs 2,4-D did not show any severe histopathological atterations (Charles *et al.* 1996).

A decrease in the cellularity was observed histologically in the thymus in mice exposed to 3,4-Dichloropropionamilide (propainl) (Cuff *et al.* 1996).

2.8.2 Pesticides

Rabbits treated with DDT, carbaryl, carbofuran, methyl parathion had decreased counts of activated lymphocytes in the lymph nodes, reduced number of germinal centres in the spleen and more pronounced atrophy of the cortex of the thymus (Street and Sharma, 1975).

The following changes were noticed in cockerels which were fed on a diet containing different concentrations of lindane for a period of 90 days. Liver showed degenerative and necrotic changes. Lymphoid follicles were necrotic and hyperplastic changes were also evident. Degenerative changes were noticed in the adrenal cortex whereas no changes were observed in the thyroid (Varshney *et al.* 1986).

One day old chicks, fed with subacute doses of DDT (10 mg of 5 per cent W.P./bird) showed degenerative changes in the

liver and in the renal tubules of the kidney. The absorptive layers of the intestine were disrupted (Ramalingam, 1987).

The *in vitro* cytopathic effects of cypermethrin on goat kidney cell culture consisted of rounding of cells, reduction in their size and balooning degeneration. There was also vacuolation of the cytoplasm, syncitial cell formation, pyknosis, karyorrhexis of the nuclei, decrease in cellular density and pleomorphism (Gupta *et al.* 1989).

Significant decrease in mature lymphocyte subpopulations which were greater in thymus than in the spleen were noticed in mice exposed to sodium methyldithocarbamate (Pruett *et al.* 1992).

2.8.3 Skin hypersensitivity to DNCB and PHA

The histological appearance of the skin sensitisation in delayed type hypersensitivity to DNCB in chicken was characterised by the infiltration of large number of granulocytes, especially heterophils and some eosinophils, as well as large number of monocytes (Haynes and Chubb, 1987).

Balakrishnan (1992) observed diffuse congestion and haemorrhages with infiltration of mononuclear cells along with lymphocytes and macrophages. The intensity of the reaction was more in the control ducks than in the ducks fed an

aflatoxin at 24 h. The intensity of the reaction reduced during 48 h and 72 h.

The cell-mediated immune response to PHA-M histologically revealed congestion and oedema of capillaries, infiltration of monocytes, macrophages and lymphocytes, during the first 24 h in both control and aflatoxin fed ducks, but the control group showed more cellular response than the treatment groups (Balakrishnan, 1992).

In the DNCB hypersensitivity skin test, a cellular response consisting of oedema and infiltration of heterophils, lymphocytes, monocytes and macrophages was observed histologically in the skin lesions of control birds whereas the response was slightly reduced in ochratoxin fed broiler chicken (Remadevi *et al.* 1996).

There was highly significant reduction in the skin thickness of OA fed birds in the cutaneous response to DNCB. There was also decreased response to PHA-M in OA fed birds (Farshid and Rajan, 1996).

2.9 Ultrastructural pathology

Maul *et al.* (1971) found a doubling of the average number of nuclear pores when lymphocytes were stimulated with phytohaemagglutinin. Karasek *et al.* (1972) suggested that an

increase in the number of perichromatin granules may be an indicator of abberations in protein synthesis.

Ultrastructurally liver changes were noticed in the mice prenatally exposed to the cholinesterase inhibitor, carbofuran. Electron microscopy revealed a dose related increase in the sinusoids filled with debris and in the number of actively phagocytizing Kupfer cells. The controls showed mostly empty sinusoids with few apparently inactive Kupfer cells. The debris was composed of free ribosomes, smooth ER and proteinaecous material (Hoberman, 1978).

During activation of lymphocytes, it was observed that there was transformation of heterochromatin to euchromatin. During the blastic transformation, the nucleus enlarged two to four fold, most of the heterochromatin was converted to euchromatin and polyribosomes were seen in the cytoplasm. Disaggregation of polyribosomes was described as an indication of impaired export protein production or depressed endogenous protein production (Ghadially, 1982).

Lalithakunjamma (1987) opined that mitochondrial damage could initiate a series of cellular changes resulting in the death of cells, defective growth and proliferation.

The ultrastructural studies of the bursa in ochratoxin fed quails showed large number of lymphoid cells with

cytological alterations indicative of cell death. Some of the cells showed only a lytic cytoplasm with a bit of condensed nucleus. The packing of lymphocytes was very loose with large spaces between them indicating that there had been cell loss. Definite indications of transformation into plasmacytoid series were lacking (Farshid, 1995).

On ultrastructural studies of the spleen in the above work lymphoid cells and macrophages with large vacuolated areas and ruptured plasma membrane were seen. Many lymphoid cells showed a homogenous organellar free cytoplasm. The nucleus of some of the lymphoid cells were represented only by the envelop in which the outer and inner membrane could not be distinguished and only some floculant aggregates of chromatin were seen (Farshid, 1995).

Ultrastructural alterations were not uniform in the lymphoid cells of the thymus. Cells with blastoid features as well as those with mature characteristics were seen. Most of these cells showed condensation of the nucleoli as a compact mass along with chromatin particles. In a few, there was segregation of the granular and fibrillar components forming delineated structures. Both in the immature and mature cells, clumping of chromatin along the inner nuclear membrane was often found. Fragmentation of the nucleus was a constant feature. Occasionally, the perinuclear cisterna was found

very prominent. Vacuoles of different sizes were found in some cells. Eventhough, a few free ribosomes were noticed in the cytoplasm, presence of rough surfaced ER was not a feature of these cells. Lysosomes were very scanty. The thymic tissue had increased amount of fibrous tissue which was oedematous in many locations (Farshid, 1995).

Vyas (1997) observed membrane alterations, mild to severe mitochondrial changes, ribosomal detachment and fragmentation of rough endoplasmic reticulum, nucleolar and nuclear changes indicative of cell damage, ultrastructurally in studies conducted on the effect of ochratoxin and mercury and cadmium metals on the lymphoid organs in ducks.

Materials and Methods

MATERIALS AND METHODS

The experiment was designed to study the following:

1. Teratogenic and embryotoxic studies on exposure to carbofuran and 2,4-D (2,4-dichlorophenoxy acetic acid) in duck embryos with special reference to the lymphoid system.

2. Immunotoxic effects of sub-lethal doses of carbofuran and 2,4-D with particular reference to the humoral and cell-mediated immune system.

3.1 The agrochemicals

Carbofuran (Technical grade) was procured from Rallis India. 2,4-D was procured from the local market.

3.2 Teratogenic and embryotoxic studies

Five hundred hatching eggs were procured from the Government Regional Duck Farm, Niranam. The eggs were fumigated and the surface was cleaned with an antiseptic lotion. The eggs were randomly divided into four groups, A, B, C and D.

Treatment

Group A: Eggs were immersed in 0.5 per cent aqueous solution of carbofuran for 30 seconds.

Group B: Eggs were immersed in 1 per cent aqueous solution of 2,4-D for 30 seconds.

Group C: Eggs were immersed in distilled water for 30 seconds.

Group D: Eggs were not given any treatment and served as control.

The eggs were incubated at 37°C at a relative humidity ranging from 70 to 75 per cent. The eggs were candled on the 4th day and all infertile eggs were removed. The eggs were further candled once in two days and the dead embryos were removed and examined for any gross abnormality.

Six embryos from each group were sacrificed on the 15th and 21st day of incubation. The embryos were weighed and examined for any gross abnormalities. The embryos were dissected and the lymphoid organs like the bursa, spleen and thymus were collected and weighed. The lymphoid organs were fixed in 5 per cent neutral buffered formalin for histopathological studies. Very small bits of spleen, thymus

and bursa were also collected in 3 per cent glutaraldehyde for ultrastructural studies.

The remaining embryos were allowed to hatch and the hatchability was recorded. The hatched out ducklings were weighed, sacrificed and collected the lymphoid organs. The bursa, spleen and thymus were weighed and representative samples of tissues were fixed in 5 per cent neutral buffered formalin and 3 per cent glutaraldehyde for histopathological and ultrastructural studies respectively.

3.3 Immunotoxic studies

3.3.1 Experimental birds

One hundred randomly selected one month old ducklings of both sexes were procured from the duck farm Niranam. The ducklings were maintained on standard diet free of aflatoxin.

The ducklings were randomly divided into six groups A_1, A_2, B_1, B_2 and C_1 and C_2 each group consisting of 16 birds.

3.3.2 Feeding of the chemicals

Group A was administered carbofuran in water orally at a dose rate of 0.06 mg/kg body weight daily till they were sacrificed.

Group B was administered 2,4-D, in water orally at a dose rate of 0.1 mg/kg body weight daily till they were sacrificed.

Groups A_1 and B_1 were fed the chemicals for 6 weeks and groups A_2 and B_2 for 10 weeks.

3.3.3 Humoral immune response

3.3.3.1 The ducklings were weighed before the commencement of the experiment and at fortnightly intervals during the experiment.

3.3.3.2 The haematological parameters like haemoglobin, total leucocyte count (TLC), differential leuckocyte count (DLC), serum protein, albumin, globulin, albumin-globulin ratio were estimated before commencement and on the 1st, 3rd and 5th fortnights.

Haemoglobin was estimated by Sahli's method (Schalm *et al.* 1975). The TLC and DLC were determined as per the method described by Sastry (1983). Total protein in the serum was estimated by the Biuret method colorimetrically (Weichselbaum, 1946) and albumin was estimated by Bromcresol green method (Doumas *et al.* 1971). The kit was procured from Boehringer, Mannheim. The globulin was estimated by substracting the value of albumin from the total protein. Albumin-globulin ratio was also calculated.

3.3.3.3 The birds of A_1, B_1, and C_1, were primed with LaSota strain of Ranikhet disease vaccine after three weeks of the treatments and administered R_2B strain of RD vaccine after one week of priming. The blood was collected after 14 days for estimation of antibody titre.

The birds of groups A_2, B_2 and C_2 were primed with LaSota strain of RD vaccine at the 7th week of the treatment and R_2B strain of RD vaccine after one week of priming. The blood was collected after 14 days for estimation of the antibody titre.

Haemagglutination and Haemagglutination inhibition tests were carried out using the microtitre method as described by Gulka *et al.* (1982) using 0.25 ml volume of virus and serum in two fold dilutions to monitor the antibody titre.

3.3.3.4 Serum protein fractions

Polyacrylamide Gel Electrophoresis (PAGE)

The pooled serum samples were subjected to PAGE to study the serum protein fractions. The PAGE was performed as per the method described by Davis (1964).

3.3.4 Cell-mediated immune response

3.3.4.1 Delayed type hypersensitivity to DNCB

The response to DNCB after 3 weeks and 7 weeks of feeding the chemicals was determined by the method adopted by Valsala *et al.* (1981)

Approximately 4-5 cm^2, relatively featherless elliptical area was chosen on the left and right side of the abdomen. The areas were first cleaned with alcohol and then 0.25 ml of the sensitizing dose of 1 per cent DNCB (Loba, India) in acetone was applied to each site. This was dropped slowly drop by drop and allowed to evaporate quickly by blowing. The area was demarcated by holding a metal ring on to the skin at the site. The metal ring was kept at the site until the solution evaporated. The birds were challenged with 1 per cent DNCB in acetone on the 14th day.

The reaction was assessed by measuring the skin thickness at the site of the test with the help of a vernier caliper. The measurement of the skin at the site of application was done at 24, 48 and 72 h post challenge. The challenged area of the skin was examined for erythema, induration and vesication. Skin biopsies were taken at each 24, 48 and 72 h post challenge and were fixed in 5 per cent buffered neutral formalin. Paraffin sections were cut at 4 μ thickness and

stained with Haematoxylin and Eosin stain (Scheeham and Hrapchak, 1980) and the histological changes were studied.

3.3.4.2 Delayed type hypersensitivity to PHA-M

Cutaneous hypersensitivity to PHA-M as described by Rajan *et al.* (1982) was assessed in six ducklings after 3 weeks and 6 weeks of feeding the chemicals.

Five micrograms of PHA-M (Difco Lab, USA) were dissolved in 0.1 ml distilled water. The feathers over the left and right side of the abdomen were plucked. Two sites were marked for injecting PHA-M. The thickness of the skin was measured using a vernier caliper. At each site, 0.05 ml of distilled water containing 2.5 μ of PHA-M was administered intradermally. The skin thickness at these sites were measured at 24, 48 and 72 h. Skin biopsies were taken at the same intervals and fixed in neutral buffered formalin for histopathological studies.

3.3.4.3 Leucocyte migration inhibition test (LMIT)

LMIT as described by Mecoy *et al.* (1976) with minor modifications was conducted in the 3rd, 6th, 8th and 10th week of feeding the chemicals.

Five millilitre of blood was collected from 6 ducks from the jugular vein in sterile syringe containing 20 IU of sodium

heparin per ml of blood and then transferred to clean sterile tubes.

Four millilitre of ficoll paque (Pharmacia, Sweden) was taken in each tube, over which 5 ml of blood was slowly layered from the wall of the tubes. The tubes were centrifuged in refrigerated centrifuge at 600xg for 20 minutes.

The supernatent was removed gently by a Pasteur pipette and resuspended in RPMI-1640 tissue culture medium (Sigma). Leucocytes so separated were washed in RPMI-1640, thrice at 300xg for one minute each. The cell concentration was adjusted to $2x10^7$ viable leukocytes per ml. The capillaries (1 mm diameter) were charged with the cell suspension, and one end of the capillary tube was sealed with plasticin, capillaries were centrifuged at 300xg for 5 minutes. Each capillary was cut with a file at 1/3 of the way below the cell pellet-liquid interface. The capillaries were immediately placed with the help of non-toxic grease in the migration chamber (Laxbro, India). The capillaries were charged with RPMI 1640 medium antigen (8 HA units of ND antigen) and other chambers without antigen for the replicate samples. The chambers were covered with coverslips, with the help of non-toxic grease and incubated on level shelf at 37°C for 18 h in a humid chamber.

The areas were examined under low magnification (40x). The migration index values were calculated as per the formula given below:

$$M.I. = \frac{\text{Area of spread of leukocytes in the presence of Ag}}{\text{Area of spread of leukocytes in the absence of Ag}} \times 100$$

3.3.4.4 Graft Vs. Host Reaction (GVHR)

GVHR was assessed on the 6th and 10th week of administration of the chemicals. The method described by Singh *et al.* (1990) was adopted.

Thirty millilitre of blood was collected from five two month old chicken through the cardiac route in sterile syringe containing 20 IU of sodium heparin per ml of blood, and then transferred to clean sterile tubes. Four millilitre of Ficoll paque was taken in each tube over which 3 ml of blood was slowly layered from the wall of the tube. The tubes were centrifuged in refrigerated centrifuge at 600xg for 20 minutes. The lymphocytes separated were then washed three times with chilled RPMI 1640 tissue culture medium at 300xg for 10 minutes each, and finally prepared as 15 per cent (v/v) suspension. An aliquot (0.2 ml) of cell suspension was injected intravenously into six ducks of A, B and C. The

inoculated birds were weighed and sacrificed 96 h later and their spleens were weighed individually.

The spleen index was calculated by the following formula.

$$\text{Spleen index} = \frac{\text{Spleen weight in g}}{\text{Body weight in g}} \times 100$$

3.4 Gross and histopathology

Birds died as well as the birds which were sacrificed were subjected to detailed post mortem examination and gross pathological changes if any, were recorded. Tissues from the spleen, thymus, bursa, liver, kidney and brain were fixed in 10 per cent neutral buffered formalin and were processed by the routine method and embeded in paraffin. Sections were cut at 4-5 μ thickness and stained with haematoxyline and eosin as described by Scheeham and Hrapchak (1980).

3.5 Ultrastructural studies

The three per cent glutaraldehyde fixed tissues were washed three times in phosphate buffer (pH 7.4) and post fixed in 1 per cent buffered osmium tetroxide (Sigma, USA) at 4°C for 2 h. They were then dehydrated in graded acetone and embedded in spurr, low viscosity embedding resin (Bio-Rad

Microsciences Division, USA). Ultrathin sections were taken on copper grids and stained with uranyl acetate and lead citrate (both Sigma products, USA) and examined in an Electron Microscope (Hitachi 600 A) at 75 KV and the electron micrographs were taken.

3.6 Statistical analysis

Statistical analysis of the data were carried out using analysis of variance technique as given by Snedecor and Cochran (1967). Design adopted were completely randomised design.

Results

RESULTS

4.1 Studies on the embryo

The effects of the agro-chemicals on the development of the duck embryos were studied. The embryos were sacrificed on the 15th and 21st day and hatched out ducklings on the 28th day. The percentage of defects noticed on the 15th and 21st days are given in Table 1. The studies conducted on the 15th day revealed early embryonic mortality of 33.36 per cent in the group B treated with 2,4-D and 26.7 per cent in the group A treated with carbofuran whereas group C treated with distilled water and group D (control) had 13.3 per cent each.

In the group A and B 13.3 per cent and 6.6 per cent of the embryos respectively had stunted growth. Generalised oedema was present in 20 per cent of 2,4-D treated embryos and 13.3 per cent of carbofuran treated embryos.

Observations on the sacrificed embryos on the 21st day revealed a mortality of 26.7 per cent in the B-group and 20 per cent in group A whereas 6.6 per cent was noticed in C and D. In the groups `A' and `B' 13.3 per cent showed mild to moderate degrees of stunted growth. Generalised oedema especially at the region of the head and neck was noticed in

26.7 per cent of the `B' group and in 20.4 per cent of the `A' group whereas 6.6 per cent of the embryos from group `C' also showed oedematous changes.

Percentage of defects and the hatchability recorded on the 28th day are shown in Table 2.

Dead in shell was more in group A (33.33%) whereas it was 31.41 per cent in group `B'. Group `C' and `D' showed 13.9 per cent and 10 per cent dead in shell respectively. Dwarf embryos were present in 6.5 per cent of the group `B' and in 3.3 per cent of the group `A' (Fig.1 and 2). Whereas there was no dwarf embryos noticed in group `C' and D. Generalised oedema was noticed in 10 per cent, 12.9 per cent and 2.7 per cent in groups A, B and C respectively (Fig.3). Some of the embryos of group A and B also showed tendency for herniation of the yolk sac (Fig.4).

Hatchability recorded were 48.8 per cent, 50 per cent, 66.6 per cent and 70 per cent in groups A, B, C and D respectively.

4.1.1 Body weight and organ weight

The mean body and lymphoid organ weight of the embryos sacrificed on the 15th day are given in Table 3.

Table 1. Percentage of defects noticed in the embryos sacrificed on day 15 and 21

Group	Embryo mortality		Stunted growth		Generalised oedema		Other defects
	15	21	15	21	15	21	
A	26.7	20.0	13.3	13.3	13.3	20.4	
B	33.36	26.70	6.60	13.30	20.0	26.7	
C	13.33	-	-	-	6.6	6.6	
D	13.30	6.6	-	-	-	-	

Table 2. Percentage of defects noticed in the embryos sacrificed on the 28th day

Group	Dead in shell	Dwarf embryo	Generalised oedema	Other defects	Hatchability
A	33.33	3.3	10.0	Omphalitis	48.8
B	31.41	6.5	12.9	Omphalitis	50.0
C	13.9	-	2.7	-	66.6
D	10.0	-	-	-	70.0

The body weight and the lymphoid organ weight did not show any significant difference between the different groups on the 15th day.

The mean body weight and lymphoid organ weight of the embryos sacrificed on the day 21 are given in Table 4.

There was no significant difference noticed among the groups compared with the control, in the body weight, and weight of the bursa and thymus. But the spleen of the group `A' showed significant reduction in the weight at 5 per cent level.

The mean of the body and lymphoid organ weight of the embryos sacrificed on the 28th day are given in Table 5 and Fig. 5, 6, 7 and 8.

The body weight of group `A' showed significant reduction compared to the control at 1 per cent level of significance. Spleen of group `A' and `B' showed significant reduction in weight at 1 per cent level whereas C and D groups did not show any statistically significant variation.

The weight of the bursa did not show any significant difference between the different groups. The thymus of group `A' and `B' showed significant difference at 5 per cent level

Table 3. Averages (Mean±SE) of the body and lymphoid organ weight of the embryos sacrificed on the 15th day

Group	Body weight (g)	Spleen (mg)	Bursa (mg)	Thymus (mg)
A	11.18± 0.71	11.11± 0.93	20.27± 1.09	36.26± 1.26
B	11.58± 0.74	11.33± 0.77	20.90± 0.90	35.35± 1.15
C	11.65± 0.94	11.80± 0.84	21.30± 0.63	35.81± 1.04
D	11.58± 0.71	11.90± 0.33	21.68± 0.66	36.35± 1.16

Table 4. Averages (Mean±SE) of the body and lymphoid organ weight of the embryos sacrificed on the 21st day

Group	Body weight (g)	Spleen (mg)	Bursa (mg)	Thymus (mg)
A	21.00± 3.52	15.928± 0.828 *	35.591± 2.323	54.361± 2.506
B	23.966± 1.74	17.380± 1.112	35.825± 1.202	55.741± 2.134
C	23.050± 1.136	17.495± 0.873	37.425± 2.820	57.203± 1.420
D	23.650± 2.70	17.611± 0.740	36.466± 0.786	57.55± 1.859

* ($P<0.05$)

Table 5. Averages (Mean±SE) of the body and lymphoid organ weight of the embryos sacrificed on the 28th day

Group	Body weight (g)	Spleen (mg)	Bursa (mg)	Thymus (mg)
A	37.155± 1.351 **	25.038± 0.932 **	51.983± 1.043	62.258± 1.769 *
B	37.91± 1.36	25.861± 1.651 **	52.827± 0.792	61.895± 0.893 *
C	38.07± 1.496	26.518± 1.054	52.438± 1.635	63.46± 1.580
D	38.35± 1.270	26.687± 1.521	54.102± 1.519	64.203± 1.042

* (P<0.05)
** (P<0.01)

Table 6. Averages (Mean±SE) of the body weight recorded before the commencement and at the first, third and fifth fortnight

Group	Before the experiment	1st F.N. (g)	3rd F.N. (g)	5th F.N. (g)
A	347.916± 45.798	504.167± 54.181	739.583± 50.518 *	1121.429± 41.905 **
B	358.333± 45.643	502.917± 35.385	796.25± 39.435	1272.714± 54.80
C	355.00± 37.295	533.083± 35.155	805.833± 39.418	1284.375± 35.197

* (P<0.05)
** (P<0.01)

Fig: 5 Average body weight of the embryos sacrificed on the 28th day

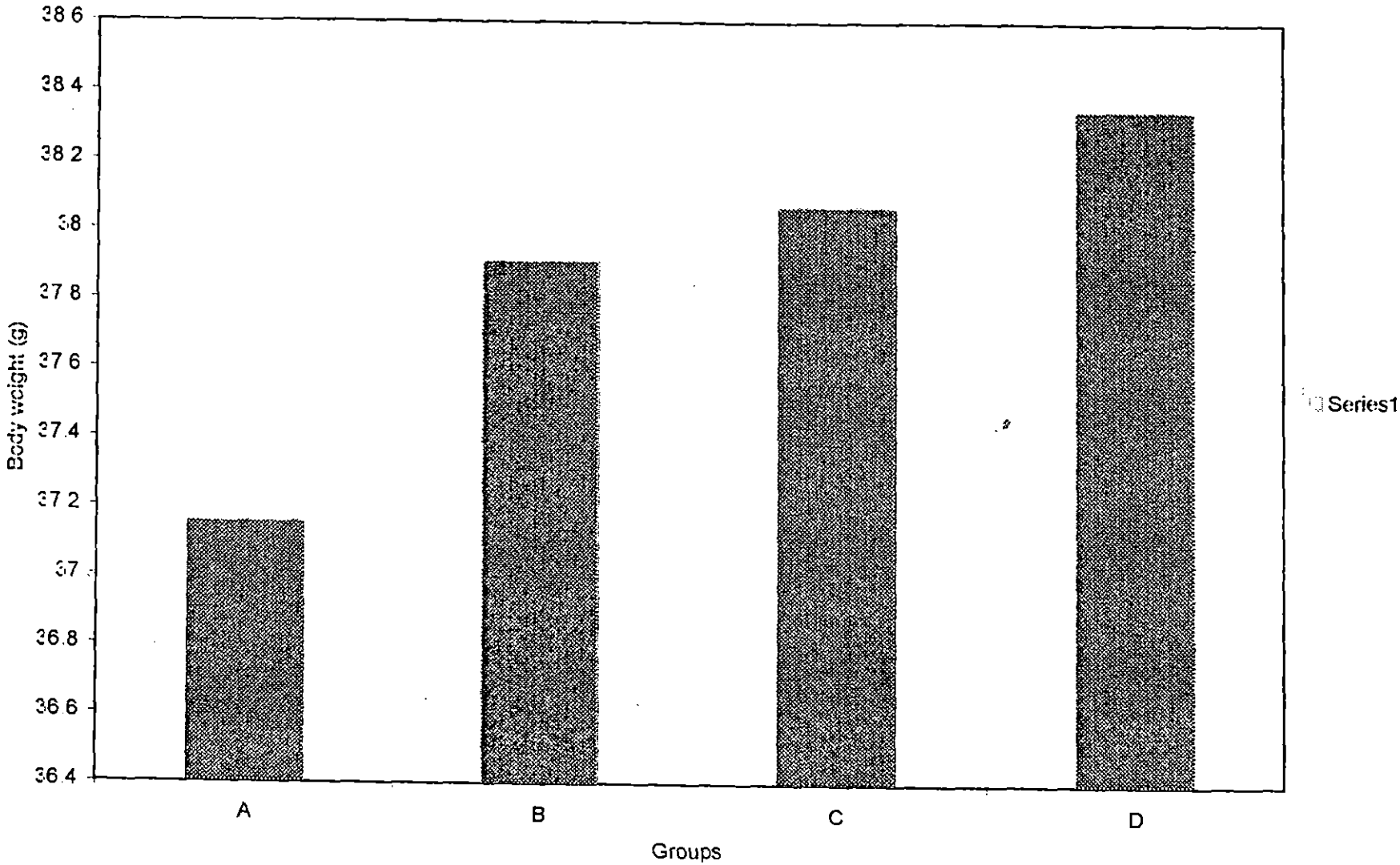


Fig: 6 Average weight of the spleen of embryos on the 28th day

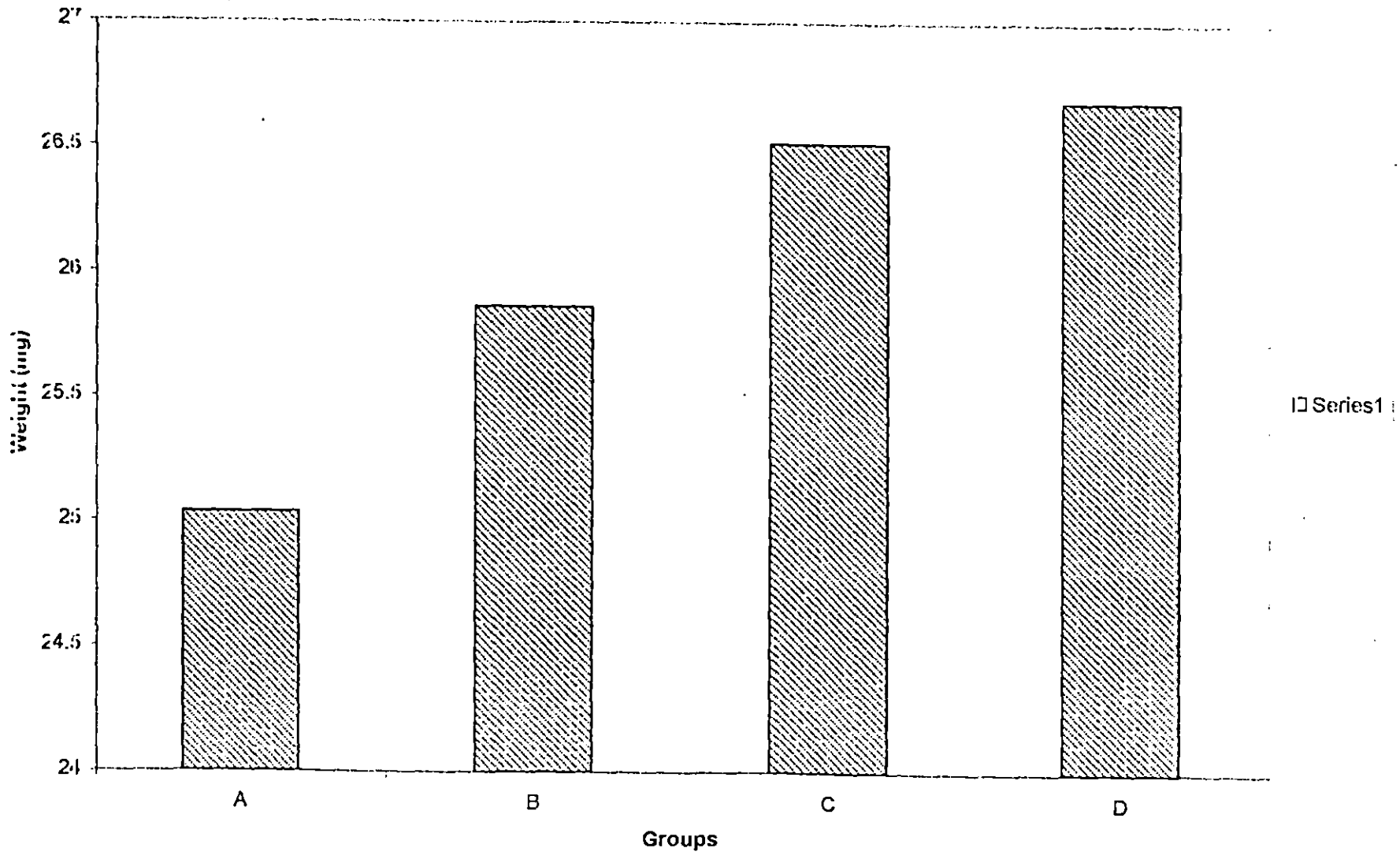


Fig.7 Average weight of the bursa of embryos on the 28th day

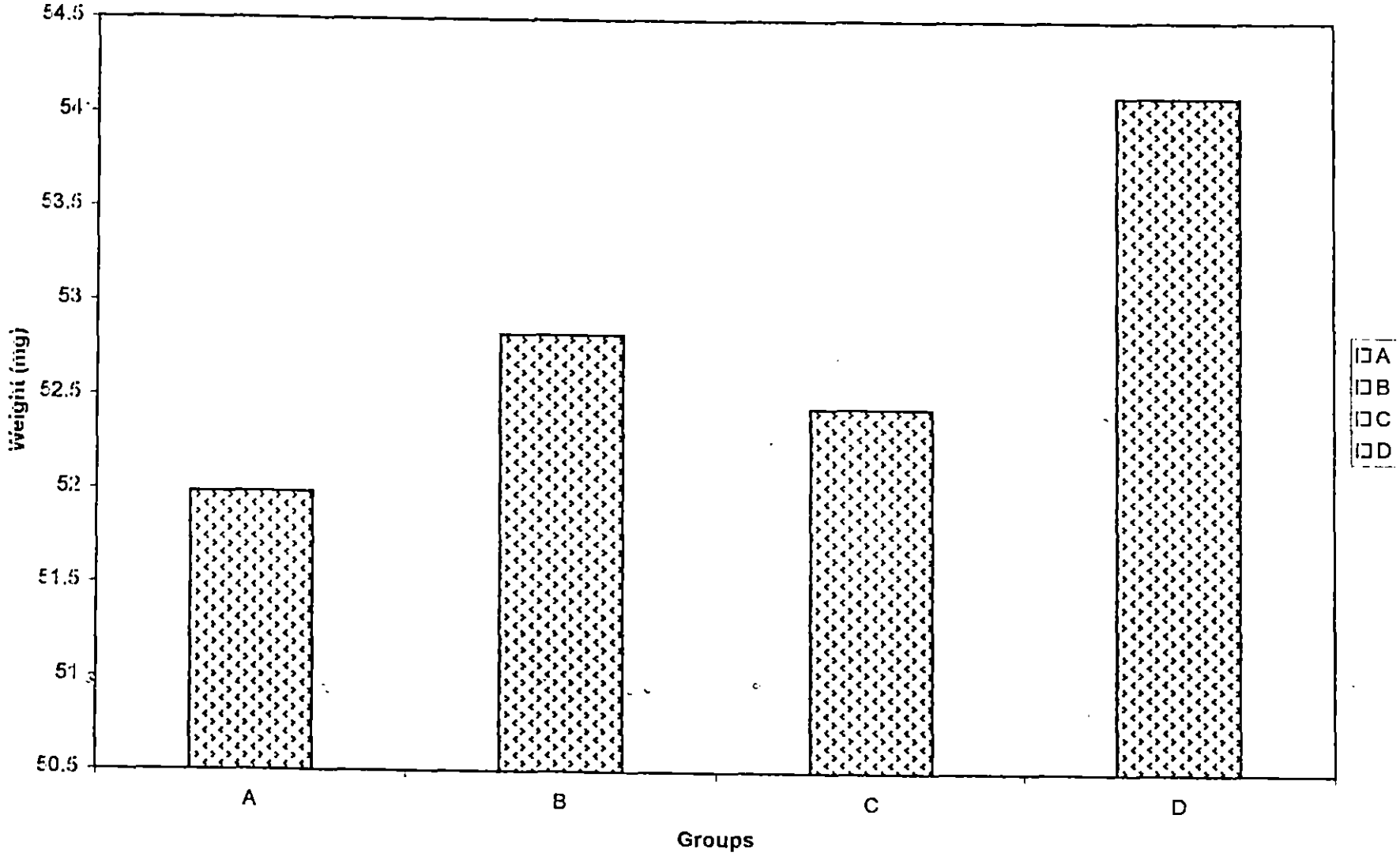
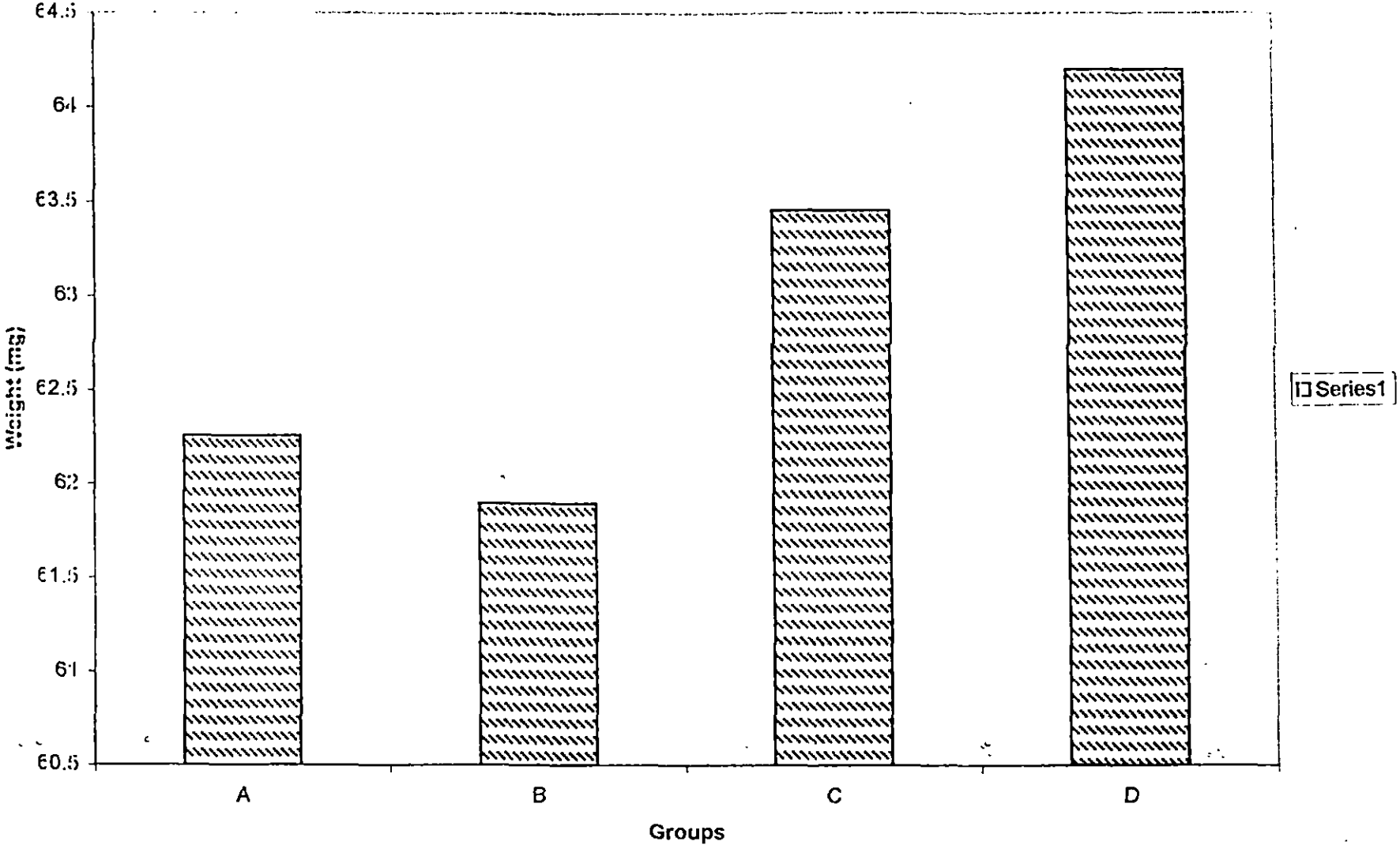


Fig: 8 Average weight of the thymus of embryos on the 28th day



when compared with `C' and `D'. But it was not significant at 1 per cent level.

4.1.2 Gross and histopathology

Bursa of Fabricius

The bursa of Fabricius did not show any difference in the size. No gross difference was noticed between A, B, C and D groups on day 15, 21 and 28.

Histologically, the bursa of all the groups on the days 15 and 21 revealed very less cellularity and the scattered lymphoid cells showed tendency to dissociate. But on the day 28, the bursa of the group `A' showed interstitial oedema, and the lymphoid cells were very loosely arranged (Fig.9). Some of the follicles showed moderate degree of lymphoblastic activity. The epithelial cells showed vacuolar degenerative changes and tendency for denudation. Mild degree of stromal proliferation was also evident.

Spleen

There was no gross morphological alterations between the different groups on days 15, 21 and 28.

The spleen from the embryos sacrificed on day 15 and 21 showed very less cellularity histologically. The trabeculae

were not well developed. Spleen from the embryos of group `A' sacrificed on the 28th day revealed very scanty lymphoid cells. There was no evidence of germinal centre in the follicles. The walls of the blood vessels appeared relatively thickened compared to the control.

Spleen from the embryos of the `B' group sacrificed on the 28th day showed evidence of germinal centre in the follicles but the lymphoid cells were very few. However, it was more when compared with that of the group A.

Spleen from the embryos of group `A' sacrificed on the 21st day showed mild degree of congestion.

Thymus

Grossly, the thymic chain was reduced in size in the group `A' and `B' compared to the control. But no gross pathological changes were noticed.

The cellularity of the thymus was very less in all the groups and no histological changes could be appreciated on day 15 and 21.

The thymus on day 28 from group `A' showed oedematous changes and very few lymphoid cells (Fig.10). Vacuolar degenerative changes were observed in the epithelial cells.

The thymus from embryos of group `B' sacrificed on the 28th day showed more number of lymphoid cells compared to `A' group and the Hassal's corpuscles showed tendency for hyalinization. Mild degree of oedematous changes was also evident.

4.1.3 Ultrastructure

Bursa of Fabricius

The number of cells and the ultrastructural changes were more prominent on the 28th day than on the 15th and 21st day.

Group A

The nucleus revealed condensed dense heterochromatin. Tendency for the fragmentation of the nuclear membrane was evident. Numerous membrane bound vesicles, some of which were surrounded by ribosomes and electron lucent content were present. Mitochondria were swollen with granular content inside. The cristae showed lytic changes. The cytoplasm had large number of ribosomes compared to Group B (Fig.11).

Group B

A group of small to large lymphocytes and lymphoblasts with densely stained heterochromatin were present. The cells showed tendency for separation with large intercellular space.

The cytoplasm was granular and electron dense. Some of the lymphocytes showed vacuoles with electron lucent content (Fig.12).

Lymphoblastic cells with abundant cytoplasm was noticed. Abundance of ribosomes was noticed giving a dense granular appearance to the cytoplasm. The nucleus of some of the cells showed pyknotic changes. The nuclear membrane of some of the cells were irregularly invaginated. The nuclear pores were intact. Some of the cells showed electron dense spherical structures. Nucleolus was not prominent.

At a higher magnification, the cytoplasm appeared prominent with many ribosomes. The mitochondria were swollen and showed signs of cristolysis. Some of the mitochondria were devoid of any cristae (Fig.13). Fragmentation of the rough endoplasmic reticulum was evident at places. The bursa of the groups C and D did not show any ultrastructural changes.

Thymus

Group A

Dark stained condensed heterochromatin was evident at the nuclear membrane. Euchromatin was less. The nuclear membrane showed invaginations. The cytoplasm revealed free ribosomes. A few vesicles with dense filamentous as well as granular

structures were present near the golgi zone. The mitochondria showed moderately dense granular content. The cytoplasmic membrane showed bleb formation at places and the discontinuity in the cellular membrane caused the letting out of free ribosomes (Fig.14).

Group B

Margination of heterochromatin with less of euchromatin was noticed. The number of nuclear pores were also found to be less (Fig.15).

Mitochondria of varying shapes from round, oval and elongated, were noticed. The cristae of the mitochondria was densely packed with less of matrix. The cytoplasm also showed membrane bound vesicles of varying shapes with electronlucent content. Dense accumulation of ribosomes were present at places.

The control groups C and D did not show any ultrastructural changes.

Spleen

Group A

Margination of the heterochromatin at the nuclear membrane was noticed in the lymphocytes. Uniformly distributed

euchromatin in the nucleus showed tendency for clumping which were more electron dense. Nuclear pores were normally distributed.

The cytoplasm of the lymphocytes contained moderate amounts of free ribosomes. The most severe changes were noticed in the mitochondria which were swollen and the cristae of some of them were partially lysed (Fig.16). Many of the mitochondria contained membrane bound electron dense vesicular structures of varying shapes. The vesicles were either empty or contained electron lucent content. The endoplasmic reticulum showed fragmentation. The cytoplasm contained vesicular structures with ribosomes surrounding it. Some of the vesicles were electronlucent while some others were empty. The golgi zone was densely stained. The cytoplasmic membrane also showed evidence of fragmentation.

Group B

A few macrophages with phagocytosed haemoglobin were seen. Various stages of disintegrated electron dense haemoglobin could be seen. A few numbers of lysosomes were also noticed. The nucleus contained prominent nucleoli and nucleolar associated condensed chromatin. The karyoplasm was uniformly granular and numerous nuclear pores were evident.

The lymphoid cells in various stages of development, separated by wide intercellular space as well as large reticular cells were seen. The nucleus of the lymphoid cells had heterochromatin marginated at the nuclear membrane. The reticular cells had irregular nucleus. Dense granules and vacuoles were present in the cytoplasm of the reticular cells.

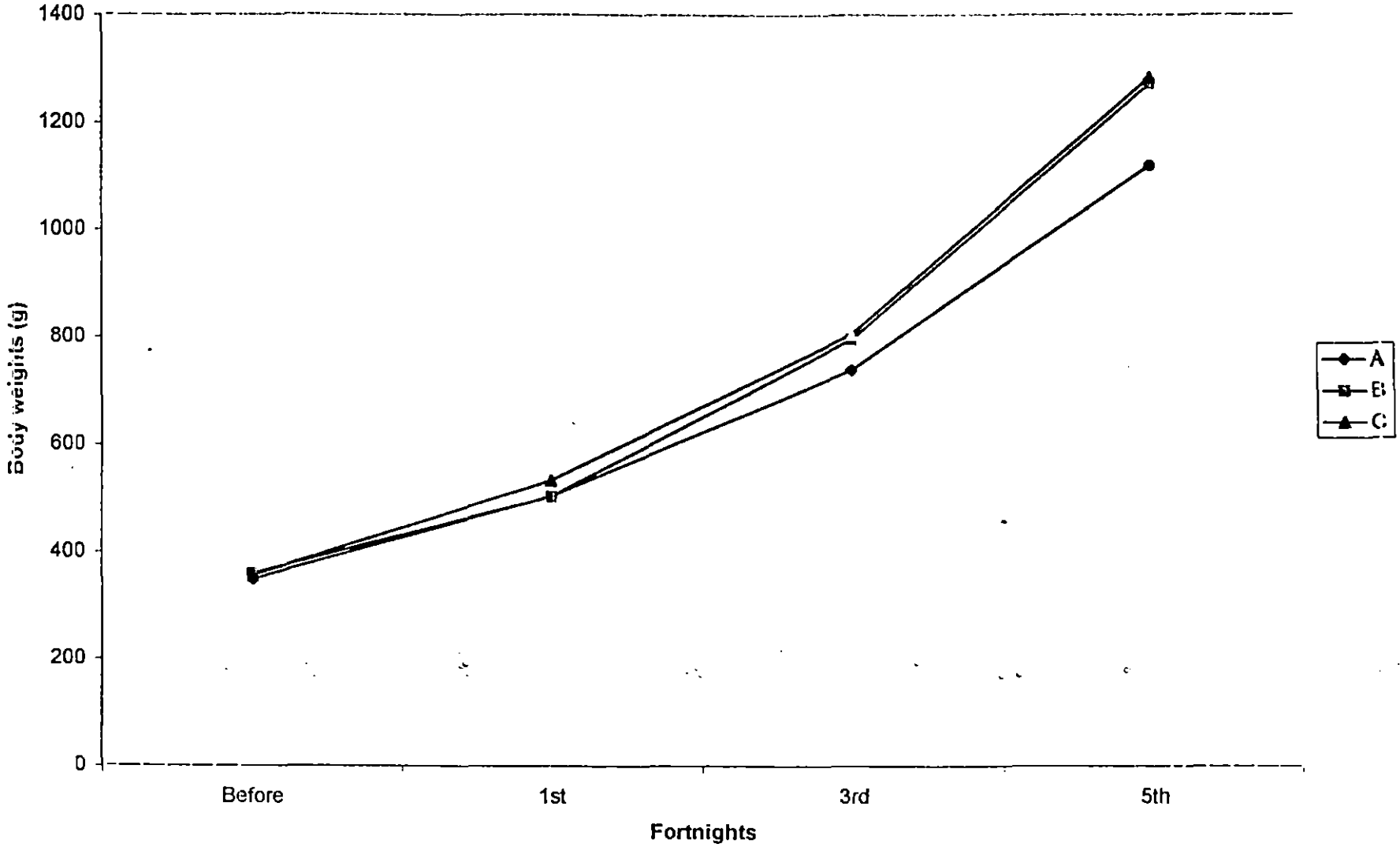
4.2 Humoral immune response

4.2.1 Body weight

The average body weights recorded before the commencement and at the 1st, 3rd and 5th fortnight are given in Table 6 and Fig.17.

The difference in the mean of the body weights of different groups was not statistically significant before the commencement and at the end of the first fortnight of the experiment. The mean of the group `A' showed significant reduction at 5 per cent level at the end of the third fortnight and at 1 per cent level at the end of the fifth fortnight when compared with the control group. Group `B' did not show any significant difference in the body weight recorded at the third and fifth fortnight when compared with the control.

Fig.17:Average body weight of the ducks recorded at the 1st, 3rd & 5th fortnight



4.2.2 Haematological evaluation

4.2.2.1 Leucocyte count

The average of the haematological values like the Total Leucocyte Count (TLC), Differential Leucocyte Count (DLC) and haemoglobin are given in Table 7. The leucocyte count did not show any significant difference between the groups before the commencement and at the end of the third fortnight of the experiment.

The mean of the TLC of group `A' showed significant difference at 5 per cent level when compared with the control at the end of the fifth fortnight. Group `B' showed an increase in the TLC, but it was not significant statistically when compared with the control.

4.2.2.2 Differential leucocyte count

The lymphocyte count in all the groups remained in the normal range, eventhough slight variations were observed between groups.

Group `A' showed slight increase in the percentage of lymphocytes at the end of the third fortnight, but it came down at the end of the fifth fortnight.

Group `B' showed slight reduction in the percentage of lymphocytes at the fifth fortnight of the experiment whereas it was almost the same at the end of the third fortnight.

The percentage of heterophils in all the groups before the commencement and at the third and fifth fortnight of the experiment were within the normal range. But the percentage of heterophils showed an increasing trend at the fifth fortnight in group `A' and `B'.

Eosinophils, monocytes and basophils remained in the normal range in all the groups during the third and fifth fortnight of the experiment.

4.2.2.3 Haemoglobin

The haemoglobin level showed a decreasing trend in the groups `A' and `B' during the third and fifth fortnight of the experiment whereas in the control group the level of the haemoglobin remained steady.

4.2.3 Serum protein, albumin, globulin and albumin:globulin ratio

Average of the serum protein, albumin, globulin and albumin:globulin ratio (A:G) are given in Table 8 and Fig.18, 19 and 20.

Table 7. Average (Mean±SE) of the haematological values of the treatment and control groups

Groups	Before the experiment							3rd F.N.							5th F.N.						
	TLC/ cu mm	H	E	B	M	L	Hb	TLC/ cu mm	H	E	B	M	L	Hb	TLC/ cu mm	H	E	B	M	L	Hb
A	26840.00± 3265.00	31.9± 3.4	1.40± 1.07	0.1± 0.3	4.0± 1.8	63.60± 2.9	10.45± 0.6	25600.00± 3806.42	30.0± 2.9	2.5± 1.6	0.0±	2.4± 0.8	65.0± 3.2	10.5± 0.4	24833.33± * 4262.24	36.7± 1.7	2.8± 0.9	0.0±	2.0± 0.6	58.50± 2.3	9.08± 0.38
B	25950.00± 4004.70	32.4± 1.7	1.7± 0.8	0.2± 0.4	1.5± 0.8	64.3± 1.82	11.0± 0.57	26490.00± 4309.7	32.9± 2.1	1.7± 0.7	0.1± 0.3	1.2± 0.8	64.1± 2.3	10.5± 0.4	28250.00± 3061.8	36.7± 2.7	0.7± 0.8	-	0.7± 0.8	62.3± 3.3	10.17± 0.41
C	26200.00± 3224.90	34.4± 3.02	1.0± 0.7	0.3± 0.5	3.1± 0.9	61.2± 2.93	11.02± 0.41	26350.00± 2981.8	34.9± 2.68	0.6± 0.5	0.3± 0.4	2.7± 0.9	61.5± 2.4	11.4± 0.3	27670.00± 2422.12	34.0± 2.2	0.7± 0.8	0.3± 0.5	2.6± 0.5	62.3± 2.06	11.66± 0.26

* ($P<0.05$)

The serum protein did not show any significant difference between the treatment and control groups at the end of the first and third fortnights. But there was reduction in the serum protein in groups `A' at the end of the third fortnight when compared with the first fortnight. The mean values of serum protein showed significant reduction at 5 per cent level in the case of group `A' when compared with the groups `B' and `C' at the end of the fifth fortnight.

The serum globulin level did not vary significantly between the groups at the end of the first and third fortnight. However, the serum globulin level of the group `A' was slightly elevated compared with the other two groups, but this was not statistically significant when compared with the value of the first fortnight. But the serum globulin level showed significant reduction in the group `A' at 1 per cent level and in the group `B' at 5 per cent level when compared with the control at the end of the fifth fortnight.

There was no significant difference between the different groups in the level of albumin at the end of the first fortnight and third fortnight whereas a reduction in the albumin was noticed in the group `A' at the end of the fifth fortnight at 5 per cent level, but the difference between the `B' group and the `C' groups was not statistically significant.

Table 8. Average (Mean±SE) of the serum protein, albumin, globulin and A:G ratio an the 1st, 3rd and 5th fortnights

Group	IFN				3rd FN				5th FN			
	SP	Ab	Gl	A:G	SP	Ab	Gl	A:G	SP	Ab	Gl	A:G
A	3.64± 0.40	1.46± 0.13	2.55± 0.19	0.57 0.10	3.60± 0.39	1.44± 0.11	2.63± 0.19	0.54± 0.10	3.47*± 0.16	1.40*± 0.11	2.45**± 0.14	0.65± 0.06
B	3.54± 0.31	1.53± 0.07	2.54± 0.28	0.60± 0.09	3.55± 0.34	1.53± 0.05	2.57± 0.25	0.59± 0.08	3.62± 0.35	1.60± 0.06	2.59*± 0.19	0.61± 0.09
C	3.59± 0.19	1.57± 0.08	2.61± 0.20	0.60± 0.04	3.65± 0.18	1.59± 0.07	2.60± 0.07	0.61± 0.04	3.80± 0.15	1.60± 0.05	2.71± 0.07	0.59± 0.03

* ($P<0.05$)
** ($P<0.01$)

Fig:18 Average of serum protein at the 1st, 3rd & 5th fortnight

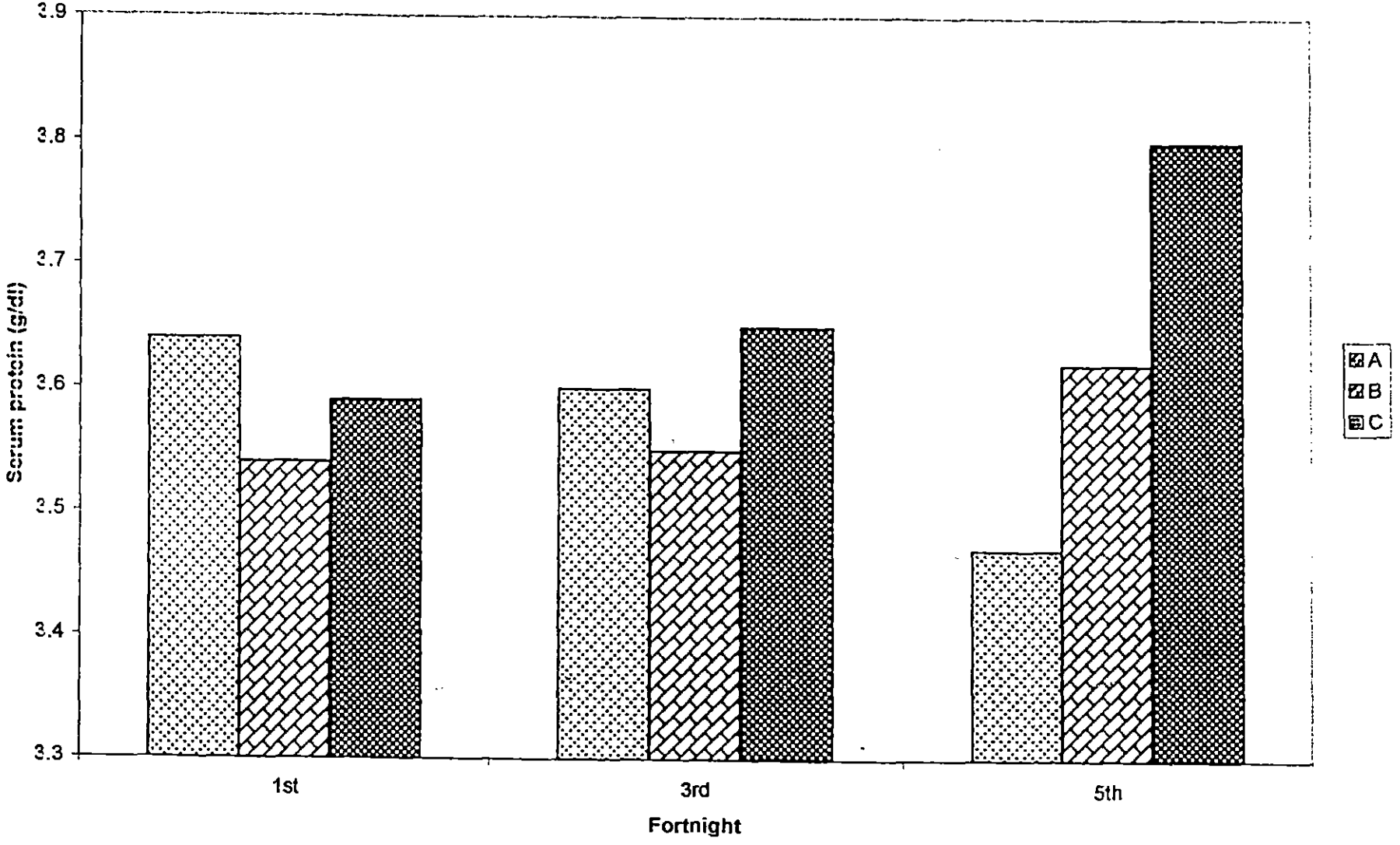


Fig:19 Average of serum globulin at the 1st, 3rd & 5th fortnight

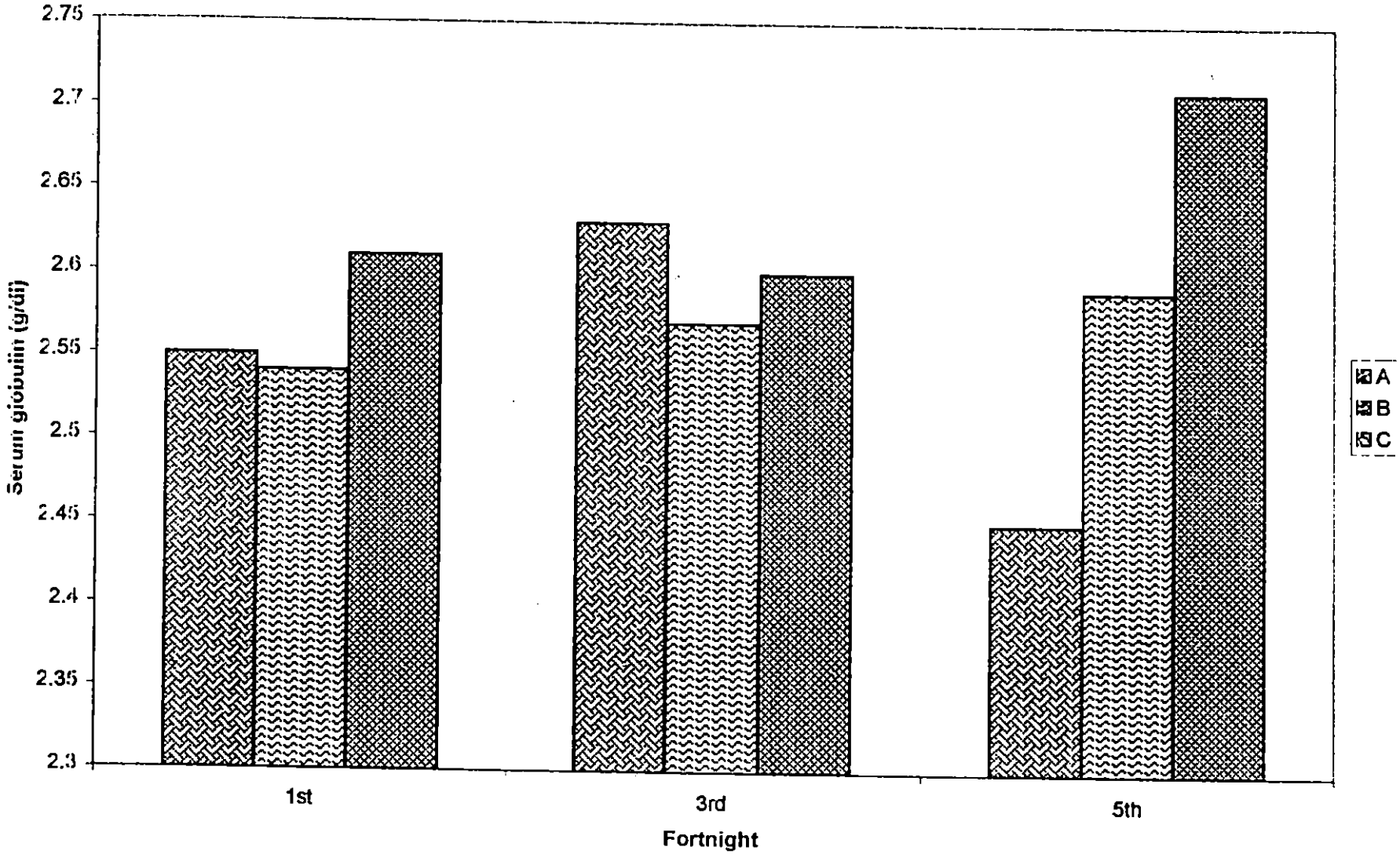
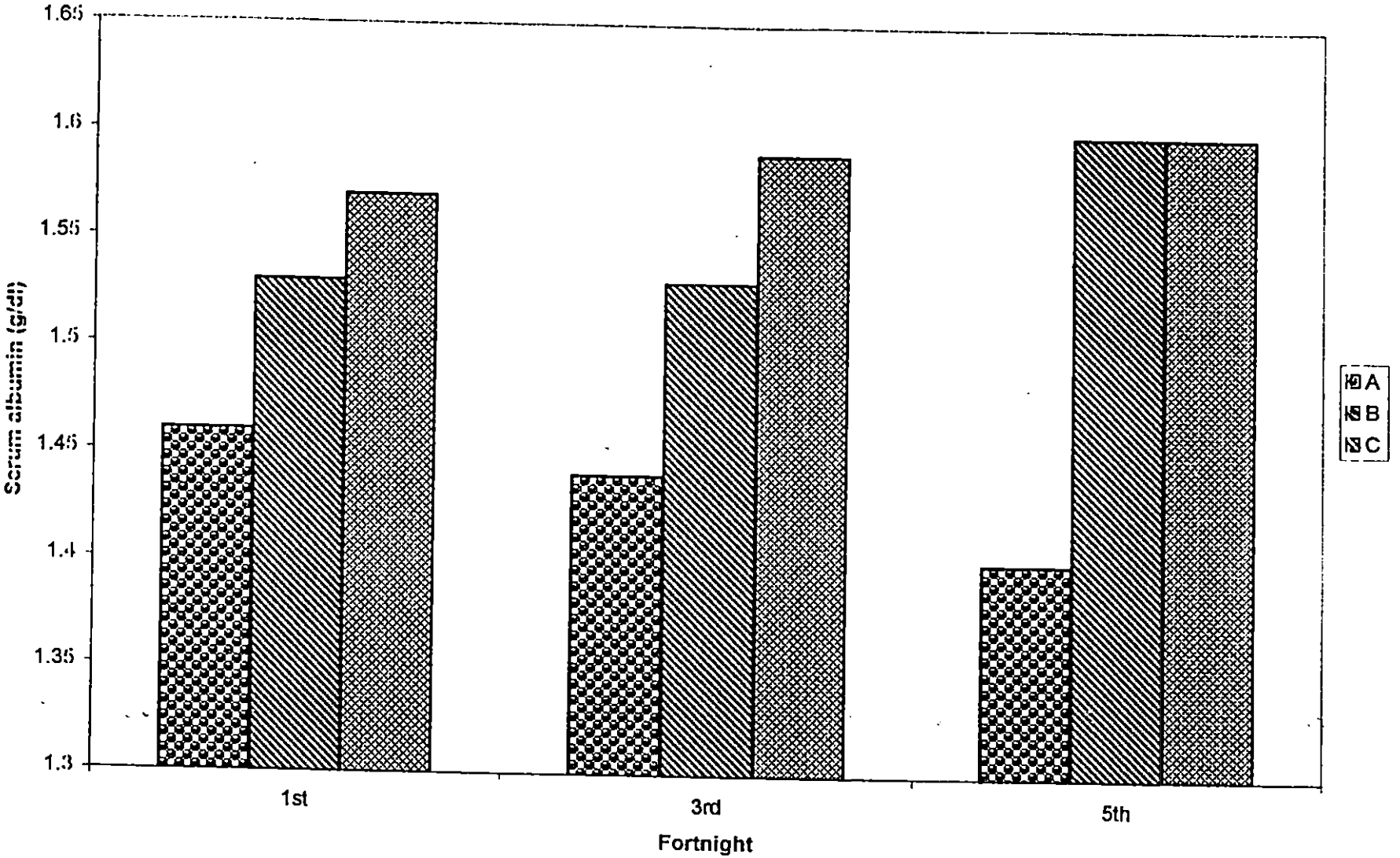


Fig:20 Average of serum albumin at the1st, 3rd & 5th fortnight



The serum albumin:globulin ratio was found to be more in the case of `B' and `C' groups compared to the `A' group at the end of the first and third fortnight. But at the end of the fifth fortnight, there was an increase in the A:G ratio in group `A' when compared with group `B' and `C'. But the difference was not statistically significant at any of the time period.

4.2.4 Antibody titre

Average of log2 values of haemagglutination inhibition titre in the treated and control ducks inoculated with NDV is given in Table 9 and Fig. 21.

The H.I. titre against NDV at the end of 6 weeks of the experiment did not show any significant difference between the control and the treatment groups, but the titre was more in group `A' and `B' when compared with the control.

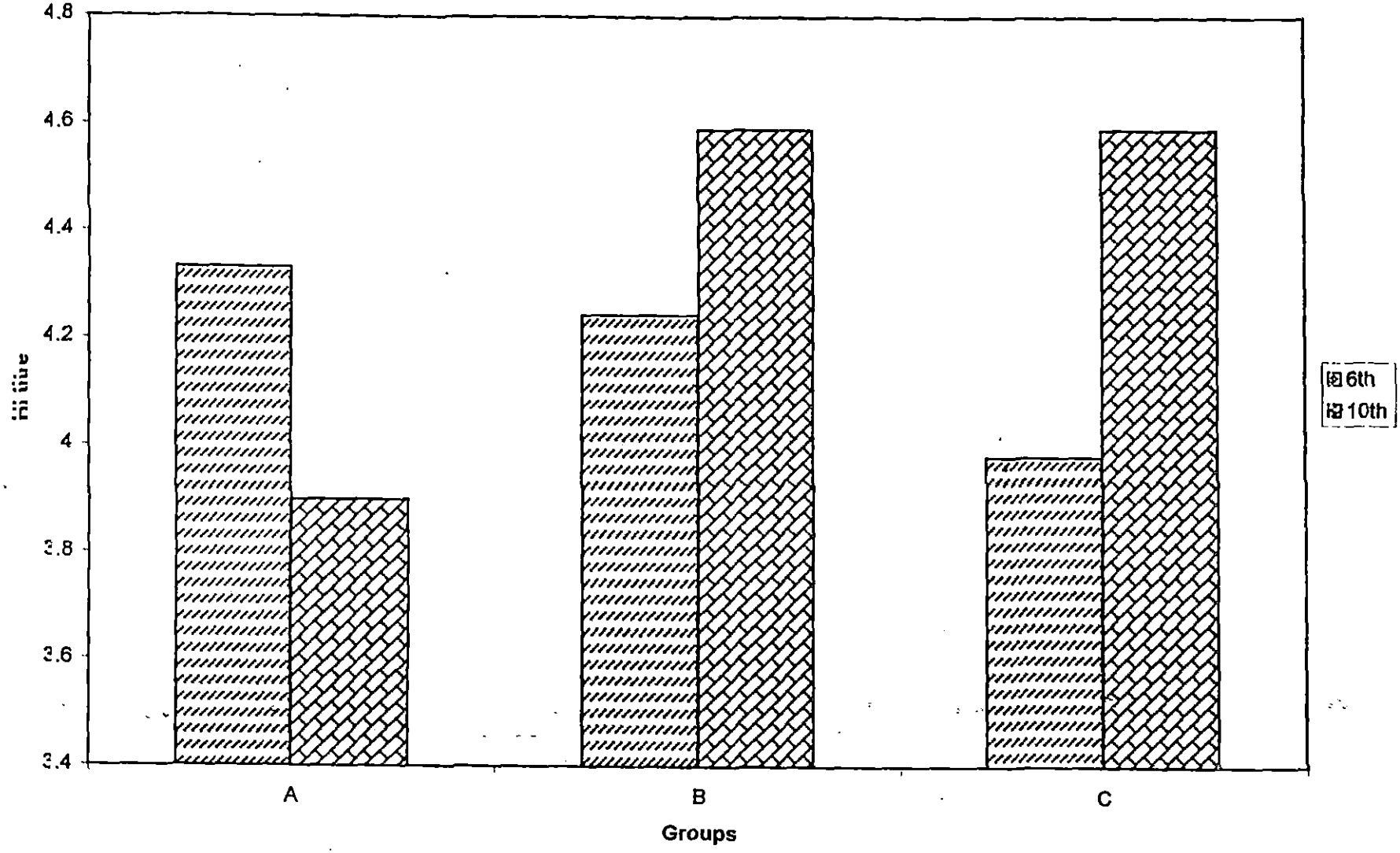
The H.I. titre against NDV at the termination of 10 weeks of feeding the chemicals showed significant reduction in the group A at 1 per cent level whereas group `B' and `C' had the same titre.

Table 9. Average (Mean±SE) of HI titre (log2 units) in the treated and control ducks inoculated with NDV

Group	After 6 weeks of treatment	After 10 weeks of treatment
A	4.332 ± 0.490	3.898 ± 0.358 **
B	4.245 ± 0.245	4.591 ± 0.359
C	3.980 ± 0.320	4.591 ± 0.359

** ($P<0.01$)

Fig:2] Average HI titre (log2 units) at the 6th & 10th week



4.2.5 Average of organ weights

The average of the weight of the body and the lymphoid organs like the spleen, thymus and bursa at the end of the 6th week and 10th week are given in Table 10.

6th week

The mean of the body weight of birds sacrificed at the end of the 6th week showed significant reduction in the body weight of birds of group `A' at 5 per cent level when compared with the control. Group `B' did not vary significantly with the control eventhough the weight was less.

The mean of the weight of the spleen of group `A' and `B' was less when compared to the control, but it did not differ significantly (Fig.22).

The average weight of the bursa was the lowest in group `A', whereas the weight of the bursa of group-B was slightly less when compared to the control group. But the difference in the mean weight of the bursa at the end of the 6th week was not statistically significant (Fig.23).

The average weight of the thymus showed significant reduction in the weight at 5 per cent level in the case of birds sacrificed from group A whereas the weight of the thymus

of group `B' did not vary significantly when compared with the control (Fig.24).

10th week

The average body weight at the end of the tenth week showed significant difference. The mean of group `A' showed significant reduction at 1 per cent level when compared with the control. The mean of the group `B' showed significant reduction at 5 per cent level.

The average weight of the spleen of group `A' showed significant reduction at 1 per cent level whereas the spleen weight of the group `B' showed reduction at 5 per cent level when compared with the control (Fig.22).

The average weight of the bursa from group `A' showed significant reduction at 1 per cent level when compared with the control. Though the average weight of the bursa from group `B' was reduced when compared with the control, it was not significant statistically (Fig.23).

The average weight of the thymus at the end of the tenth week showed significant reduction at 1 per cent level in group `A' and `B' when compared with the control (Fig.24).

Table 10. Averages (Mean±SE) of the weight of the body and lymphoid organs at the end of the 6th and 10th week

Group	6th week				10th week			
	Body weight (g)	Spleen (mg)	Bursa (mg)	Thymus (mg)	Body weight (g)	Spleen (mg)	Bursa (mg)	Thymus (mg)
A	778.55±* 26.42	1.176± 0.085	1.408± 0.062	2.581±* 0.113	1090.38** 42.94	1.378±** 0.070	1.607±** 0.079	2.913±** 0.115
B	785.35± 31.75	1.182± 0.089	1.442± 0.047	2.728± 0.119	1239.40±* 55.68	1.435±* 0.085	1.706± 0.100	3.039±** 0.064
C	800.34± 40.65	1.262± 0.089	1.459± 0.113	2.752± 0.136	1300.39± 35.31	1.505± 0.020	1.754± 0.055	3.217± 0.139

* (P<0.05)
** (P<0.01)

Fig: 22 Average weight of spleen at the end of the 6th and 10th week

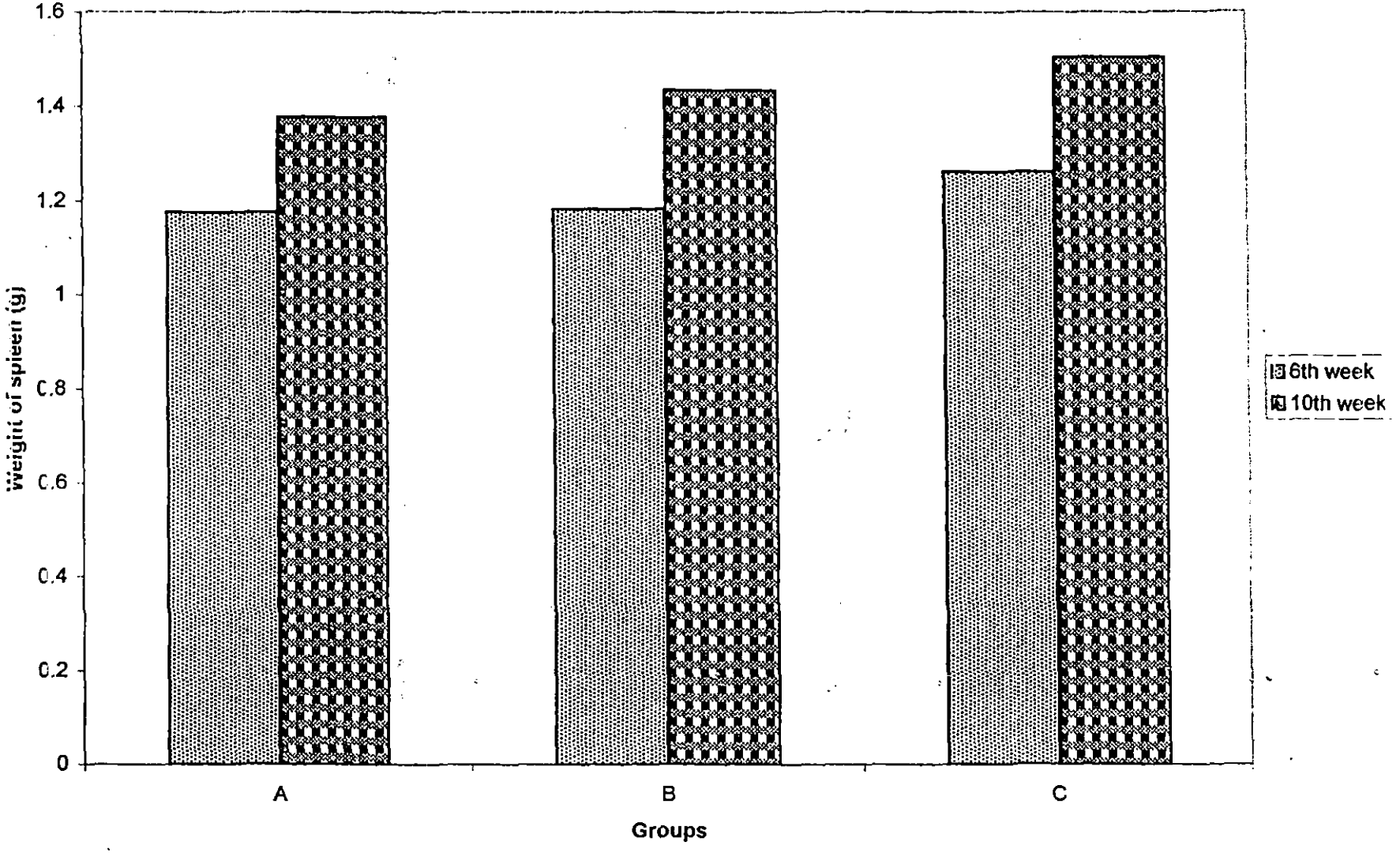


Fig:23 Average weight of bursa at the end of the 6th and 10th week

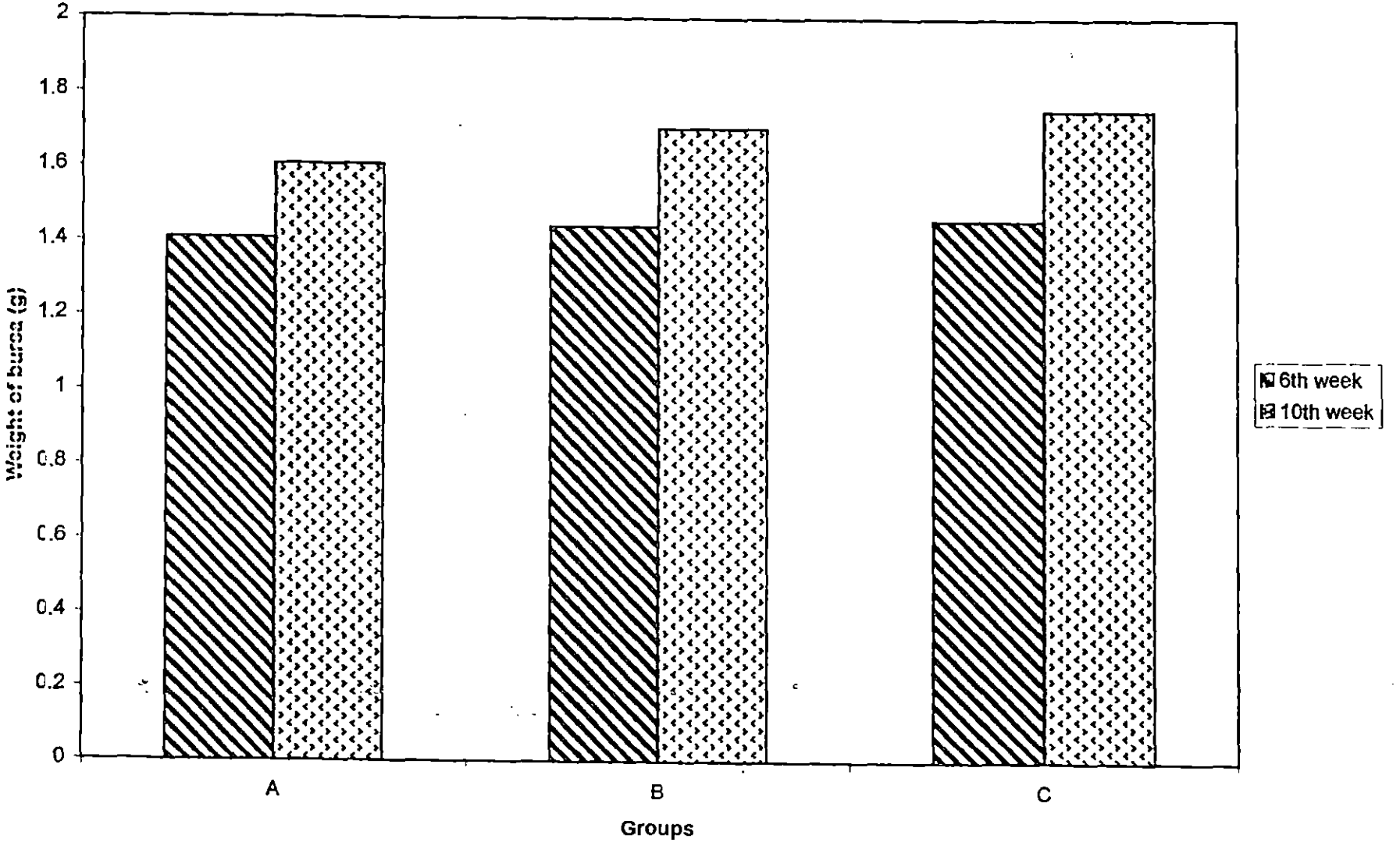
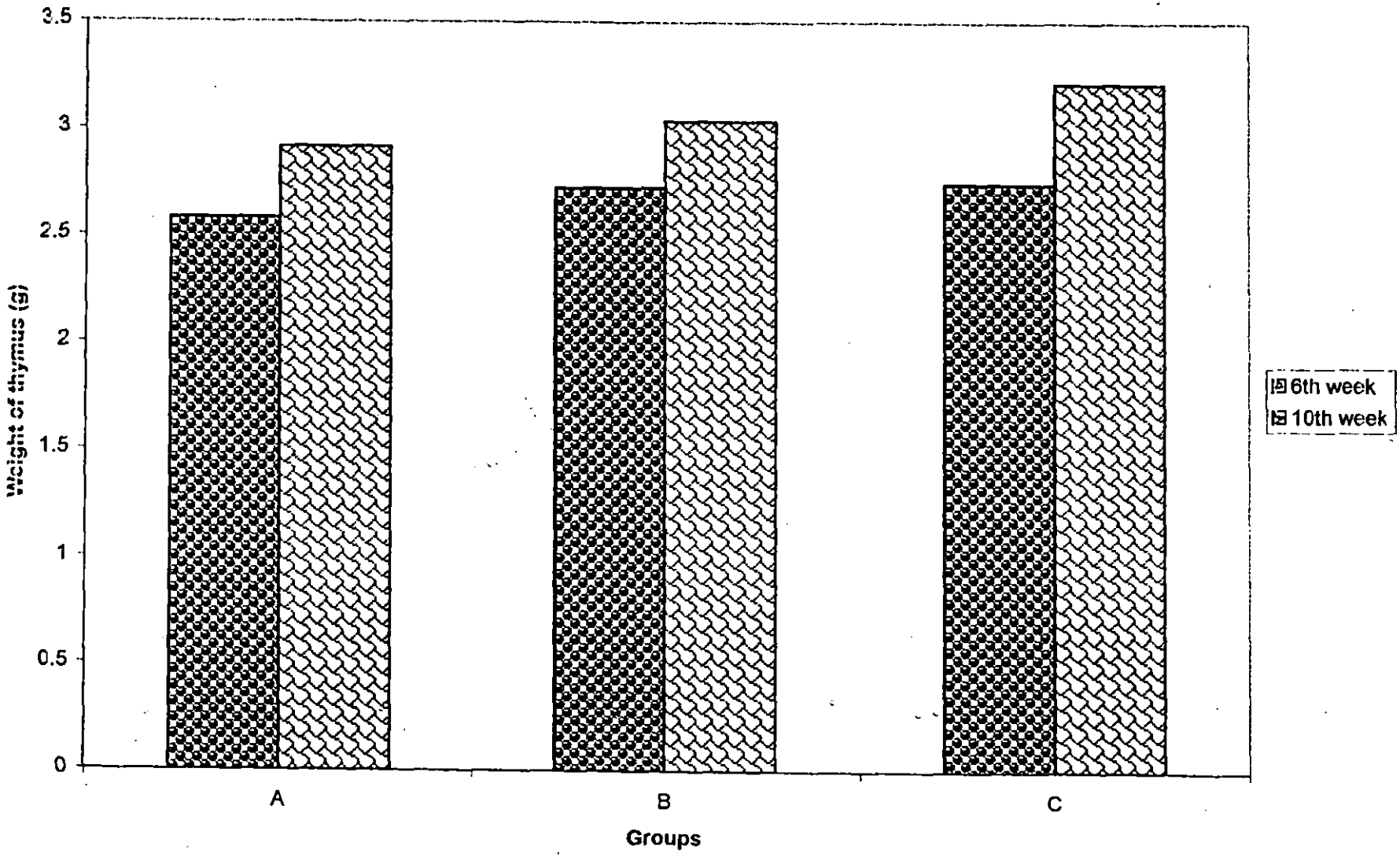


Fig:24 Average weight of thymus at the end of the 6th and 10th week



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The result of the PAGE is shown in Fig.25 and 26.

The different bands obtained were albumin, α-globulin, ß globulin and Γ-globulin. No difference could be appreciated between the treatment and the control groups after 6 weeks of the experiment. But all the globulin fractions were less in the group `A' and `B' when compared with the control after ten weeks of the experiment.

4.3 Cell-mediated immune response

4.3.1 Skin sensitivity to DNCB

Skin sensitivity to DNCB and PHA was evaluated to assess the influence of agro-chemicals on the cell-mediated immune response.

Response to DNCB and PHA were evaluated after three and seven weeks of feeding the chemicals. The cutaneous response was measured by measuring the thickness of the skin at 24 h, 48 h and 72 h. The visible physical alterations on the skin were also noted. Biopsies were taken and subjected to histopathological examination.

The average (mean±SE) of the thickness of the skin at 24 h, 48 h and 72 h in response to DNCB after 3 weeks and 7 weeks of the experiment are given in Table 11.

After 3 weeks

The skin showed erythematous changes with formation of blisters in some of the birds in all the groups at 24 h. On measuring the thickness of the skin using the vernier calipers, it was found that the thickness was more in the control group compared to the two treatment groups and the difference was significant at 5 per cent level.

The erythematous changes were reduced on the 48th and 72nd h and the difference in the thickness was not found to be statistically significant (Fig.27).

After 7 weeks

Grossly, the skin showed erythematous changes after 24 h of the challenge with DNCB in all the groups, which started reducing after 48 h and 72 h. On measuring the skin thickness, the skin of the control birds showed increased thickness compared to the treatment groups after 24 h, 48 h and 72 h, but it was found to be significant only at 24 h at 1 per cent level and at 48 h at 5 per cent level (Fig.28).

4.3.2 Skin sensitivity to PHA

The average of the skin thickness in response to PHA-M after 3 weeks and 6 weeks of the experiment is given in Table 12.

Table 11. Average (Mean±SE) thickness of the skin in response to DNCB - $A_1B_1C_1$ and $A_2B_2C_2$

Group	Before challenge	24 h	48 h	72 h
A_1	0.62± 0.15	* 1.69± 0.18	1.15± 0.14	0.83± 0.09
B_1	0.55± 0.08	* 1.975± 0.21	1.15± 0.13	0.675± 0.12
C_1	0.60± 0.20	2.36± 0.34	1.932± 0.31	1.00± 0.30
A_2	0.70± 0.06	** 1.65± 0.11	* 1.45± 0.11	0.90± 0.08
B_2	0.59± 0.29	** 1.78± 0.15	* 1.54± 0.19	0.95± 0.03
C_2	0.72± 0.05	2.25± 0.21	1.94± 0.26	1.25± 0.29

* ($P<0.05$)
** ($P<0.01$)

Fig:27 Average thickness of skin in response to DNCB at the end of the 3rd week

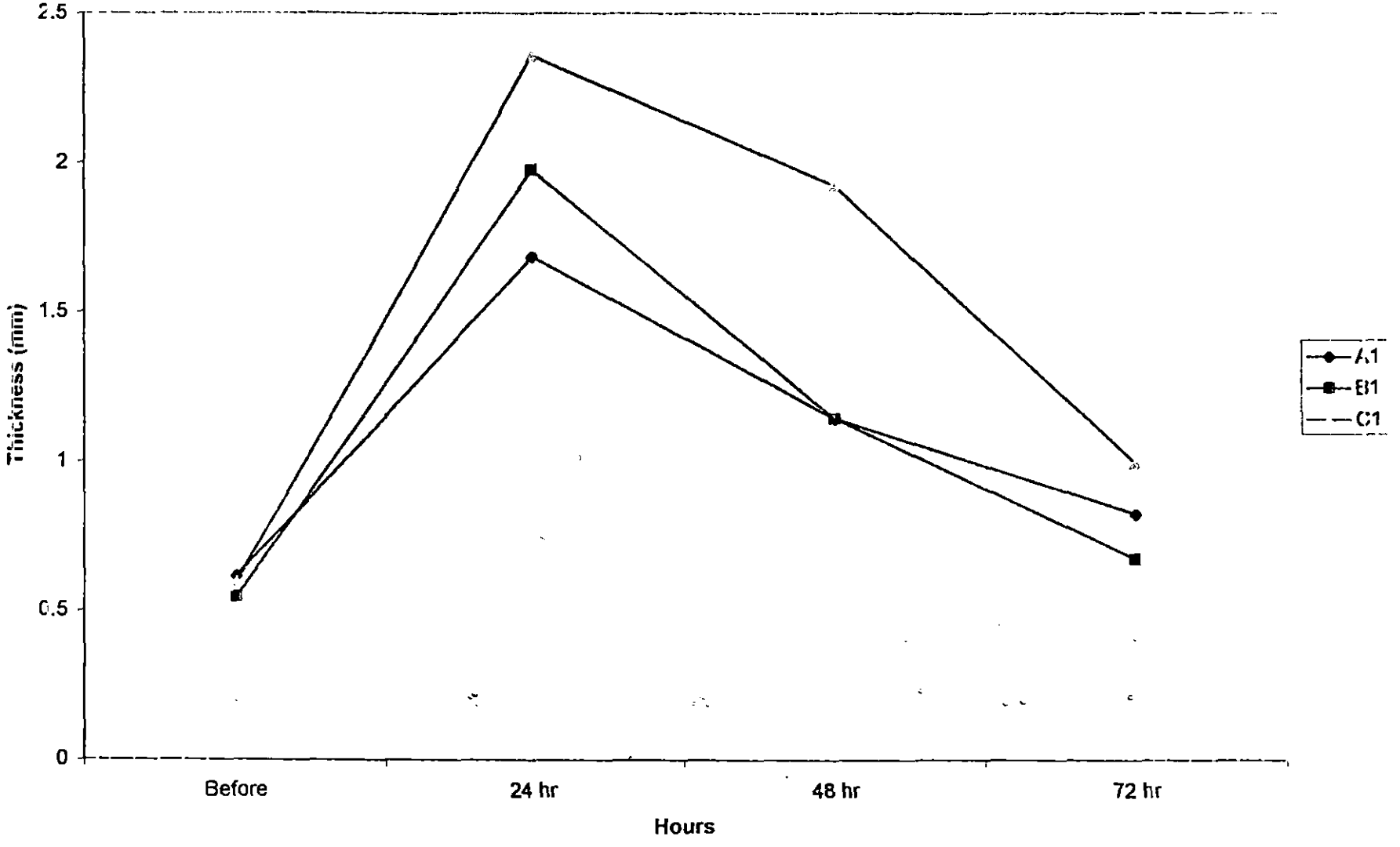


Fig: 28 Average thickness of skin in response to DNCB at the end of the 7th week

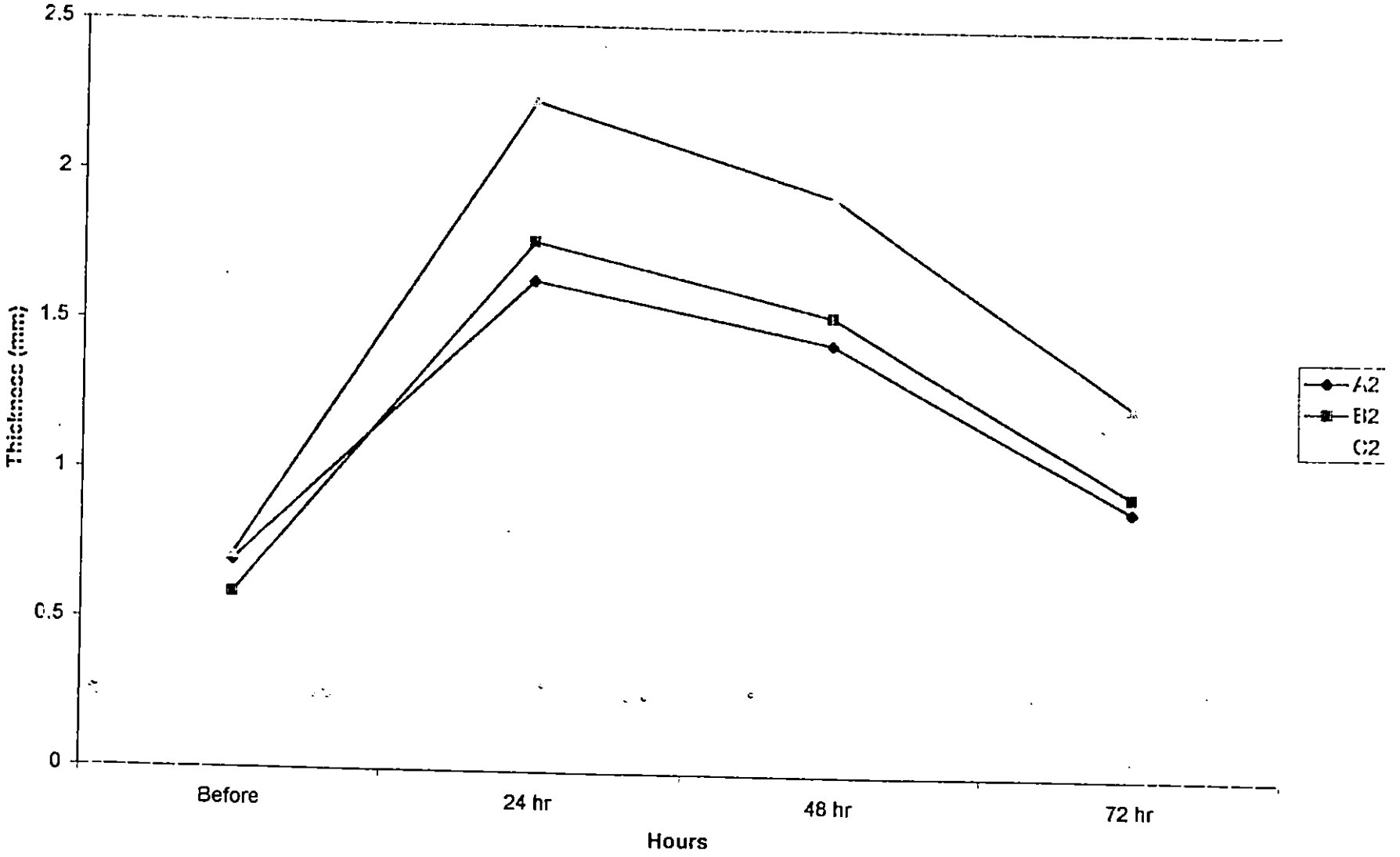


Table 12. Average (Mean±SE) thickness of the skin in response to PHA-M - $A_1B_1C_1$ and $A_2B_2C_2$

Group	Before commencement (mm)	24 h (mm)	48 h (mm)	72 h (mm)
A_1	0.67± 0.21	* 1.675± 0.16	1.48± 0.23	0.95± 0.07
B_1	0.52± 0.05	* 1.625± 0.10	1.50± 0.09	0.98± 0.10
C_1	0.475± 0.05	1.915± 0.09	1.64± 0.08	1.10± 0.05
A_2	0.575± 0.05	** 1.52± 0.09	* 1.34± 0.08	1.08± 0.05
B_2	0.58± 0.06	** 1.54± 0.07	* 1.38± 0.07	1.10± 0.05
C_2	0.58± 0.04	2.19± 0.23	1.66± 0.23	1.21± 0.23

* ($P<0.05$)
** ($P<0.01$)

Fig;29Average thickness of the skin in response to PHA-M at the end of the 3rd week

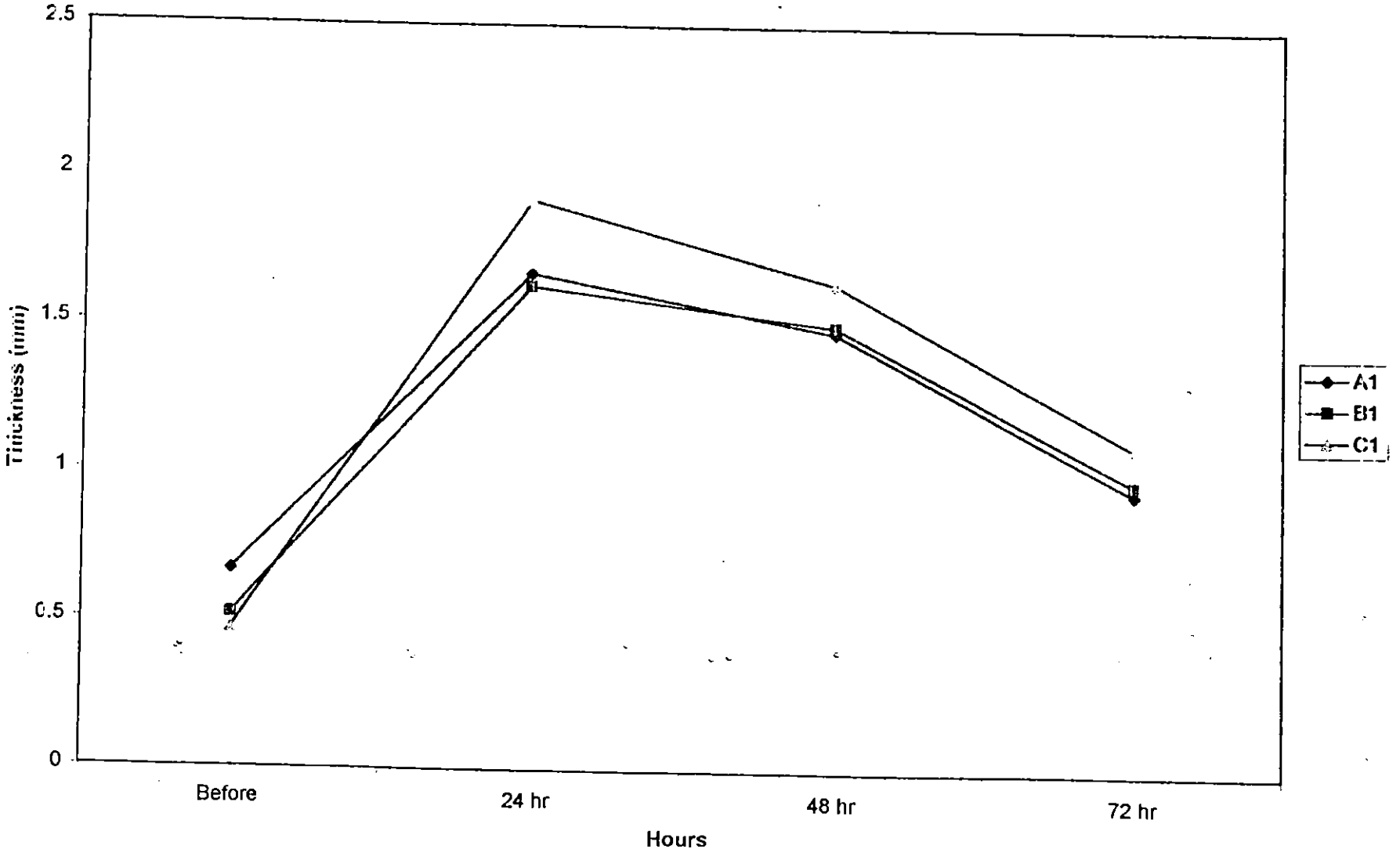
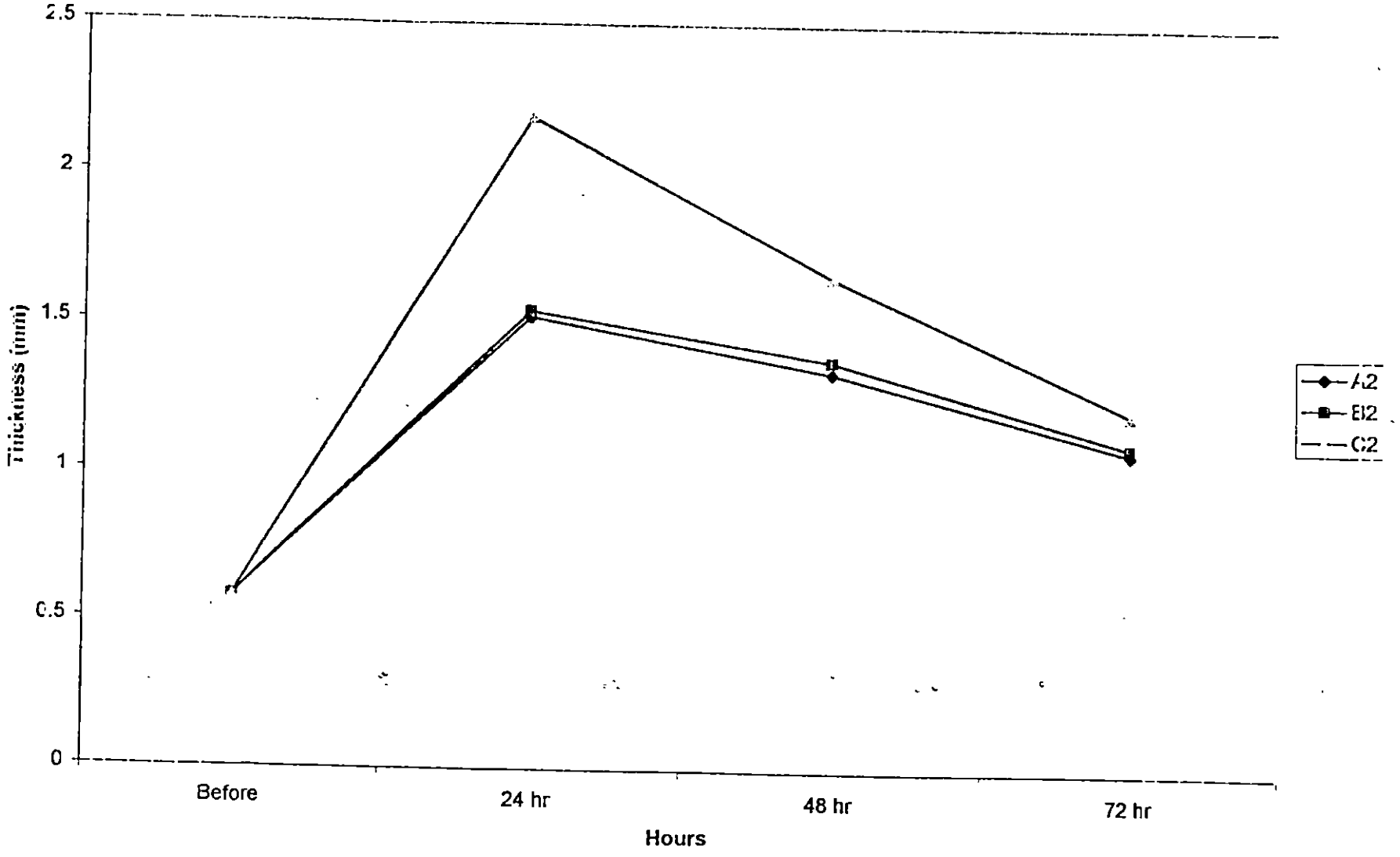


Fig:30 Average of thickness of skin in response to PHA-M at the end of the 6th week



After 3 weeks

Grossly, the skin did not show any erythematous change, but an increase in the thickness of the skin was noticed. The increase in the thickness was statistically significant at 5 per cent level at 24 h in the C1 group when compared with A1 and B1. At 48 h and 72 h, though the thickness was more in the control group, the difference was not statistically significant (Fig.29).

After 6 weeks

The maximum increase in the thickness was noticed at 24 h and it started decreasing at 48 h and 72 h. The increase in the thickness was significant in the control group when compared with the treatment group at 1 per cent level at 24 h and at 5 per cent level at 48 h. At 72 h, though the thickness was more in the control group, it was not statistically significant (Fig.30).

4.3.3 Leucocyte migration inhibition test - Migration indices

The average (mean±SE) of migration indices on the 3rd, 6th, 8th and 10th week of the experiment is given in Table 13.

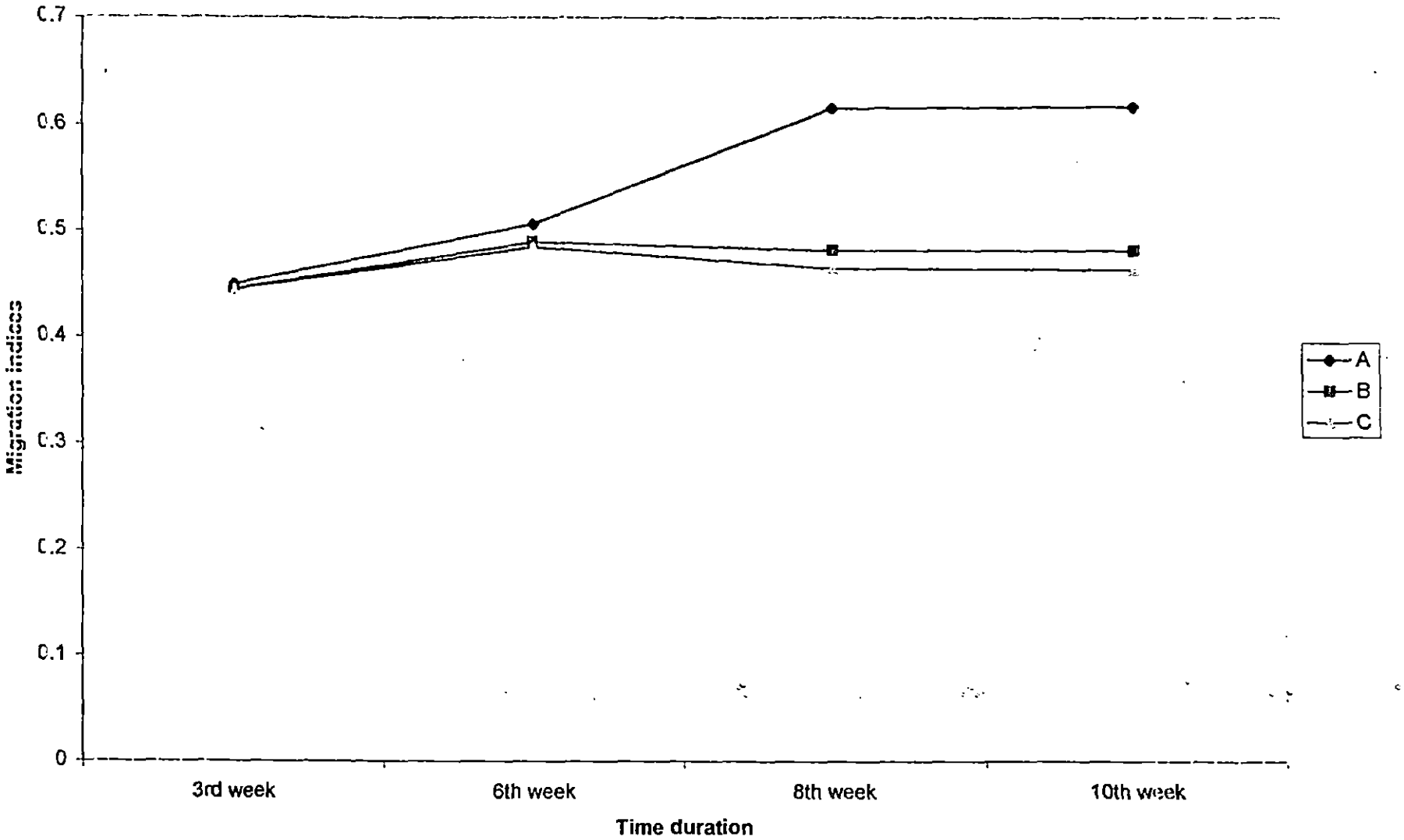
There was an increase in the migration indices in group A1 and B1 on the 3rd and 6th week of the experiment. But the

Table 13. Average (Mean±SE) of the migration indices of the experimental and control groups

Group	3rd week	6th week	8th week	10th week
A	0.450± 0.006	0.507± 0.017 *	0.616± 0.054 **	0.618± 0.050 **
B	0.445± 0.011	0.490± 0.011	0.482± 0.014 *	0.482± 0.013 *
C	0.445± 0.012	0.485± 0.013	0.465± 0.016	0.464± 0.018

* ($P<0.05$)
** ($P<0.01$)

Fig:3)Average of migration indices of the experimental and the control birds



increase was significant only on the 6th week in group A at 5% level, whereas the increase in group `B' was not significant compared with the control. At the 8th and 10th week of the experiment, the increase was significant at 1 per cent level in group `A', whereas it was significant only at 5 per cent level in group `B'. But the difference between the data recorded on the eighth and tenth week was not very much appreciable (Fig.31).

4.3.4 Graft Vs Host Reaction (GVHR - Spleenic indices)

The average (mean±SE) of the spleenic indices at the end of the sixth and tenth week of the experiment is given in Table 14.

The mean of the spleenic indices was found to be significantly different between the groups at the end of the sixth and tenth week of the experiment. The treatment groups revealed an increased spleenic index compared with the control group. But within the groups, the spleen index of the group `B' was found to be more compared to the group `A' (Fig.32).

4.3 Gross and histopathology

4.3.1

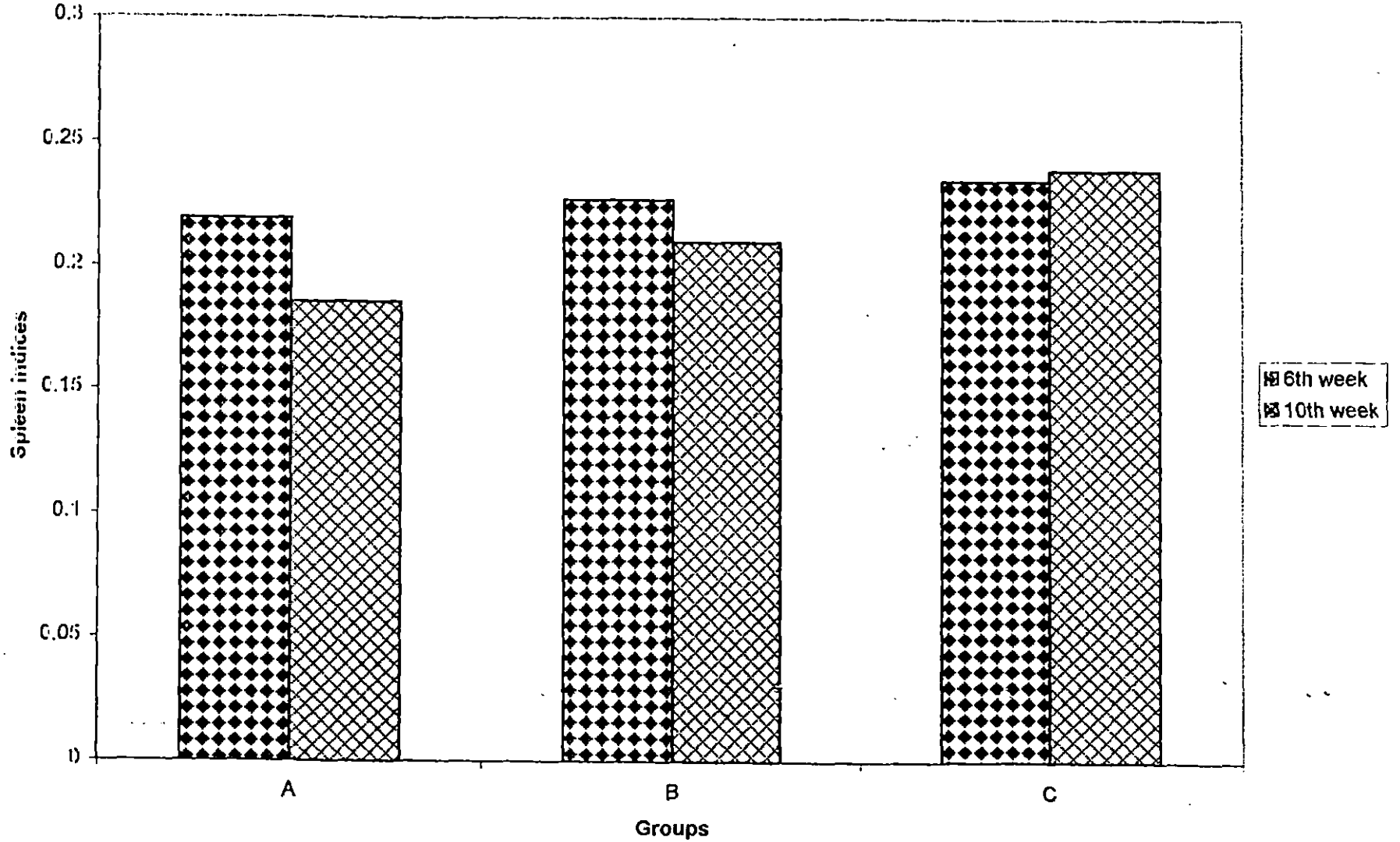
The birds maintained in three separate cages groupwise, appeared active and the feed intake was normal.

Table 14. Average (Mean±SE) of the spleen indices at the end of the 6th and 10th week

Group	6th week	10th week
A	0.219 ± 0.013 *	0.185 ± 0.009 **
B	0.227 ± 0.002 *	0.210 ± 0.020 **
C	0.235 ± 0.013	0.239 ± 0.013

* (P<0.05)
** (P<0.01)

Fig:32 Average of spleen indices of the experimental and the control birds



During the first six weeks of the experiment, three birds died in group A, two birds in group B and one bird in the control group. The post mortem examination revealed moderate degree of congestion of the visceral organs in birds of group `A' and the eyes showed slight tendency for development of corneal opacity. The bird from the group B showed moderate degree of congestion of the intestinal mucosa and in one case, intussusception of the intestine was noticed. The control bird did not show any gross pathological changes except for mild degree of congestion of the visceral organs. There was no mortality from the sixth week to the tenth week of the experiment.

Twelve birds from each group were sacrificed after six weeks of the experiment. No apparent gross pathological changes could be detected in the liver, kidney, heart and intestine in birds of any of the groups. Lungs showed mild congestion.

4.3.2 Bursa - A_1, B_1 and C_1

Grossly, the bursa from A1 group did not show any pathological changes, but the size appeared to be smaller compared to the B1 and C1 group. The bursa from A1 group appeared to be more tense and firm compared to B1 and C1. There was no gross difference in the bursa of B1 and C1 groups.

The histological picture of the bursa from the A1 group showed lymphoid follicles of varying size. The follicles were few in number and smaller in size. The follicles were devoid of active germinal centre without any evidence of lymphoblastic activity. The tendency for stromal proliferation was evident. The interfollicular area showed slight oedematous changes. The epithelial lining at places was eroded (Fig.34). In some of the bursa the follicles were seen separated from each other by loose stroma and the follicles were of varying shape. The interfollicular area showed oedematous changes.

The follicles of the bursa from the birds of the B1 group had more lymphoblastic activity when compared to the A1 group. Follicles in the bursa of B1 and C1 groups were uniform, with large number of lymphoid cells. The bursal stroma was intact (Fig.35).

4.3.3 Bursa - A_2, B_2 and C_2

There was no appreciable gross pathological changes noticed in the bursa of the ducks sacrificed at the end of the 10th week. The bursa from some of the birds in the A2 group appeared smaller in size and the consistency of the bursal wall was more firm compared to the B2 and C2 group.

Histologically, the changes were more pronounced in A2. Some of the bursa revealed large follicles with very less of lymphoblastic activity. The interfollicular tissue revealed severe stromal hyperplasia (Fig.36). The musculature of the vessels was relatively thick and there was reduction of the lumen. The epithelial cells showed hyaline changes. Follicles of the bursa of some of the birds were of varying size and shape without much lymphoblastic activity (Fig.37).

Bursa from group B2 showed large follicles with active germinal centre. The epithelial tissue showed signs of secretory activity (Fig.38).

The bursa from the group C2 were normal with large uniform follicles with active germinal centre showing lymphoblastic activity (Fig.39).

4.3.4 Thymus - A_1, B_1 and C_1

Grossly, the thymic chain did not show any pathological changes except that the thymus from group A, was slightly smaller in size compared with B1 and C1.

Histologically, the thymus from group A, showed mild to moderate degree of congestion and the lymphoid cells were loosely arranged. Slight tendency for fibrous tissue proliferation was evident (Fig.40).

The thymus had differentiated lymphoid follicles mainly in the cortex in the control (C1) and B1 group. The medulla contained less number of lymphocytes.

4.3.5 Thymus - A_2, B_2 and C_2

The histological changes were more prominent in the thymus of birds sacrificed at the end of the 10th week.

The thymus of group A2 revealed marked depletion as well as necrotic changes of the lymphoid cells. Blood vessels were congested and thickened (Fig.41). Oedema was also occasionally seen. Hassel's corpuscles were seen frequently. Some sections of the thymus showed severe degree of fibrous tissue proliferation and was devoid of lymphoblastic activity (Fig.42). The sections also revealed hyalinized Hassel's corpuscle and necrotic debris in the parenchyma. Some of the blood vessels were dilated.

The thymus from B2 group showed moderate numbers of lymphocytes loosely arranged (Fig.43) compared to the control group. Reticular cell proliferation was also noticed. Moderate degree of interfollicular oedema was also evident. Hassel's corpuscles were also present.

The thymus from the C2 group showed follicles with active germinal centre and lymphoid activity.

4.3.6 Spleen

Grossly, the spleen from the different groups did not reveal any pathological changes after 6 weeks of the experiment. But the spleen from the experimental groups showed mild to moderate degree of congestion after 10 weeks.

Histologically, the spleen from the B1 and C1 group revealed normal structure, whereas mild degree of vacuolar degenerative changes were noticed in the lymphoid cells of the A1 group. The lymphoid cells were sparse in the germinal centre, which was encircled by a thick band of fibrous tissue. The sinusoids were moderately engorged. Moderate degree of oedematous changes was also noticed (Fig.44).

Spleen from the B2 group showed moderate degree of congestion. The germinal centre was moderately active. The blood vessels revealed endothelial proliferation as well as thickening of the vessel wall. Mild degree of degenerative changes of the lymphoid cells were also noticed (Fig.45).

The spleen from the A2 group revealed depletion and necrotic changes of a more severe degree compared to the group B2. The blood vessel walls were markedly thickened almost occluding the lumen. The lymphoid cells in the follicles were scattered and necrotic. The parenchyma had a washed out appearance devoid of the lymphoid cells (Fig.46).

4.3.7 Liver, kidney and brain

The liver, kidney and brain from the treatment and the control groups did not reveal any gross pathological changes.

Histologically, the liver and kidney showed mild degree of congestion and fatty changes at the end of the sixth week of the experiment (A1 and B1). A few of the liver specimens from the A2 group showed fatty change of the hepatic cells.The lobular architecture was not disturbed. Mild degrees of vacuolar degenerative changes of the renal epithelial cells were also noticed.

There was no conspicuous histological changes noticed in the brain except for moderate dilatation of perivascular space and occasional perineuronal oedema in the A2 group. The B2 group showed mild degree of gliosis and neurons with hyperchromatic nucleus.

The control group did not reveal any histological changes.

4.3.8 Histological changes in the skin response to DNCB and PHA

The sections of the skin showed pronounced oedema, congestion and inflammatory changes in all the groups after

24 h. The oedematous changes were almost uniform, but the infiltrated cells were more in the control group and consisted of plasma cells, lymphocytes and macrophages. The cellular content was less in the treatment groups especially in the A2 and B2 groups. The inflammatory changes were almost of the same pattern in DNCB and PHA. The infiltration subsided by the 48th and 72nd h, but the cells were more in the control group.

4.4 Ultrastructural pathology

The lymphoid tissues like the bursa, spleen and thymus were subjected to ultrastructural studies at the end of the sixth and tenth week of the experiment.

The ultrastructural changes noticed were pronounced in the treatment groups and the alterations were more severe in birds sacrificed after ten weeks of the experiment.

4.4.1 Bursa of Fabricius - Ultrastructural changes

After 6 weeks (A1 B1 C1)

A1 group

Bursa from group A1 revealed large number of lymphoid cells, some of which were lymphoblasts. Some of the cells had

more of heterochromatin whereas in some cells, the chromatin was condensed. The nucleus was of varying size and shape. The nuclear membrane was thickened and electron opaque. Small round vesicles were also noted associated with the condensed chromatin. Evagination of the nuclear membrane was also evident. The cytoplasm appeared granular. The changes were prominent in the mitochondria. Some of the lymphoid cells had spherical mitochondria with lysed cristae. The cytoplasm contained a few vacuoles. The endoplasmic reticulum was prominent at places. The intercellular membrane was found to be intact (Fig.50).

B1 group

Mature lymphocytes with more of euchromatin was noticed. Lymphoblastic activity was less. The cytoplasm was granular with electron dense mitochondria. The intercellular space showed separation between the cells. The reticular cells with irregular nuclear membrane was also noticed (Fig.51).

The control group (C1) showed mature lymphocytes along with lymphoblastic cells and a few cells in stages of mitosis could be seen.

10th week (A2, B2, C2)

A2 group

Organellar damages were more pronounced. Chromatin was condensed at the nuclear rim and the nucleus was in abberant shapes (Fig.52). The nuclear membrane was fused and thickened at places. The nuclear pores were less when compared with the control. Perichromatin granules could also be seen in the karyoplasm. The mitochondria were swollen and cristolysis was evident. Electron dense condensed mitochondria could also be noted in some sections. The endoplasmic reticulum showed extensive fragmentation and cystic dilatation (Fig.53). Fragmented endoplasmic reticulum was present in the cytoplasm with the ribosomes attached to it. Degranulation of the endoplasmic reticulum was also seen at places. Lipid droplets were noticed in the cytoplasm (Fig.54). Electronlucent membrane bound vesicles were also seen.

B2 group

The ultrastructural changes were less severe compared to the A2 group. The chromatin condensation was prominent. Nuclear membrane was thickened with condensation of chromatin and with formation of vesicles at places. Mitochondria were swollen and the cristolysis was evident resulting in granular

matrix content. A few of the cells showed densely packed moderately electron dense mitochondria. Moderate degree of fragmentation of the endoplasmic reticulum was evident. The cytoplasm was generally granular in texture. But the desmosomes were intact (Fig.55).

The group C2 did not reveal any ultrastructural changes in the bursa.

4.4.2 Thymus

Ultrastructurally, the changes were more pronounced in the A group.

A1 group

The nucleus of the lymphocytes showed condensed chromatin in the karyoplasm. The nuclear membrane was thickened and fused at places. The mitochondria were slightly swollen and the cristae were elongated extending to about three fourth of the mitochondrial width. But almost all the mitochondria showed slight tendency for lysis of the cristae. The cytoplasm was highly granular with fragmented endoplasmic reticulum at places. Electron dense irregular lysosomes and fragmented endoplasmic reticulum could also be seen (Fig.56).

B1 group

The ultrastructural changes were not very prominent in the thymus of the B1 group compared to the A1 group. The number of lymphocytes were more compared to A1. Many of the nucleus had heterochromatin. Mitochondria of varying shapes although showed slight tendency of lysis, were almost intact with electron dense granular matrix and cristae. The cytoplasm was highly granular. The intercytoplasmic membranes were intact. The endoplasmic reticulum was scanty.

10th week

A2 group

The number of lymphocytes were very few compared to the A1 group. The cells generally showed necrotic changes. The nucleus showed condensation of chromatin at the nuclear membrane. The nuclear membrane showed tendency for bleb formation. Nuclear membrane was fused at places and formation of few vesicles was evident at the nuclear rim. Karyoplasm contained perichromatin granules. Very few nuclear pores could be noticed. Many of the cells showed a washed out appearance. The reticular cells showed more necrotic changes than the lymphoid cells with cytoplasmic extensions. The cytoplasm was loosely granular. The mitochondria showed moderate degree of swelling and lysis of the cristae (Fig.57).

Many of the cells contained vacuoles with electronlucent contents as well as tubular fragmented endoplasmic reticulum. In some other areas, dilatation of the endoplasmic reticulum was a conspicuous feature. Fragmented endoplasmic reticulum studded with ribosomes could also be noticed. Disintegration of polyribosomes was also evident. Lipid inclusions were also seen (Fig.58).

B2 group

The ultrastructural changes were almost similar to the B1 group, but the changes were more pronounced in the B2 group. Perichromatin granules were present in the karyoplasm. The mitochondria were markedly swollen with lysis of the cristae. Some of the cells showed dissolution of organelles. Disintegration of cytoplasmic membrane was also noticed at places (Fig.59).

The control groups C1 and C2 did not show any prominent ultrastructural changes.

4.4.3 Spleen

Ultrastructural studies of the spleen revealed many predominant areas of plasma cells, macrophages and heterophils.

A1 group

In many areas, macrophages and lymphocytes with densely condensed chromatin in the karyoplasm were noticed. Perichromatin granules could also be seen. At places, the nuclear membranes were fused. Numerous tubular fragmented endoplasmic reticulum were seen. The endoplasmic reticulum was predominantly of the smooth type. Vacuoles of varying sizes, some of them studded with ribosomes were seen. Mitochondria were severely swollen with lysis of the cristae. The fragmented endoplasmic reticulum was also dilated at places. Electron dense, darkly staining particles were also seen. The cytoplasm was granular (Fig.60 and 61).

The ultrastructural changes in the lymphoid cells were moderate mainly affecting the mitochondria. The nucleus contained a prominent nucleoli with condensed chromatin. The mitochondria were spherical and showed lytic changes. In some areas, cells revealed necrotic changes, devoid of any organelles.

B1 group

Large number of plasma cells with cart wheel shaped chromatin pattern and abundant rough endoplasmic reticulum were noticed. Endoplasmic reticulum revealed dilatation at

places with electronlucent content. The lymphocytes did not reveal any significant ultrastructural changes (Fig.62).

A2 group

Certain sections revealed lymphocytes with severe necrotic changes of the cells resulting in the dissolution of the organelles. Lymphocytes with bizzare nuclear shape and electron dense pyknotic nucleus could be seen. Certain cells revealed completely lysed mitochondria, along with electron dense mitochondria. The cytoplasm was generally devoid of the organelles (Fig.63).

Round electron dense bodies could be seen. The endoplasmic reticulum showed severe fragmentation with electronlucent contents. The nuclear chromatin of the lymphoid cells was markedly dense and condensed.

At a higher magnification, the part of the nucleus was highly granular and perichromatin granules could be seen. Mitochondria was severely swollen with lytic changes. Mitochondrial matrix was granular. Some of the mitochondria also showed granular ring shaped structures inside the matrix (Fig.64).

The endoplasmic reticulum was scanty. Remnants of fragmented endoplasmic reticulum was studded with ribosomes.

Numerous vacuoles of varying sizes with electronlucent content was seen in the cytoplasm.

B2 group

Heterophils with electron dense granular bodies of different size and shape were seen. The cytoplasmic membrane showed extrusions at places. Electron dense granules were seen in the cytoplasm (Fig.65).

The lymphoid cells had approximately equal quantities of euchromatin and heterochromatin. The mitrochondria of varying sizes were seen. Some of them showed lytic changes whereas others were intact. The cytoplasm showed numerous vacuoles of different sizes studded with ribosomes.

Plasma cells at higher magnification showed mitochondria with severe degree of swelling and lytic changes of the cristae. The matrix contained filamentous remnants of the cristae. Moderate degree of fragmentation and dilatation of the endoplasmic reticulum with electronlucent content was seen. Disaggregation of polyribosomes was also evident (Fig.66).

C1 and C2 groups

The control group did not show any ultrastructural changes. The cells mainly consisted of lymphocytes along with macrophages and plasma cells.

Fig.1. Embryo-28th day control and `A' group showing stunted growth

Fig.2. Group `A'-Embryo-21st day - Dead stunted embryo


CONTROL A
28 DAY

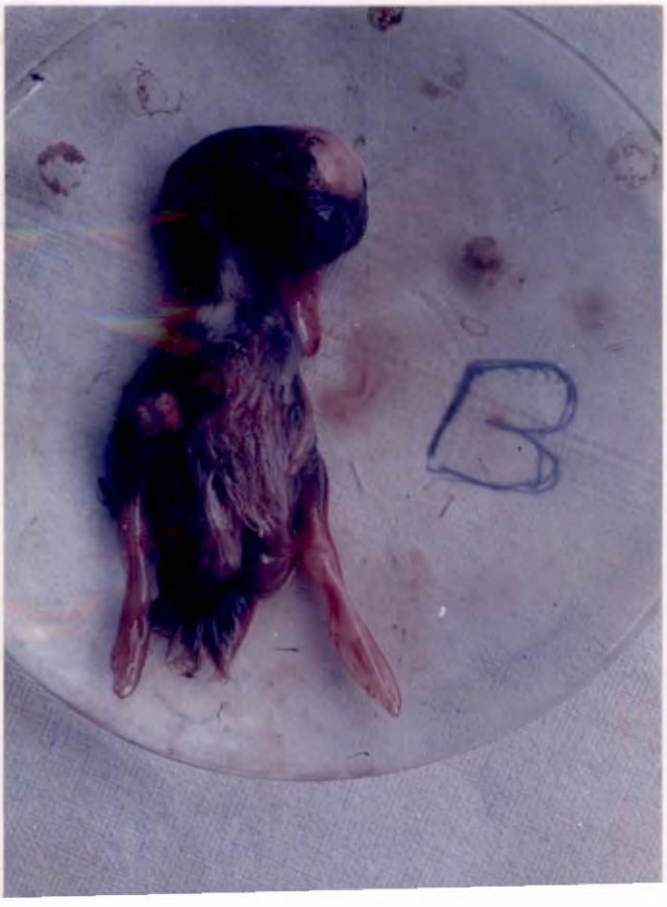
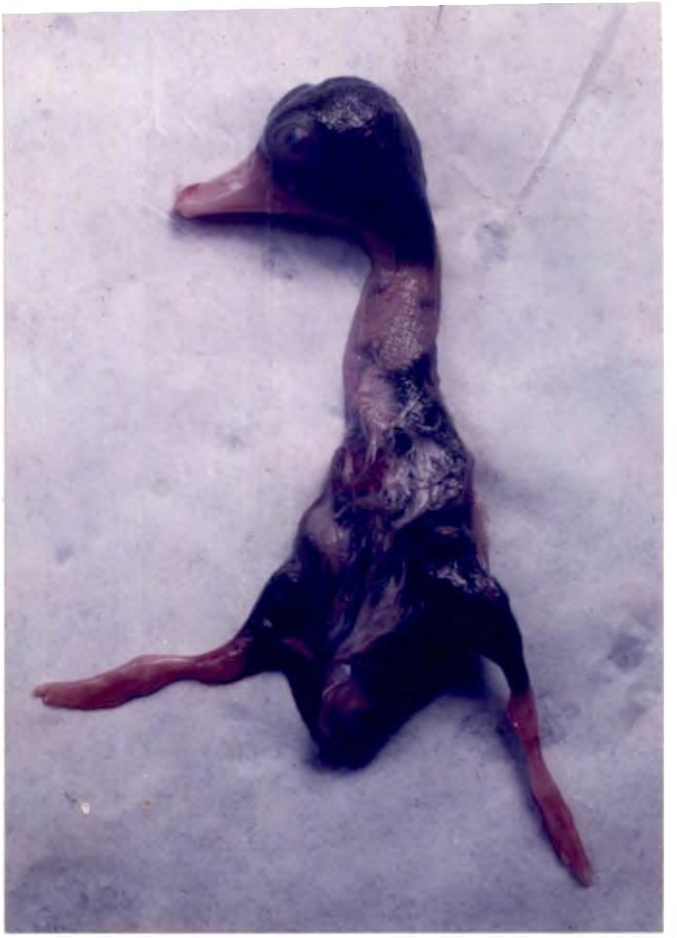

B

Fig.3. **Embryo-28th day- Group A showing generalised oedema**

Fig.4. **Embryo-28th day - Group B showing oedema of the head and neck and hermiation of the yolk sac**



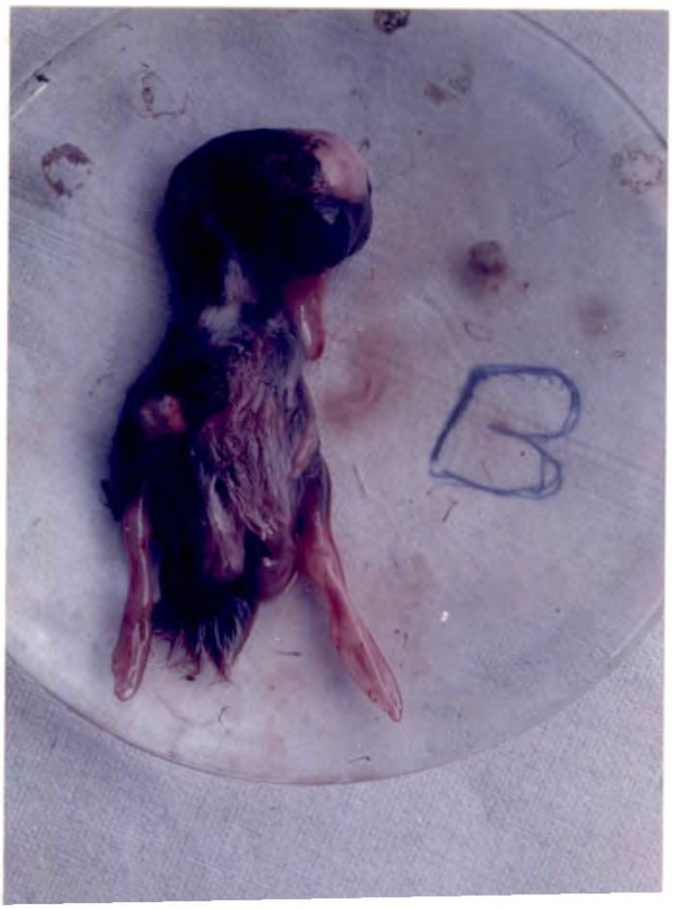
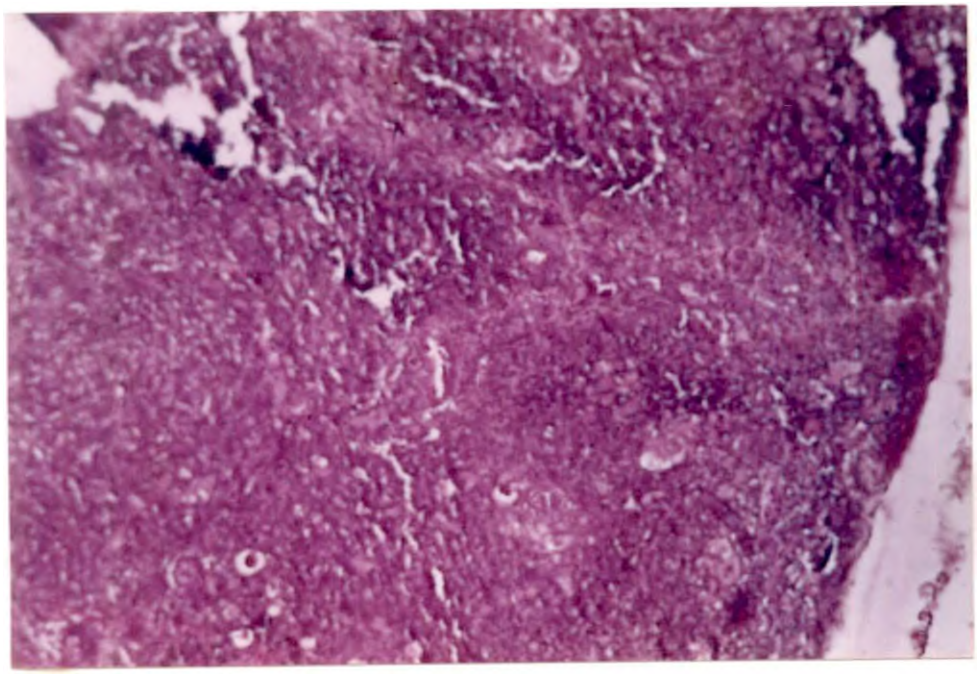

B

Fig.9. Embryo-28th day-Group A - Bursa showing loosely arranged lymphoid follicles with oedematous changes and scattered lymphocytic cells. H&E-200

Fig.10. Embryo-28th day-Group A - Thymus lack of lymphoblastic activity H&E-200



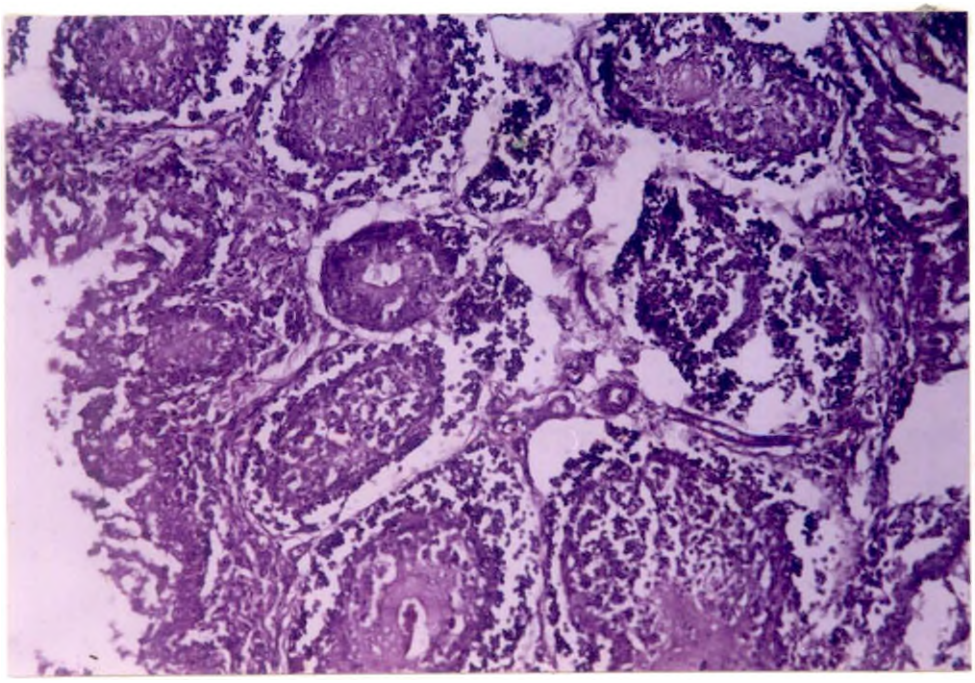


Fig.11. Electron micrograph - Embryo A Gr. Bursa showing lymphocytes and lymphoblasts with dense heterochromatin. x8000

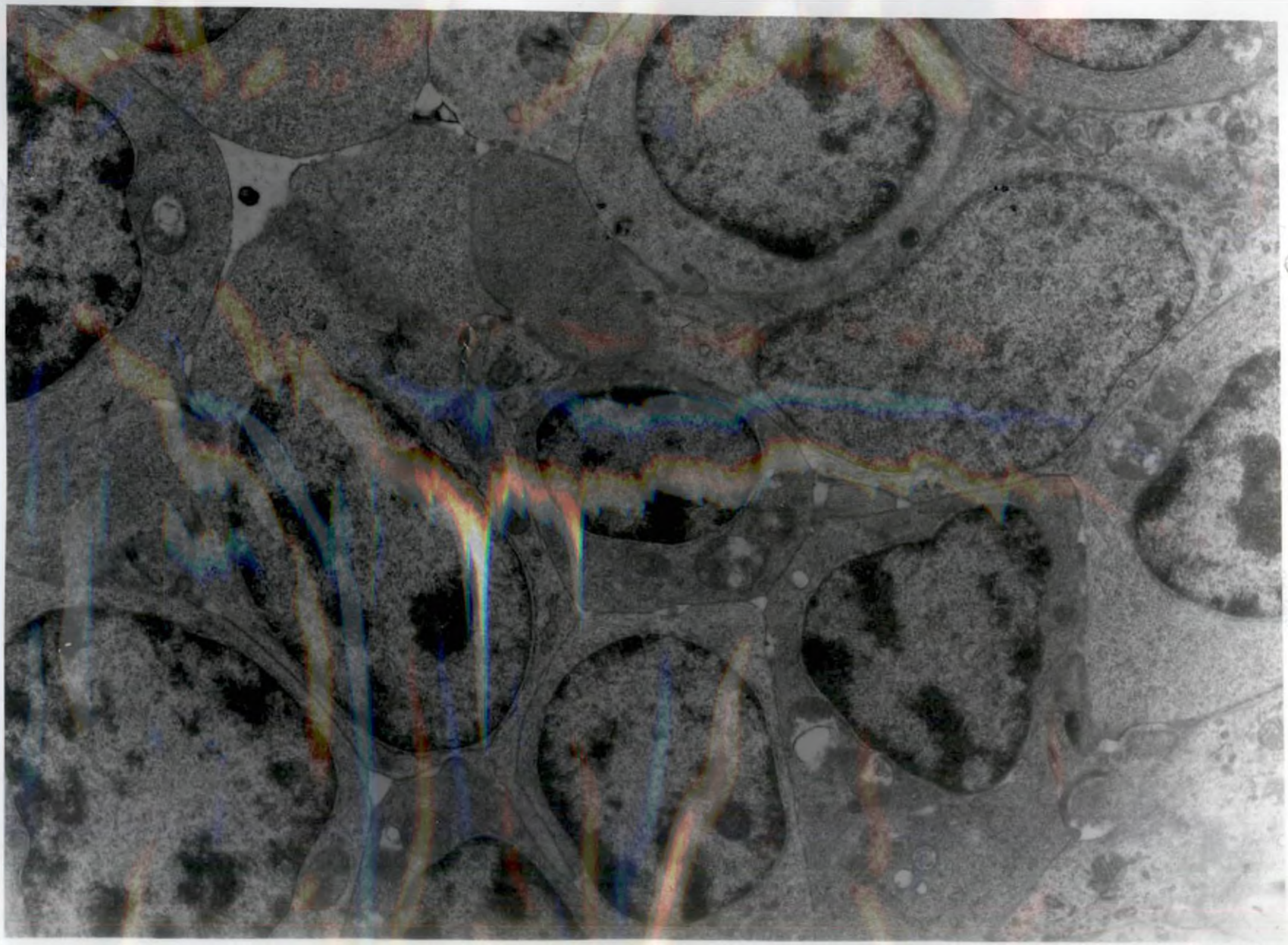


Fig.12. **Electron micrograph - Embryo B Gr. Bursa: Lymphoblasts with indented nuclear membrane and granular cytoplasm. Separation of the lymphoid cells seen. x6000**

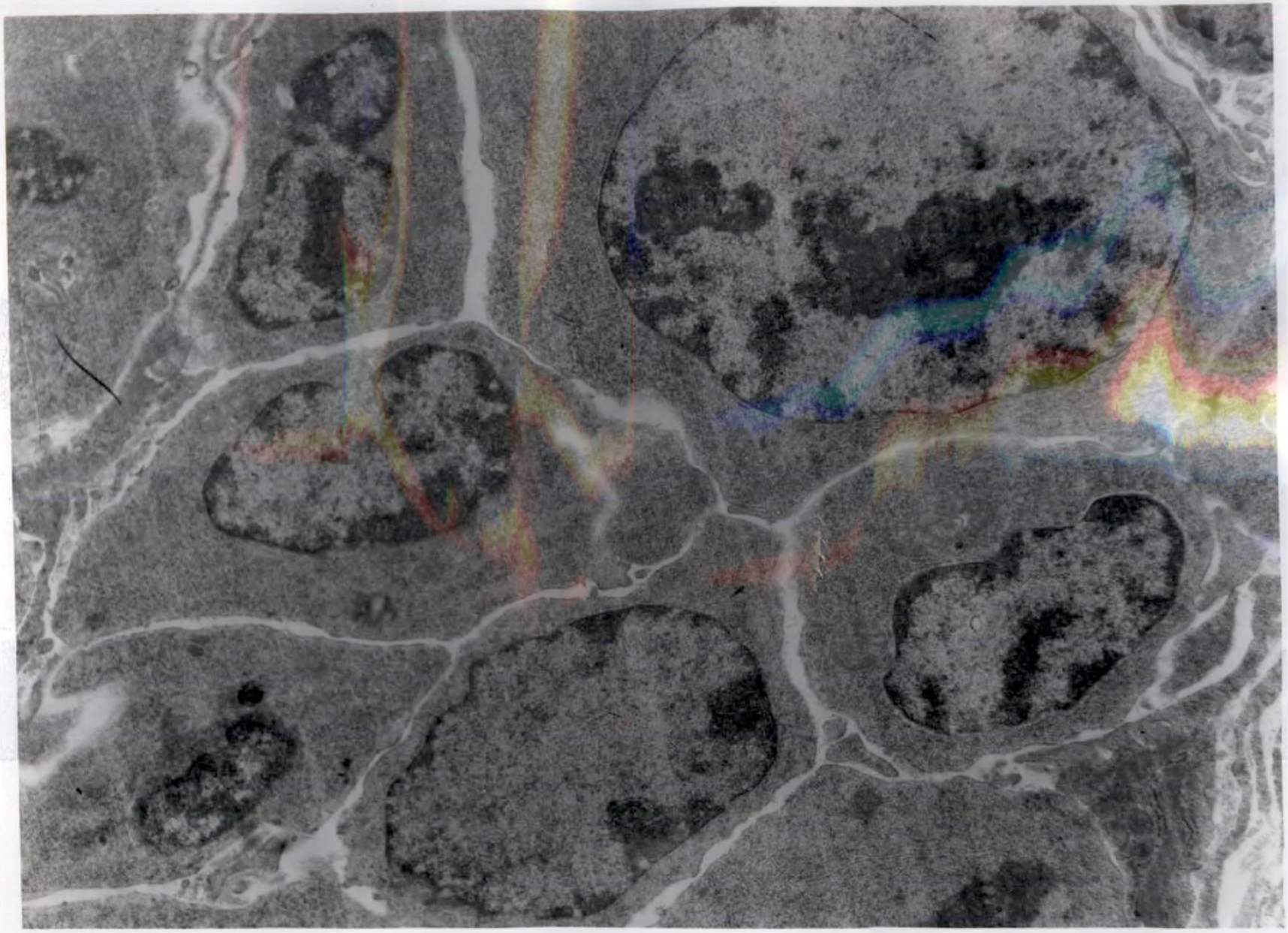


Fig.13. Electron micrograph - Embryo B Gr. Bursa: Lymphocyte showing granular cytoplasm and mitochondria showing cristolysis. x12000

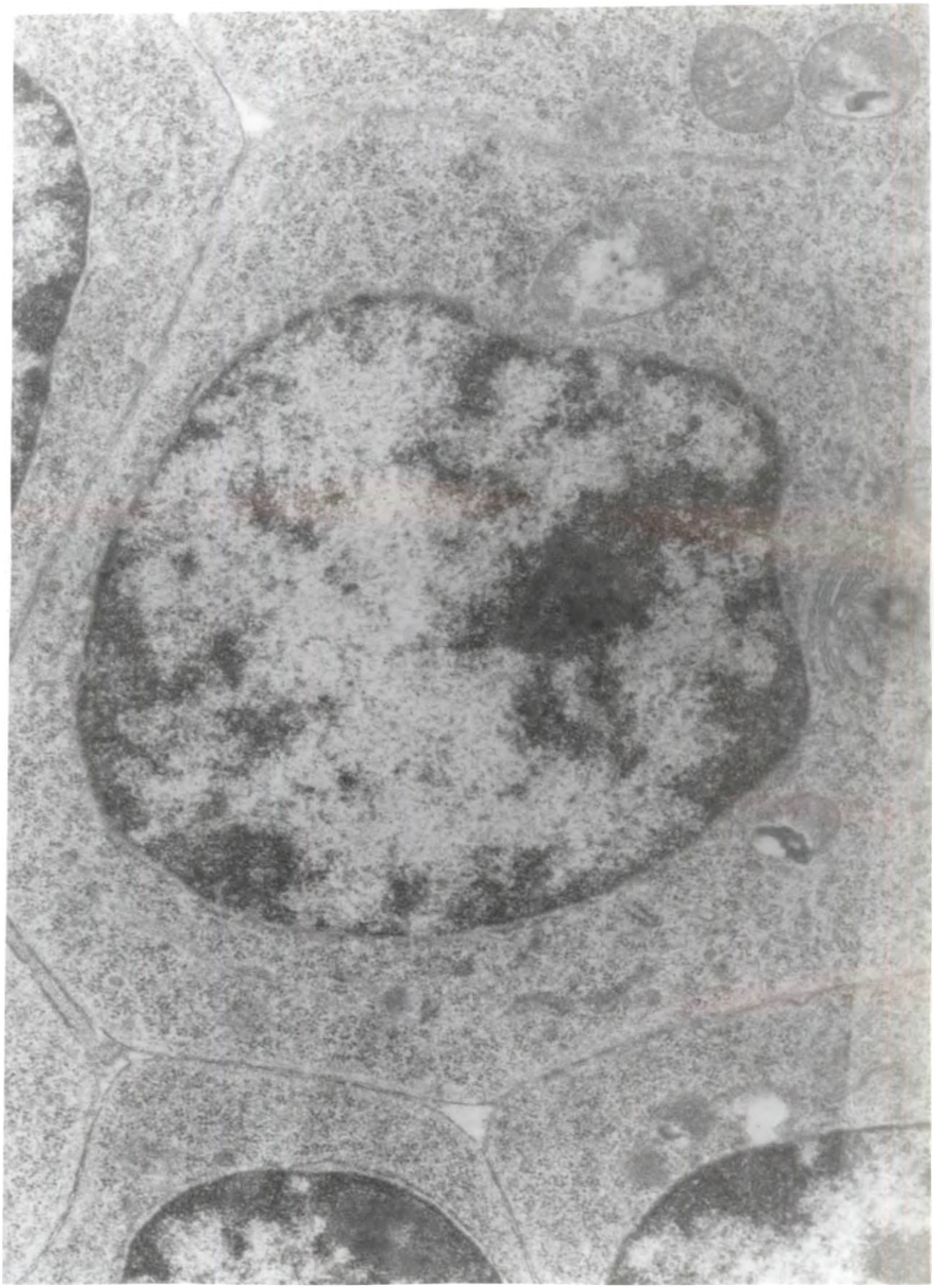


Fig.14. **Electron micrograph - Embryo A Gr. Thymus: Dark stained heterochromatin, dense mitochondria and disruption of cellular membrane. x30000**

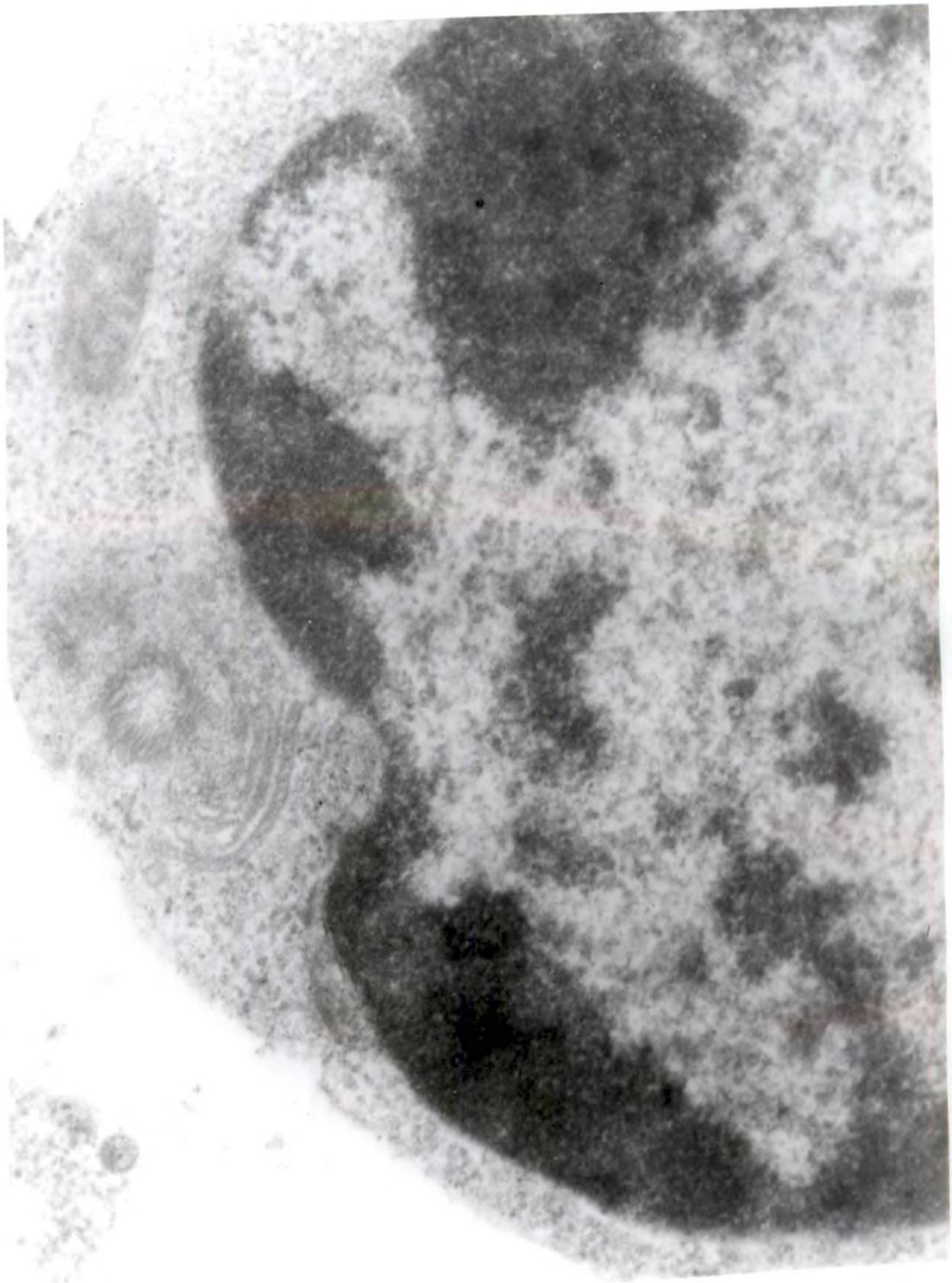


Fig.15. Electron micrograph - Embryo B Gr. Thymus: Separation of cells with increased intracellular space. x5000

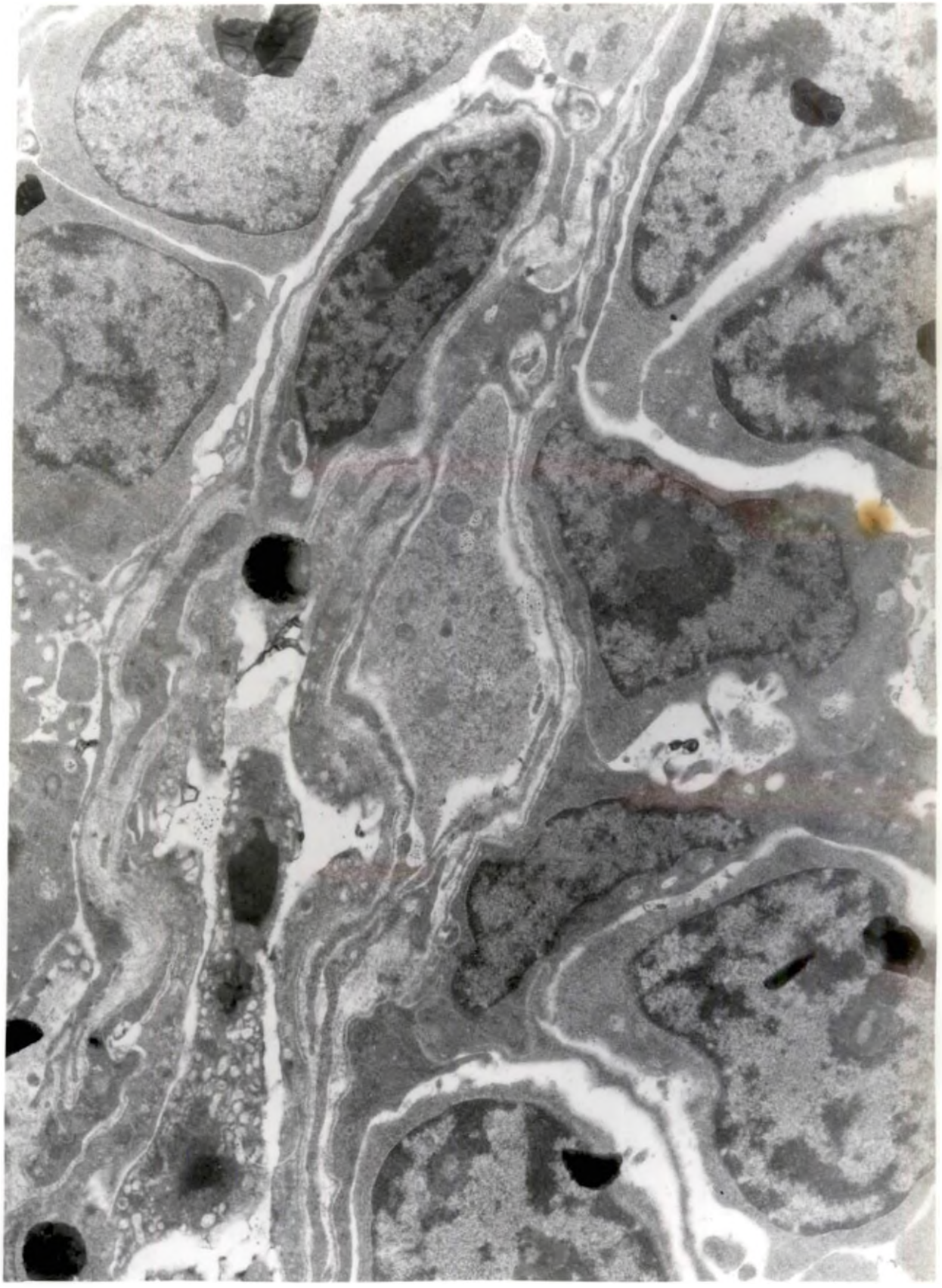


Fig.16. **Electron micrograph - Embryo A group. Spleen: Lymphocyte and a heterophil with electron dense granules. Mitochondria of varying size with cristolysis and other vacuolar changes. x10000**

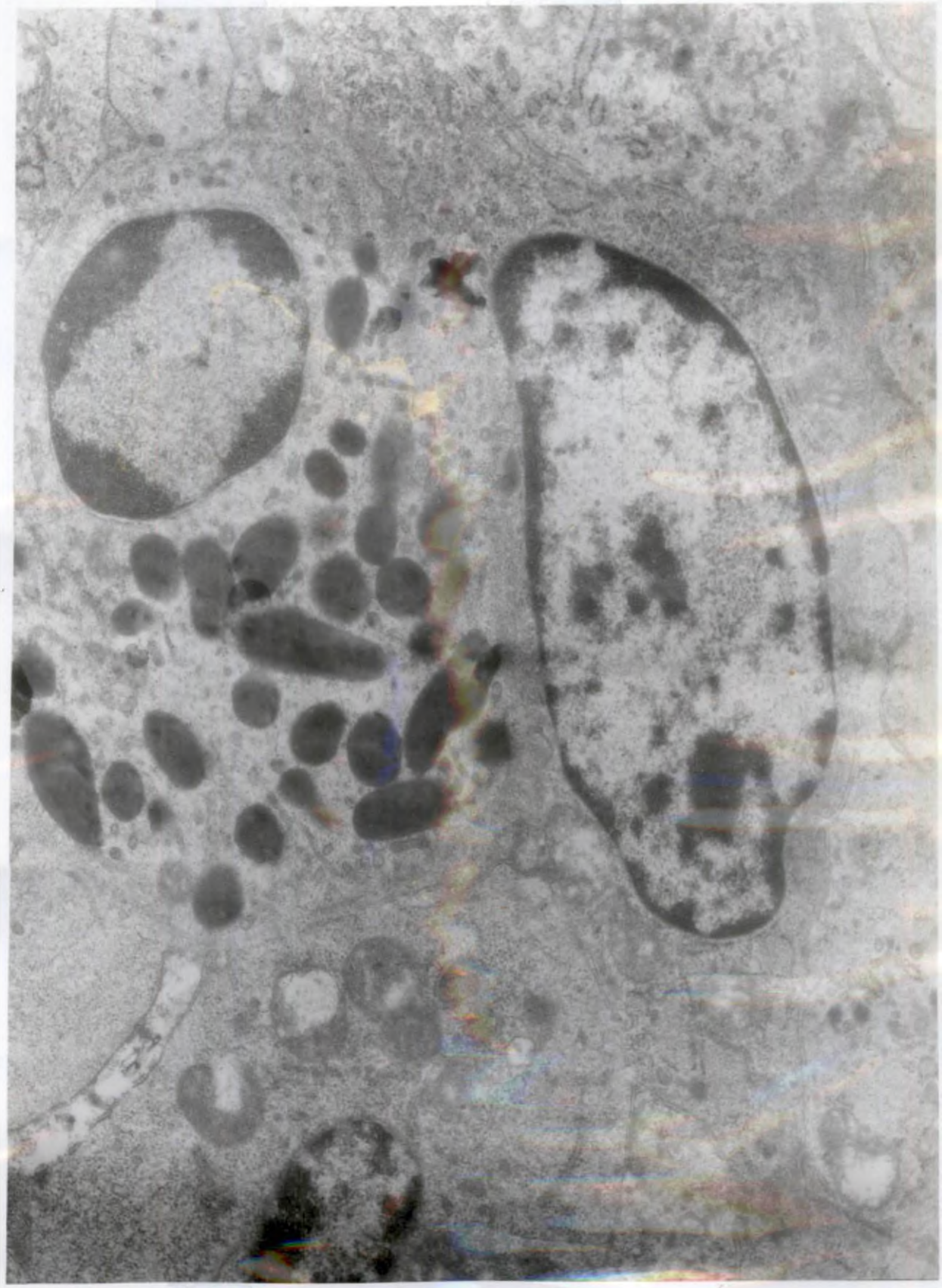
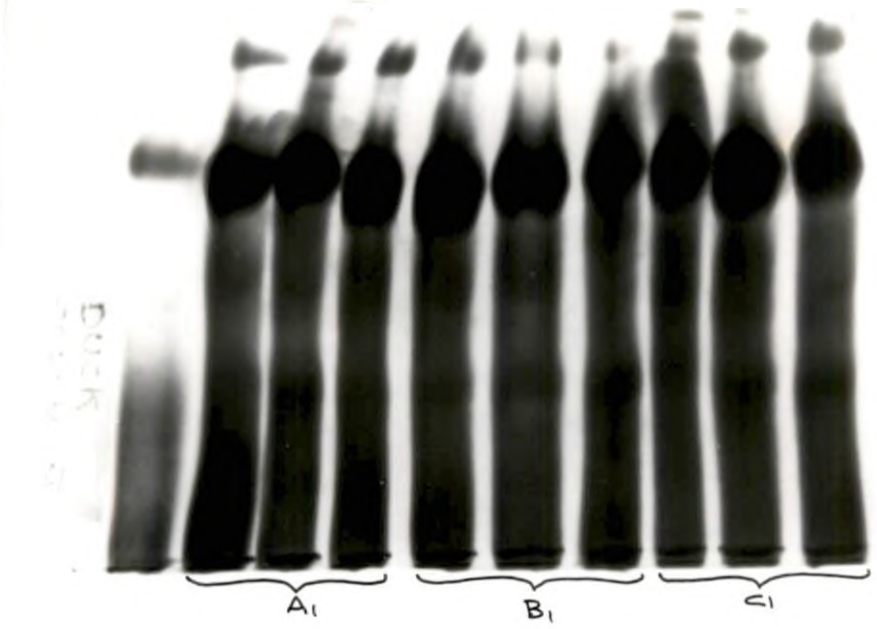


Fig.25. Electrophoretic patterns in groups A_1, B_1 and C_1

Fig.26. Electrophoretic patterns in groups A_2, B_2 and C_2


A1
B1
C1

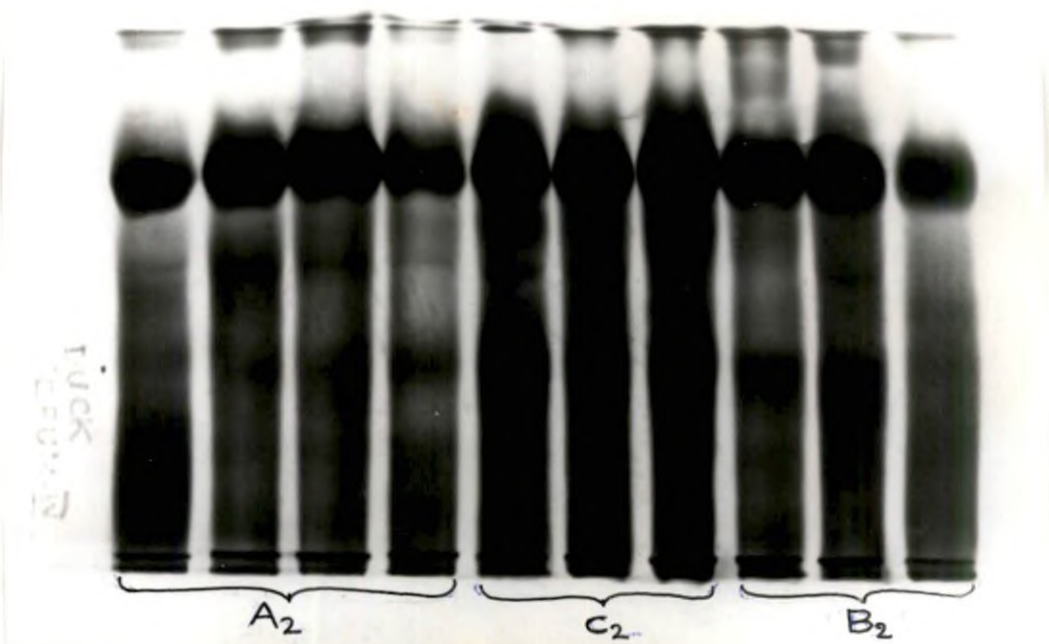
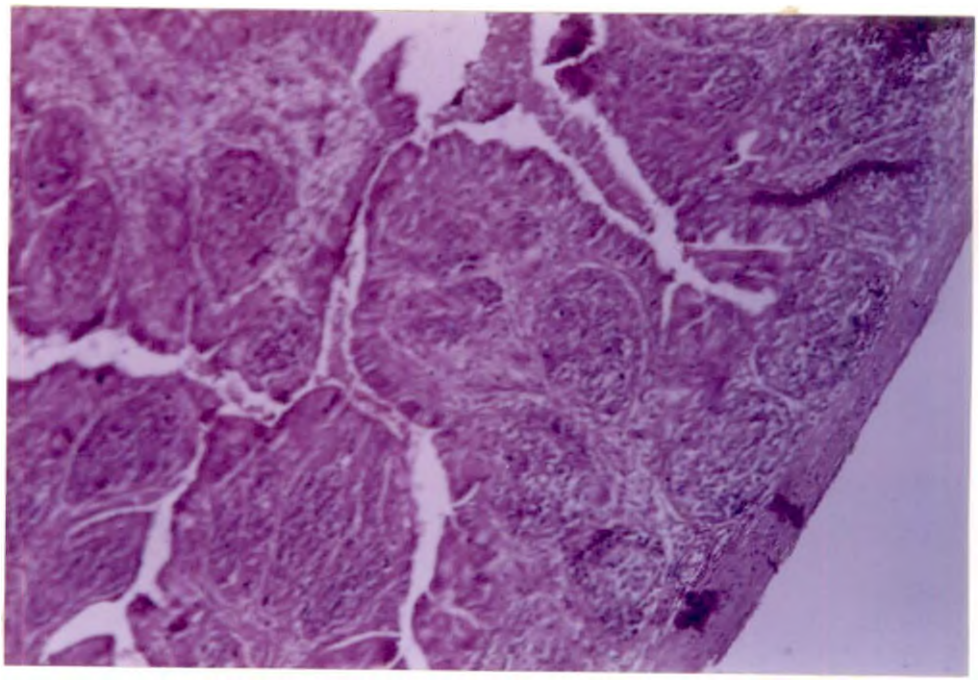

A2
C2
B2

Fig.34. Group A_1 Bursa: Erosion of epithelial cells and interfollicular oedema. H&Ex200

Fig.35. Group B_1 Bursa: Uniform sized follicles with moderately active germinal centre and mild vacuolar degenerative changes and debris in the lumen. H&Ex400



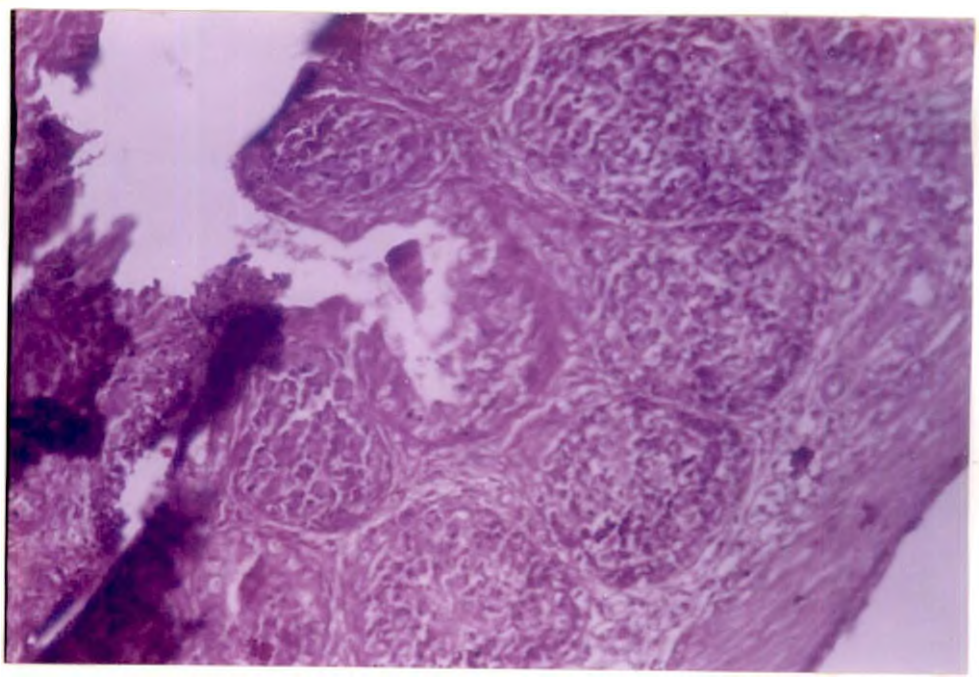
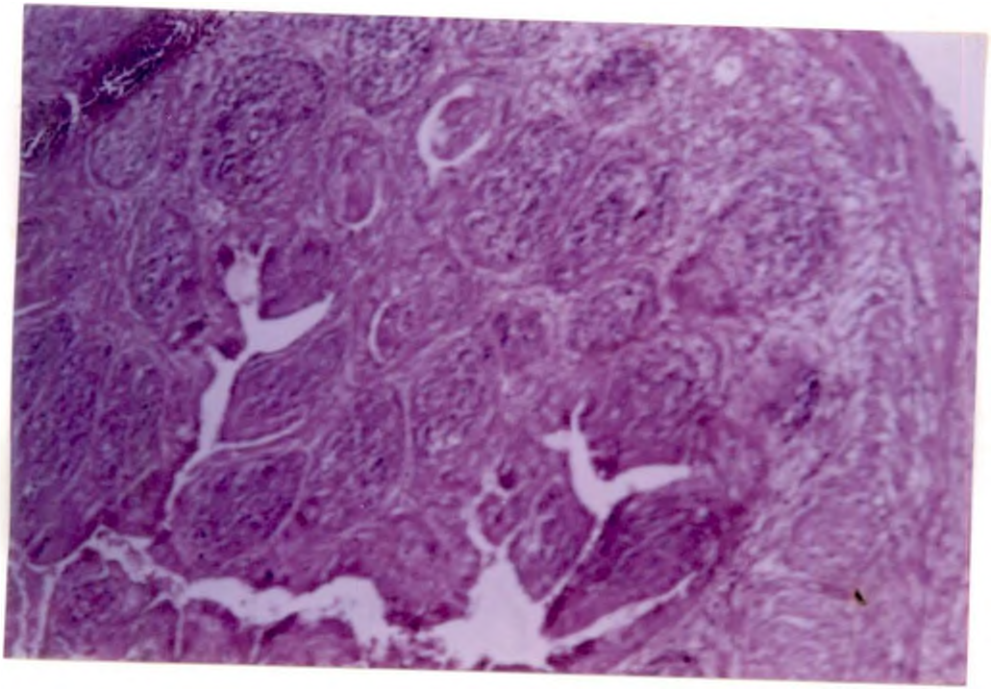


Fig.36. Group A_2 Bursa: Depleted follicles with severe stromal fibrosis. H&Ex400

Fig.37. Group A_2 Bursa: Follicles of varying sizes with severe interfollicular stromal hyperplasia. H&Ex200



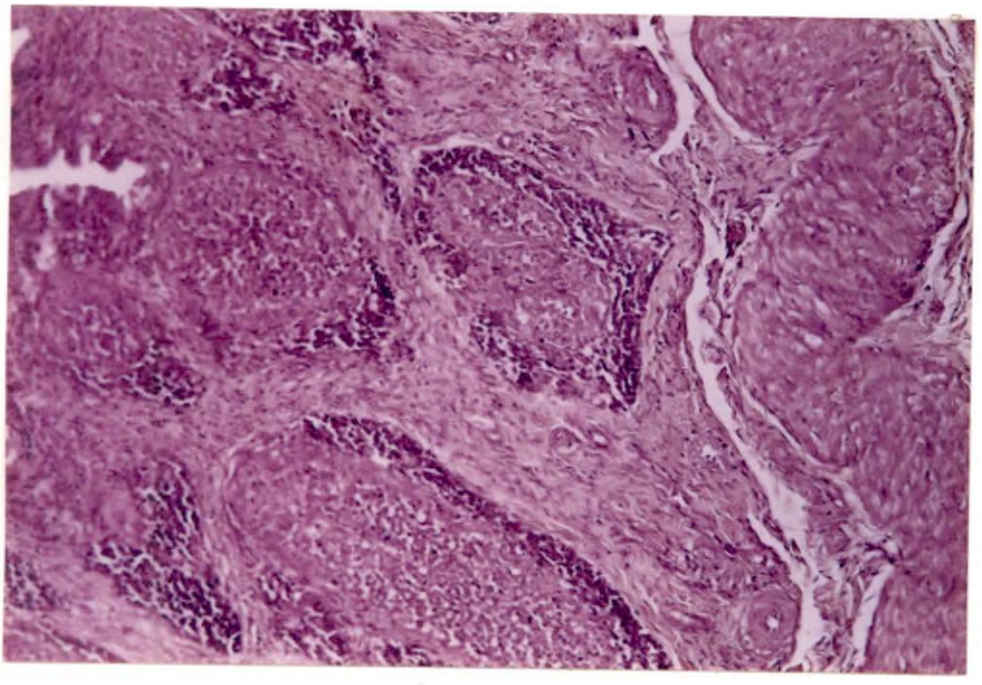
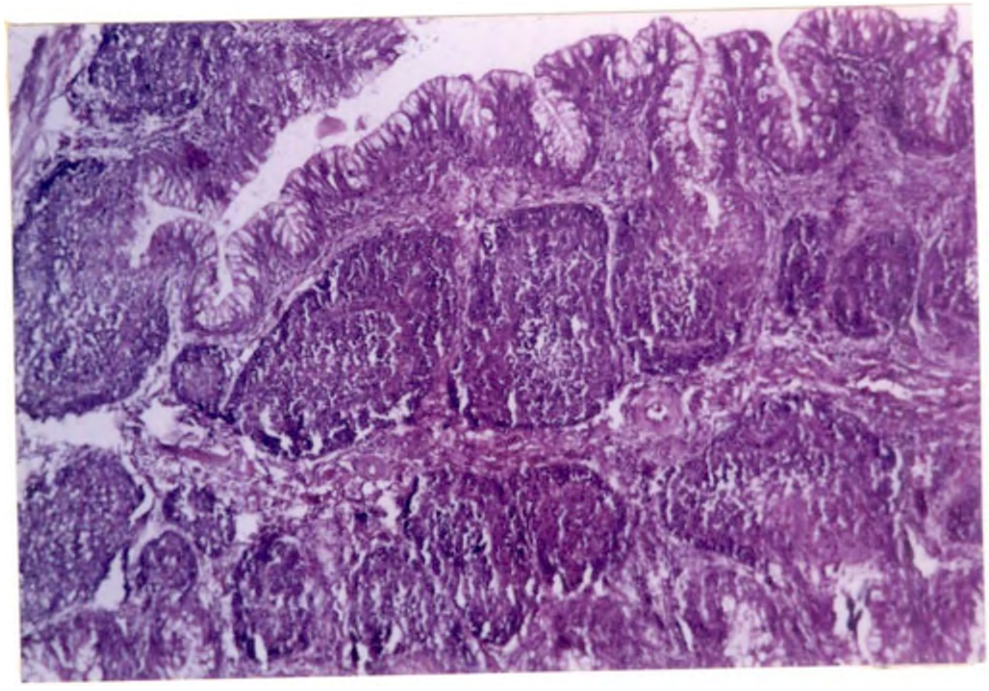


Fig.38. Group B_2 Bursa: Follicles with depleted germinal centre and epithelial cells showing secretory activity. H&Ex400

Fig.39. Group C_2 Bursa: Normal follicles. H&Ex400



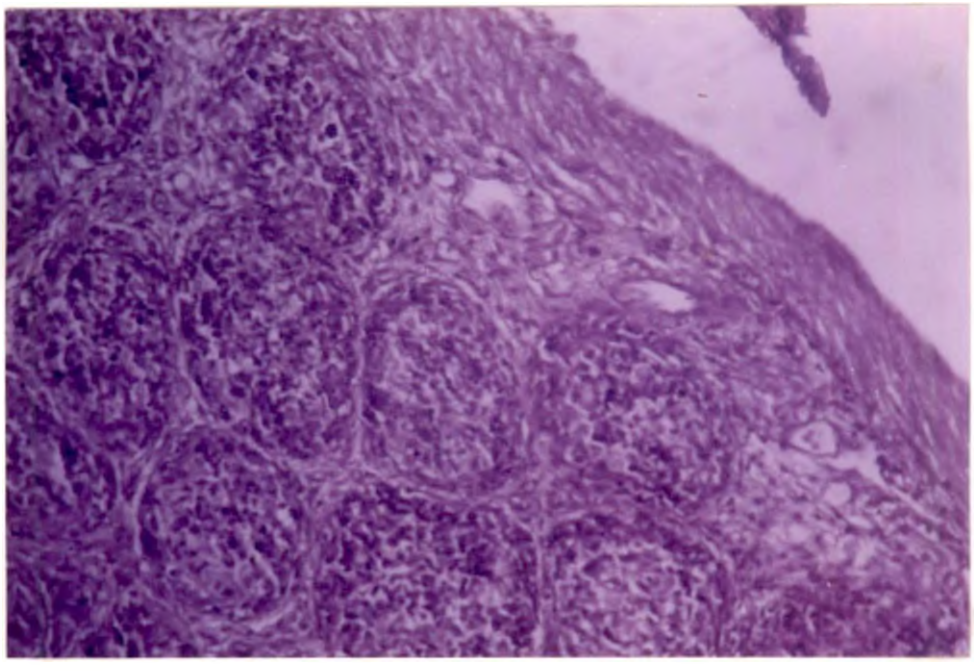
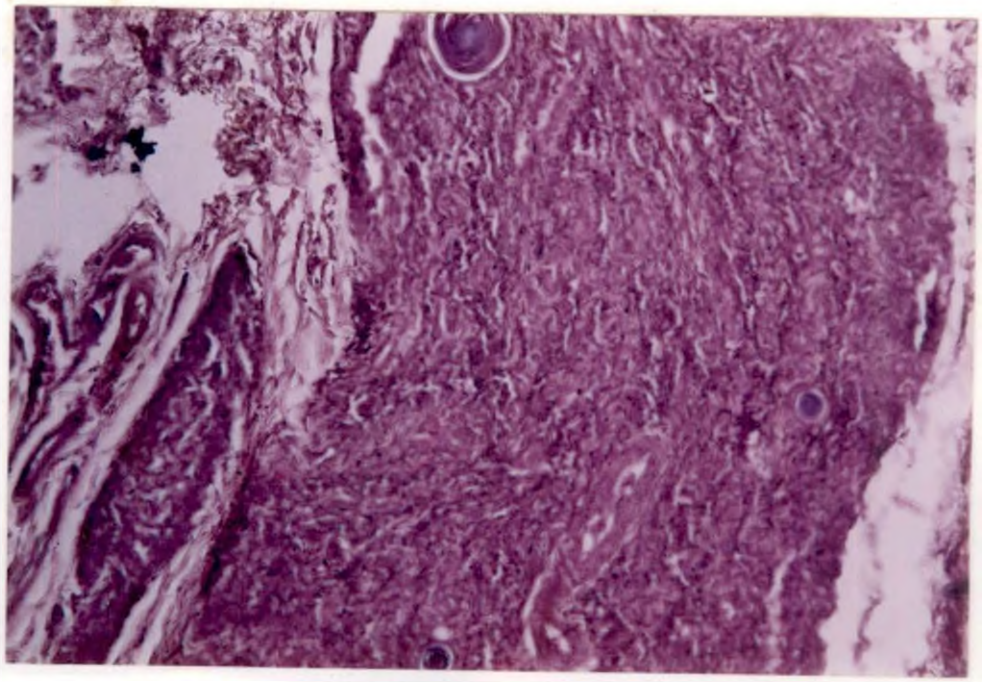


Fig.42. Group A_2 Thymus: Severe degree of fibrous tissue proliferation. H&Ex200

Fig.43. Group B_2 Thymus: Loosely arranged lymphoid cells. H&Ex200



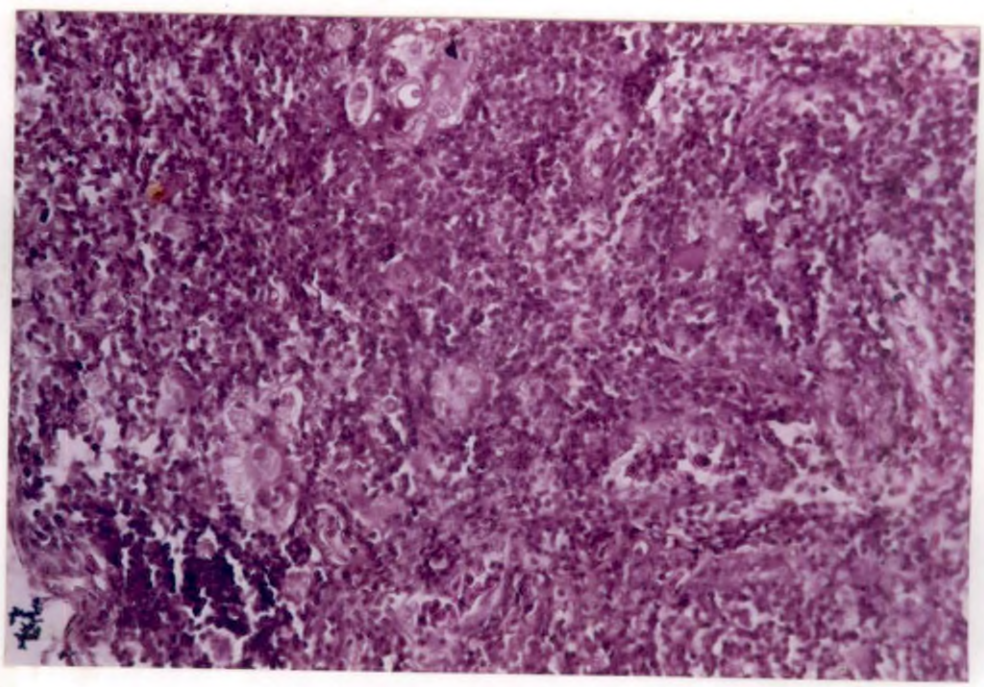
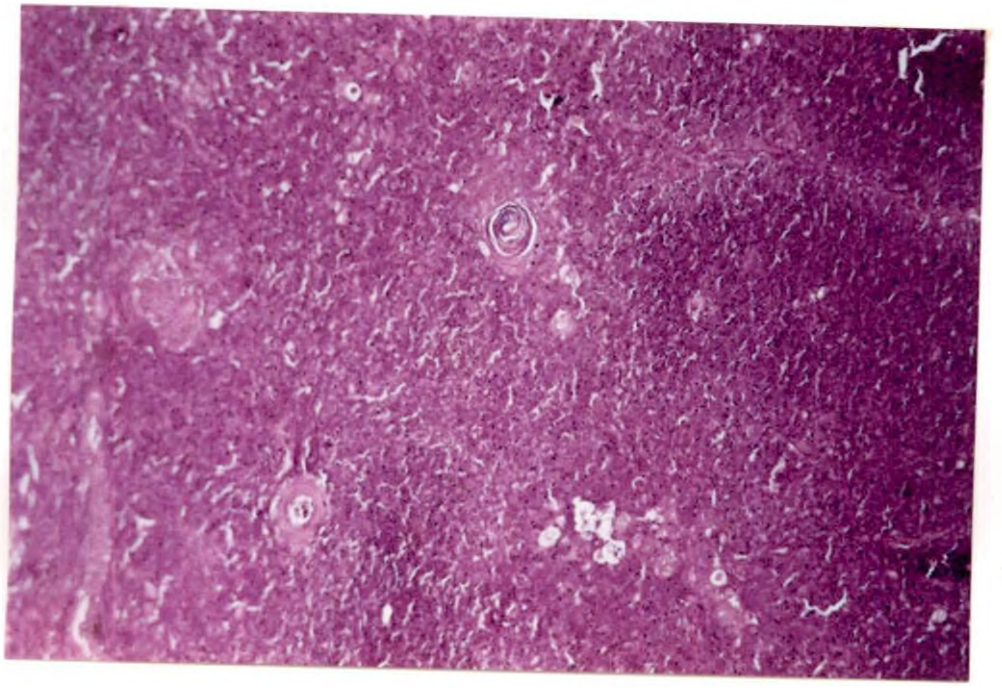


Fig.40. Group A_1 Thymus: Mild degree of congestion and loosely arranged lymphoid cells H&Ex200

Fig.41. Group A_2 Thymus: Marked depletion of lymphoid cells and necrotic changes along with oedema. H&Ex200



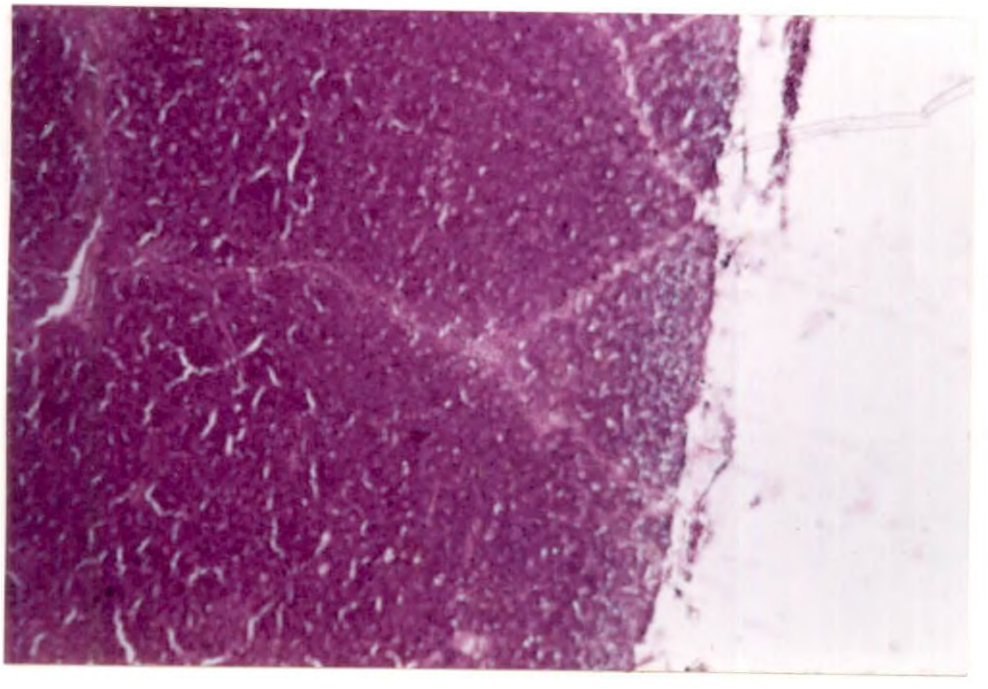
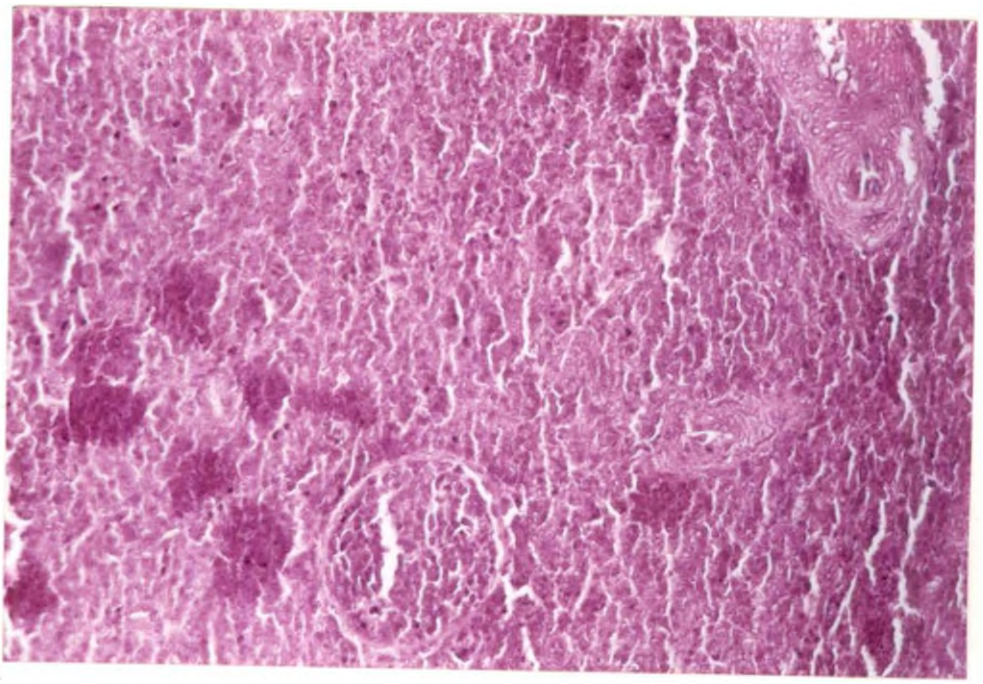


Fig.44. Group A_1 spleen: Sparse lymphoid cells in the germinal centre with thick fibrous tissue in the periphery. H&Ex200

Fig.45. Group B_2 spleen: Depleted germinal centre encircled by thick fibrous tissue. Oedema is also evident. H&Ex400



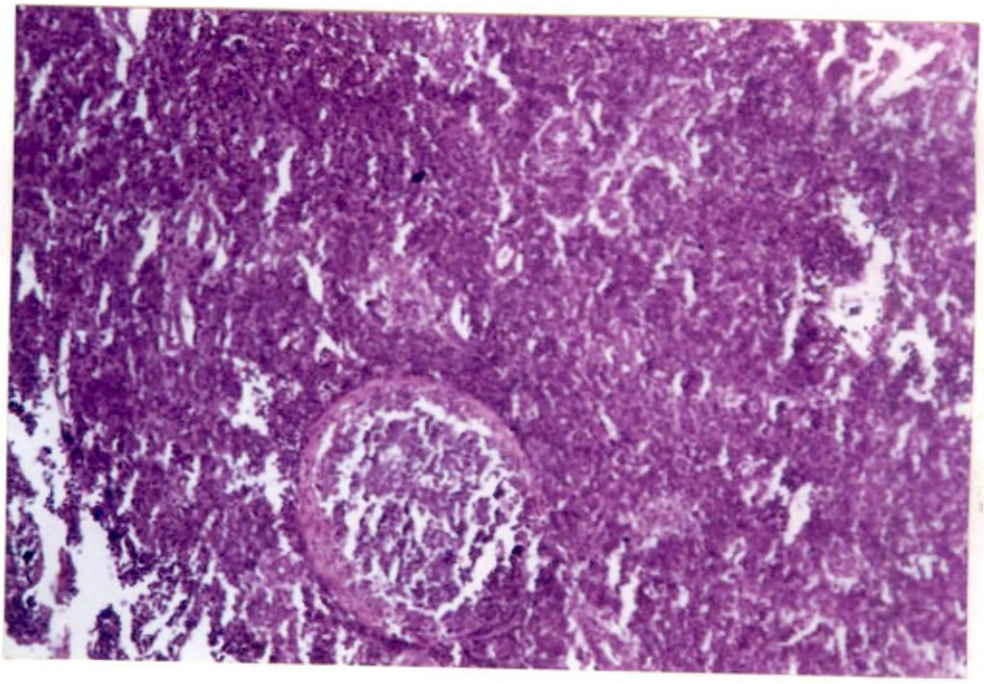
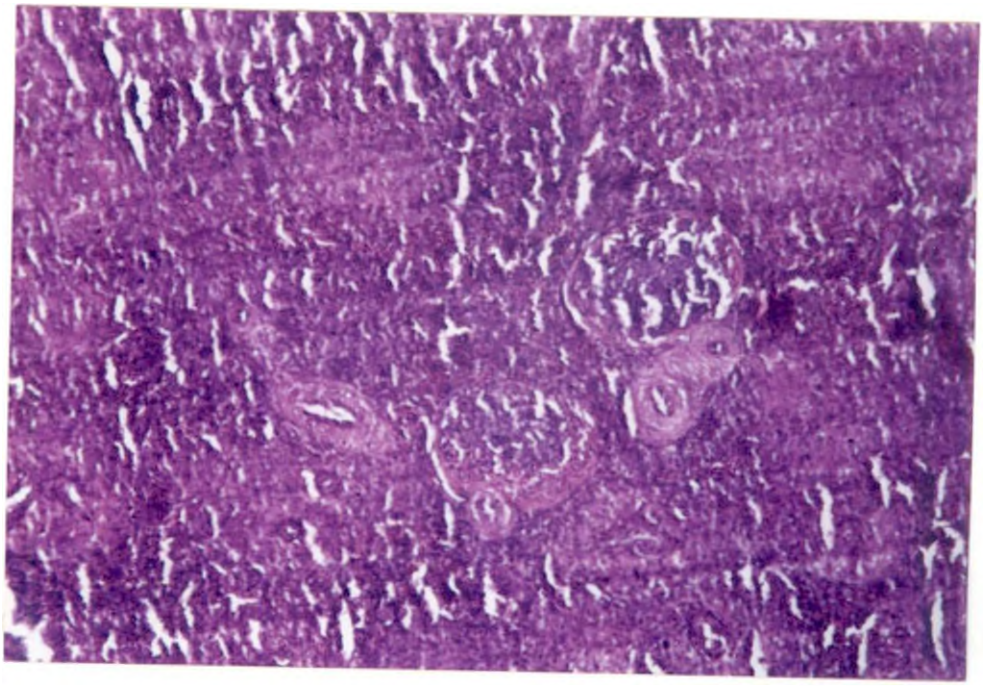


Fig.46. Group A_2 spleen: Thickened blood vessel wall H&Ex200

Fig.47. Group C_2 24 h skin response to DNCB, infiltration of plasma cells and lymphocytes H&Ex200



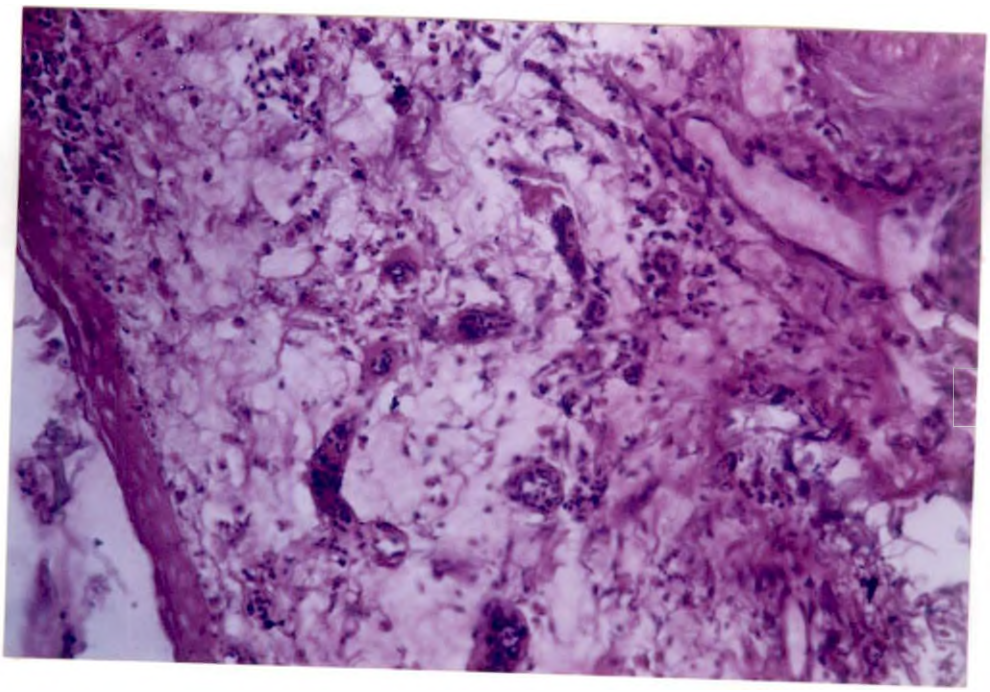
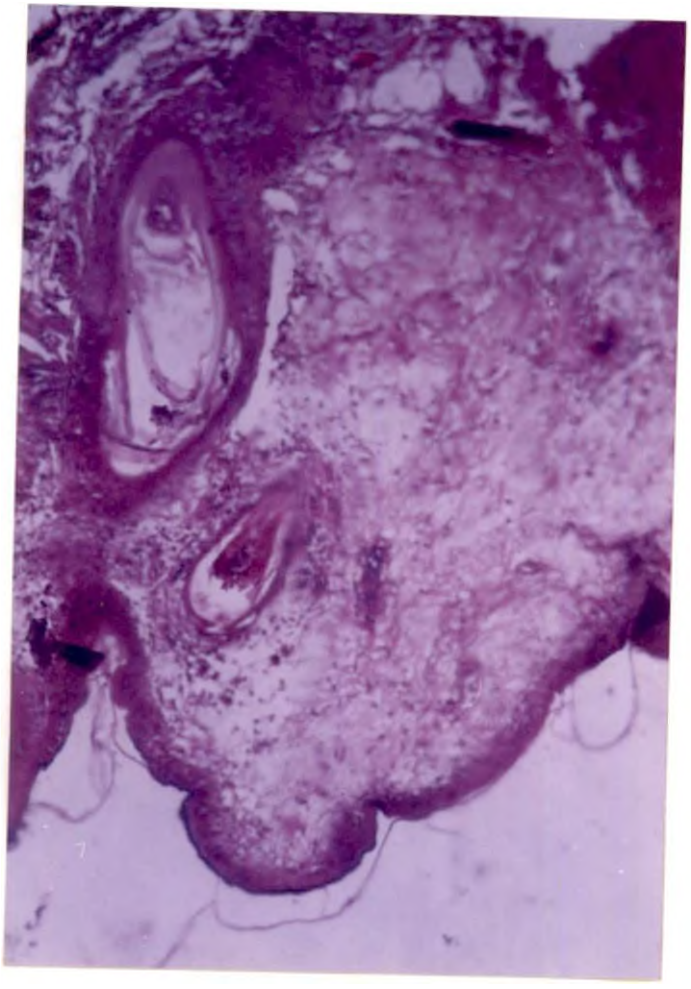


Fig.48. Group C_2 24 h skin response to PHA-M, Infiltration of plasma cells, heterophils and lymphocytes H&Ex200

Fig.49. Group A_2 24 h skin response oedematous changes with less intensity of infiltration H&Ex200



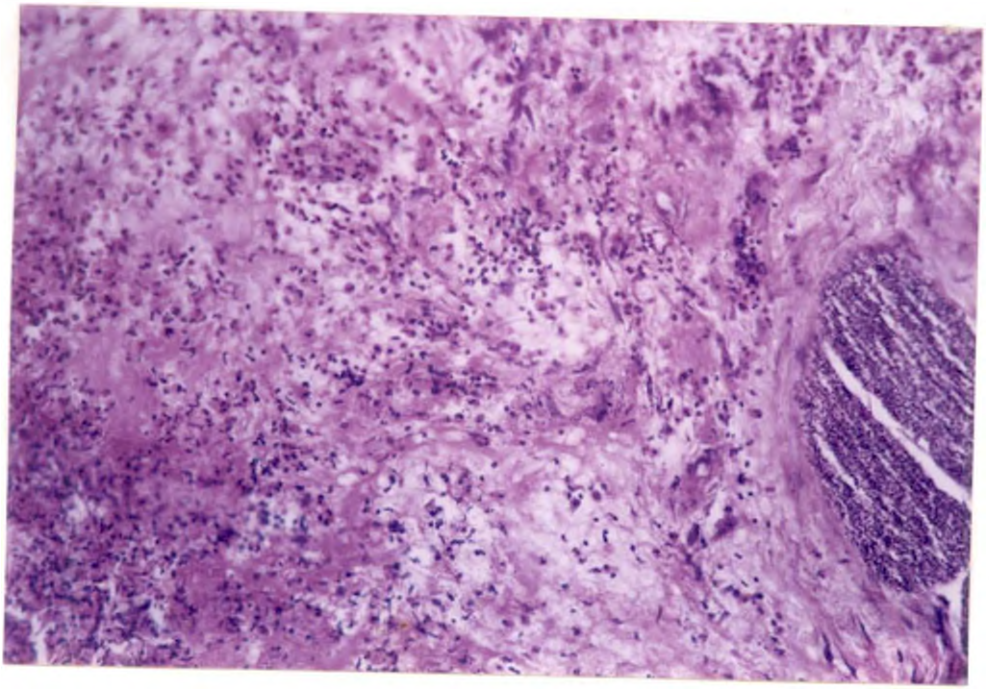


Fig.50. Electron micrograph Gr. A_1 Bursa: Lymphocytes and lymphoblasts - Evagination of the thickened nuclear membrane. Crystolysis of the swollen mitochondria. x8000

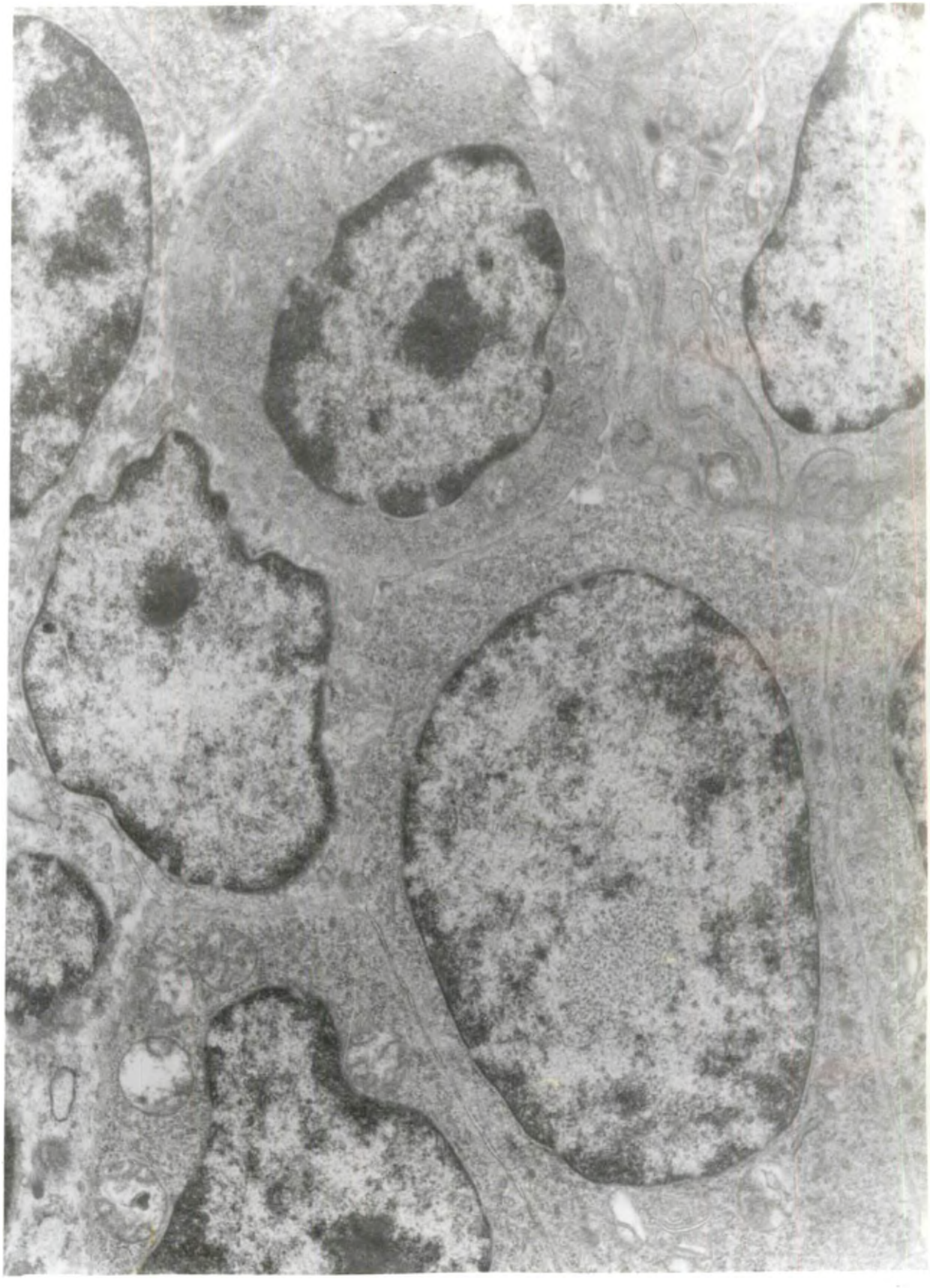


Fig.51. Electron micrograph Gr. B_1 Bursa: Lymphocyte predominant area. Intercellular space showing tendency for separation. x8000

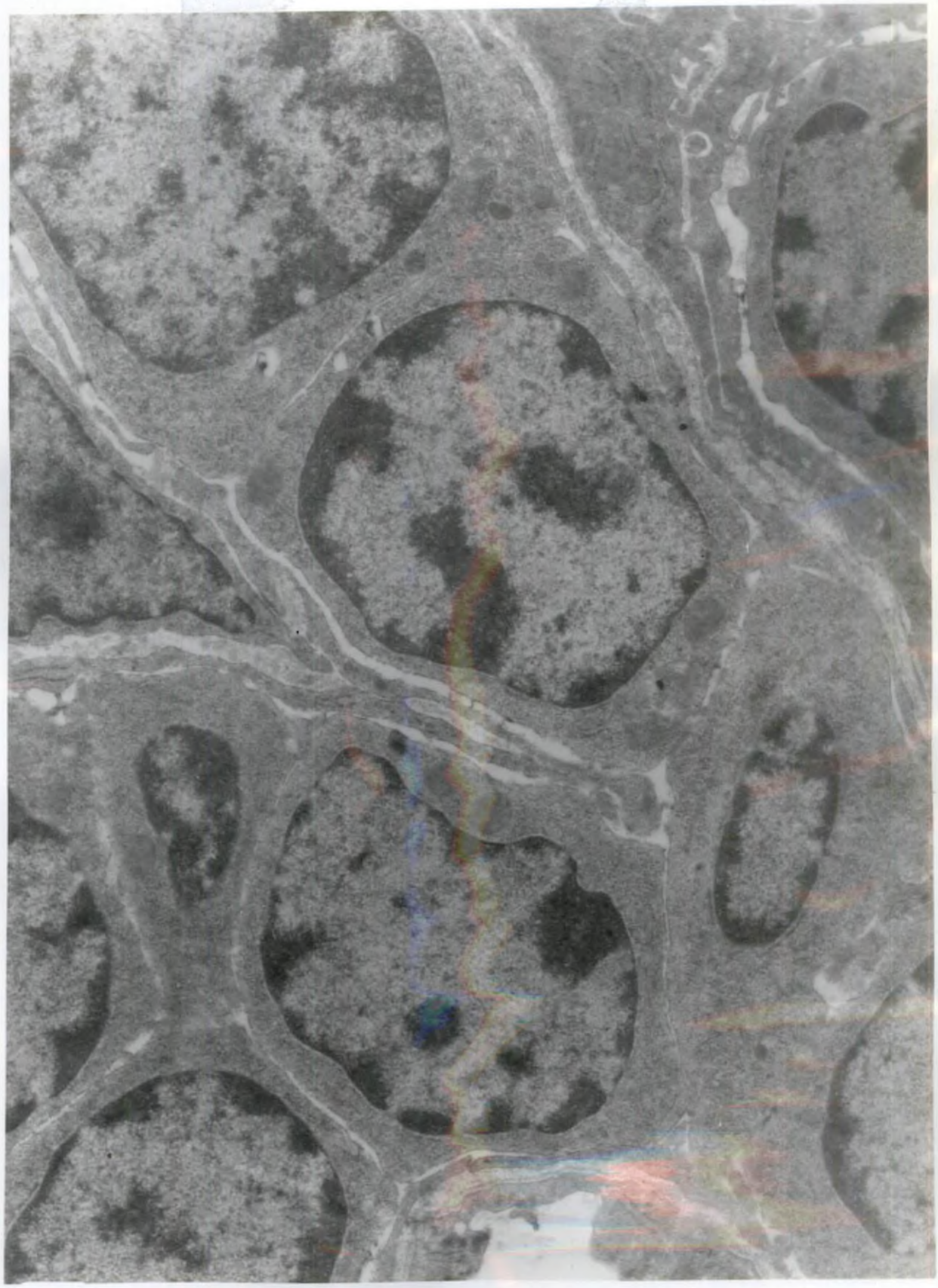


Fig.52. **Electron micrograph Gr. A_2 Bursa: Lymphocytes with bizzare shape of the nucleus and dissolution of cytoplasmic organelles. Perichromatin granules also seen. x10000**

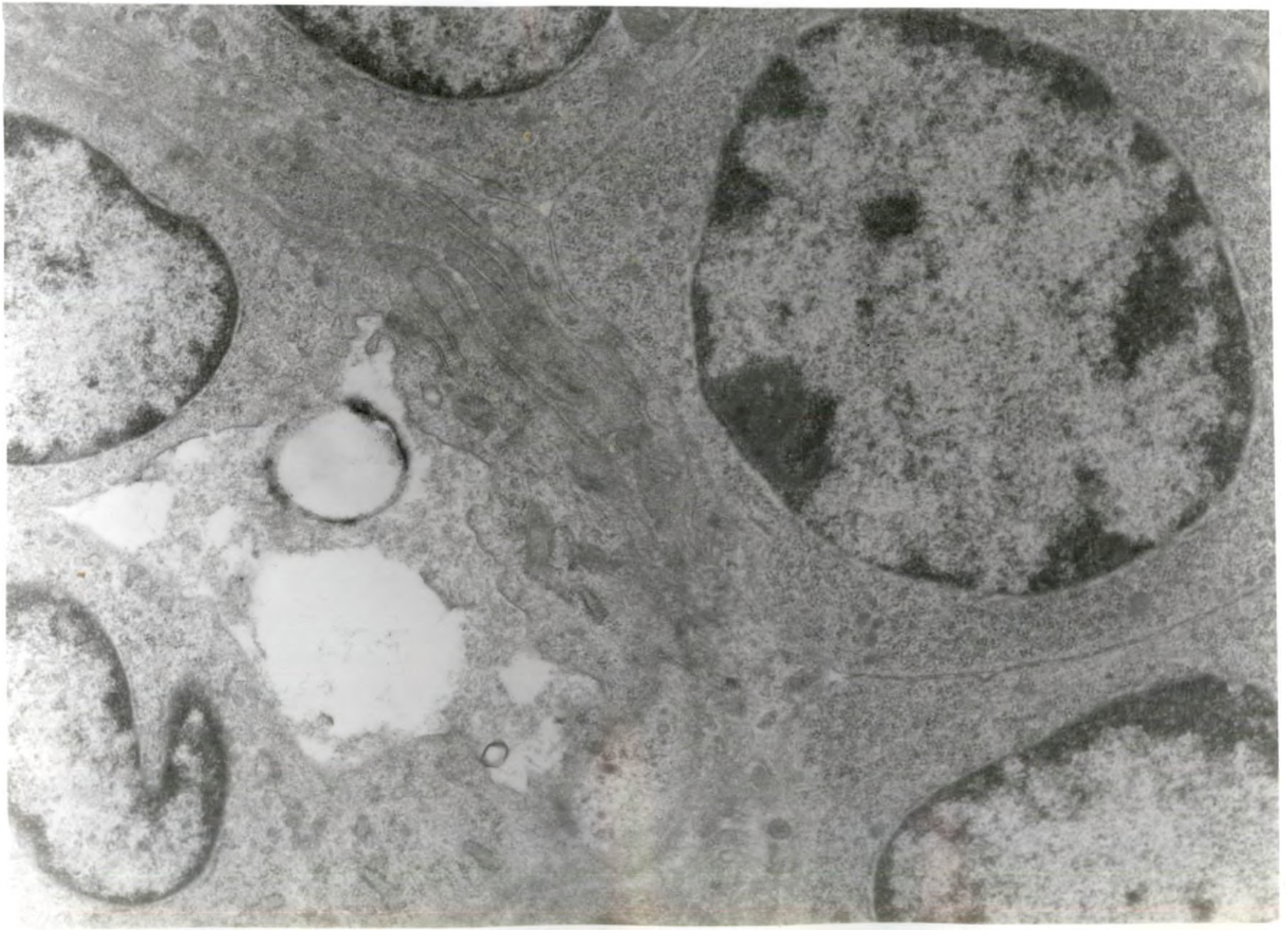


Fig.53. **Electron micrograph. Gr. A_2 Bursa: Lymphocyte with perichromatin granules. Membrane bound vesicles and lipid droplets seen. x20000**

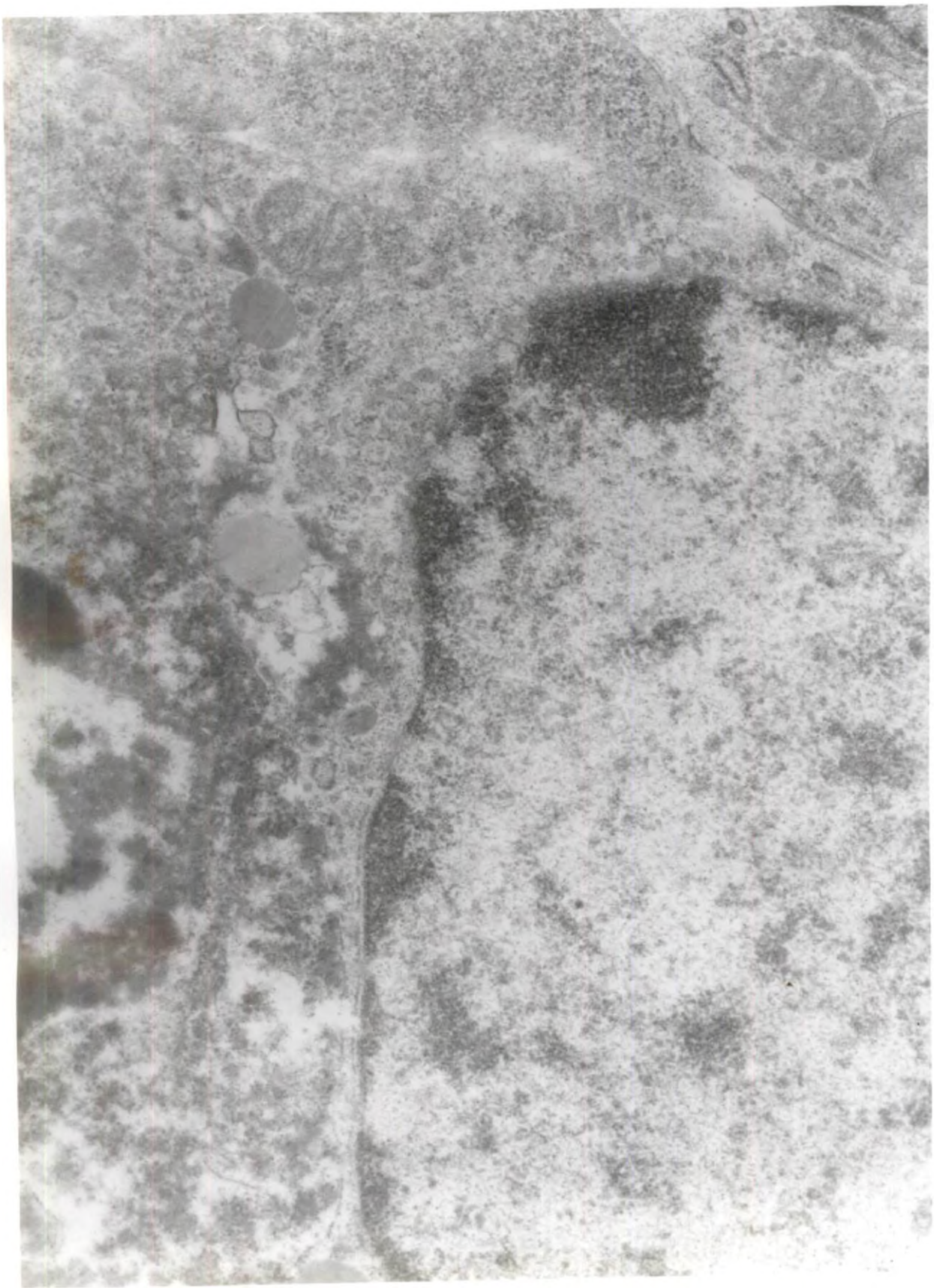


Fig.54. Electron micrograph. Gr.A_2 Bursa: Lymphocyte with perichromatin granules and less number of nuclear pores. Cristolysis of mitochondria and lipid inclusions seen. x10000

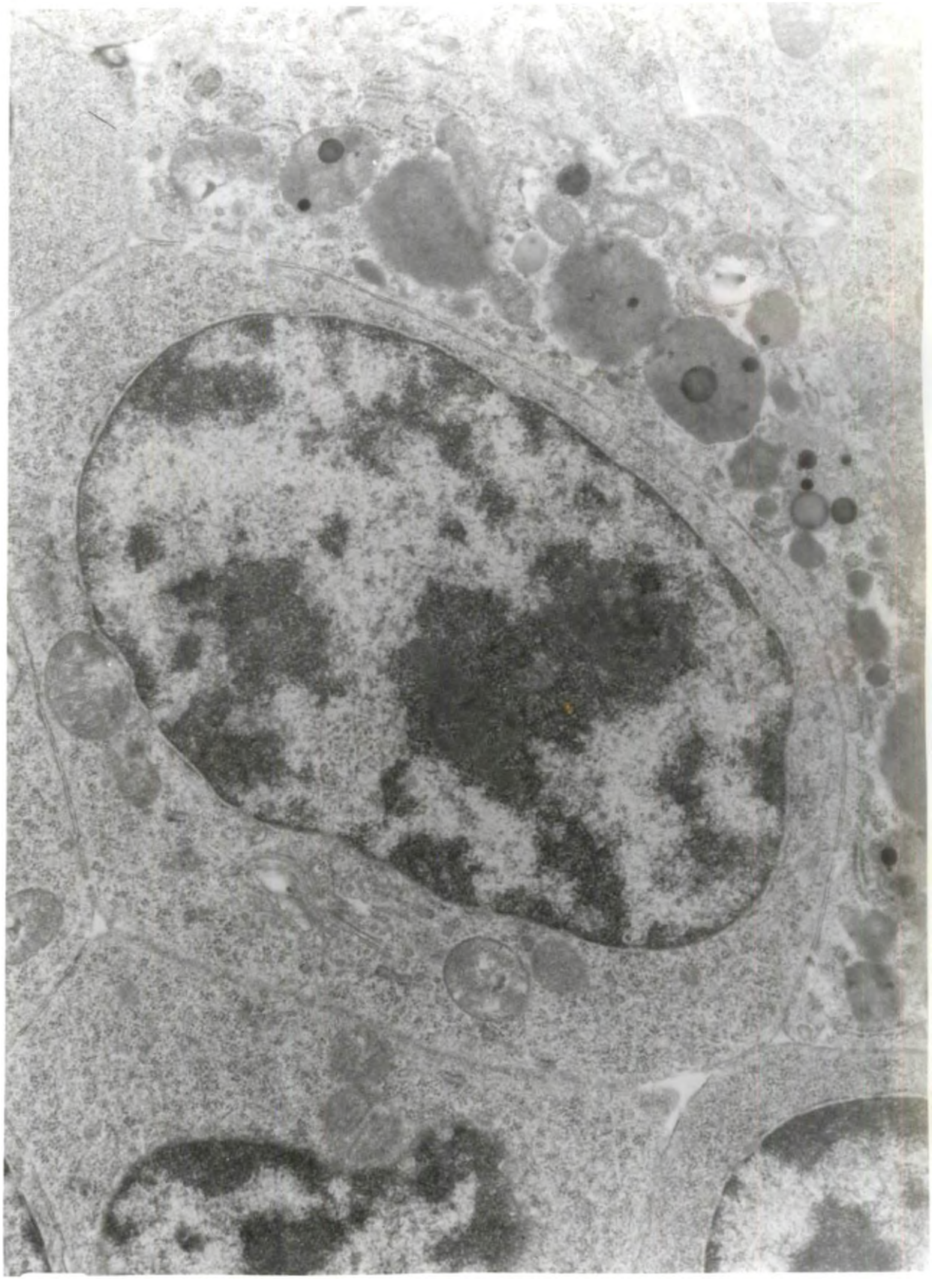


Fig.55. Electron micrograph. Gr.B_2 - Bursa: Lymphocyte with condensed chromatin. Densely packed moderate electron dense mitochondria. Granular cytoplasm. x10000

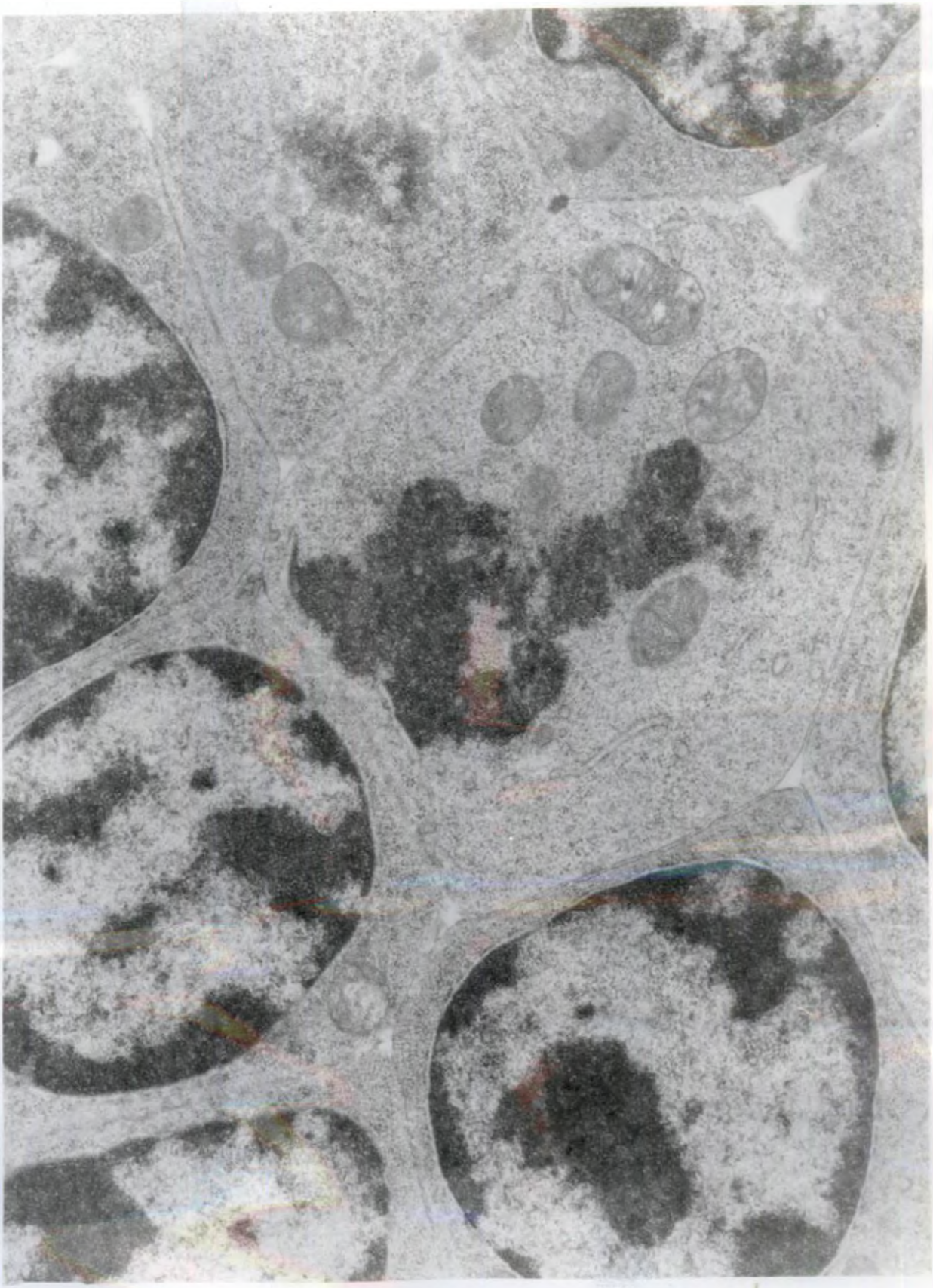


Fig.56. Electron micrograph. Gr.B_1 Thymus: Lymphocytes with electron dense heterochromatin. Very few nuclear pores seen. Electron dense mitochondria. x20000

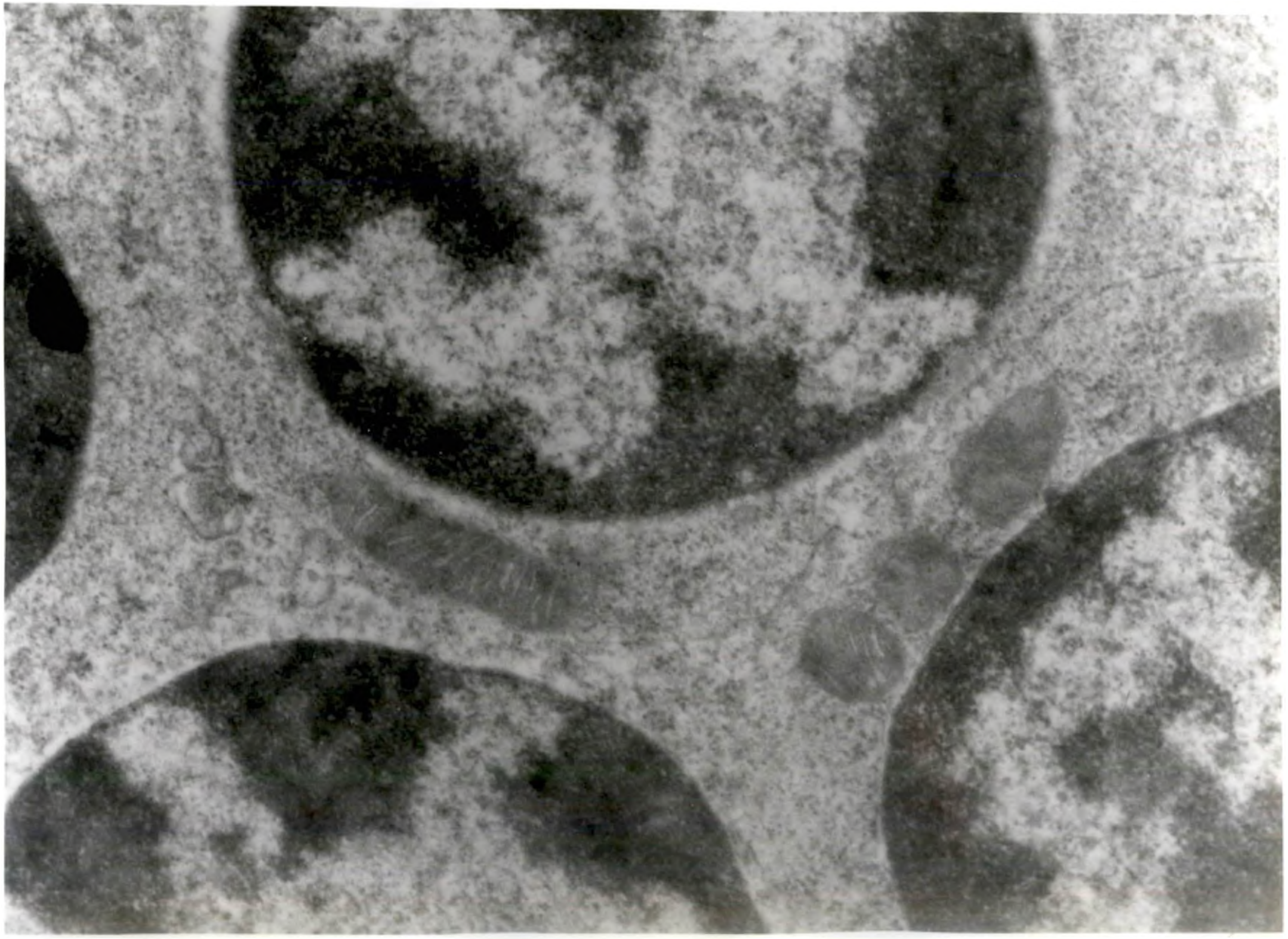


Fig.57. Electron micrograph. Gr.A_2 Thymus: Lymphocyte with condensed chromatin at the nuclear rim, swollen mitochondria, fragmented and dilated endoplasmic reticulum. x10000

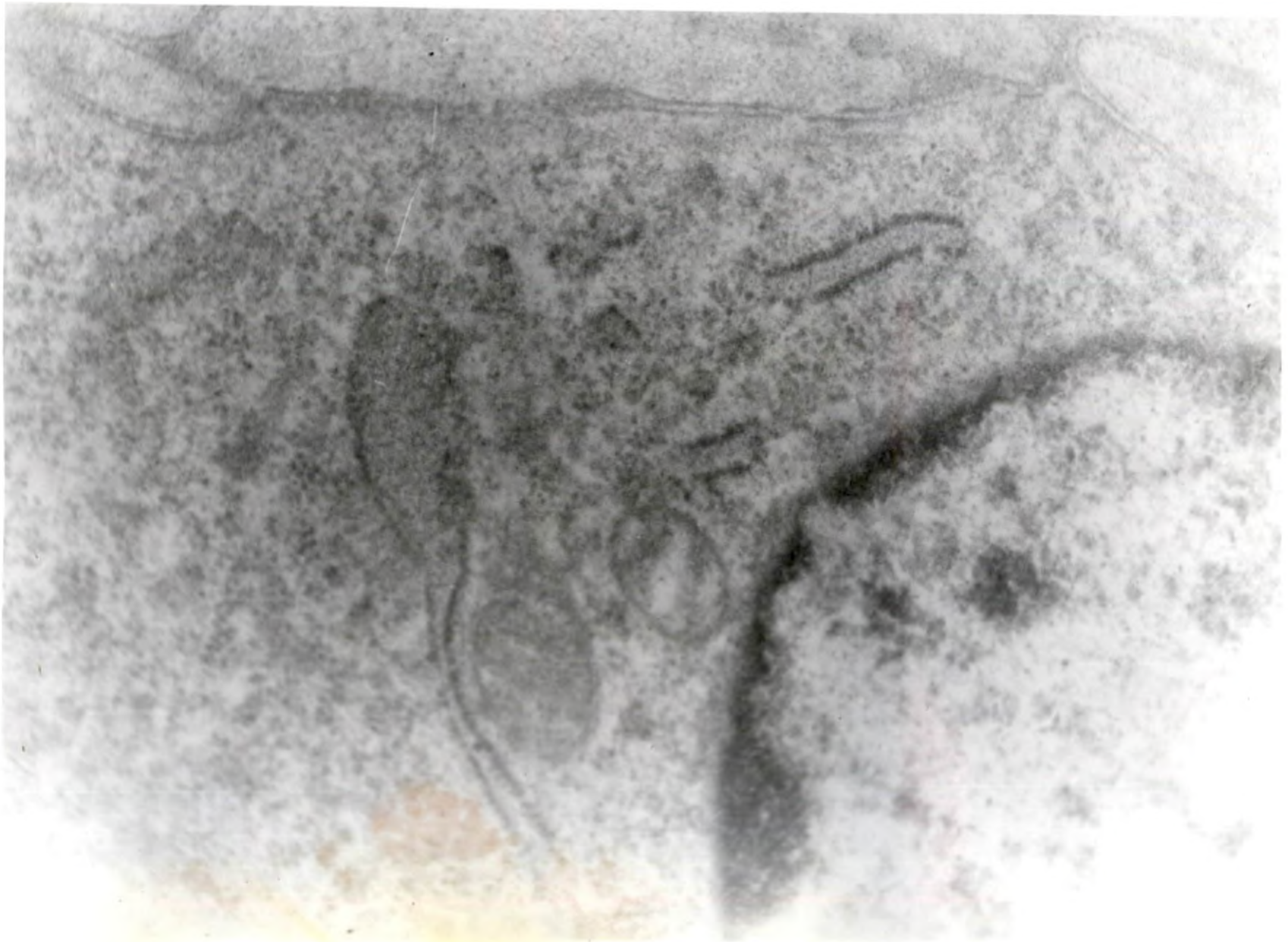


Fig.58. **Electron micrograph. Gr.A_2 Thymus: Lymphocyte - highly electron dense condensed chromatin, swollen mitochondria with lysed cristae. Fragmented dilated endoplasmic reticulum. x10000**

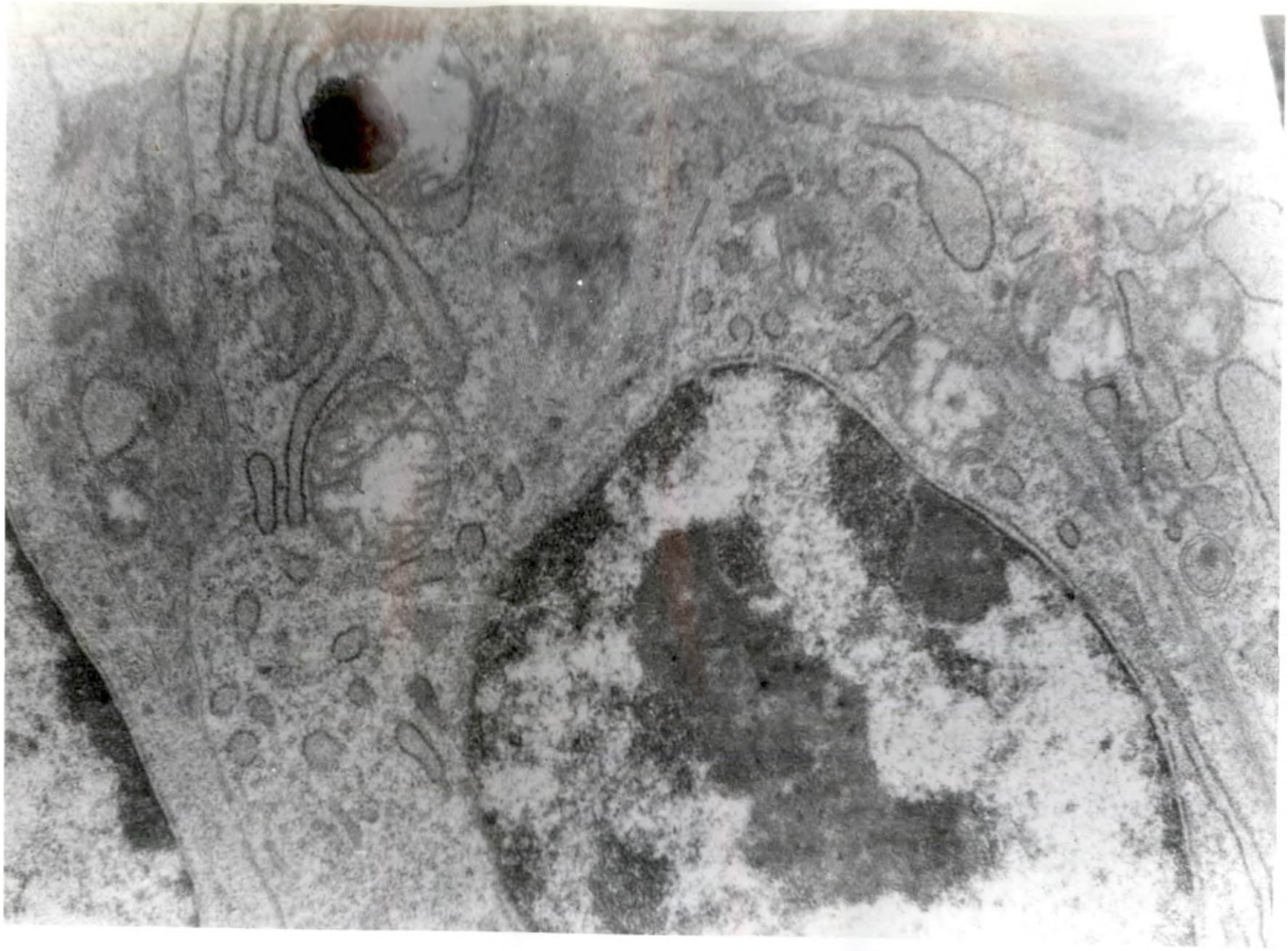


Fig.59. **Electron micrograph. Gr.B_2 Thymus: Lymphocyte - condensed chromatin and perichromatin granules, swollen mitochondria with lysed cristae disruption of cytoplasmic membrane seen. x10000**

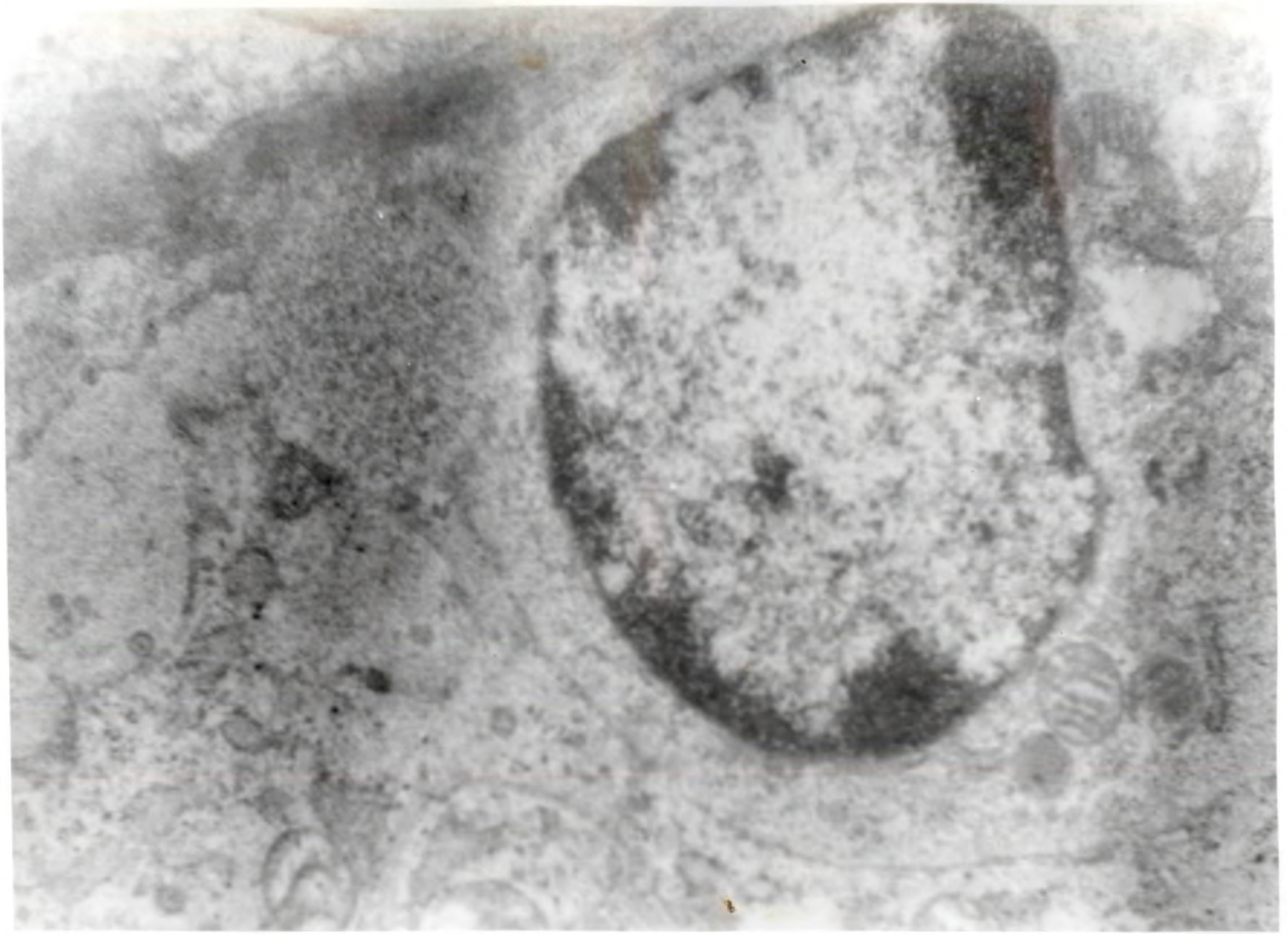


Fig.60. **Electron micrograph. Gr.A_1 spleen: Lymphocyte with electron dense heterochromatin. Fusion of nuclear membrane. Fragmented endoplasmic reticulum and swollen mutochondria. x10000**

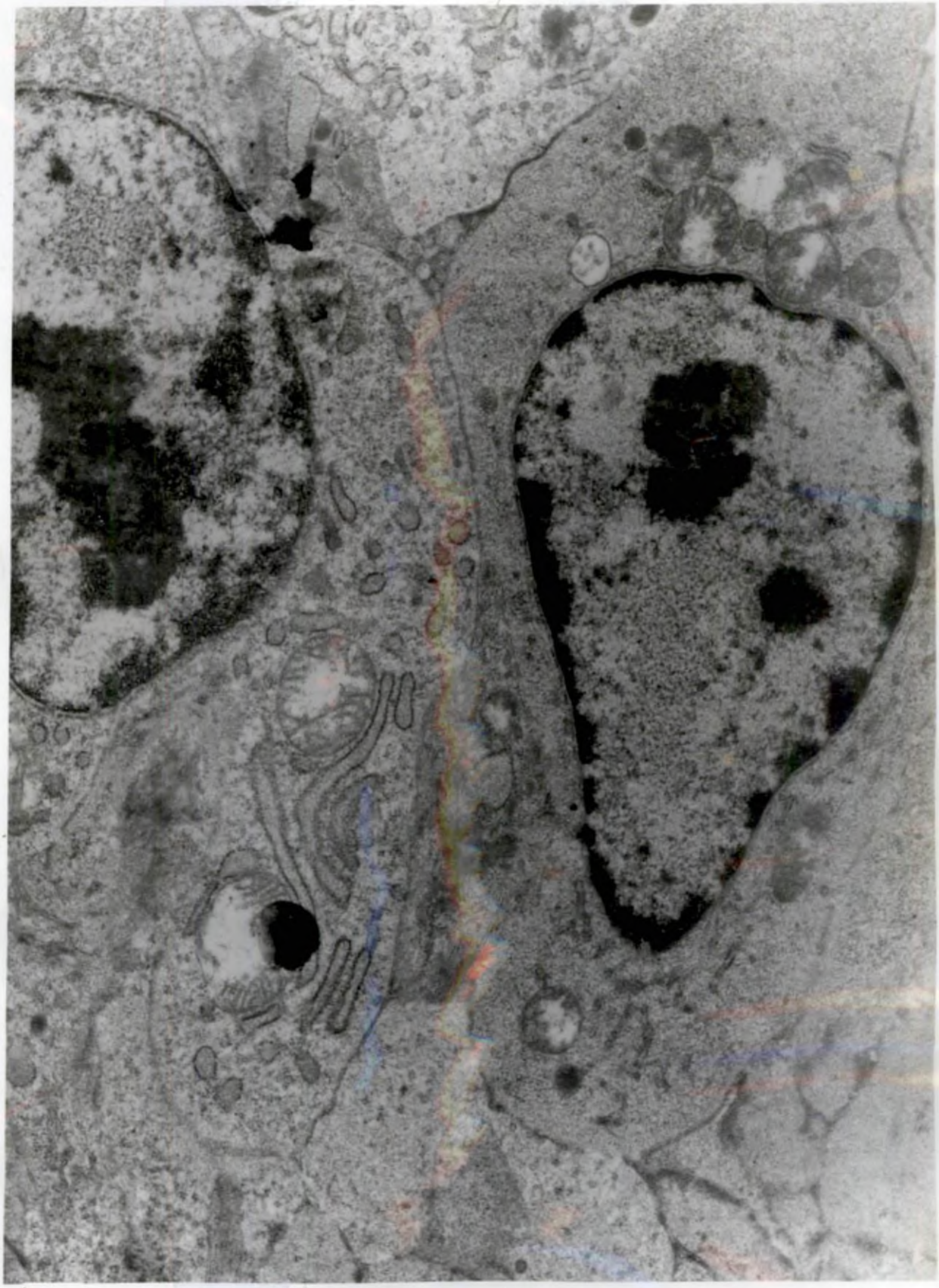


Fig.61. **Electron micrograph. Gr.A_1 spleen: Macrophage with condensed chromatin, condensed mitochondria. Disruption of cellular membrane and erythrophagocytic activity are also seen. x6000**

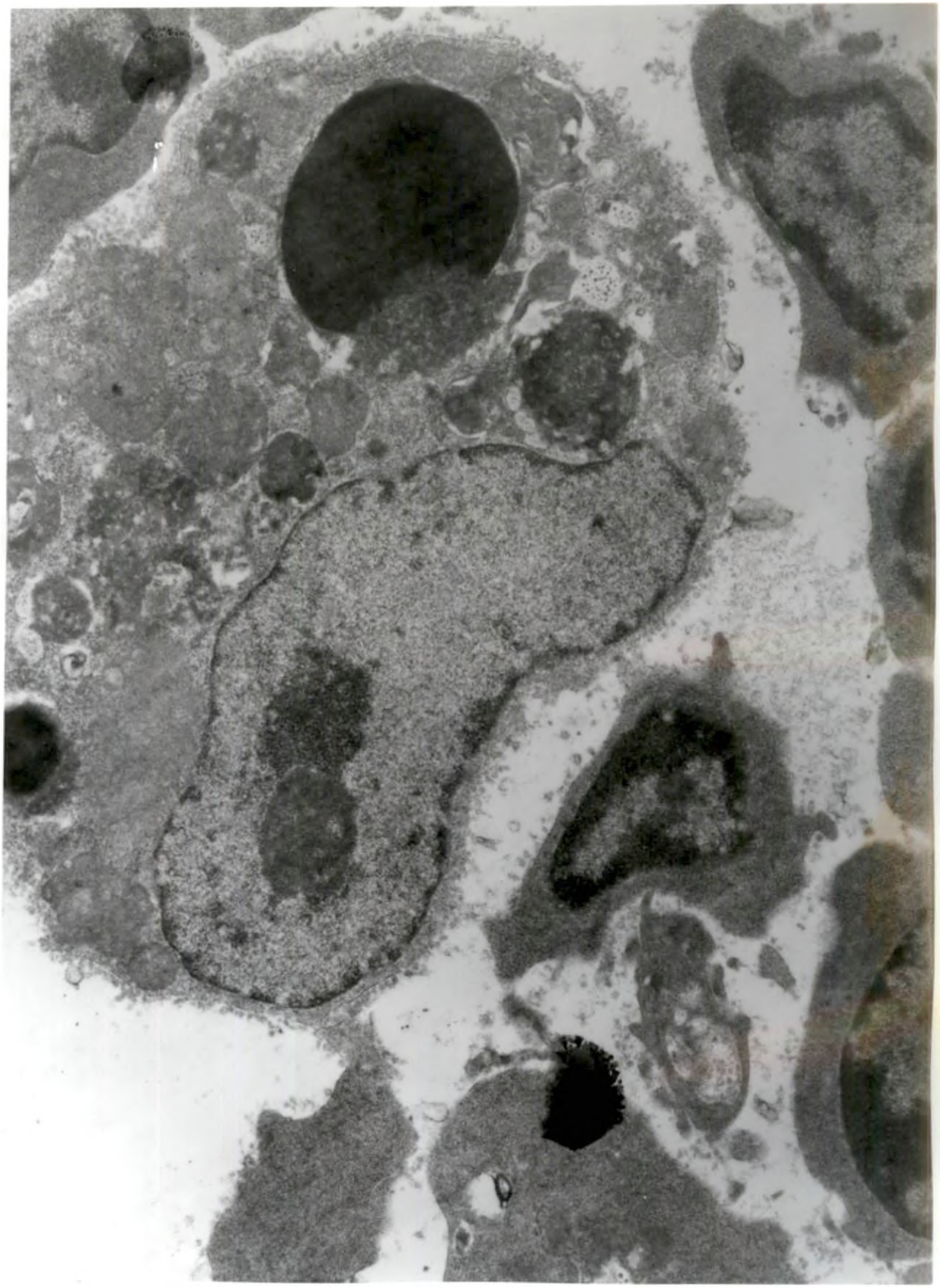


Fig.62. **Electron micrograph. Gr.B_1 spleen: Plasma cell predominant area with abundant endoplasmic reticulum showing dilatation at places. x8000**

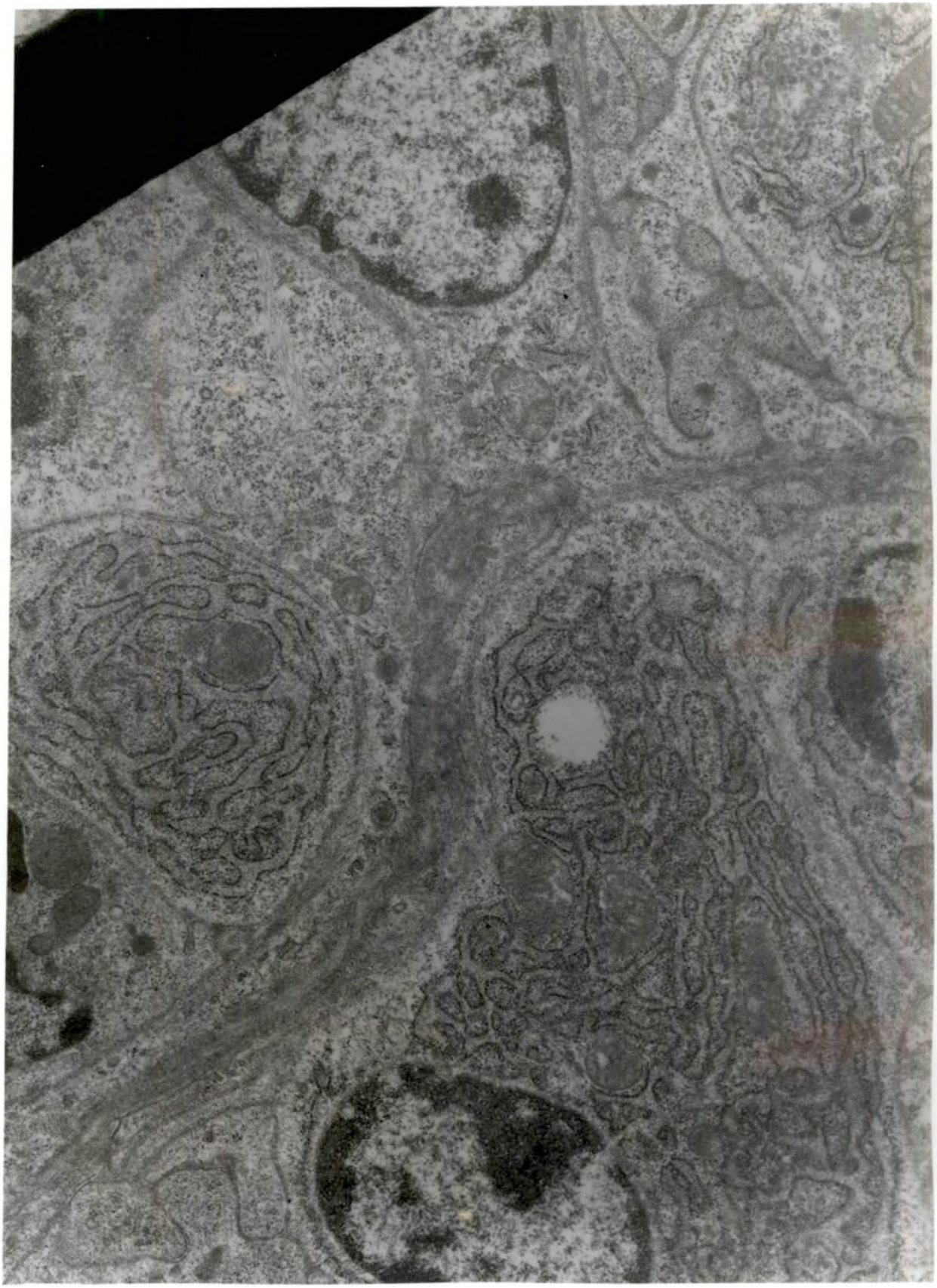


Fig.63. Electron micrograph, Gr.A_2 spleen: Group of lymphocytes with necrotic changes - bizzare nucleus, dissolution of cytoplasmic organelles X6000

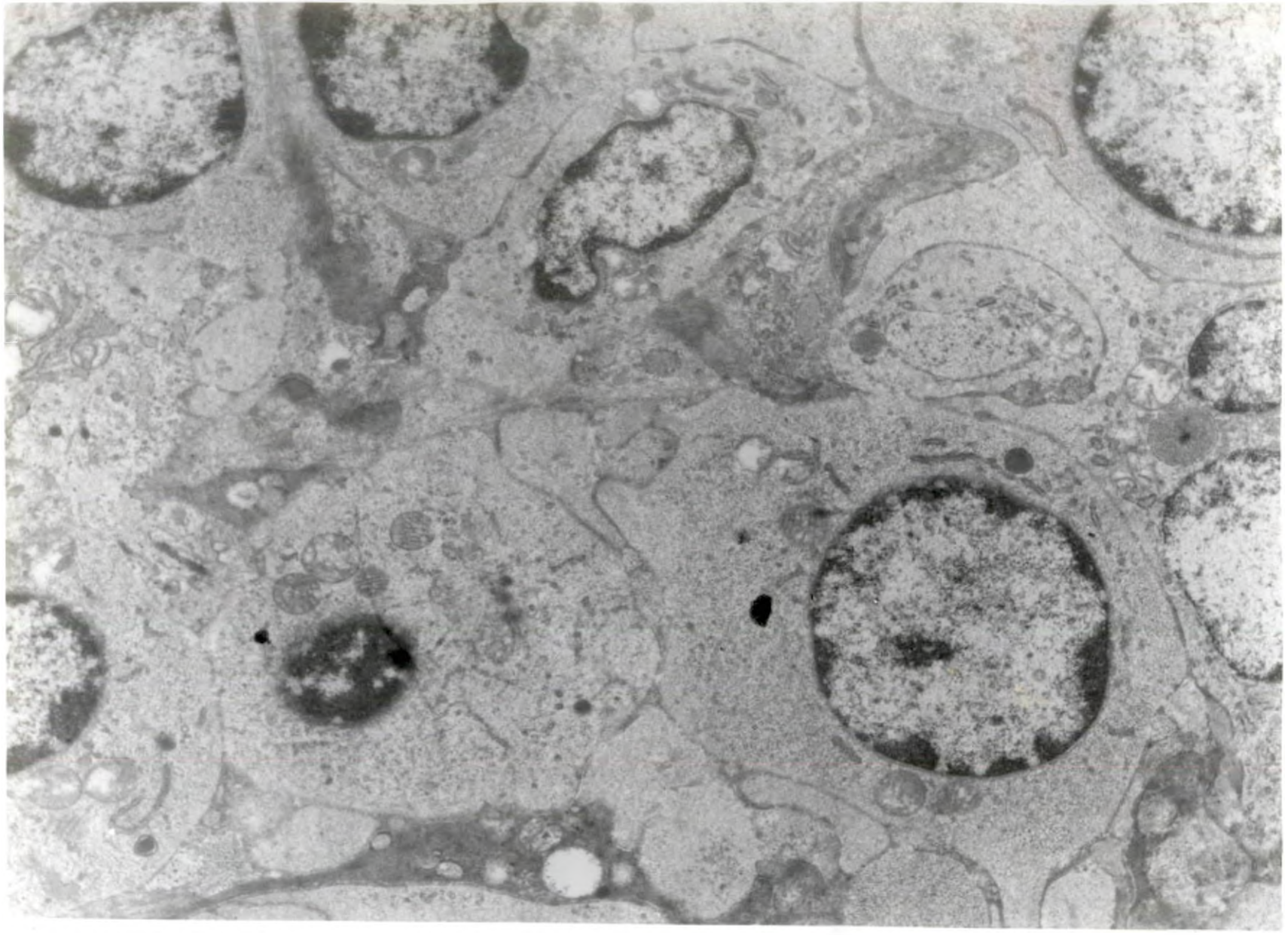


Fig.64. **Electron micrograph Gr.A_2 spleen: Lymphocyte with perichromatin granules, cristolysis swollen mitochondria and granular ring structure in the mitochondrial matrix. x20000**

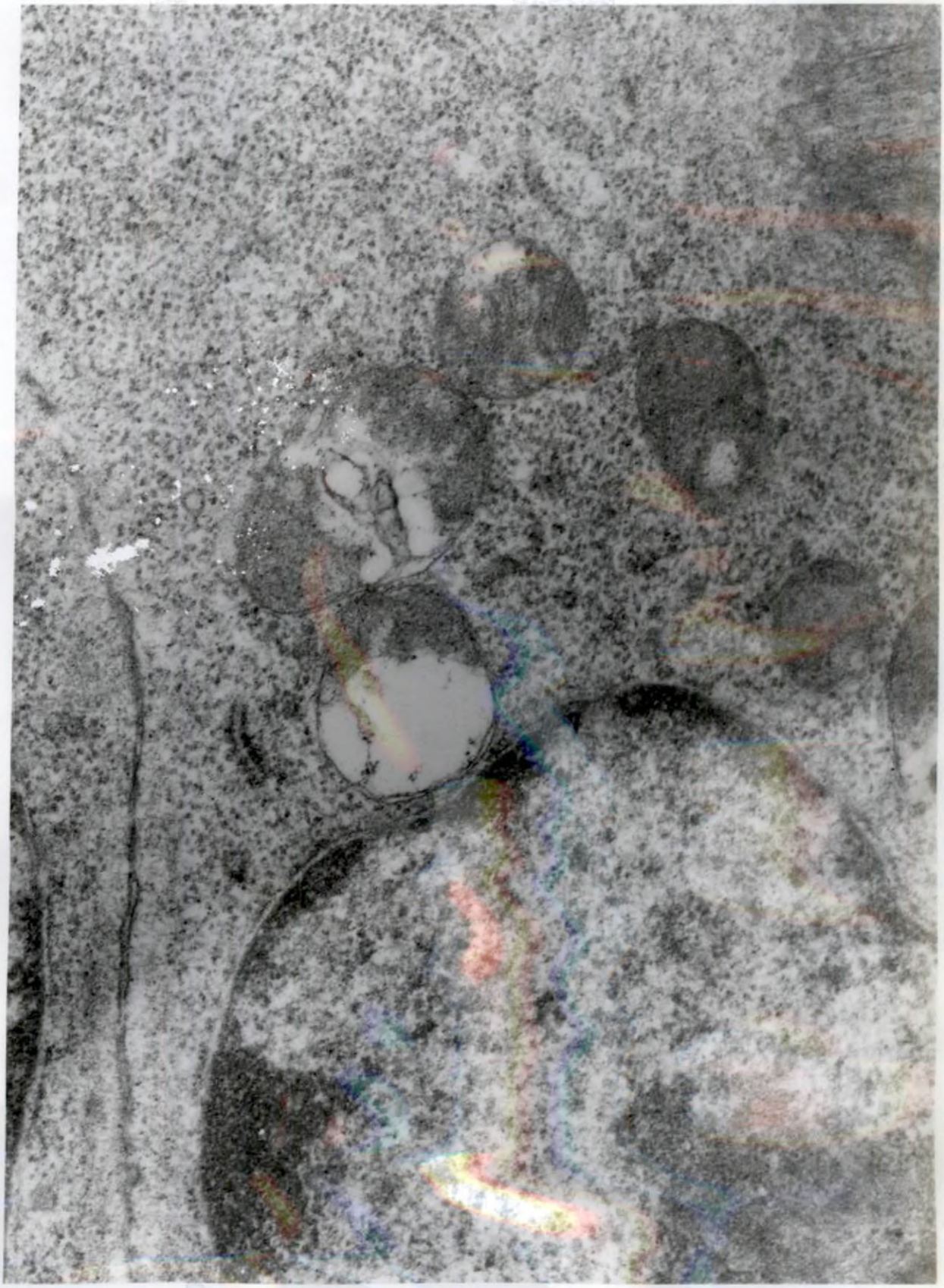


Fig.65. **Electron micrograph. Gr.B_2 spleen: A heterophil with electron dense granules and extrusions of nuclear membrane. x12000**

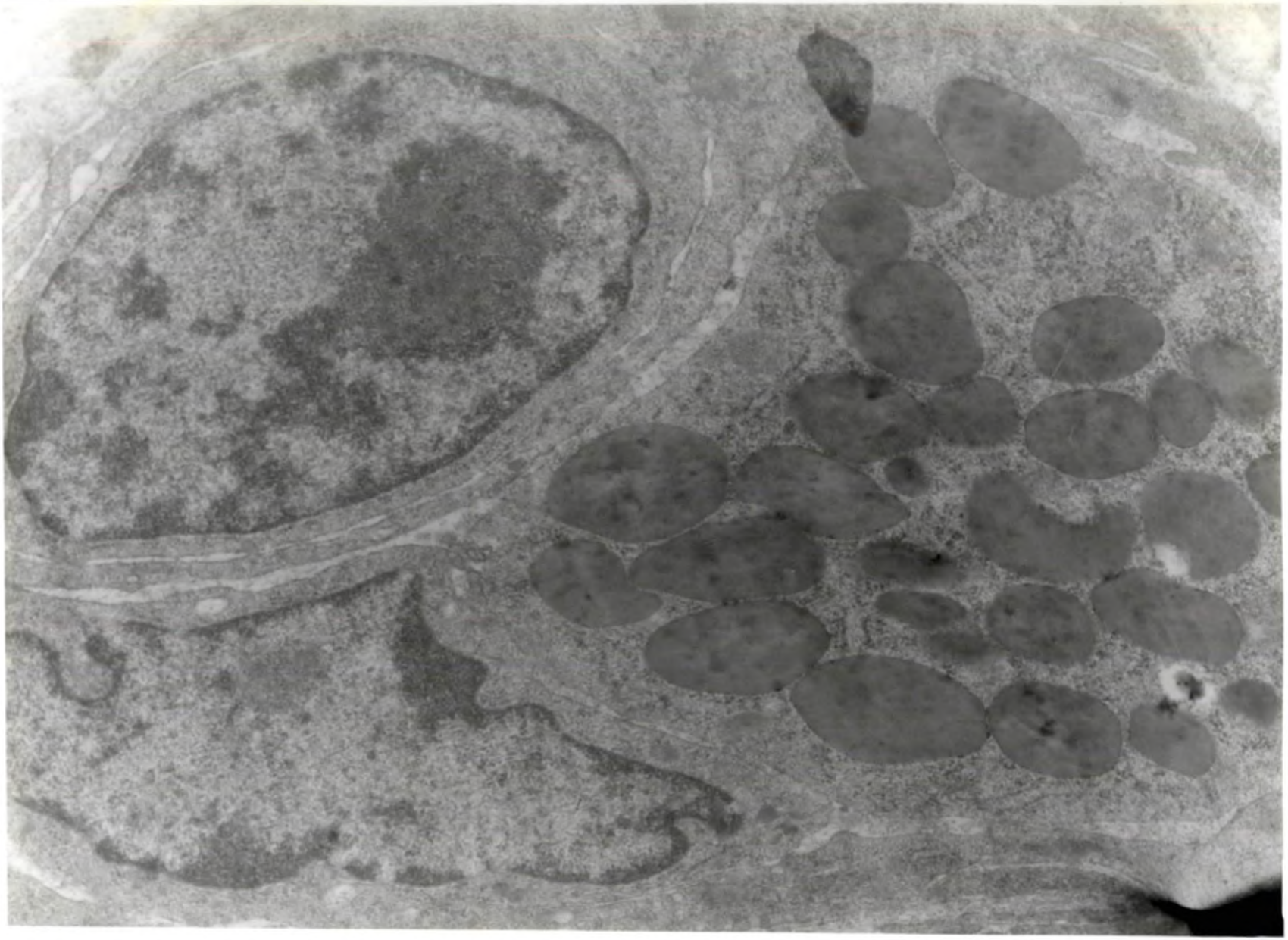
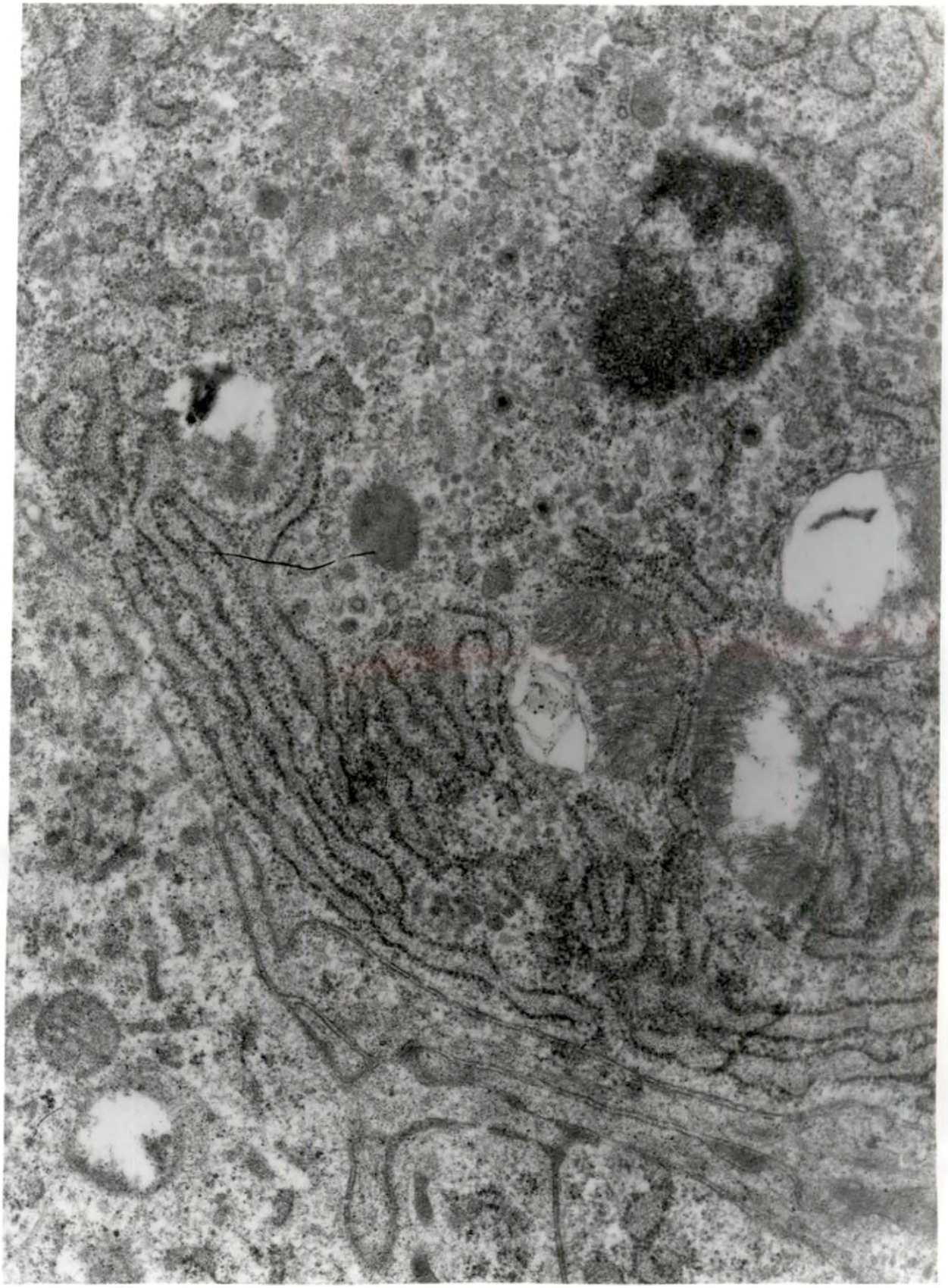


Fig.66. **Electron micrograph. Gr.B_2 spleen: Plasma cell with swollen mitochondria. Cristolysis is evident. Disaggregation of polyribosomes, and fragmented endoplasmic reticulum. x15000**



Discussion

DISCUSSION

The experiment was designed to study the teratology and embryotoxic effects of carbofuran and 2,4-D in the duck embryos with special reference to the immunopathological effects of the above compounds taking ducklings as the experimental model.

5.1 Studies on embryos

The studies on the embryo exposed to the agro-chemicals mainly consisted of documentation of the data on the weight of the embryo, lymphoid organs like the bursa, spleen and thymus on day 15, 21 and 28 of incubation. The embryos were examined for teratologic effects and other pathologic conditions. Another aspect of the study was the assessment of the histologic and ultrastructural features of the lymphoid organs like the spleen, bursa and thymus on day 15, 21 and 28.

Embryo mortality was found to be more in the B group exposed to 2,4-D on day 15 and 21. This was significantly high when compared to the control group and the group dipped only in distilled water. Stunted growth was more in group A (13.3%). Generalised oedema was more in group B (20%). Oedematous changes were more in group B on the 21st day of incubation (26.7%). On the 28th day, hatchability was found

to be reduced in group A and B. The data suggested that 2,4-D is relatively more embryotoxic than carbofuran.

At the dosage employed there was marked embryo mortality. There was also poor hatchability and oedematous changes in the embryo. These observations have relevance and significance as the presence of these chemicals in the feed can certainly cause loss in hatching operations. However, at the dosage employed there were no teratological abnormalities.

The teratological studies conducted with carbofuran by Mc Carthy *et al.* (1971) and Cambon *et al.* (1979) did not reveal any teratological abnormalities in rats, rabbits and dogs. The results obtained, therefore, are in agreement with the above observations. It is pertinent to point out that some of the studies conducted with certain fungicides (Maneb) revealed highly teratogenic abnormalities at all concentrations (Maci and Arios, 1987).

There was no significant effect on the body weight and lymphoid organ weight on the 15th and 21st day. There was however, reduction in the weight of the bursa. There was reduction in the body weight of group A on the 28th day. The thymus and the spleen of group A and B showed reduction in the weight on the 28th day. This demonstrated the mild immunotoxic effect of 2,4-D and focussed attention on the possible immunosuppressive effect of the chemicals.

Alekshashina *et al.* (1973) found that a large single dose of 2,4-D did reduce the growth and survival rate of the foetus and daily administration of 0.5 mg/kg reduced the growth, and 0.1 mg/kg did not cause any effect. The findings of this study are in agreement with the findings of Alekshashina *et al.* (1973) that 0.1 mg/kg daily did not cause any reduction in the weight of the spleen and thymus on the 28th day. Thymic atrophy has been reported between the second and seventh day post exposure in mice exposed to a herbicide propanil by Cuff *et al.* (1996). The studies on other herbicides like 2,4,5-T revealed that it affects the foetal weight, increased the incidence of cleft palate and embryolethality in mice (Holson *et al.* 1992). The herbicide 3,4 dichloropropionanilide (propanil) induced significant thymic atrophy (Cuff *et al.* 1996). In the present study, a reduction in the weight of the thymus was observed, but the reduction was not marked. Hence 2,4-D should be considered as less embryotoxic when compared with the reports of other herbicides like 2,4-5-T and propanil.

No teratological effects were noticed in the embryos on days 15, 21 and 28 dipped in the aqueous solution of 2,4-D. This is in agreement with the observations of Binns (1970) that no congenital malformations occurred in lambs receiving 2000 mg of 2,4-D during the first 30, 60 and 90 days of gestation. Aleksashina *et al.* (1973) also reported that 2,4-D

was not teratogenic at 0.5 mg/kg and 0.1 mg/kg body weight in rats.

Konstantinova *et al.* (1975) could not produce embryo mortality or any malformations with 2,4-D with an oral dosage of 80 mg/kg body weight in rats. However, teratologic changes were noticed in the hamster and teratogenic and embryotoxic effects in mice with 2,4-D but only with a high dosage rate of 110 mg/kg/day (Collins and Williams, 1971). It would, therefore, appear that there is species variation in the susceptibility to this chemical.

There was no significant effect on the body weight and lymphoid organ weight on the 15th and 21st day. There was, however, reduction in the weight of the spleen of group A on day 21. The bursal weight did not show any significant change during any of the time intervals studied. The histological studies of the lymphoid organs did not reveal any microscopic lesions on day 15 and 21. The lymphoid elements were loosely arranged in all the groups.

The bursa on the 28th day from group A revealed oedematous and degenerative changes characterised by vacuolation of the epithelial cells and the lymphoid elements. This was supported by the ultrastructural alterations seen in the mitochondria and the nuclear membrane. The bursa from group B also showed ultrastructural changes affecting the

mitochondria, and the endoplasmic reticulum. These subtle but significant sub-cellular changes gave proof to the surmise that 2,4-D is certainly embryotoxic and immunotoxic.

The histological changes in the spleen from the embryos sacrificed on the 28th day revealed less lymphoid activity in group A and B when compared with the control group. But the changes did not suggest a picture of degenerative or necrotic changes of the structure and hence it has to be presumed that the agrochemicals may be affecting the genesis of the lymphoid elements.

The ultrastructural changes in the spleen from group A demonstrated changes in the mitochondria. An early degenerative change was visible. The appearance of the organellae and the nuclear membrane indicated a structural damage and perforce a functional incompetency. The membrane bound vesicles with electronlucent content and ribosomes indicated the activity of the cell.

The spleen from the group B revealed macrophages with phagocytic activity and well formed lysosomes. This indicated the functional state of the phagocytic activities. The lymphoid cells in various stages of development with more of heterochromatin also pointed out to the active state of the spleen. Eventhough, the functional state of the lymphoid cells and macrophages were evident, the number of lymphoid cells in

the light microscopic examination was less compared to the normal.

The ultrastructural and histological changes in the spleen pointed out that group A was more damaged than the group B. Though the cells of the spleen in group B revealed the functional state, the number of cells were few compared to the control. These observations clarified the immunosuppressive effect of the agro-chemicals. These are observations which will have far reaching consequences. The low but significant adverse effect of the agro-chemicals on the immunocompetent cells of the spleen will have certainly a bearing on the immunological competency. The subdued immunological profile in such situations will lead to breakdown of immunity and precipitation of disease outbreaks.

The thymus was reduced in weight and the histological and the ultrastructural changes seen in the groups A and B established that there was immunotoxic effect in this organ by these chemicals.

The teratogenic and embryotoxic effect of the two agro-chemicals were assessed by exposing the embryos to the acqueous solution of the chemicals. The results obtained by the gross pathological examination, microscopical examination and ultrastructural studies proved that the chemicals at this concentration are not teratogenic. The histological and

ultrastructural studies however, suggested a mild degree of toxic changes of the lymphoid organs which correlates with the reduction in the body weight and also the reduced weight of the thymus and spleen. The mitochondrial damages as seen from the ultrastructural studies and the consequent histological changes could initiate a series of cellular changes resulting in death of cells, defective growth and functional depression and this in turn may cause immuno-deficiencies in ducklings hatched out of these contaminated eggs. Therefore, caution should be exercised to control the exposure of the ducks to the agro-chemicals in order to avoid the residues in the egg and consequent immunotoxicity in the embryo. The poor hatchability and disease outbreaks in the ducklings will certainly cause severe economic loss to the farmers.

5.2 Studies on ducklings

Sub-toxic level of the agro-chemicals did not cause any clinical manifestation except that there were isolated cases of development of corneal opacity in group A and B which recovered later by the supplementation of vit. A to all the groups. This may probably be attributed to the malabsorption caused by changes in the gastro-intestinal tract due to the agro-chemicals. The mortality was more in group A and B compared to the control during the first 6 weeks of the experiment, eventhough it was not a markedly noticeable

mortality rate. The absence of lesions in the visceral organs indicated that the sub-toxic level of the agro-chemicals administered was not sufficient enough to cause any gross pathological effects. In acute cases of poisoning by 2,4-D, rats showed irritation of the stomach and congestion of the lungs (Rowe and Hyâs, 1951). Congestion of the visceral organs was reported in man (Neelsen *et al.* 1965, Dudley and Thaper, 1972). In acute poisoning by carbofuran, there was hyperaemia and degenerative lesions in the visceral organs in rams (Topalski *et al.* 1987).

As part of the haematological evaluation, the total leucocyte count, differential leucocyte count and haemoglobin were estimated. The haematological values were found to be in the normal range till the end of the third fortnight while TLC, showed difference at 5 per cent level in group A at the end of the third fortnight. Group B did not reveal any significant difference.

The notable finding in DLC was an increasing trend in the heterophil count at the end of the fifth fortnight in both the treated groups. The lymphocytes were more in group A at the end of the third fortnight. But the percentage of lymphocytes were reduced at the end of the fifth fortnight in both treatment groups. The haemoglobin was markedly reduced in

group A whereas it showed decreasing trend at the end of the fifth fortnight in group B compared with the third fortnight.

The findings of this study revealed that there was no significant pathological effects caused by the agro-chemicals at the levels used, if exposed for a short duration of two weeks or 4 weeks. The decrease in the total leucocyte count could be a reflection of reduction of lymphocytes in the fifth fortnight. Haemoglobin was also found to be decreased at the end of the fifth fortnight.

Previous workers have reported (Singh *et al.* 1989) low blood values by feeding agro-chemicals like fenthion indicating cellular damage in the haemopoietic organs of birds leading to haemolytic anaemia. These studies were conducted with a higher level of chemicals. The herbicide propanil has also been shown to be myelotoxic to early haemopoietic cells (Blyler *et al.* 1994).

The results obtained in this study indicated that the chemicals damaged the haematopoietic organs resulting in anaemia and the decreased lymphocyte count at the end of the fifth fortnight. This is further evidence to show that immunosuppression is possible if the duration of exposure is prolonged.

Moregaonkar et al. (1994) reported lowered haemoglobulin levels as a result of depressed erythropoiesis on feeding a carbamate insecticide at 500, 1000 and 1500 ppm for 45 days and they also found higher heterophil count in chicks on the 7th day and opined that the heterophilia may be consequent to the toxic damaging effect on the visceral organs and lowered lymphocytic and monocytic counts as a manifestation of immunosuppression.

The level of serum protein and albumin did not show any variation till the end of the third fortnight. The serum globulin level was elevated in the third fortnight. However, this was not significant. A significant reduction at the end of the fifth fortnight in group A, and a significant reduction at 5 per cent level in group B was recorded. The changes in the level of the serum protein, globulin and albumin is a pointer to the damaging effect of the chemicals at the above dosage on prolonged exposure. The effect of carbofuran was more severe than 2,4-D. This observation points out the suppressive effect of these chemicals on the humoral immune response.

The antibody titre against RD antigen, did not show any significant difference at the end of 6th week, but the titre was more in group A. This finding along with increased lymphocytes and the globulin level at the end of the sixth

week points to a slight stimulatory effect on the humoral immune system by the carbofuran group of pesticides, whereas the values of group B did not vary much from the observations of the control group. This finding is in consonance with the observation of Rozakoweski (1979) and Vijayan *et al.* (1990) who recorded immunostimulation following insecticide exposure. This observation deserves special attention in the context of the report made by Ercegovich (1973) that certain organophosphorous chemicals particularly malathion in rabbits have the potential to stimulate immunologic reactions through the production of haptenic determinants by protein binding and also specific antibodies for protein conjugate. The above fact has to be confirmed by further studies. Does it hold true in the case of carbofuran also, is a mute point to be clarified by further detailed investigation. However, it may be noted that immunostimulation was observed only during the initial stage when there was no immunotoxic effect. In the later stages of exposure to agro-chemicals there was immunosuppression.

Pruett *et al.* (1992) observed that sodium methyl dithiocarbonate on oral administration to mice at 300 mg/kg/day for 3, 5, 10 and 11 days did not suppress the antibody production.

The study revealed reduced antibody titre in group A at the end of the 10th week suggesting immunosuppression whereas it was not observed in group B when compared with the control. This observation indicated that carbofuran acts as an immunosuppressant on long exposure. This has significance as the birds may get a contaminated diet or water for a long duration in a particular agroclimatic region.

The result of the study are in agreement with the opinion of Street and Sharma (1975) that the variation in the humoral immune response might result from the type and route of antigen administration and also duration of the insecticide treatment. Most of the results of the studies on agro-chemicals which revealed immunosuppression were observed in trials conducted with higher level of exposure to the agrochemicals.

Studies made on the effect of several widely used pesticides, Wiltrout *et al.* (1978) noted that the pesticides had the potential to suppress an ongoing primary humoral response when administered at a single dose. But however, there are conflicting reports on the effects of repeated dosing with these chemicals, prior to immunisation (Pruett *et al.* 1992). The humoral response to bacteriophage in DDT-pretreated rabbits was seen to lag than that of the control, however, by the 3rd and 4th weeks, the antibody

titres were similar but in contrast to this, the host defense by mice treated with carbofuran in the diet for varying times was seen to diminish with increasing duration of the pesticide treatment (Fan *et al*. 1978). This is in agreement with the findings of this study.

Weights of the body and lymphoid organs have been used as one of the general tests employed for immunotoxicological evaluation of environmental chemicals at the termination of a routine immunotoxicity study. The size of the lymphoid organ is easier to estimate during gross examination than at microscopy. In the present study conducted, there was no significant difference in the body weight at the end of the first fortnight of the experiment. But a duration related reduction was noticed in the body weight in the group A since the reduction was significant at 5 per cent level after the third fortnight and at 1 per cent level at the end of the fifth fortnight. But group B did not show any significant difference at the third and fifth fortnight. This observation is in agreement with the observation of Khera and Mc Kinely (1972) who did not find any difference in the normal weight gain in post natal studies conducted in rats with 2,4-D. Studies conducted earlier with 2,4-D at 5000 ppm, 1000, 50 and 10 ppm dose levels revealed that it inhibited the growth in chicks while reduction caused by 5 ppm was not significant (Whitehead, 1973). But sub-chronic studies conducted in dogs

at the dose level of 1.0 mg/kg/day and above resulted in reduction in body weight gain (Charles *et al.* 1996).

The weight of the lymphoid organs like the spleen, bursa and thymus did not differ significantly except for the reduction in the weight of the thymus from group A at 5 per cent level at the end of the sixth week.

The reduction in body weight was more in birds after ten weeks of the experiment in group A and B when compared with the control. But the reduction was less in group B compared with group A.

The average weight of the spleen, bursa and thymus from group A was significantly less at 1 per cent level when compared with the control. The weight of the spleen from group B also showed reduction in the weight at 5 per cent level when compared with the control. The bursa of group B did not reveal any significant variation from the control group. The thymus from group B showed significant reduction at 1 per cent level when compared with the control.

The weight of the lymphoid organs was indicated as one of the general tests employed for immunotoxicological evaluation of environmental chemicals (Sharma, 1982). A decrease in the weight of the thymus was observed at both the periods which is in consonance with the observation of Pruett *et al.* (1992).

They reported that sodium dithiocarbamate at 300 mg/kg/day for 3, 5, 10 or 14 days decreased thymus weight at all time points.

Various workers have reported conflicting observations on the body weight and organ weight in different species of animals like mice, dogs and chicks after the administration of 2,4-D. Generally it is seen that experiments with low dose as 5 ppm for 8 weeks have not caused any substantial reduction in the body weight but at 10, 50 and 100 ppm, significant reduction in food intake and growth was noticed (Hansen *et al*, 1971; Khera and Mc Kinely, 1972; Whitehead, 1993; Charles *et al*. 1996).

The effects of pesticides on the cell-mediated immune responses have received relatively little direct attention. Skin sensitivity to DNCB and PHA has been documented as a reliable test to assess the cell-mediated immune system. There was decreased response to DNCB and PHA-M in both the treatment groups compared with the control. The increase in the thickness was much appreciable at 24 h and it started reducing at 48 h and 72 h. DNCB caused erythematous changes in the skin. The increase in the thickness of the skin followed almost the same pattern in both DNCB and PHA-M. The result of the study proved that the cell-mediated immune response is affected by the carbofuran and 2,4-D.

Many workers employing delayed type hypersensitivity reaction have demonstrated lowered CMI in pesticide toxicity like cypermethrin in goats (Jha *et al.* 1988) and femitrothion in rats (Kunimatsu *et al.* 1996).

Olefir (1973) demonstrated impairment of T-cell dependent immunological process, in rats receiving single or long term dosing with carbamate insecticide.

Increased migration indices were noticed at all points of time interval in both the treatment groups, when compared with the control. The results of the LMIT, therefore, provides proof for the assumption that the the cell-mediated immune system is adversely affected even at the sub-chronic doses of the agro-chemicals like carbofuran and 2,4-D. The increase in the migration indices was progressive in the early stages, but it was not very much appreciable between the eighth and the tenth week of the experiment.

LMIT has been identified as a significant test to assess the cell-mediated immune response in many of the infections (Tompkins, 1970; Falecka, 1979; Kantoch *et al.* 1979; Chandrasekhar *et al.* 1989) in leptospirosis (Ramakrishna *et al.* 1990) and in mycotoxicosis (Balakrishnan, 1992; Farshid and Rajan, 1996). However, from the literature scanned no reports could be traced pertaining to the application of this technique to assess the CMI in pesticide toxicity studies.

Increase in the migration indices in the present study is an indication of an impairment in the lymphokine production by the sensitized lymphocytes following an interaction with the antigen.

The results of GVHR also showed suppression of the cell-mediated immune response, the suppression being more when the duration of exposure was prolonged. The observation that there is reduction in the CMI response as a result of the administration of low doses of carbofuran and 2,4-D has great relevance and practical value. In order to keep the animal system immunologically active caution should be exercised to monitor and manage the environmental pollution so that the animals and birds will not have access to these chemicals at an intolerable level.

The bursa from the birds of A1 and A2 groups was more tense and firm compared to the B1, B2, C1 and C2 groups. The firmness of the bursa could be attributed to follicular degeneration and interfollicular stromal proliferation in the A1 group and more extensive stromal tissue hyperplasia in the A2 group. The blood vessels also showed thickening of the musculature.

Grossly, the thymic chain of A1 and A2 was comparatively smaller in size. The histological changes noted were mainly

loosely arranged lymphoid cells and fibrous tissue proliferation.

The histological changes were more pronounced in the treatment groups sacrificed at the end of 10 weeks. A2 group showed marked depletion and necrotic changes of the lymphoid components. Thickened blood vessel wall and oedema were seen associated with fibrous tissue proliferation. B2 group showed moderate degree of interfollicular oedema. The changes were indicative of a progressive damage to the lymphoid elements as well as generalised pathological changes in the thymus. The reduction in the CMI could be related to these histological changes.

The spleen also showed progressive degenerative changes, the changes being more pronounced in the treatment groups sacrificed after ten weeks.

Group A2 revealed more severe lympholytic changes. In the B2 group however changes were moderate. This observation clarified that carbofuran was much more immunotoxic than 2,4-D and the changes were time dependant.

The histological findings are in agreement with the observations of Street and Sharma (1975) in rabbits. Rabbits treated with carbofuran had decreased counts of activated lymphocytes in the lymphnode, reduced number of germinal

centres in the spleen and more pronounced atrophy of the cortex of the thymus. Pruett *et al.* (1992) also observed decreased mature lymphocyte subpopulations in the thymus and in the spleen, in mice exposed to sodium methyl dithiocarbanate. Varshney and Bagha (1986) also observed necrotic and hyperplastic changes of the lymphoid follicles in a toxicological evaluation of dietary lindane.

Charles *et al.* (1996) could not observe any severe histopathological alterations in an experimental study in dogs with 2,4-D at doses of 1.0, 3.75 and 7.5 mg/kg/day. But thymic atrophy and a decrease in the cellularity were reported in the studies conducted with herbicides like pepanil (Cuff *et al.* 1996). Faith *et al.* (1986) reported characteristic depletion of lymphocytes in the cortex in 2,3,7,8 tetra chloro-di-benzo-p-dioxin (TCDD) toxicity.

The liver from the A1 group revealed mild degree of fatty degeneration and congestion. The degenerative changes were more extensive in the A2 group compared with A1. The degenerative lesions in the group B were more pronounced in the renal epithelial cells. The changes were more extensive in the B2 group. In fatal cases of poisoning in man by 2,4-D degenerative lesions in the brain ganglion cells, kidney tubules and liver were reported (Nielsen *et al.* 1965; Dudley and Thapar, 1972). It would therefore, appear that these

agro-chemicals have a hepatotoxic effect also at the dose level employed, although mild in nature.

Mineralised deposits observed in the renal pelvis of rats by Kociba *et al.* (1979) in the toxicological evaluation of 4-5T were not observed in this study. This again prove that the extent of renal damage caused by 2,4-D in less compared to herbicide like 2,4,5-T.

Severe degenerative changes of the internal organs were noted by Topalski *et al.* (1987) in rams died off acute poisoning by carbofuran. In the present study only very mild degeneratiive changes were seen since only low doses were used.

The ultrastructural alterations noticed in the treatment groups were more pronounced in the birds sacrificed after ten weeks of the experiment compared to the 6th week. The intensity and the ultrastructural alterations observed at the sixth week and tenth week supported the intensity of histological changes observed at the sixth week and the tenth week.

The ultrastructural picture of the bursa from birds sacrificed after six weeks of the experiment revealed large number of lymphoid cells which also consisted of lymphoblasts. This pointed out that the bursa was in an active state.

However, there was indications of mild pathological changes like chromatin condensation and more of heterochromatin in many of the cells and also thickening of the nuclear membrane.

The cytoplasmic changes were mainly localised in the mitochondria which showed tendency for lysis of the cristae. The degranulation of the endoplasmic reticulum was evident. These changes could well be correlated with the degenerative changes of the lymphoid cells observed in the histological sections. Nuclear and cytoplasmic changes were more severe in the A2 group with abberrant nuclear shapes and nuclear membrane damages like thickening and fusion at places. Perichromatin granules also could be seen in the group A2. The swollen mitochondria with lytic changes and degranulated and fragmented endoplasmic reticulum in the A2 group strongly suggest that the carbofuran is more cytotoxic to lymphoid cells as the duration of the exposure is prolonged.

Lymphoid cells with lymphoblastic activity and intact reticular cells with mild degree of oedema causing separation of the cells were the initial changes in the bursa of B group. As the duration of exposure of 2,4-D prolonged, mild to moderate degenerative changes were seen in the endoplasmic reticulum and mitochondria.

In the thymus the ultrastructural changes were more prominent in the group A and the intensity of changes were

more in the group A2 than in the group A1. Necrotic changes of the lymphoid as well as the epithelial cell components were seen. Nuclear membrane damages like fusion, thickening and accumulation of perichromatin granules are all indications of cellular damage at the unstrastructural level. Dilatation and fragmentation of the endoplasmic reticulum and swollen mitochondria with lytic changes of the cristae were seen. These are distinct indications of cellular toxicity.

The ultrastructural findings in the thymus in group B were very mild compared to the group A.

Ultrastructural changes observed in the spleen in all the groups were mild compared with the bursa of Fabricius and thymus.

The changes were more pronounced in the A2 group. Macrophages contained dense chromatin. The endoplasmic reticulum was mainly smooth type and showed dilatation and fragmentation. Mitochondria were swollen.

The plasma cells showed severe necrotic changes with dissolution of the organelles. Electron-dense mitochondria were also encountered. Nuclear membrane damages were also noticed. These distinct but subtle subcellular changes in the plasma cell is certainly a morphological indication of functional impairment. Synthesis and delivery of the

macroglobulins will be certainly affected leading to low humoral immunity.

The organellar changes like condensation of mitochondria, vacuolation and disruption of the cellular membrane in the macrophages could lead to derangement in the engulfing and processing of the antigen. This is a morphological indication of impaired cell-mediated immunity.

In the B1 and B2 groups, the heterophils had normal cytoplasmic extrusions and electron dense granules in the cytoplasm. The mitochondria of the lymphoid cells showed mild to moderate lytic changes.

The literature scanned did not reveal reports on ultrastructural studies of the lymphoid organs from birds exposed to pesticides.

Hoberman (1979), in an ultrastructural study of the liver of mice exposed to carbofuran observed sinusoids filled with debris consisting of free ribosomes, smooth endoplasmic reticulum and proteinaecous material. Active phagocyting Kupfer cells were also noticed. In the present study, free ribosomes were frequently seen in the lymphoid organs indicative of degranulation. Disaggregation of polyribosomes observed in this study could be an indication of impaired

'export ' protein production or depressed endogenous protein production as described by Ghadially (1982).

In many of the lymphoid cells of the B group and C group, an equal proportion of heterochromation and euchromatin were seen. According to Ghadially (1982) this can be considered as a stage of activation of the lymphocyte and transformation of heterochromatin to euchromatin occurs during activation of lymphocytes.

Various studies conducted have supported the view that accumulation of perichromatin granules in the nucelus reflects a state of suppressed protein synthesis. The perichromatin granules observed in this study mostly in birds of the treatment group sacrificed after 10 weeks is a definite pointer to the suppression of protein synthesis.

A reduction in the nuclear pores was also observed in the treatment group. This observation clarified that there was impaired cellular activity, since nuclear pores are potential pathways of nucleo-cytoplasmic exchanges. This observation gain significance and relevance when viewed with the report of Maul *et al.* (1971) who found doubling of the average number of pores when lymphocytes were stimulated with phytohaemagglutinin.

In the present study, varying intensity of pathological changes in the mitochondria and endoplasmic reticulum membrane system were a consistent finding proportionate to the duration of the exposure. These changes observed conforms with the opinion of Lalithakunjamma (1987) that the mitochondrial damage could initiate a series of cellular changes resulting in the death of cells, defective growth and proliferation. It has also been pointed out that the mitochondria that are damaged would cause a depression in ATP production inducing the failure of the Na pump at the cell membrane. This in turn could lead to flooding of water which may account for the pronounced dilatation of the endoplasmic reticulum and the vacuolar changes observed.

The mitochondrial enzymes are localised in the outer membrane, inner membrane, space between the membrane and the matrix. Hence it is to be inferred that consistent mitochondrial swelling and cristolysis affects the metabolic activity of the cells, leading to impaired cellular activity.

Dilatation and vesiculation of the rough endoplasmic reticulum can be due to an ingress of water or storage of secretory products. In the present study, the dilated cystic endoplasmic reticulum were filled with electronlucent content indicating that the secreted products have diluted with flooding of water.

The ultrastructural changes observed in this investigation were almost identical with the observations like loose packing of lymphocytes, lack of transformation into plasmocytoid series, ruptured plasma membrane, organellar free cytoplasm, chromatin clumping and fragmentation of the nucleus, observed in mycotoxicosis by Farshid (1995) and mild to moderate mitochondrial changes, ribosomal detachments, fragmentation of rough endoplasmic reticulum, nucleolar and nuclear damages indicative of cell damages in ochratoxicosis, mercury and cadmium toxicity observed by Vyas (1997). However, the changes were of a comparatively less intensity in the toxicity due to agrochemicals observed in the investigation. According to them these changes in the immunopotent cells lead to immunotoxicity and lowered immunological profile in mycotoxicosis and metal toxicity. It must also be pointed out that the ultrastructural changes appear to be basically the same for all types of toxicity like mycotoxins, metal toxicity and also agro-chemicals. The variation is only in the intensity based on the dosage and duration of exposure.

By the assessment of the structural and functional state of the humoral and the cell-mediated immune system employing variety of clinical, histological and ultrastructural markers, it was established that carbofuran had immunotoxic effect even when exposed for short duration. It was further clarified

that it was dose and time dependent. It was demonstrated that 2,4-D also produces immunotoxicity but to a lesser degree when the exposure is for a prolonged duration.

The demonstration of immunotoxicity by carbofuran and 2,4-D and clarifying its nature in this investigation focusses attention on the need for monitoring and managing the environment to prevent pollution by these agro-chemicals in order to safe guard animal and human health. Since these two agro-chemicals are widely used in agricultural operations the problem has great significance.

Summary

SUMMARY

The study was designed with the objective of assessing the teratologic, embryotoxic and the immunopathologic effects in ducks of two agrochemicals, carbofuran - a pesticide and 2,4-D, a herbicide used extensively in agricultural operations.

The studies on the embryo were carried out by dipping the embryonated eggs in aqueous solution of the chemicals and studying the pathological effects on the 15th, 21st and 28th day of incubation. The parameters studied included the gross pathological changes, embryo mortality, weight of the body and the lymphoid organs like the bursa, spleen and thymus. The lymphoid organs were subjected to histopathological and ultrastructural studies.

The study revealed significant embryo mortality in the treatment groups. Generalised oedematous changes and stunting of growth were also encountered more in the treatment groups. On the 28th day, hatchability was found to be reduced and dead in shell were more in the groups treated with agro-chemicals.

On the 21st day, there was reduction in the weight of the spleen in group A. On the 28th day, the body weight of group A was found to be reduced.

The thymus and spleen showed reduction in weight in the treatment groups on the 28th day. The reduction was more in embryos of group A.

Grossly, the lymphoid organs like the spleen, bursa and thymus did not show any significant changes, but histologically, it was noted that the lymphoid components were less and loosely arranged with mild degree of oedema and stromal proliferation. The changes were prominent in group A compared to group B.

Ultrastructurally, the lymphoid cells were less and the changes were mainly confined to the mitochondrial membrane systems. The lymphoid cells were mainly with heterochromatin indicative of latent stage of activity. The changes were more or less of the same pattern in the treatment groups, but the intensity was more in the group A.

The body weights recorded at fortnightly intervals of ducks fed on the agrochemicals showed that in the case of carbofuran, there was a time dependent reduction in the body weight when compared with the control group whereas the body weight was not affected in the group fed on 2,4-D.

The haematological evaluation revealed a decreased total leukocyte count in group A. Differential leucocyte count did not show any wide fluctuations except that in group A, average

lymphocyte count was more at the third fortnight whereas it was decreased at the end of the fifth fortnight. Heterophils showed an increasing trend at the end of the fifth fortnight in A and B groups. Haemoglobin level was found to be lower in both the treatment groups compared to the control.

There was no change in the serum protein, globulin, albumin, albumin-globulin ratio after six weeks of feeding the chemicals, but they were found to be reduced at the end of the 10th week.

The HI titre against NDV showed a slight increase after six weeks of experiment. It was reduced in group A after 10 weeks, whereas in groups B and C it remained the same.

The body weight of ducks found to be reduced and it was more in birds after 10 weeks of the experiment. The reduction was noticed more in group A than in group B.

The average weight of the spleen, bursa and thymus were less in the group A after ten weeks of the experiment. The weight of the spleen and thymus from group B was reduced, but the weight of the bursa was not found to be affected when compared with the control group.

The cell-mediated immune response was assessed using the skin hypersensitivity test to DNCB and PHA, Leucocyte

migration inhibition test (LMIT) and Graft vs. Host Reaction (GVHR).

The results of the skin sensitivity to DNCB and PHA proved that the cell mediated immune response was suppressed in the treatment groups compared to the control and the suppression was more in the group exposed to the chemicals for longer duration. Histological evaluation of the dermal inflammatory response demonstrated that the response was less in the treated groups.

Assessment of the cell-mediated immune response using LMIT and GVHR also showed that the cell-mediated immune response was suppressed in both groups A and B, but the intensity of suppression was found to be more in the group A compared to the group B and also the suppression was more pronounced when the exposure period was prolonged.

Grossly, the lymphoid organs did not show any significant pathological changes. The visceral organs like the liver showed degenerative changes at the end of ten weeks in group A and nephrotic changes in group B. The changes were more pronounced in the groups A2 and B2.

In the bursa there was stromal proliferation and low lymphoblastic activity and this was associated with varying shape and size of the follicles. The changes were more in

group A compared to group B and the intensity of the changes were more in the A2 and B2 groups than in A1 and B1 groups.

The thymus revealed necrotic changes of the lymphoid cells in the A2 group. The fibrous tissue proliferation was also evident. Interfollicular oedema and reticular cell proliferation were seen in the B2 group.

The spleen also showed necrotic changes leading to depleted germinal centre. The follicles were encircled by fibrous tissue. The changes were more pronounced in the A2 group compared to B2 group.

Ultrastructurally, the lymphoid organs like the bursa, thymus and spleen revealed mild degree of lytic and oedematous changes of the mitochondria in the A1, B1 groups. The number of lymphoid cells were less when compared with the control group. The abundance of heterochromatin was indicative of inactivity. The nuclear membrane damages leading to fusion and thickening the nuclear membrane and reduction in the number of nuclear pores, all indicated the reduced metabolic activity of the cell. The accumulation of perichromatin granules in the groups A2 and B2 was considered as definite indication of suppression of protein synthesis. The changes were more pronounced in groups A2 and B2 affecting the mitochondria as well as the endoplasmic reticulum. The degranulation of ribosomes were prominent. Nicrotic changes

of the lymphoid cells resulted in the reduced number of lymphoid cells in the A2, B2 groups. The A1 and A2 group showed more intense changes compared with the B1 and B2 groups.

The investigation undertaken revealed that these agro-chemicals are not teratogenic at the dose levels used. However, it was confirmed by the gross, histological and ultrastructural studies that these chemicals are embryotoxic. Employing the various elegant immunological techniques, the immunological profile was assessed and it was clarified that the ducklings which hatch out from the contaminated eggs could be immuno deficient and therefore, the response to the vaccines may not be adequate to give sufficient protection. Hence breakdown of immunity and disease outbreaks may occur.

The agro-chemicals, carbofuran and 2,4-D were mildly toxic at the dose levels administered and the intensity was dose and time dependent. From the results obtained it was concluded that carbofuran is more toxic than 2,4-D.

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IMMUNOPATHOLOGICAL RESPONSE OF THE DUCK *(Anas platyrrhyncos domesticus)* TO SUBLETHAL DOSE OF SELECTED AGRO-CHEMICALS

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ABSTRACT OF A THESIS

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ABSTRACT

The study was conducted with the objective of assessing the teratologic, embryotoxic and immunopathologic effects of two agro-chemicals carbofuran and 2,4-D used in agricultural operations, on duck embryos and ducklings.

The embryological studies included the observation on the embryonic defects and weight of the lymphoid organs along with the histopathologic and ultrastructural studies of the lymphoid organs like the bursa, spleen and thymus.

The results showed that the contamination of the eggs with the above chemicals caused moderate deleterious effects on the lymphoid system, though no teratologic effects could be observed. The histological and ultrastructural changes also confirmed the deleterious effects at the tissue and cellular level.

The immune system of the ducks exposed to the above chemicals for two different durations were assessed using a battery of tests. The humoral immune system was assessed based on the body weight, lymphoid organ weight, haematological evaluation, serum biochemical evaluation and the evaluation of HI titre against NDV.

The cell-mediated immune system was assessed by the skin reactivity tests to DNCB and PHA. LMIT and GVHR were also employed to assess the cell-mediated immune system. The histological and ultrastructural studies were also conducted after six weeks and ten weeks of the experiment. The assessment of the immunological profile employing these elegant tests revealed that these agro-chemicals caused mild but significant suppression of the cell-mediated and humoral immune response.

By this investigation it was clarified that these agro-chemicals have mild to moderate degree of immunotoxic effect when exposed for a short duration, but the changes were severe when exposed for longer duration. From the studies, it was demonstrated that the immunotoxic effect of carbofuran is more compared to 2,4-D (herbicide) and the changes were dose and time dependent.