THE IMMUNOLOGICAL AND PATHOLOGICAL CHANGES IN POULTRY INDUCED BY OCHRATOXIN A

PRABHAKER DWIVEDI

B.Sc., B.V.Sc. & A.H., M.V.Sc.

A thesis presented for the degree of

Doctor of Philosophy

of the University of Edinburgh

1984

TO

MY PARENTS, MRS. RAJ DULARI

AND MR. CHINTAMANI DUBE WITH

RESPECT AND DEEP ADMIRATION

CONTENTS

CHAPTER Page

CHAPTER Page

DECLARATION

This is to certify that I, Prabhaker Dwivedi have carried out the investigation reported herein and have composed this thesis. Fields of collaboration have been fully identified.

Prabhaker Dwivedi

ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. R.B. Burns for his keen interest, encouragement and cooperation throughout these studies. I am also grateful to Dr. J.E. Phillips, my University Supervisor, for his invaluable advice and critical appraisal of the manuscript.

I am greatly indebted to Dr. D.W.F. Shannon, Director of the Poultry Research Centre, for granting me the facilities to undertake this work, also to Dr. W.G. Siller, Head of the Department and Dr. P.A.L. Wight, for their support, keen interest and expert advice.

Mr. M.H. Maxwell introduced me to the methods of electron microscopy and Dr. S.R.I. Duff guided me in the bone studies; I extend my gratitude to both of them. Other people who have, at times, assisted with special techniques are: Dr. C.C. Whitehead (biotin studies), Mr. R. Talbot (plasma steroid hormone analysis), Miss Jean Robb, East of Scotland College of Agriculture (mycotoxin analysis), Dr. M.R.A. Morgan, Food Research Institute (mycotoxin analysis and gift of antiserum) and Dr. T.J. Simpson, Department of Chemistry, University of Edinburgh (gift of some Ochratoxin A); to them I am indebted. The excellent technical assistance which was provided, from time to time, by Mrs. J. Johnston, Mr. R.S. Wilkie, Miss I. Anderson, Mr. M. Lynch, Mr. G. Robertson and Miss S. Spence, is gratefully acknowledged.

My special thanks must go to Messrs R.K. Field, N.J. Russell and E. Armstrong of the Photographic and Graphics Sections at the PRC, for their outstanding work, and to Mrs. Lesley Burns for the exemplary way in which, under constant pressure, she prepared the final typescript.

My gratitude is due to Dr. and Mrs. N.K. Bagariafor their consistent support, and to Dr. and Mrs. K.V. Krishna and Mr. A.M. Singh for encouragement. I need hardly

mention my profound appreciation of the continued support from my wife, Kusum and children, Sandeep, Alka and Archana. I extend my special thanks to my 11 year old daughter Alka for making a fair copy of the entire thesis for final typing. I am grateful to my uncle Mr. R.P. Dube for his constant support. I am obliged to him and Mr. R.S. Misra for looking after my son during my absence from India.

It would, of course, have been impossible for me to undertake this work without leave from the Indian Council of Agricultural Research, and without the grant from the Association of Commonwealth Universities, London. I am, therefore, indebted to both these authorities.

Mr. D. Waddington advised and helped with statistical methods, for which I am grateful.

ABSTRACT

The mycotoxin ochratoxin A (OA) can contaminate a wide variety of feedstuffs and be nephrotoxic to domestic animals and poultry. The objective of this work was to study the clinical, pathological and immunological effects induced by feeding OA to broilers and turkeys from hatch as well as its effects on production characteristics and teratogenicity in quail. Some basic immune mechanisms were studied.

OA caused growth depression in broilers and turkeys but not in quail. There was a loss of carotenoid pigments in the shank of broilers.

The principal pathological effects were in the kidney especially in the proximal convoluted tubules (PCT) where the mitochondria were the organelles most sensitive to OA-damage although other intracellular elements were involved together with a marked increase in lysosomal activity. OA localised within PCT and glomeruli. Ring-form mitochondria in the PCT and the accumulation of glycogen in the liver were considered to be of diagnostic significance.

In the lymphoid organs, widespread degeneration was a consistent feature of OA-toxicity, suggesting a direct effect on immunity. OA induced a marked depression of both humoral and cell-mediated immune (CMI) responses in broilers and turkeys. Total serum levels (IgG, IgM, IgA) were reduced in broilers but only IgM levels were depressed in turkeys. Tissue immunoglobulins were also depressed. Delayed hypersensitivity responses were reduced in broilers and turkeys. Graft-versus-host reactions, evaluated by a chick embryo splenomegaly test, were depressed in OA-fed broilers.

It was not possible to elicit a distinct Arthus reaction in turkeys in contrast to broilers.

In general, granulocytes, particularly heterophils and eosinophils, and vascular endothelial cells appeared to play a significant role in CMI responses in poultry.

OA-contaminated feed resulted in reduced egg production, fertility and hatchability in a dose-related fashion in Japanese quail and an increase in embryonic mortality and a number of developmental defects.

All abbreviations used in the text of this thesis are listed on page346which can be folded out for ease of reference.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Importance and General Introduction

Mould fungi are one of the most ubiquitous types of microorganism in the environment. A few are pathogenic to poultry and livestock resulting in mycoses, but the majority grow saprophytically on a wide range of substrates and are among the most common causes of spoilage of human and animal feedstuffs. When grown under suitable conditions, moulds produce secondary metabolites, termed mycotoxins, some of which are very toxic to livestock and poultry. Many moulds possess the potential to produce one or more mycotoxins in cereal grains and feedstuffs. Poisonings caused by ingestion or inhalation of or in some cases dermal contact with mycotoxins present in mould-damaged food are known as mycotoxicoses. Mycotoxins are not necessarily eliminated from feedstuffs by cooking or processing practices, and hence constitute a potential threat to animal and human health.

Although toxic metabolites from mouldswere recorded as early as the Middle Ages, the possibility that fungi growing in feedstuffs could produce highly toxic metabolites was largely ignored until the outbreak of 'turkey-x disease' in East Anglia and Southern England in 1960, when at least 100,000 turkeys (Blount, 1961) died after consuming a diet incorporating Brazilian groundnut meal, which contained a highly toxic fungal metabolite, chiefly produced by Aspergillus flavus and now known as aflatoxin. Since then extensive research efforts have led to the discovery of many other mycotoxins, whose synthesis, natural occurrence and toxic properties have been documented (Wyllie and Morehouse, 1977).

Mycotoxicoses have been categorised (Pier, Richard and Cysewski, 1980a) as acute primary, chronic primary and

secondary. Acute primary conditions are characterised by specific lesions in the target tissues whereas in chronic primary mycotoxicoses there may be no specific lesions to be seen except reduced growth and production. Secondary mycotoxicoses may result from the intake of a low concentration of mycotoxin and cause impairment of immunogenesis and lowering of resistance, predisposing the host to secondary infection or diseases.

Of the mycotoxins known to be produced in feedstuffs, aflatoxin, ochratoxin and trichothecenes are the most prevalent and the most important. Ochratoxin A is primarily nephrotoxic (Krogh, 1978; Elling, 1981), whereas aflatoxin B_1 is hepatotoxic (Siller and Ostler, 1961; Goldblatt, 1969) and trichothecenes affect the nervous, haematopoeitic and digestive systems (Mirocha, Pathre and Christensen, 1979; Pier, 1981). All these mycotoxins are known to cause diseases in poultry under natural and experimental conditions (Austwick, 1977; Peckham, 1977; Joffe, 1977; Hamilton, Huff, Harris and Wyatt, 1982; Robb, Kirkpatrick and Norval, 1982). Other mycotoxins causing renal damage are citrinin (Mehdi, Carlton and Tuite, 1981, 1983, 1984) and oosporein (Pegram and Wyatt, 1981; Pegram, Wyatt and Smith, 1982; Manning and Wyatt, 1984).

Recently considerable attention has focussed on mycotoxins because of the alleged use of trichothecenes as a chemical warfare agent (yellow rain) in Southeast Asia, Afghanistan and Iran. Yellow rain, which hit the headlines in the international press, journals and other public media, continues to be a controversial issue. There is disagreement as to whether the "yellow rain" contained trichothecenes (Mirocha, 1982; Heyndrickx, 1984) or was coloured because of faecal material from honey bees containing large amounts of pollen (Ashton, Meselson, Robinson and Seeley, 1983; Nowicke and Meselson, 1984). Seagrave (1981) has given an elaborate account of his journey and investigation of the

affected areas in his book, 'Yellow rain: a journey through the terror of chemical warfare'.

Ochratoxin A (OA), a most potent nephrotoxin, chiefly produced by Penicillium viridicatum and Aspergillus ochraceus, has been found commonly as a natural contaminant of cereals and feedstuffs in many countries of Europe, in the United States and Canada, and in almost all countries where attempts to detect the toxin have been made (Krogh, 1978). In the United Kingdom, cereal grains and compounded animal and poultry feedstuffs have been reported to be contaminated with OA (Shreeve, Patterson and Roberts, 1975; Clarke and Niles, 1978; Syrett, 1978; Anon., 1980; Benham, 1982). Recently, in a comprehensive survey of 4,000 samples of animal feedstuffs comprising cereals, compounded feeds, hay and silage during 1976 to 1979, OA was detected more frequently than other mycotoxins, sometimes in association with citrinin, sterigmatocystin and zearalenone. Aflatoxin B, was rarely found (Buckle, 1983). In Taiwan, 27 to 63 per cent of mixed feeds for pigs, chickens, ducks and fishes were found to be contaminated with OA (Chang, 1980). OA production could occur even in the absence of visible mould growth (Stuart and Bedell, 1982) or even after treatment of feed with propionic acid, a mould inhibitor (Buckle, 1982, 1983).

OA has been responsible for spontaneously occurring Mycotoxic Porcine Nephropathy (MPN) in Norway, Denmark and Sweden (Elling and Møller, 1973; Rutqvist, Björklund, Hult, Hökby and Carlsson, 1978) and Ireland (Buckley, 1971). Experimentally OA has produced renal disease in all the homoiothermic animals to which it has been administered (Pier, 1981). In poultry, spontaneously occurring OA-induced nephropathy (Elling, Hald, Jacobsen and Krogh, 1975) as well as natural field outbreaks involving 970,000 turkeys, 70,000 laying hens and 12 million broilers have been reported with OA levels ranging from 0.3 to 16 parts per million (ppm) in

the feed incorporating contaminated corn. Mortality reached as high as 59 per cent in one of the episodes in turkeys (Hamilton et al.,1982). Recently, OA caused fatal gastroenteritis in rabbits, chickens and dogs due to ingestion of naturally mouldy bread containing as high as 80 ppm OA and 9.6 ppm ochratoxin B, produced by Aspergillus ochraceus (Visconti and Bottalico, 1983). OA has been found to be extremely toxic to broilers causing growth depression, blood coagulopathy, anaemia, bone abnormalities and type-x glycogen storage disease (Huff, Wyatt, Tucker and Hamilton, 1974; Huff, Chang, Warren and Hamilton, 1979a; Huff, Doerr, Hamilton, Hamann, Peterson and Ciegler, 1980; Warren and Hamilton, 1980a; Doerr, Huff, Hamilton and Lillehoj, 1981). In laying hens egg production was reduced (Page, Stewart, Wyatt, Bush, Fletcher and Brown, 1980; Prior, Sisodia and O'Neil, 1981; Tohala, 1983).

OA has been reported to cause teratogenic effects in mice, rats and hamsters (Hayes, 1981; Arora, 1982). Embryotoxic and teratogenic effects have been studied in chickens also by inoculation of OA in 2 to 4-day-old embryos (Veselá, Veselý and Jelinek, 1983). However, teratogenic effects remain to be demonstrated in poultry following natural oral consumption, though the passage of OA into the eggs of laying hens (Juszkiewicz, Piskorska-Pliszczyńska and Wiśniewska, 1982) and Japanese quail (Piskorska-Pliszczyńska and Juszkiewicz, 1979) after OA administration has been reported. Carcinogenicity of OA has not been proven beyond doubt (IARC, 1976) and it is still categorised as a suspected carcinogen.

Mycotoxins, especially aflatoxins have been shown to impair the immune response, acquired immunity and resistance of mammals and birds making them more susceptible to bacterial, viral and parasitic diseases (Pier, Richard and Thurston, 1980b). However, no authentic information is available on the effect of OA on the immune response in poultry, though the few reports of OA on the immune response

in mice and other animals are either contradictory or inconclusive (Richard, Thurston, Deyoe and Booth, 1975a; Patterson, Shreeve, Roberts, Berrett, Brush, Glancy and Krogh, 1981; Prior and Sisodia, 1982; Creppy, Størmer, Röschenthaler and Dirheimer, 1983). In poultry lymphoid depletion in the bursa of Fabricius (Peckham, Doupnik and Jones, 1971; Huff et al., 1974), thymic regression (Chang, Doerr and Hamilton, 1981), leucocytopenia (Chang, Huff and Hamilton, 1979), impaired phagocytic ability of heterophils (Chang and Hamilton, 1980) and reduced complement activity (Campbell, May, Huff and Doerr, 1983) after OA treatment suggest an immunosuppressive effect. Hamilton et al. (1982) observed an increased incidence of air sacculitis in several field outbreaks of ochratoxicosis in broilers and turkeys suggesting the possibility of immunosuppression.

The presence of OA in eggs and edible tissues of poultry (Elling et al., 1975; Juszkiewicz et al., 1982), and pigs (Krogh, Elling, Hald, Larsen, Lillehoj, Madsen and Mortensen, 1976b; Krogh, 1977a; Rutqvist, Björklund, Hult and Gatenbeck, 1977), meat products, retail wheat flour, corn flour, bakery products, cereal and cereal products, corn oil, beans, beer, coffee beans etc. in many parts of the world (Krogh, 1980; Chelkowski and Goliński, 1982; Pepeljnjak and Blaževic, 1982; Stack, Mislivec, Gibson and Pohland, 1982) including Britain (Lindsay, 1981; Cooper, Wood, Chapman and Williams, 1982; Norton, Toule, Cooper, Partington and Chapman, 1982; Buckle, 1983) should be a matter of great concern because of their possible effect on human health, particularly when there has been increasing evidence for a causal association of OA with Endemic (Balkan) nephropathy, a fatal kidney disease affecting nearly 10 to 12 per cent of the population in certain areas of Bulgaria, Yugoslavia and Romania(Barnes, Austwick, Carter, Flynns, Peristianis and Aldridge, 1977; Krogh, 1977b; Cooper, 1979; Elling, 1981).

The economic importance of OA-induced losses has not been estimated precisely, but it runs into several million dollars due to MPN alone in Denmark. It has been calculated that pigs showing gross lesions had a lowered growth rate to the value of 10 dollars per pig (Krogh, 1982). Though the prevalence of MPN and OA-induced avian nephropathy in the U.K. has not been studied, Prof. Krogh is worth quoting, "The frequency with which the feed in the U.K. was contaminated with OA, it could almost be predicted that MPN must be as prevalent in this country as in Denmark, whether one wishes to face it or not." Similarly, in tropical and developing countries, including India, where climatic and storage conditions are very conducive to fungal growth and OA production, virtually no information is available on the epidemiology and association of OA with livestock and poultry health.

Thus, despite widespread OA contamination of feed and its association with natural disease outbreaks in poultry, there is a paucity of information on the immunological, detailed pathological and ultrastructural changes with which this agent is associated in poultry. Most of the studies conducted in broiler chicks (and a few in turkey poults and quail) deal with the clinical and biochemical changes induced by short-term exposure, usually up to 3 weeks, of graded doses of OA. There are, then several aspects of avian ochratoxicoses, where much still remains to be understood and which require detailed investigation. A better understanding of the disease process and the effect of OA on the liver, kidney and lymphoid (immunocompetent) organs and on the humoral and cell-mediated immune (CMI) responses should help in identifying the host-mycotoxin interaction and assessing the problem of ochratoxicosis under field conditions, leading to an awareness, to improved methods of diagnosis and to eventual control.

1.2 Objectives of the Study

The present studies were conducted on ochratoxin A, a commonly occurring and potent nephrotoxic mycotoxin, by feeding it in the diet in sublethal or even subclinical doses, to broilers, turkeys and quail with the following objectives in view.

I To study detailed clinical, pathological and ultrastructural changes in the kidney, liver and lymphoid organs of broilers and turkeys after OA treatment.

II To make a comparative study of the ultrastructural changes induced in broilers and turkeys up to 8 and 10 weeks of age.

III To examine OA-induced skeletal changes in young birds.

IV To determine the effect of OA on immunoglobulin levels in sera and in tissues.

V To evaluate and define delayed hypersensitivity employing tuberculin and phytohaemagglutinin (PHA), contact hypersensitivity (employing dinitrochlorobenzene, DNCB) and Arthus reactions in normal and OA-fed broilers and turkeys, thus assessing the effect of OA on CMI responses. Basic immune processes, hitherto not known, were also studied, particularly in turkeys.

VI To study the effect of OA on graft-versus-host reactions, a measure of CMI response, in broilers employing a splenomegaly assay in chick embryos.

VII To investigate the effect of OA on growth, production, hatchability and particularly teratogenicity in quail, fed subclinical levels of OA in the diet, the natural mode of OA-

poisoning under field conditions.

VIII To demonstrate the localisation of OA in the kidney, liver, muscles and the gut (jejunum) and to relate it to pathological changes.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1 General and Historical Background

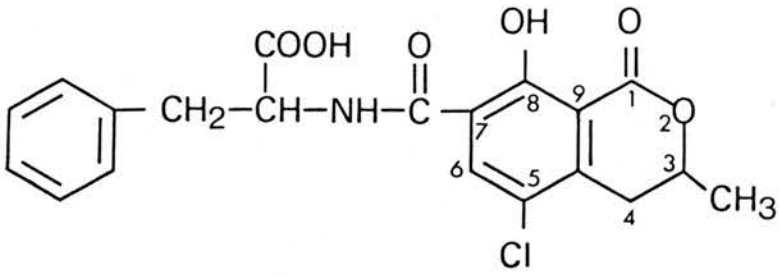
General reviews and books encompassing various aspects of mycotoxins and mycotoxicoses, including ochratoxins, include those of Ciegler and Lillehoj (1968), Ciegler, Kadis, and Ajl (1971), Newberne (1974), Purchase (1974), Wogan (1975), Rodricks, Hesseltine and Mehlman (1977), Wyllie and Morehouse (1977), Jemmali (1978), CAST (1979), Eaker and Wadström (1979), Mirocha *et al*. (1979), National Academy of Sciences (1979), WHO (1979), Ciegler and Bennett (1980), Pier *et al*. (1980a), Pier (1981), Scanes, Ottinger, Kenny, Balthazart, Cronshaw and Jones (1982), Smith (1982), Ciegler, Burmeister and Vesonder (1983) and Moreau (1984). Reviews dealing specifically with ochratoxins are those by Steyn (1971), Applegate and Chipley (1973), Harwig (1974), Chu (1974), and Carlton and Krogh (1979). Only aspects relevant to the present study will be presented here.

Ochratoxins have attracted considerable attention in recent years. Whereas aflatoxins were recognised as a result of attempts to determine the aetiology of a recognised disease outbreak (Allcroft and Carnaghan, 1962), the ochratoxins were identified during the course of an investigation into the toxicity of moulds from South African cereal and legume crops (Scott, 1965a, 1965b). The identification, during the investigation, of toxicogenic strains of *Aspergillus ochraceus* Wilhelm, which were fatal when fed as corn cultures to ducklings, rats and mice, led to the isolation and chemical characterisation of the ochratoxins. The main toxic component of the culture extract was found to be ochratoxin A (Van der Merwe, Steyn, Fourie, Scott and Theron, 1965a; Van der Merwe, Steyn,and Fourie, 1965b), but in the course of the work the dechlorinated and ethyl ester derivatives of OA, ochratoxin

B (OB) and ochratoxin C (OC) respectively, were isolated (Van der Merwe et al., 1965b). Subsequently, Steyn and Holzapfel (1967a) confirmed these metabolites and isolated the methyl esters of OA (OA-M) and OB (OB-M). OA and OB as well as their ester derivativeshave also been chemically synthesised (Steyn and Holzapfel, 1967b; Roberts and Woollven, 1970). Ochratoxin-D (4-hydroxyochratoxin) has also been isolated (Hutchinson, Steyn and Thompson, 1971), though not characterised.

As well as A. ochraceus, other moulds of the genus Aspergillus are known to produce OA. These include A. alliaceus, A. melleus, A. ostianus, A. petrakii, A. sclerotiorum and A. sulphureus. (Lai, Semeniuk and Hesseltine, 1968; Hesseltine, Vandegraft, Fennell, Smith and Shotwell, 1972). Several species of Penicillium, namely P. viridicatum Westling, P. commune, P. cyclopium, P. variable, P. purpurescens and P. palitans also produce ochratoxins (Van Walbeek, Scott, Harwig and Lawrence, 1969; Scott, Van Walbeek, Harwig and Fennell, 1970; Scott, Van Walbeek, Kennedy and Anyeti, 1972; Ciegler, Fennell, Mintzlaff and Leistner, 1972). Though A. ochraceus is considered to be the principal producer of ochratoxin A (Chu, 1974), P. viridicatum has been shown to be a common source of naturally occurring ochratoxin A in grains (Scott et al, 1972), particularly in the United Kingdom (Buckle, 1983).

Ochratoxin A, which is more toxic than other ochratoxins, is a colourless, crystalline compound, moderately soluble in organic solvents with a molecular formula of $C_{20}H_{18}ClNO_6$, and the chemical structure, 7-carboxyl-5-chloro-8-hydroxyl 3, 4-dihydro-3-R-methyl isocoumarin linked to L-β-phenylalanine. Its physico-chemical properties have been summarised by Steyn (1971), Chu (1974), Harwig (1974) and Wyllie and Morehouse (1977).



Ochratoxin A

OA is a stable compound and can be stored in ethanol in the refrigerator for more than a year (Chu and Butz, 1970), though it is unstable in light (Neely and West, 1972). Harwig, Chen and Collins-Thompson (1974) found that 53 per cent of the OA content of contaminated beans was not affected by blanching, salting and heat processing in tomato sauce for one hour. The toxin is fairly heat stable and a large proportion survived autoclaving in cereals for up to 3 hours (Trenk, Butz and Chu, 1971). OA has been shown to bind to bovine serum albumin (Chu, 1971) a property which has been exploited for the production in rabbits of antisera to OA (Chu, Chang and Hinsdill, 1976; Elling, 1977b; Whittaker, Fuller and Morgan, 1981) for the demonstration of OA in tissues by means of the immunofluorescent staining technique (Elling, 1977b) and also for the detection and analysis of OA in cereals, feedstuffs and tissues by the microtitre plate enzyme linked immunosorbent assay (ELISA)(Morgan, Matthew, McNerney and Chan, 1982).

2.2 Natural Occurrence

2.2.1 Ochratoxin-producing fungi

Ochratoxin-producing fungi are ubiquitous (Hesseltine et al., 1972; Raper and Fennell, 1965). A. ochraceus has been isolated from stored grains (Christensen and Kaufmann, 1969; Ichinoe, Udagawa, Tazawa and Kurata, 1970).

Toxicogenic strains of A. ochraceus have been isolated from a variety of food and feedstuffs including peanuts (Doupnik and Peckham, 1970), cereal and sorghum (Scott, 1965a; Christensen, Nelson, Mirocha and Bates, 1968), black and red peppers (Christensen, Fanse, Nelson, Bates and Mirocha, 1967; Christensen et al., 1968), adzuki beans, pepper flour (Natori, Sakaki, Kurata, Udagawa, Ichinoe, Saito and Umeda, 1970), red beans and spices (Udagawa, Ichinoe and Kurata, 1970), brewery hops (Van Walbeek, Scott and Thatcher, 1968), hay (Still, 1973), rice (Udagawa et al., 1970; Yamazaki, Maebayashi and Miyaki, 1970), corn (Christensen and Kaufmann, 1969; Richard, Tiffany and Pier, 1969), pecans (Doupnik and Bell, 1971), dried fish (Udagawa et al., 1970), soyameal and poultry feed (Bacon, Sweeney, Robbins and Burdick, 1973; Connole, Blaney and McEwan, 1981), Brazil nuts (Van Walbeek et al., 1968), cured ham (Escher, Koehler and Ayres, 1973), smoked meat products (Pepeljnjak and Blaževic, 1982), coffee beans (Levi, Trenk and Mohr, 1974; Stack et al., 1982), and bread (Visconti and Bottalico, 1983).

Penicillium viridicatum has been isolated from wheat (Scott et al., 1970, 1972), dried white beans, peanuts, pelleted dairy ration, chicken feed (Scott et al., 1972), corn (Krogh and Hasseleger, 1968; Carlton, Tuite and Mislivec, 1968; Carlton and Tuite, 1970; Still, 1973), barley (Buckle, 1983), ham (Van Walbeek et al., 1969) and sausage (Ciegler et al., 1972; Mintzlaff, Ciegler and Leistner, 1972).

It has been shown that ochratoxin B, citrinin and penicillic acid can all be produced by ochratoxin A-producing fungi (Scott, 1977; Ciegler, 1972; Scott et al., 1972; Bacon et al., 1973).

2.2.2 Ochratoxin A

The first reported natural occurrence of OA was in a

low grade sample of corn analysed in a survey in the United States (Shotwell, Hesseltine and Goulden, 1969). Since then contamination of cereals, animal feedstuffs and mixed diets have been reported from various countries (Table 1), including Japan (Uchiyama, Isohata and Takeda, 1976; Sugimoto, Minamisawa, Takano, Sasamura and Tsuruta, 1977). Sometimes multiple contamination with other mycotoxins such as, citrinin, sterigmatocystin, ochratoxin B, zearalenone or aflatoxin B, has also been observed (Shotwell, Hesseltine, Vandegraft and Goulden, 1971; Scott et al., 1972; Krogh, Hald and Pedersen, 1973; Buckle, 1983).

Since no extensive countrywide survey on the natural occurrence of ochratoxins has been made in any country other than Denmark (Krogh, 1978), the total extent of OA contamination of food and feeds cannot be assessed at present. Table 1 and Table 2 give an indication of the extent of contamination in a wide variety of feedstuffs and animal products used for animal, poultry and human consumption. In the United Kingdom, the majority of earlier reports (Clarke and Niles, 1978; Syrett, 1978; Anon., 1978; Lindsay, 1978; Anon., 1980; Alderman, 1981; Benham, 1982) suggest frequent contamination of edible commodities with OA, though a quantitative estimate was not recorded. In a routine survey of mycotoxins in animal feedstuffs in England and Wales, Buckle(1983) found OA in 12.8 per cent of cereals, even at low moisture content; barley and wheat in particular, had a high concentration of OA. Even when other mycotoxins, such as citrinin, sterigmatocystin and zearalenone were present, OA was invariably present as well at a higher concentration. OA has been found, particularly in cereals, with frequencies up to 14 per cent (Krogh, 1978). The presence of OA in retail flour (Osborne, 1980) particularly in the U.K. to the extent of 6.25 ppm (Richardson, Flude, Patterson, Mackenzie and Wakefield, 1978), and bread (Osborne, 1980) up to 80ppm (Visconti and Battalico, 1983),

and its natural occurrence in cereals (Table 1), animal products and processed food (Table 2) poses a threat to public health.

2.3 Ochratoxicosis

2.3.1 Naturally occurring ochratoxicosis

Spontaneously occurring nephropathy associated with OA in poultry was reported in Denmark in 1975 when Elling et al. (1975) detected microscopic lesions in 4 out of 14 kidneys showing gross lesions. The thoracic muscles of birds with renal lesions contained residues of OA.

The first authenticated naturally occurring outbreaks of ochratoxicosis in poultry were reported by Hamilton et al. (1982) who investigated five independent episodes of ochratoxicosis in about 970,000 turkeys, two episodes in 70,000 laying hens and two episodes in 12,000,000 broiler chickens in the United States. The turkeys had symptoms of decreased feed conversion and mortality ranging from 21 to 59 per cent and there was an OA content of 2 to 16 ppm in the contaminated diet. The broilers had poor growth and laying hens showed poor production with OA levels ranging from 0.3 to 4ppm in the diet. These episodes were traced to contaminated corn in 8 episodes and to corn gluten meal in one episode. All the species of birds developed a nephropathy and it was possible to reproduce the disease in young turkeys by feeding the suspect diet. Other toxins and chemicals were not involved.

A lethal gastroenteritis due to feeding naturally mouldy bread to chickens, rabbits and dogs has been reported by Visconti and Bottalico (1983) from Italy. The mouldy bread was contaminated with A. ochraceus and contained 80 ppm OA and 9.6 ppm OB, the highest contamination level recorded in a naturally mouldy feed.

Table 1. Natural occurrence of OA in cereals and other feedstuffs

Country	Year	Commodity	No. samples examined	No. positive & (% affected)	OA level (ppb)*	Reference
U.S.A.	1967	Corn	283	1 (0.35)	110- 150	Shotwell et al. (1969)
	1968	Corn	293	3 (1.02)	83- 166	Shotwell et al. (1971)
	1971	Barley	127	18 (14.2)	10- 40	Nesheim (1971)
	1973	Barley	180	(13.0)	10- 37	Fischback and Rodricks (1973)
	1974	Coffee beans	267	19 (7.1)	20- 360	Levi et al. (1974)
	1976	Wheat (red winter)	291	3 (1.0)	5- 115	Shotwell, Goulden and Hesseltine (1976)
	1976	Wheat (red spring)	286	8 (2.8)	5- 115	
Canada	1968	Heated grains	29	18	30-27000	Scott et al. (1972)
		Mixed feed	3	2	20- 530	
		Dried white beans		3	20- 210	
		Peanut	1	1	4900	
		Wheat (red spring)	4	2	20- 100	Scott et al. (1970)
	1971-74	Wheat	95	6	30- 6000	Prior (1976)
		Hay	95	1	30	
	1975-79	Barley, wheat oat, rye	148	4	30- 4000	Prior (1981)
		Forage	108	1	30- 4000	
		Corn	19	2	30- 500	
	1979-82 (Jan.)	Oat and barley (dairy cattle)	51	2	48- 380	Abramson, Mills and Boycott (1983)
		Feed, wheat, oat and barley (beef cattle)	51	1	5900	
		Feed (poultry)	51	1	140	
Denmark	1973	Barley and oats (swine)	33	19 (57.6)	28-27500	Krogh et al. (1973)
	1978	Malt barley	50	3 (6.0)	9- 189	Krogh (1978)
Sweden	1972	Barley and oat	84	7 (8.3)	16-409.5	Krogh, Hald, Englund, Rutqvist and Swahn (1974b)
	1976	Barley	269	17 (6.3)	up to 20	Hökby, Hult, Gatenbeck and Rutqvist (1979)
Yugoslavia	1971-75	Corn, wheat, barley	47	6 (12.8)	5- 90	Krogh, Hald, Plestina and Ceovic (1977)
	1975	Corn	20	2	25- 100	Munk and Topolko (1975)
	1975-76	Corn	191	(26)	490	Balzer, Bogdanić and Mužic (1977)

Table 1 continued

Country	Year	Commodity	No. samples examined	No. positive & (% affected)	OA level (ppb)	Reference
Australia	1971-80	Goat feed	25	1	70000	Connole et al. (1981)
France	1973-74	Maize	463	12 (2. 6)	15- 200	Galtier (1975)
Poland	1974	Barley, wheat, rye oat, maize	150	8 (5. 3)	50- 200	Juszkiewicz and Piskorska-Pliszczyńska (1976)
	1976	Mixed feed	203	10 (4. 9)	10- 50	Juszkiewicz and Piskorska-Pliszczyńska (1977)
	1975-80	Cereals	784	61 (7. 8)	1100	Chelkowski and Goliński (1982)
	1981	Cereals	100	6 (6. 0)	1200	
	1980-81	Mixed feed	131	2 (1. 5)	200	
Taiwan	1979	Chicken feed	499	(43)		Chang (1980)
		Pig feed		(31)		
		Fish feed		(31)		
		Duck feed	3	1		
UK	1976-79	Barley	376	51 (13. 6)	25-5000	Buckle (1983)
		Wheat	101	15 (14. 1)	25-2700	
		Oat	46	1 (2. 2)	80	
		Pigmeal	96	12 (12. 5)	25- 250	
		Pig pellets	195	8 (4. 1)		
		Cattle feed	318	1 (0. 3)		
		Poultry feed	203	6 (3. 0)	25- 150	
	1980-81	Turkey mixed feed	11	11	10- 130	Howell (1982)
		Broiler diet	2	2	10	
		Cattle feed	11	2	10	
		Pig feed	2	1	20	
	1980	Maize	29	11	50- 500	Anon. (1980)
		Corn flour	13	4	50- 500	
		Soyabean	25	9	50- 500	
		Soya products	28	6	50- 500	
		Cocoa beans	56	10	60- 500	
		Cocoa products	25	5	60- 200	
	1981	Breakfast cereals	243	12	5- 108	Lindsay (1981)

* Parts per billion

Table 2. Natural occurrence of OA in edible animal tissues, animal products and processed foods

Country	Year	Commodity	No. samples examined	No. positive & (% affected)	OA level (ppb)	Reference
A. Slaughtered animals						
Denmark	1972	Pig kidney	19	18	Up to 67	Hald and Krogh (1972)
	1975	Pig kidney	60	21 (35)	Up to 68	Krogh (1977a)
Sweden	1975	Poultry muscle	14	5	4.3-29.2	Elling et al. (1975)
	1976	Pig kidney	129	32 (25)	2- 104	Rutqvist et al. (1977)
	1976-77	Pig kidney	34	34	32- 218	Rutqvist et al. (1978)
		Liver	10	10	26- 65	
Yugoslavia	1977	Pork	12	1	5	Krogh et al. (1977)
	1979	Pig kidney	38	(6. 6)	26- 76	Pepeljnjak, Blažević and Culjak (1982a)
Hungary	1980-81	Pig kidney	122	(39)	5->100	Sandor, Glávits, Vajda, Ványi and Krogh (1982)
B. Animal products and processed foods						
UK	1982	Meat products	33	7	4	Cooper et al. (1982)
		Bakery products	8	3	80	
		Fruits and nuts	5	1	trace	
		Cheese	19	3	7	
		Sugar and confectionery	1	1	trace	
Yugoslavia	1978-80	Ham	206	(28. 9)	40- 70	Pepeljnjak and Blažević (1982)
		Bacon		(18. 9)	37-200	
		"Kulen"		(13. 3)	10- 460	
		Sausage		(12. 0)	10- 920	

No other mycotoxins were demonstrated in the bread.

Recently, an outbreak of ochratoxicosis in ducks was reported (Bodnarchuk and Kaspruk, 1984) from the Ukraine (USSR), with a 42 per cent mortality after 2-6 days of illness. The affected birds had haemorrhages in various tissues, and catarrhal gastritis and enteritis. The feed was severely contaminated with A. ochraceus as well as OA.

Field cases of suspected mycotoxicoses including ochratoxicosis in laying birds have been reported from Hungary (Ványi, Bata and Lasztity, 1980), the United States (Page et al., 1980), Canada (Abramson et al., 1983) and Britain (Munro, 1982; Howell, 1982). Huff and Hamilton (1975a) associated two field cases of under-pigmentation in broilers with an ochratoxin A-contaminated diet.

In mammals, naturally occurring cases of diseases associated with mycotoxins have been reported. Nephrotoxicity in pigs associated with the consumption of naturally contaminated barley was also recognised in Ireland (Buckley, 1971). Mortality in pigs and incoordination in a horse (Shreeve et al., 1975) due to mycotoxin contaminated cereals have been observed in the United Kingdom, but conclusive evidence of an association with OA is lacking. However, a causal association of OA with Mycotoxic Porcine Nephropathy (MPN), an endemic disease condition in Scandinavia, has been strongly suggested (Krogh, 1978; Rutqvist et al., 1977, 1978; Elling, 1981). A. ochraceus has also been implicated in bovine abortions in Wisconsin (Still, Macklin, Ribelin and Smalley, 1971).

2.3.2 Experimental ochratoxicosis

Though OA is primarily nephrotoxic, effects in other organs and tissues have been observed in various species.

The susceptibility of different species of animals varies a great deal. Broiler chicks, ducklings, turkeys, dogs, pigs and rainbow trout appear to be susceptible, whereas laboratory rodents and ruminants (sheep and cattle) are relatively resistant.

Acute toxicity of OA has been reported by several workers. The LD_{50} (median lethal dose) of OA given orally to seven-day-old New Hampshire Leghorn cross chicks was 166 µg (Chu and Chang, 1971) but in two seven-day trials using day-old White Leghorn (Babcock-300) cockerels, in which sublethal doses of OA as well as OB caused depressed growth, the LD_{50} values by the oral route were 116 µg (3.3 mg/kg body weight) and 135 µg (3.9 mg/kg) respectively. In broiler chicks, the LD_{50} for day-old chicks was 2.14 mg/kg and for 3 week-old birds 3.60 mg/kg bodyweight (Peckham et al., 1971)

Initial studies by Van der Merwe et al. (1965a) on day-old ducklings indicated that the oral LD_{50} of OA was 25 µg. Later work using a larger number of ducklings has suggested a higher value of approximately 150 µg (Purchase and Nel, 1967). Steyn and Holzapfel (1967a) also have estimated that in day-old ducklings, the oral LD_{50} of OA, OA-M and OC range between 135-170 µg.

In a comparative seven-day evaluation of the LD_{50} in three avian species at one-day-old, Prior, Sisodia and O'Neil (1976) reported values of 3.4, 5.9 and 16.5 mg/kg body weight for WLH chicks, turkeys and Japanese quail respectively. All the birds dying of acute ochratoxicosis showed symptoms of listlessness, huddling, occasional diarrhoea, ataxia and prostration. Chang et al. (1981) found the oral LD_{50} for day-old and three-week-old turkey poults to be 4.63 and 7.84 mg/kg respectively. Intraperitoneally, the values were 0.16 and 0.34 mg/kg respectively.

2.3.2.1 Young chicks

In several toxicity trials, graded dietary doses of OA up to 8 ppm have been given to young broiler chicks for three weeks from hatching. Growth was depressed at all the OA levels, the minimum growth inhibitory level being 2 ppm. There was an enlargement of the kidney, liver, crop, proventriculus and gizzard whereas the bursa of Fabricius regressed. The heart, pancreas and spleen did not alter (Huff et al., 1974). Tucker and Hamilton (1971), on the other hand,had earlier reported an enlargement of the pancreas. On the basis of these studies, these workers considered OA to be the most potent nephrotoxic mycotoxin in chickens.

The nephrotoxicity of OA was confirmed by impaired renal function as measured by phenol red clearance test, which was decreased by 15 and 31 per cent by doses of 4 and 8 ppm respectively. Plasma uric acid was increased whereas potassium levels were decreased at these dose levels. The percentage dry weight of the kidney was also increased (Huff and Hamilton, 1974; Huff, Wyatt and Hamilton, 1975). OA caused significant increases in recalcification time of clotted blood and prothrombin time in a dose related fashion but not in whole blood clotting time (Doerr, Huff, Tung, Wyatt and Hamilton, 1974). The impairment of the common clotting pathway functions during OA-toxicosis was due primarily to a reduction in fibrinogen levels in the blood, though the activity of other factors was also decreased at higher dose levels (Doerr et al., 1981). This was later associated with an increased susceptibility to bruising and "the bloody thigh syndrome" in broiler chickens (Huff, Doerr, Wabeck, Chaloupka, May and Merkley, 1983). Levels of plasma carotenoids were lowered by dietary levels of 2 ppm or more of OA (Huff and Hamilton, 1975a; Osborne, Huff, Hamilton and Burmeister, 1982).

Birds were found to be more susceptible to the cold but more resistant to heat at 4 and 8 ppm OA levels (Huff and Hamilton, 1975b). A hypochromic microcytic anaemia of iron deficiency type was observed in broilers particularly at higher dietary OA levels (Huff et al., 1979a). OA inhibited glycogenolysis, the conversion of glycogen to glucose, resulting in an accumulation of glycogen in the liver at higher OA (4 and 8 ppm) levels (Huff, Doerr and Hamilton, 1979b). This was shown to be due to the inhibition of a cyclic adenosine 3',5', monophosphate-dependent protein kinase enzyme (Warren and Hamilton, 1980a). The increased amount of glycogen during ochratoxicosis was considered to be a type-x glycogen storage disease (Warren and Hamilton, 1981).

The breaking strength of the tibia was decreased at the growth inhibitory level of 2 ppm dietary OA, but bone diameter was reduced only at OA levels of 4 ppm or higher in broilers (Huff et al., 1980). However, no histopathological lesions were detected in the epiphysis. Warren and Hamilton (1980b) observed a decreased breaking strength of the large intestine due to a decrease in collagen content at OA levels of 2 ppm and higher. Though the lipid content of the large intestine was found to be increased during OA-toxicosis at 8 ppm dietary OA, no measurable steatorrhea or decrease in bile salts or in digestive enzymes were noticed (Osborne et al., 1982)

A shock-like response, characterised by a transient decrease in both heart rate and blood pressure, could be induced in broilers when they were given an intravenous (i.v.) injection of OA after they had received dietary OA (up to 4 ppm) for three weeks (Richardi and Huff, 1983).

Galtier, Moré and Alvinerie (1976) observed prostration, cachexia and retardation of growth in ten-day-old WLH chicks given graded concentrations of OA either as

a single dose or by daily administration over ten days.

Krogh, Elling, Hald, Jylling, Petersen, Skadhauge and Svendsen (1976a) studied the effect in young fowls of giving subclinical levels of OA in the diet (0.3 and 1ppm) for 341 days. They observed impairment of glomerular and tubular functions, indicated by decreases in inulin clearance, in tubular excretion rate of para-aminohippuric acid and in urine concentrating capacity. Bitay, Glávits and Sellyey (1979) conducting a trial, in which OA was fed to broiler chickens for 6 weeks from the age of 15 days, noticed a retardation of growth and an increase in the feed consumption required to give a weight gain of 1 kg. Prior, O'Neil and Sisodia (1980) reported lower food consumption for the first 5 weeks of life and depressed growth was more marked in male than female broilers, fed dietary OA in graded doses (0 to 2 ppm) up to 8 weeks of age from hatch.

Manning and Wyatt (1984) compared the toxicity of wheat contaminated with A. ochraceus and of three different chemical forms of OA (potassium salt, sodium salt or organic acid) in broiler chicks fed from hatching for 4 weeks at the rate of 3 ppm in the diet. All OA diets caused the usual symptoms of depressed growth, dehydration, mortality varying from ten to seventeen per cent, enlargement of the liver and kidneys, decreased levels of total proteins, albumin, globulin, cholesterol and phosphorus in the serum and increased serum uric acid concentration. The potassium salt of OA was more toxic than the sodium salt in the oral LD_{50} determination.

The interaction of OA with other mycotoxins, nephrotoxic substances and coccidia have been demonstrated in young broiler chicks. In a study of the combined toxicity of dietary OA (2 ppm) and aflatoxin (2.5 ppm) in birds up to 3 weeks of age, Huff and Doerr (1981)

noticed an enhancement of the toxic effects in the form of a more marked depression of growth, greater mortality and more damage to the kidney, although OA inhibited the accumulation of lipids in the liver normally induced by aflatoxin. Further, this interaction also resulted in an increased susceptibility to bruising (Huff et al., 1983)

In an attempt to study the synergistic effects of two nephrotoxic mycotoxins, OA and citrinin, known to occur naturally in feed, Doerr and Campbell (1982) fed dietary OA (2 ppm) and citrinin (400 ppm) to broilers up to 3 weeks of age and observed enhanced toxicity which particularly affected serum protein, liver weight and growth.

Feeding dietary OA (3 ppm) and tannic acid (1.5 per cent) to day-old broiler chicks for up to 26 days caused synergistic toxicity in terms of poor weight gain, decreased carcass pigmentation and very poor efficiency of feed utilisation (Kubena, Phillips, Creger, Witzel and Heidelbaugh, 1983). A combination of OA (0 to 8 ppm) and either of two species of coccidia (Eimeria acervulina or E. tenella) produced, in broilers, a greater decrease in weight gain, increased feed conversion, and decreased plasma carotenoid levels than either of the diseases by themselves (Huff and Ruff, 1982).

OA-producing strains of Aspergillus have been fed to young chicks. Two isolates of A. ochraceus grown on corn (final OA concentrations of 7.925 and 1.05 ppm) were highly toxic to day-old Babcock B-300 cockerels causing high mortality, whereas two other isolates were moderately toxic and resulted in growth suppression and slow mortality (Doupnik and Peckham, 1970). Similarly, Nelson, Johnson, Kirby and Beasley (1982) found A. ochraceus contaminated corn (containing 800 ppb OA) when fed to 4 week-old chicks, reduced amino acid and dry matter digestibility, and energy utilisation by chicks.

2.3.2.2 Adult fowls

When OA in dietary concentrations of 0 to 4 ppm, was fed to WLH pullets from the age of 14 weeks up to 1 year, delayed sexual maturity, decreased egg production, depressed weight gain, increased feed consumption with decreased feed efficiency and severe mortality at the higher OA levels, due to kidney and liver damage, were observed. Hatchability of fertile eggs was reduced with depressed progeny performance during their first 2 weeks of life (Choudhury, Carlson and Semeniuk, 1971). Prior and Sisodia (1978) also reported a significant reduction in egg production in the second to the sixth weeks as well as in egg weights in WLH hens fed a diet containing 4 ppm OA. Similarly, Page et al. (1980), in an attempt to investigate an excessive number of egg-shell stains suspected to be due to chronic ochratoxicosis under field conditions, conducted a trial in WLH laying hens by feeding 0.5 and 1 ppm OA in the diet for 3 weeks and found decreased egg production and increased serum uric acid levels, together with an increase in egg-shell stains. However, Prior et al. (1981), in another study in WLH hens on 4 ppm dietary OA, did not find a decrease in egg production, though egg weights did decrease significantly in restricted feed intake and organoleptic trials, despite the severe growth depression caused by the reduced feed consumption.

In a study of OA toxicity in laying hens, given 0.25 to 2 ppm OA for 12 weeks, Tohala (1983), observed reduced egg weight, increased egg spots and reduced specific gravity of eggs in a dose-dependent fashion. Water consumption, mortality rate and liver weights were increased at the highest level of OA, and enlargement of the kidneys was seen at both 1 and 2 ppm OA levels. The effects of graded dietary OA levels (0.3 and 1 ppm) on renal functions have been studied (Svendsen and Skadhauge, 1976). Glomerular filtration rates, renal concentrating abilities and plasma protein concentrations, were reduced in OA-fed birds.

2.3.2.3 Chick embryos

2.3.2.3.1 Embryotoxic effect

The toxicity of ochratoxin for chick embryos has been determined. Air cell injections of OA into fertile eggs before incubation resulted in an LD_{50} value of 5-10 µg/egg at 21 days (Brown, 1970). Isocoumarin carboxylic acid (the hydrolysate of OA) was found to have an LD_{50} value of 16.96 µg/egg (Yamazaki, Suzuki, Sakakibara and Miyaki, 1971). Choudhury and Carlson (1973) found that OA was less toxic to the chick embryo when injected into the yolk sac before incubation began than when injected into the air cell during incubation, and that embryos at 6 days of age were the most sensitive. A value of the LD_{50} of 7 µg OA/egg was obtained in eight-day-old embryos injected into the yolk sac (Chu, 1974). In a comparative study of the toxicity of five mycotoxins in chick embryos an LD_{50} for OA of 10 µg/egg was obtained (Gedek, 1972). Veselý, Veselá and Jelinek (1982) studied embryonic death and the incidence of caudal trunk abnormalities in 40-hour-old WLH embryos injected subgerminally under the caudal region with 19 mycotoxins (including OA) dissolved in 30 per cent ethanol and incubated for another 24 hours. The lethal dose for OA (causing 100 per cent mortality) was found to be 0.1 µg/egg.

2.3.2.3.2 Teratogenic effect

Teratogenic effects of OA after injection of the toxin into chick embryos have been studied. Injections of OA in the air sac of embryonated eggs at days 2-4 in doses ranging from 0.5 to 7µg/egg killed the embryos. The LD_{50} for embryos at 2,3 and 4 days of age was 0.7,3 and 7 µg/egg respectively. Malformations observed in the embryos included short and twisted limbs and necks, microphthalmia, exencephaly, reduced body size and everted viscera. Embryos treated at 2 days, showed ventricular septal defects, aortic stenosis

and malformation of the valves, though in only six of 30 surviving embryos. However, no dose-response relationship was observed (Gilani, Bancroft and Reily, 1978). In an attempt to study the embryotoxic and teratogenic potential of OA alone and also in combination with citrinin, Veselá et al. (1983) injected the toxin(s) into chicken embryos on days 2, 3 and 4, either subgerminally or intraamniotically and observed maximal embryocidal and malformation effects in embryos treated with OA on day 3, at much lower doses (0.01 to 0.05 μg), when subsequently examined on day 8. Beak abnormalities were also noticed. When 4 μg of citrinin was constantly added to graded doses (0.03 - 0.5 μg) of OA, a strictly additive effect was seen.

Teratogenicity of OA has been amply shown in rats (Still et al., 1971; Moré and Galtier, 1974, 1975, 1978; Brown, Szczech and Purmalis, 1976), mice (Hayes, Hood and Lee, 1974; Arora, 1982) and hamsters (Hood, Naughton and Hayes, 1976) with similar types of malformation in the three species. Recently, a detailed pathoanatomical study has been conducted in mice by Arora (1982) to elucidate the mechanism of teratogenesis. It is interesting to note that OA has a more pronounced teratogenic potential than either aflatoxin B_1 or zearalenone.

2.3.2.4 Turkeys

In a feeding study in turkey poults fed graded doses (up to 8 ppm) of OA in the diet from hatching up to 3 weeks of age, Chang et al. (1981) observed decreased growth rate, increase in water consumption and in plasma uric acid levels, enlargement of the proventriculus and gizzard and at 4 and 8 ppm OA levels regression of the thymus but no changes in the sizes of the liver, kidney, spleen, pancreas and Bursa of Fabricius. Decreased plasma glucose and dry weight of the kidney, and increased feed conversion ratio and mortality were noticed only at 8 ppm

OA level. A leucocytopaenia, particularly lymphocytopaenia, coupled with decreased heterophils and increased basophils, was also recorded. However, total plasma protein, prothrombin time, plasma carotenoids and phenol red clearance remained unaltered. On the basis of these studies, they suggested that OA is a potent nephrotoxin for turkeys and in some respects differs in its action in this species from other avian species.

2.3.2.5 Quail

Doster, Arscott and Sinnhuber (1973) noted high mortality in Japanese quail either fed 4, 8 and 16 ppm pure OA in an experimental diet (isolated soya-bean protein-glucose monohydrate diet) or given a crude A.ochraceus culture extract in the diet, for 10 days from hatching. Birds receiving OA in either form, were emaciated, inactive, showed markedly less feathering than controls and had a depressed weight gain. Total feed intake was also reduced.

The response of Japanese quail to OA was also studied by Prior, O'Neil and Sisodia (1978) who fed up to 16 ppm of dietary OA to 5 week-old birds for 5 to 6 weeks, in an attempt to observe production characteristics. Egg weight was reduced during the 3rd week at levels of 4 ppm and more whereas feed consumption and body weight were reduced only at the 16 ppm OA level. Fertility and hatchability were also greatly reduced at this level. Prior, Sisodia, O'Neil and Hrudka (1979) further reported that the fertility of eggs was not effected by 16 ppm OA, during a 9 week feeding experiment starting from 5 weeks of age. However, eggs from females receiving 16 ppm OA had a significantly higher percentage of early embryonic deaths. In males, spermatogenic activity was not adversely affected. They (Prior et al., 1979) also studied in vitro embryotoxic effects of OA by inoculating 400, 800, 1600 or 5000 ng OA in 0.1 ml 0.1M sodium bicarbonate into the yolk of another group of fertile eggs obtained from birds

raised on the control diet. Injection of 1600 ng or more resulted in higher embryonic deaths (75-78%), during 7 days of incubation, though the vehicle 0.1M sodium bicarbonate, and lower levels of OA (400 and 800 ng) also caused 29, 25 and 24 per cent embryonic mortality respectively. However, the results were comparable with the embryotoxic effects observed during in vivo studies (60 to 64 per cent). On the basis of these studies, it was suggested that the embryotoxic effect of OA is exerted through its presence in the environment of the embryo rather than through mutagenesis, and alteration of gametes. These workers, however, did not attempt to study teratogenesis.

2.3.2.6 Domestic mammals

Ochratoxicosis has been extensively studied in several species of animal. Excellent reviews dealing with various aspects of MPN have been published by Krogh (1976, 1977a, 1977b, 1980), Elling (1977a, 1981), Carlton and Krogh (1979), and Stuart and Bedell(1982). Further studies on ochratoxicosis in swine have been conducted by Zimmermann, Carlton and Tuite (1979a, 1979b), Madsen, Hald, Lillehoj and Mortensen (1982a), Madsen, Mortensen and Hald (1982b), Meisner and Krogh (1982), Mortensen, Madsen and Hald (1982), Pepeljnjak et al. (1982a), Sandor et al. (1982), Mortensen, Hald, Larsen and Madsen (1983) and Szemeredi and Agüero (1983). Studies on ochratoxicosis have been reported also in dogs (Szczech, Carlton and Tuite, 1972; Szczech, Carlton and Tuite, 1973a, 1973b; Szczech, Carlton and Hinsman, 1974; Kitchen, Carlton and Sansing, 1974; Kitchen, Carlton and Hinsman, 1977a; Kitchen, Carlton and Tuite, 1977b, 1977c), in sheep (Munro, Scott, Moodie and Willes, 1973; Pettersson and Kiessling, 1976), in cattle (Still, 1973; Ribelin, Smalley and Strong, 1973; Pier, Cysewski, Richard, Baetz and Mitchell, 1976; Hult, Teiling and Gatenbeck, 1976; Patterson et al., 1981) and in goats (Ribelin, Fukushima

and Still, 1978; Nip and Chu, 1979).

2.4 Pathology

In most species the target organ of OA appears to be the kidney, though other systems, such as the liver, gastrointestinal tract, lymphoid organs, skeletal system, haematopoietic tissues, and reproductive organs, can also be affected.

2.4.1 Gross changes

Gross lesions reported in ochratoxicosis appear to be neither characteristic nor consistent.

In young chicks, the gross lesions observed after short-term exposure to OA were emaciation, dehydration, a dry and firm gizzard sometimes with erosions on the horny luminal surfaces, proventricular mucosal haemorrhages (Doupnik and Peckham, 1970; Peckham et al., 1971; Huff et al., 1974) and catarrhal enteritis (Chu and Chang, 1971). In broilers as well as in turkeys affected with ochratoxicosis under both natural and experimental conditions the kidneys were pale, swollen and enlarged and later changed from the normal mahogany colour to a tan colour (Hamilton et al., 1982). Visceral gout with white fleck-like deposits in the kidneys, ureters, heart, pericardium, liver and spleen of chicks which died (10 out of 32) after acute oral dosing with OA, has been recorded (Peckham et al., 1971). Prior et al. (1976) also reported visceral gout in the hearts of WLH chicks which died, but not in young turkeys and quail . The liver could be enlarged, pale and friable or haemorrhagic, sometimes with an hypertrophied gall bladder (Chu and Chang, 1971; Huff et al., 1974; Galtier et al., 1976). Diminution in the size of the bursa of Fabricius in broilers (Huff et al., 1974) and of the thymus in turkeys (Chang et al., 1981) has also been noted. Bones became more flexible and rubbery or in severe cases (8 ppm

OA) they became brittle in broilers after feeding dietary OA at 2 ppm and above (Huff et al., 1980). No gross lesions were reported in other organs.

Quail chicks had pale tan to bright orange coloured livers with focal haemorrhages and sometimes oedemaafter OA exposure (Doster et al., 1973). The kidneys and other organs were not examined. In ducklings, fatty livers have been reported (Van der Merwe et al., 1965a) and in adult ducks catarrhal gastritis, enteritis and haemorrhages in various tissues were noticed in natural outbreaks (Bodnarchuk and Kaspruk, 1984).

In adult birds, usually no specific gross lesions were observed (Krogh et al., 1976a). However, pullets and laying hens were emaciated and had pale tan to yellow-coloured livers with focal haemorrhages (Choudhury et al., 1971). Pale combs, abnormal livers and pale kidneys were reported after feeding 1 to 2 ppm OA to hens for 12 weeks, in addition to egg-shell stains (Tohala, 1983). Under-pigmentation of the carcass has been recorded (Huff and Hamilton, 1975a; Hamilton et al., 1982).

2.4.2 Microscopic changes

Microscopic changes in the kidney are usually characteristic of OA toxicity and are frequently seen in the proximal convoluted tubules (PCT). Tubular dilatation, swelling of the tubular epithelial cells, localised necrosis, desquamation of the tubular basement membrane and the presence of proteinaceous material in the lumen have been observed in chicks (Peckham et al., 1971; Huff et al., 1975; Prior et al., 1976; Gylstorff and Rolf, 1978). Fatty changes, microhaemorrhages and the presence of hyaline casts have been noticed by Galtier et al. (1976) but not by other workers. The presence of urates, scattered heterophils (Peckham et al., 1971), tubular necrosis and an "impression" of intercellular

œdema (Huff et al., 1975) have been reported. The changes were usually confined to PCT but distal convoluted tubules (DCT) could also be affected; the alterations were dose-dependent (Huff et al., 1975).

In the liver, hepatic cells were enlarged and vacuolated (Prior et al., 1976), and there was an accumulation of glycogen in the cytoplasm, particularly at the periphery of the liver lobes in broilers at higher levels of dietary OA (4 and 8 ppm) (Huff et al., 1979b). Other changes reported in the liver were the presence of necrotic foci, haemorrhages, inflammatory cells (Peckham et al., 1971; Galtier et al., 1976) and adenomatous hyperplasia of hepatic parenchyma (Pepeljnjak, Mužic and Herceg, 1982b).

Swollen nuclei, bile duct proliferation and diffuse, sometimes severe, vacuolation of hepatocytes have been noticed in quail chicks (Doster et al., 1973). In young ducklings, during acute poisoning, there were fatty changes with occasional intranuclear vacuolation without necrosis and increased hepatic mitotic activity (Theron, Van der Merwe, Liebenberg, Joubert and Nel, 1966). There was no increase in hepatic glycogen in duckling. In chick embryos given 25 µg OA per egg, there was necrosis and marked vacuolation of hepatocytes with no fat or glycogen being detected in the vacuoles (Yamazaki et al., 1971).

Other changes observed in chicks were suppression of haematopoiesis in the bone marrow and depletion of lymphoid elements from the spleen and bursa of Fabricius (Doupnik and Peckham, 1970; Peckham et al., 1971; Gylstorff and Rolf, 1978). Despite gross changes, microscopic examination of the bone did not reveal any abnormality (Huff et al., 1980; Pepeljnjak et al., 1982b). Pepeljnjak et al. (1982b) reported lysis and pyknosis of ganglion cells in the brain and nervous signs have been seen in chicks given high doses of OA (Huff et al., 1974).

In adult birds, no consistent lesions have been reported in the kidney. Tohala (1983) did not detect any histological lesions in the kidneys of hens fed OA (up to 2 ppm) for 12 weeks. However, lesions of degeneration and necrosis followed by regeneration were seen in the PCT and DCT of chickens fed 0.3 and 1 ppm OA in the diet for 341 days from hatching. Ballooning and enlargement of tubular epithelial cells with the presence of bizarre mitotic figures were also frequently observed. The brush border was slightly reduced in the PCT though the glomeruli were normal. Neither renal vascular lesions nor interstitial fibrosis, seen in spontaneously occurring nephropathy (Elling et al., 1975), were noticed in any of the groups (Krogh et al., 1976a). No significant changes in the liver were seen by Krogh et al. (1976a), though Choudhury et al. (1971) observed the presence of a large number of heterophils in the interstitial areas of the liver of hens fed up to 4 ppm OA from 14 weeks to one year of age. They failed to detect changes in the kidney.

Detailed studies of gross and microscopic changes in the kidney during ochratoxicosis A have been carried out in pigs (Carlton and Tuite, 1970; Szczech et al., 1973c; Krogh, Axelsen, Elling, Gyrd-Hansen, Hald, Hyldgaard-Jensen, Larsen, Madsen, Mortensen, Møller, Petersen, Ravnskov, Rostgaard and Aalund, 1974a; Krogh, 1978; Elling, 1977b, 1981) and in dogs (Szczech et al., 1973b; Kitchen et al., 1977b).

2.5 Ultrastructural Changes

Young ducklings are the only birds in which OA-induced ultrastructural changes have been studied. Hepatic changes were reported in ducklings given 100 µg OA and studied for up to 24 hours (Theron et al., 1966). These included increased fatty vacuolation in the hepatocytes, mitochondrial swelling and granularity, reduction in the

amount of endoplasmic reticulum (ER), the formation of isolated dilated vesicles, focal loss of ribosomes from ergastoplasmic membranes, and increased numbers of free ribosomes in the cytoplasm.

Renal ultrastructural alterations have been studied in detail only in Beagle dogs which had been given oral doses of 0.3 mg OA/kg body weight for 11-15 days. These doses produced cytomorphological alterations primarily in the endomembrane system of the epithelial cells of the PCT. Increased amounts of smooth-surfaced membranes, and a few basilar infoldings of the plasma membrane were observed. The mitochondria appeared mostly normal. Changes seen in the interstitium included separation between tubular basement membranes, an increased number of interstitial cells, well developed secretory apparatus, and the presence of collagen and amorphous material. The DCTs were slightly affected and contained more lipid droplets (Szczech et al., 1974). When dogs were fed OA (0.1 to 0.2 mg/kg body weight) for 14 days, cytoplasmic vacuolation and myelin figures were noticed in the PCT epithelial cells, together with a reduction in the height of the cells, in addition to cytoplasmic disarray involving the ER and mitochondria. Similar changes were seen in a combined toxicity involving OA and citrinin but more areas of the tubules such as the DCTs and collecting tubules were affected (Kitchen et al., 1977c).

In pigs, fed 200 ppb OA in the diet for 3 months, the ultrastructural changes at the early stage were characterised by a reduced brush border and atrophy of the epithelial cells of the PCT, together with large apical vesicles and an increased number of lysosomes. Mitochondria were reduced in number in the basal part of the cell and often lacked cristae and the basement membranes were unevenly thickened (Elling, 1977a).

2.6 OA Residues in Tissues

After oral administration of OA to fowls, there was a faster absorption and wider distribution of the toxin in the tissues together with a quicker elimination than in pigs and rabbits (Galtier, Alvinerie and Charpenteau, 1981). In chickens given low OA levels for 341 days, OA residues have been demonstrated in the kidney, liver and muscles with values ranging from 49.5 to 9.1ppb, but not in eggs (Krogh et al., 1976a). In WLH hens fed up to 4 ppm OA for 6 weeks OA was detected in the kidney (36.8-106.9 ppm (sic)), liver (26.3-72.6 ppm (sic)), red muscle (8.0-20.8 ppm (sic)) and white muscle (4.6-15.9 ppm (sic)) in a dose-related fashion, but not in fat, skin and eggs (Prior and Sisodia, 1978). These residues persisted in the kidney and liver even 48 hours after the withdrawal of OA-contaminated feed. In broilers fed up to 2 ppm OA in the diet for 8 weeks, OA residues were detected in the kidney (41 ppb) and liver (24 ppb), but not in red muscle and fat, only at the highest OA dose (Prior et al., 1980). Detectable levels of OA disappeared from the liver within 24 hours and from the kidneys within 48 hours after withdrawal of the mycotoxin-contaminated feed. Reichmann, Blaney, Connor and Runge (1982) reported the detection of OA residues in kidneys (3.0-10.0 ppb) and livers (1.5-2.5 ppb) from 3 week-old broiler fowls given 1 ppm OA in the diet for 5 weeks, though no adverse effects were seen on the growth response, or feed intake, on the liver or kidney, or on blood uric acid and enzymes.

Juszkiewicz et al. (1982) studied the tissue deposition and passage of OA into eggs after giving a single oral dose (1.0, 5.0 and 10.0 mg/kg body weight) or feeding OA-contaminated diets (2.5 and 10 ppm) for 7 days to Plymouth Rock laying hens. Twelve hours after a single dose of 1 mg OA/kg body weight, residues of OA were found in all tissues examined (blood cells, plasma, muscle, liver, kidney and ovarian follicles), the greatest amounts being

in the kidney and liver (8.6-8.7 ppb). The quantity in tissues increased with increasing dosage of OA, and was 27.2 ppb in the kidneys and 4.5 ppb in ovarian yolk at 5 mg OA/kg, and 95.2 ppb in the kidney, 82.6 ppb in liver, 47.4 ppb in plasma and 15.7 ppb in ovarian yolk at 10 mg/kg body weight. In hens fed 2.5 ppm dietary OA mycotoxin residues were detected on the second day of a 7 day trial only in the liver (3.0-5.5 ppb) and kidney (3.8-4.6 ppb), where as at 10 ppm OA level, OA residues were found in the kidney (4.0-5.9 ppb), liver (3.8-5.2 ppb), plasma (4.0-10.2 ppb), ovarian yolk (0.9-1.7 ppb), though not in the blood cells. In egg yolk and muscles OA was detected at levels of 1.2 and 2.3 ppb respectively only after 4 days of feeding OA at 10 ppm. OA residues were still present in the kidney, muscles, ovary and eggs 3 days after withdrawal of the OA-contaminated diet, but not in eggs laid on the 5th, 6th and 7th day after removal of the feed. After administration of high single doses of OA to Japanese quail, residues of the toxin have been detected in tissues and eggs (Piskorska-Pliszczyńska and Juszkiewicz, 1979).

OA residues have also been detected in the kidney, liver, muscles and fat of pigs given OA (Krogh et al., 1974a, 1976b; Shreeve, Patterson, Pepin, Roberts and Wrathall, 1977; Madsen et al., 1982b; Mortensen et al., 1983), in tissues of calves (Patterson et al., 1981) and in cow's milk (Still, 1973).

Thus OA residues in tissues of domestic animals, including poultry and eggs, pose an important problem, especially as OA is known to induce severe teratogenic effects (Arora, 1982), and renal and hepatic tumours (Kanisawa and Suzuki, 1978) in mice.

2.7 Effect of OA on the Immune Response

The effects of mycotoxins on immunity and resistance have recently been reviewed and summarised (Richard, Thurston and Pier, 1975b, 1978; Pier, Richard and Thurston, 1979; Pier et al., 1980a, 1980b; Pier, 1981).

The direct effects of OA on humoral and cellular immune responses have not been studied in avian species, though there is circumstantial evidence suggesting OA might be an immunosuppressant in the fowl. Chang et al. (1979) reported a leucocytopaenia in broiler chicks which they considered to be primarily a lymphocytopaenia and to a lesser extent a monocytopaenia. Chang and Hamilton (1980) studied the effects of graded doses of dietary OA (up to 8 ppm) on the in vitro phagocytic, locomotory and bactericidal capacity of heterophils from broiler chicks. After 3 weeks of OA treatment, both the directed and undirected locomotion of heterophils and their mean phagocytic activity were impaired at 4 and 8 ppm OA levels.

The reduced phagocytic activity was associated with the heterophils themselves and not due to a serum factor, such as complement. The ability of heterophils to kill engulfed bacteria (Enterobacter cloacae) was not impaired at 4 ppm OA level.

Campbell et al. (1983) fed broiler chicks OA at 2 ppm alone as well as mixed with 2.5 ppm aflatoxin in the diet for 21 and 38 days from hatching. Though the effects with OA alone, were marginal, aflatoxicosis and ochratoxicosis in combination resulted in hypoproteinaemia, lymphocytopaenia, heterophilia, reduced complement activity and a decreased relative weight of the bursa of Fabricius. However, antibody titres to Brucella abortus and sheep red blood cells (SRBC), and the phagocytic ability of heterophils were not affected by either treatment.

Suppression of bone marrow activity and lymphoid depletion from the spleen and bursa of Fabricius in young chicks (Doupnik and Peckham, 1970; Peckham et al., 1971; Gylstorff and Rolf, 1978) and regression of the thymus in turkey poults (Chang et al., 1981) after OA treatment have been reported, suggesting a possible immunosuppressive effect.

While investigating naturally occurring outbreaks of ochratoxicosis Hamilton et al. (1982) observed an increased incidence of air sacculitis, caused by a secondary infection of Escherichia coli in turkeys and broilers. The birds did not respond to therapy with antibiotics to which the E. coli had been found sensitive by in vitro testing. When the diet was changed, antibiotic therapy became effective. The cases of air sacculitis were thought to be due to an impaired immune response caused by OA.

Studies have been conducted in laboratory animals to delineate the effects of OA on the immune system. Richard et al. (1975b), in a pioneering study, reported that OA given to guinea pigs at the rate of 0.45 mg/day for 4 weeks did not affect complement activity or antibody response to Brucella abortus antigen, although OA significantly lowered the level of β-globulin in the serum. Similarly Patterson et al. (1981) did not find any significant alterations in the levels of serum IgA, IgM, IgG_1 and IgG_2 in Friesian calves fed 390 to 540 ppb OA, or 320 to 500 ppb OA and 12-13 ppb aflatoxin B_1.

Murine humoral responses to SRBC, particularly those mediated by IgM were reduced by 50 per cent after intraperitoneal injections of very low doses of OA (0.005 µg/kg body weight). On the basis of these studies Haubeck, Lorkowski, Kölsch and Röschenthaler (1981) considered OA to - to a potent immunosuppressive agent. The immunosuppression caused by OA could be prevented by phenylalanine, because the toxic effect of OA was considered to be due to its

competitive inhibition of the phenylalanyl transferase ribonucleic acid (t-RNA) synthetase catalysed reactions of protein and RNA synthesis (Creppy, Lugnier, Beck, Röschenthaler and Dirheimer, 1979a; Creppy, Lugnier, Fasiolo, Heller, Röschenthaler and Dirheimer, 1979b). In a later study (Creppy, Lorkowski, Röschenthaler and Dirheimer, 1982) the production of an IgG response was also inhibited in mice in a dose-related fashion by injections of 1 μg and 2 μg OA/kg body weight. However, contradictory results have been reported by Prior and Sisodia (1982) in 8-10 week-old mice, which were either fed 4 ppm OA in the diet for 7 weeks or injected with 5 mg OA/kg body weight intraperitoneally (i.p.) for 50 days (very high doses as compared with previous studies). Neither oral nor intraperitoneal administration of OA affected the response of mice to SRBC. On the other hand, although the oral administration of OA did not depress antibody titres to killed Brucella abortus, intraperitoneal injections of OA caused a significant depression of antibody titres. They postulated that the synthesis of IgM rather than of IgG was suppressed.

It has been further shown (Creppy et al., 1983) that 4(R)-4-hydroxyochratoxin A, a metabolite of OA, was as strongly immunosuppressive as OA in mice, since in doses of 1 μg/kg body weight it resulted in an 80 to 93 per cent suppression of IgM-and IgG-synthesising spleen lymphocytes. On the other hand, ochratoxin α, another metabolite was ineffective.

An effect of OA on cell mediated immune (CMI) responses has been strongly suspected (Pier et al., 1980a), but not proven. In an in vitro study, OA in concentrations of 20 to 1667 μM, gave up to 56 per cent inhibition of the migration of macrophages obtained from guinea pigs, indicating a possible suppressive effect on CMI (Klinkert, Lorkowski, Creppy, Dirheimer and Röschenthaler, 1981).

From the literature it is evident that studies on pathological and immunological aspects of ochratoxicosis, particularly in poultry, are either incomplete or inconclusive, and sometimes even contradictory, indicating a need for further study.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

The main part of the work is based upon five experiments involving the feeding of diets contaminated with ochratoxin A to broilers and turkeys. A summary of the experimental design in these trials is given in Table 3. Details of a sixth experiment conducted in quail are presented separately (see section 3.1.2). The detailed composition of some of the reagents used in the various tests and procedures employed in this work are given in the Appendix.

3.1 Experimental Birds

3.1.1 Broilers and turkeys

Commercial one-day-old broiler chicks (Marshall's Broiler Breeder, Strain M4) and one-day-old British United Turkeys (BIG5 Strain) were used. Birds in all the experiments were kept in battery brooders except in Experiment V, in which turkey poults were floor-brooded. The birds were usually fed a PRC starter ration. The turkey poults used in Experiment V were given a semi-synthetic diet computed by Dr C.C. Whitehead. In all the experiments, the diet was fed as mash rather than as crumbs or pellets. All the birds were kept under continuous illumination and feed and water were provided ad libitum. At 4 weeks of age, the birds were moved to grower cages. The chicks used in the Experiment I were from a broiler-pullet flock.

3.1.2 Japanese quail

The early sexual maturity and small size of Japanese quail make them excellent experimental subjects.

Groups of 46, 41 and 41 one-day-old Japanese quail

Table 3. Experimental Design

Experiment No.	Experimental birds	Number of birds in group (♂,♀)	Dietary OA level (ppm)	Age when killed (weeks)	Duration of experiment	Parameters investigated*
I	Broilers	22(11,11) 20(11, 9) 10(6, 4)	0 2 4	3	3 weeks	1,2,5,6,7,8,11,12a
II	Broilers	22(13, 9) 20(14, 6)	0 2	16	16 weeks	1,2,3,4,5,6,13a,13b,13c,15
III	Broilers	11(5, 6) 11(5, 6)	0 4	2,4,6,8,10	10 weeks	1,2,3,5,6,7,8,9a,10,12a,12b,14,16,17,18
IV	Turkeys	11(7, 4) 11(7, 4)	0 4	2,4,6,8	8 weeks	1,2,3,5,6,7,8,9a,10,12a,14
V	Turkeys	27(13,14) 27(12,15)	0 4	4,10	10 weeks	1,2,3,5,6,7,8,9b,11,12a,13a,13b,13c,15,17,18

*CLINICAL AND PATHOLOGICAL

1. Clinical signs and growth response
2. Differential leucocyte count
3. Blood pyruvate carboxylase activity and biotin level
4. Plasma hormone levels
5. Organ weight
6. Gross pathology
7. Histopathology
8. Ultrastructural changes
9a. Bone and toe ash content
9b. Patho-morphological changes in bone
10. OA residues in tissues

IMMUNOLOGICAL

11. Total immunoglobulins in serum
12a. Immunofluorescent (IF) demonstration of immunoglobulins in tissues
12b. IF demonstration of OA in tissues
13a. Delayed hypersensitivity (DH) response to avian tuberculin
13b. DH response to phytohaemagglutinin (PHA)
13c. DH response to bovine serum albumin (BSA)
14. Contact hypersensitivity to dinitrochlorobenzene (DNCB)
15. Arthus reaction
16. Graft-versus-host (GVH) reaction
17. Histopathology/histochemistry of skin
18. Ultrastructural study of skin

bred and maintained at the PRC, of both sexes were fed diets containing 0, 4 and 8 ppm OA respectively for a period of 11 weeks. There were 22, 23 and 23 males and 24, 18 and 18 females at 0, 4 and 8 ppm OA levels respectively. Weight gains of the birds were recorded at weekly intervals. The oil content of the feed was reduced to enable easier and better mixing with OA. Some birds were, however, killed for other purposes at 4-5 weeks of age.

3.2 Experimental Diets

The composition of the experimental diets (Experiments I-VI) is given in Table 4. Before they were mixed with OA, all the diets were examined by Miss Jean Robb of the Department of Microbiology, East of Scotland College of Agriculture, for mycotoxin analysis and for evidence of microbial and fungal contamination.

3.2.1 Preparation of OA-contaminated diet

Pure ochratoxin A was purchased from Makor Chemicals Ltd., Jerusalem and from Calbiochem, San Diego (U.S.A.) or Sigma Chemical Company (U.S.A.). About 150 mg of OA was kindly supplied by Dr T.J. Simpson, Department of Chemistry, University of Edinburgh.

Solutions of OA in either chloroform or ethanol were thoroughly mixed with 200g diet to give a first premix which was then dried to facilitate evaporation of the solvent. This premix was then used to prepare a second premix, by thorough mixing with a further 2.5kg diet for an hour in a Rotamixer, Dales Pharmaceuticals Ltd., U.K., (drum capacity of 5kg), giving 40 revolutions per minute. This final premix, if necessary, was further mixed in a 10kg capacity feed mixer to give the final

Table 4. Composition (g/kg) of experimental diets

INGREDIENTS	Experiment I	Experiments II and III	Experiment IV	Experiment V		Experiment VI	
	0-3 weeks+	0-16 weeks, 0-10 weeks	0-10 weeks	0-4 weeks	4-10 weeks	0-6 weeks	6-11 weeks
Maize	300	330	371	-	371	290	520
Barley	10	-	-	-	-	-	-
Wheat	245	250	175.5	656	175.5	162	140
Soyabean meal	220	298	247.30	110	247.30	400	100
Herring meal	50	23	141	120	141	128	100
Grass	50	-	-	-	-	-	50
Meat and bone	-	46	31.9	-	31.9	-	-
Limestone	5.3	20	5	14	5	5	50
Dicalcium phosphate	21.7	-	20	12	20	-	10
Vit. Mix No. 3*	-	-	-	3.75	-	-	-
Vit. Mix No. 4**	2.5	2.5	-	-	-	-	-
Vit. Mix No. 6***	-	-	2.8	-	2.8	5	2.5
Mineral Mix No. 5****	2.5	2.5	2.8	2.5	2.8	5	2.5
Salt	2.5	2.5	3	3	3	3	2.5
Choline chloride 50%	-	0.2	1.2	-	1.2	3	-
Vegetable (corn) oil	-	28	-	20	-	10	-
Yeast	-	-	-	-	-	-	25
Isolated soyabean protein ++	-	-	-	60	-	-	-
Analysis (%)							
Crude protein	20.3	23	25.4	26.5	25.4	28.3	19.7
Ether extract	2.7	3.7	4.3	4.2	4.3	3.22	3.6
Calculated available biotin (µg/kg)	-	112	218	55	218	-	-
Calcium	1.18	1.2	1.52	1.23	1.52	0.80	2.41
Phosphorus	0.69	0.5	1.05	0.70	1.05	0.54	0.62

+ Age range of birds during experiment

++ FP-950 (FPD-Hypak, Food Production Developments Ltd, London)

* Included at 2.5 g/kg diet supplied (/kg diet): Vitamin A 6 x 10^3 i.u., vitamin D_3 8 x 10^2 i.u., vitamin E 0.025 g, menaphthone 0.001 g, riboflavin 0.004 g, nicotinic acid 0.028 g, pantothenic acid 0.01 mg.

** Included at 2.5 g/kg diet supplied (kg/diet): vitamin A 2 x 10^6 i.u. vitamin D_3 6 x 10^2 i.u., vitamin E 0.025 g, menaphthone 0.001 g, riboflavin 0.004 g, nicotinic acid 0.028 g, pantothenic acid 0.01 g

*** Included at 2.5 g/kg diet supplied (/kg diet): vitamin A 1 x 10^4 i.u., vitamin D_3 1.5 x 10^2 i.u., vitamin E 0.03 g, menaphthone 0.004 g, riboflavin 0.01 g, pyridoxine 0.005 g, vitamin B_{12} 2 x 10^{-5} g, folic acid 2 x 10^{-3}g, biotin 1 x 10^{-4} g, pantothenic acid 1.6 x 10^{-2} g, nicotinic acid 0.05 g, thiamine 0.004 g

**** Included at 2.5 g/kg diet supplied (/kg diet): $CuSO_4.5H_2O$ 3.5 x 10^{-3} g, KIO_3 4 x 10^{-4}g, $FeSO_4.7H_2O$ 0.08 g, $MgCO_3$ $Mg(OH)$ $2.3H_2O$ 0.03g, $MnCO_3$ 0.01 g, ZnC 0.05 g.

concentration desired. The prepared diets were analysed by Miss Robb and also in some experiments (III and IV), by Dr M.R.A. Morgan, Food Research Institute, Norwich.

3.2.2 Safety precautions

Strict safety measures were taken during the handling of OA and in the preparation of toxic diets, as follows:

1. Handling of pure OA and preliminary mixing of the diet was done in a restricted and isolated room and in a fume cupboard after donning protective clothing (disposable plastic coats, masks, gloves and shoes). In later experiments, an air-filter unit (Airstream Dustmaster, Racal Safety Ltd., England) was used as a mask.

2. A 5 per cent bleach solution (sodium hypochlorite) was used to wash surfaces, instruments and equipment. Brooder, cages and droppings were decontaminated with bleach.

3. Protective masks and gloves were worn when handling OA-treated diets and birds.

4. Wherever possible, experimental birds were kept separately and all the containers, brooders and cages were labelled with an appropriate warning.

5. Protective clothing, dead birds, faecal material and other contaminated materials were incinerated after inactivation of the toxin in bleach solution.

3.3 Experimental Procedure

Experimental birds were maintained on the OA-

contaminated diet, and clinical signs, mortality and weekly weight changes were recorded. The birds were killed by an overdose of pentobarbitone sodium. All the birds whether they were killed or had died were carefully examined for the presence of gross lesions.

Various organs (liver, kidney, spleen, thymus, bursa of Fabricius, proventriculus, heart and, in some cases, Harderian gland) were removed, blotted dry and weighed.

3.4 Histology and Histochemistry

Pieces of kidney, liver, spleen, thymus, bursa of Fabricius, Peyer's patch, caecal tonsil, small intestine and Harderian gland were collected and fixed in 10 per cent formol saline. Tissues were dehydrated in ethanol, cleared in CNP30, Best obell Chemical Products Ltd., Surrey (Maxwell, 1978), embedded in paraffin wax and sectioned at 5 µm. Sections were stained with haematoxylin and eosin (H and E). Sections of liver were stained with Best's carmine for the demonstration of glycogen, and kidney and liver sections were treated by the periodic-acid-Schiff (PAS) reaction (Culling, 1966) with suitable controls for the demonstration of mucopolysaccharide.

3.5 Immunofluorescence

Duplicate pieces of all those tissues collected for histology were fixed in ethanol for 24 hours and wax-embedded according to the method of Sainte-Marie (1962), sectioned at 5 µm and treated for the immunofluorescent demonstration of IgA, IgG, IgM and anti-BSA-antibody using specific labelled antisera (Miles Laboratories) by a modification (Brandtzaeg, 1981) of the sandwich method of Coons, Leduc and Connoly (1955). The prepared sections

were examined microscopically under ultra-violet illumination.

The method of Elling (1977b) was used in an attempt to demonstrate OA in tissues (kidney, liver, muscle and small intestine) from broilers (Experiment III) and quail (Experiment VI) at 10 and 11 weeks of age. Cryostat sections were cut and flooded for 30 minutes with anti-OA antiserum supplied by Dr M.R.A. Morgan, who had produced it in rabbits injected with an OA-BSA complex. After two washes in phosphate buffered saline (PBS), the sections were incubated for 30 minutes with fluorescein conjugated goat anti-rabbit antiserum, washed in PBS and mounted in buffered glycerol. In Experiment III the anti-OA-BSA antiserum was used at dilutions of 1:10 and 1:50 whereas in Experiment VI it was first precipitated with BSA and then used at a dilution of 1:5. The sections of quail tissue were initially incubated with 0.05M cyanamide (1 ethyl-3 (3 dimethyl-aminopropyl)) solution for 30 minutes to fix OA to tissue proteins and thus lessen the possibility of loss during washing (Elling, 1977b). In both experiments sections from control birds as well as from OA-fed birds were examined.

3.6 Collection of Blood

Differential leucocyte counts (DLC) were made on smears prepared from blood collected by pin pricks at the site of the brachial vein. Birds were bled at 3 weeks of age (Experiment I, 10, 10 and 8 birds on 0, 2, and 4 ppm OA), at 4 weeks (Experiments III and IV, 5 birds from each group) at 10 weeks (Experiment V, 8 and 12 birds on 0 and 4 ppm OA), at 11 weeks (Experiment VI, 8, 4 and 4 birds on 0, 4 and 8 ppm OA) and at 12 weeks (Experiment II, 9 and 7 birds on 0 and 2 ppm OA). The smears were air-dried and stained by the combined May-Grünwald and

and Giemsa method (Lucas and Jamroz, 1961).

In Experiment III, 2 ml blood (for blood cell counts) was collected from the brachial vein of 4 week-old broilers into tubes coated with sequestrene (ethylene-diaminetetraacetic acid, 1.5 mg/ml) anticoagulant. Blood was collected for plasma hormone analysis using heparin (5000 units/ml) (Boots Co. Ltd., England)as anticoagulant.

Blood samples (2-3 ml) for serum were collected and allowed to clot in tilted vials overnight at room temperature. The serum was pipetted out and stored at - 20°C.

3.7 Haematology

Blood counts. White blood cell (WBC) counts were carried out by two different methods and the mean of the results were recorded.

Blood was diluted 1:20 with diluting fluid (Natt and Herrick, 1952) and total WBC counts were made in a haemocytometer (Hawksley and Sons Ltd., England) (imporved Neubauer counting chamber). Blood samples were also diluted 1:50,000 in Isoton II (Coulter Electronics) and the total number of cells was recorded using an electronic particle counter (Fn Model, Coulter Electronics), at threshold 12, aperture 16 and attenuation 0.707.

Blood smears were prepared and stained for differential leucocyte counts. The WBC : thrombocyte ratio was estimated by counting the number of WBCs to thrombocytes in 24 fields of each stained blood smear using a 21 mm graticule under a X40 objective. Red blood cell (RBC)

counts were determined by counting the number of RBCs in 12 fields with a X100 objective. The RBC:WBC: thrombocyte ratio was thus estimated and total cell, RBC, WBC and thrombocyte counts were calculated (Maxwell, 1981). The total numbers of lymphocytes were also calculated in each blood from the differential leucocyte counts and total WBC counts.

3.8 Electron Microscopy

Electron microscopy was used to study ultrastructural changes in the tissues of broilers in Experiment I on 3, 5 and 5 birds at 0, 2 and 4 ppm OA levels respectively at 3 week of age, and for a comparative sequential study at 2 week intervals (2-3 birds) in broilers up to 10 weeks of age (Experiment III) and in turkeys up to 8 weeks of age (Experiment IV). Tissues were also taken from some turkeys at 4 weeks of age (Experiment V). Pieces of liver, kidney, spleen, thymus, bursa of Fabricius, Peyer's patches, caecal tonsils and, in a few cases, Harderian gland were collected into the following fixatives:

Liver: 1 per cent osmium tetroxide in 0.2 M sodium cacodylate buffer, pH 7.4 and 375 milli-osmole (mosm) for 1 hour at 4°C.
Kidney and spleen: 1 per cent modified Dalton's osmium tetroxide (3 per cent potassium dichromate, 2.6 per cent sodium chloride) at pH 7.4 and 330 mosm, for 1 hour at 4°C.
Other lymphoid organs: 2.5 per cent glutaraldehyde in 0.075 M sodium cacodylate buffer (pH 7.4 and 550 mosm), for 2 hours at 4°C, washed in 0.075 M sodium cacodylate buffer and 0.2 M sucrose (pH 7.4 and 340 mosm) overnight at 4°C. Post-fixation was in 1 per cent osmium tetroxide in 0.175 M sodium cacodylate buffer (pH 7.4 and 350 mosm) for 1 hour at 4°C.

Following fixation, tissues were dehydrated in increasing concentrations of ethanol, passed through Inhibisol (Maxwell, 1978) and embedded in Araldite. Sections, 1 µm thick and stained with toluidine blue, were used for orientation, if necessary. Ultrathin silver sections, 30-50 nm, were cut on an LKB ultramicrotome III, mounted on copper grids, stained with a 2 per cent alcoholic solution of uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined in an electron microscope.

3.9 Studies on Skeletal Development

Changes in the bones were studied in broilers and turkeys in Experiments III and IV and at 4 weeks of age in turkeys (Experiment V).

3.9.1 Experimental procedure

The birds were killed either at fortnightly intervals for 8 or 10 weeks (Experiments III and IV) to carry out bone and toe ash determination, or at 4 weeks of age (Experiment V) to study pathomorphological changes. The pelvic limbs from each bird were dissected and used for these studies.

In Experiments III and IV, 2 birds each were killed from both 0 and 4 ppm OA groups at 4 and 6 weeks of age. At 8 weeks of age 3 (0 ppm) and 5 (4 ppm OA) turkeys were killed. The number of broilers killed at 8 and 10 weeks of age was 2 and 3 from each group.

3.9.2 Bone and toe ash determination

To investigate the effect of OA on the mineralisation and calcification of bone, the ash content of the middle toe (Fritz and Roberts, 1968) and of the tarso-metatarsus

was determined in broilers (Experiment III) and turkeys (Experiment IV). The entire toe was used for the assay of ash content. After removing the middle toe by cutting through the joint, it was cleaned, but no tissues were removed and they were not solvent-extracted. Tarsometatarsal samples were cleaned of adherent tissues and extracted (defatted) with diethylether for 24 hours. Tarsometatarsal bones and toes were dried overnight at 95-100°C in an hot-air oven and weighed. Tarsometatarsal bones were finally ashed to constant weight at 720°C for approximately 18 to 20 hours (Huff, 1980a). Middle toes were ashed at 600°C for 4 to 6 hours to constant weight (Fritz and Roberts, 1968).

3.9.3. Patho-morphological studies

Patho-morphological changes were studied on the major pelvic limb bones, i.e. femur, tibio-tarsus and tarsometatarsus (Experiment V). The bones were carefully dissected, examined and radiographed in two planes (lateral and anteroposterior) to identify abnormalities and were then preserved in 10 per cent buffered neutral formalin.

Pathological changes were evaluated in decalcified bone sections. Bones were decalcified in formic acid-formalin, and then paraffin embedded, sectioned and stained with H and E, toluidine blue and safranin-orange fast green (Lillie, 1965; Drury, Wallington and Cameron, 1976; Duff, 1979).

3.10 Biotin Status

To assess the effect of OA on biotin status, blood pyruvate carboxylase (PC) activity and plasma biotin levels in broilers (Experiments II and III) and in turkeys (Experiments IV and V) were determined, at 3 weeks of age.

Birds in Experiment V received a semi-synthetic diet very low in biotin (Table 4).

Blood PC activity was measured (nmol $^{14}CO_2$ incorporated/ml of blood) in 1 ml of heparinised blood from each of 8 birds in each group in Experiments III and IV and from each of 16 birds in each of Experiments II and V. Blood PC activity was immediately assayed by the method of Bannister and Whitehead (1976) modified by Whitehead, Armstrong and Waddington (1982), which involves incubation of red blood cells with sodium pyruvate and $NaH^{14}CO_3$, and measurement of the amount of $^{14}CO_2$ incorporated into the oxaloacetate, as pyruvate carboxylase enzyme catalyses the formation of oxaloacetate from pyruvate by CO_2 fixation.

For an assay of blood biotin levels to be made, pooled plasma samples from each group were sent to F. Hoffmann - La Roche and Company, Basle, who carried out a microbiological assay.

3.11 Plasma Testosterone and Progesterone Levels

The effect of OA on plasma testosterone and progesterone levels in broilers of both sexes was studied at 6 weeks of age (Experiment II). Samples from 5 and 7 male birds and from 5 and 4 females receiving 0 and 2 ppm OA respectively were used.

The concentration of plasma testosterone was measured using a radioimmunoassay (Sanwall, Sundby and Edqvist, 1974) modified by Sundby, Tollman and Velle (1975). Plasma progesterone was estimated by a modified solid phase radioimmunoassay method (Dighe and Hunter, 1974; Williams and Sharp, 1978).

3.12 Analysis of OA Residues in Tissues

Tissues particularly liver, kidney and muscles (Experiments III and IV) were sent to Dr M.R.A. Morgan, for the analysis of OA residues in these tissues by an ELISA technique (Morgan et al., 1982) validated with a sensitivity detection limit of 120 pg/g.

3.13 Immunology and Serology

The effect of OA on humoral immune responses was assessed by measuring total serum immunoglobulin levels. Cell-mediated immune (CMI) responses were evaluated by cutaneous delayed hypersensitivity (DH) to a variety of antigens, contact hypersensitivity and Arthus reactions and by a graft-versus-host (GVH) response.

3.13.1 Serology

3.13.1.1 Total immunoglobulin levels in serum

Relative concentrations of IgA, IgM and IgG were determined in sera from broilers (Experiment I) and turkeys (Experiment V) by a modification of the radial immunodiffusion assay, RIA (Mancini, Carbonara and Heremans, 1965: Blythman and White, 1977), using 1.5 per cent agarose in 8 per cent saline with 10 per cent anti-chicken IgA, IgM or IgG incorporated. The wells were filled with 5 µl test serum. Immunoglobulin levels in sera from OA-treated birds, read after a 36 hour incubation, were expressed as a percentage of that determined for the control birds.

3.13.1.2 IgE levels in serum

Some serum samples were screened (Experiments I, II and V) for IgE by the Phadebas IgE paper radio-immunosorbent

(PRIST) method (Pharmacia, Sweden) as described by Burns and Maxwell (1981).

3.13.2 Cell-mediated immunity (CMI)

A GVH response assessed by a splenomegaly assay in chick embryos and DH skin tests using a battery of antigens and the Arthus reaction were used to study the effects of OA on CMI.

3.13.2.1 Hypersensitivity reactions

The experimental details of studies on hypersensitivity responses in broilers and turkeys are summarised in Tables 5a-5c. To evaluate DH responses various skin sites were tested and their comparative sensitivity to different antigens was assessed. Skin thicknesses were measured (in mm) with a micrometer, Model A02 (Schnelltaster, System Kröplin, Germany) at different time intervals after intradermal injection of the test-antigen(s). All the birds were examined for macroscopic lesions 4-6 hours after the test injections.

Table 5a. Hypersensitivity Reactions: Methodology

Antigen	Sensitising Agent (route)	Dose of Antigen (route)
AT, PPD	a) M.avium in LP 1mg/ml (i.m.)	0.1 ml (i.d.)
	b) AT, PPD in FCA 0.5 ml/0.5ml (i.m.)	0.1 ml (i.d.)
PHA - P	-	0.1 ml (100µg) in PBS (i.d.)
DNCB	1% DNCB in vehicle 20ul x 3/wk (x6)(topical)	20µl 0.3% (topical)
BSA a) Arthus reaction b) DH to BSA	0.5ml 1% BSA x4 (i.v.)	0.1 ml 1% BSA (i.d.) 0.1 ml 1% BSA (i.d.)

See footnotes Table 5c

Table 5b. Hypersensitivity reactions in broilers

Test antigen	Experiment No.	No. of birds in group NC	OA	Age (weeks)	Skin sites tested	Examination times (hours)
AT (PPD) a)M.avium primed	II	3	4	8	W,PAA, LTS,WW, DCL	0,24,48,72
b)AT(PPD) primed		3	4			
PHA-P	II	4	4	10	W,PAA, LTS, DCL	0,24,48
DNCB	III	5	5	6	C,W, PAA, LLTS, RLTS	0,24,48, 72
BSA a)Arthus reaction	II	7	7	11	LTS	0,24,48
b)DH to BSA		7	7		W,PAA, WW,DCL	0,24,48

See footnotes Table 5c

Table 5c. Hypersensitivity reactions in turkeys

Test antigen*	Experiment No.	No. of birds in group NC	OA	Age (weeks)	Skin sites tested**	Examination times (hours)
AT (PPD) a)M.avium primed	V	4	5	7	FP,ES, DCL	0,24,48
b)AT(PPD) primed		4	5			
PHA-P	V	8	10	4	FP,ES, WW,LTS, DCL	0,24,48
DNCB	IV	3	5	6	ES,LLTS, RLTS	0,24,48
BSA a)Arthus reaction	V	9	9	7	LTS	0,24,48
b)DH to BSA		9	9		FP,ES, DCL	

*AT avian tuberculin, PPD purified protein derivative, PHA-P phytohaemagglutinin (P), DNCB dinitrochlorobenzene BSA bovine serum albumin, DH delayed hypersensitivity reaction, M.avium Mycobacterium avium

NC normal (untreated) control, OA ochratoxin treated

**C comb, DCL dorsal cloacal lip, ES earskin area, FP frontal process, LTS lateral thoracic skin (apterium) LLTS left lateral thoracic skin, RLTS right lateral thoracic skin, PAA post auricular apterium, W wattle, WW wing web

FCA Freund's complete adjuvant

Corresponding areas or contralateral skin sites were used for control injections of saline or application of vehicle. Control for unpaired sites such as, the fowl comb, turkey frontal process and dorsal cloacal lip, were either the initial thickness at 0 hours or similar sites on other birds injected with diluent alone. Pieces of skin were usually collected at 48 hours for histology.

3.13.2.1.1 Delayed hypersensitivity to avian tuberculin, PPD

The birds were sensitised either with killed Mycobacterium avium, Central Veterinary Laboratory, Weybridge, Surrey (Rose and Bradley, 1977) or avian tuberculin PPD, Central Veterinary Laboratory, Weybridge, Surrey (Giambrone, Ewert, Wyatt and Eidson, 1978) by an intramuscular injection of 1 ml antigen into the leg. Two birds from each group were injected with the diluent or vehicle, liquid paraffin (LP) and Freund's complete adjuvant (FCA) as controls. All the birds were tested 3 weeks later by intradermal injection of 0.1 ml avian tuberculin, Purified Protein Derivative, PPD (25,000 i.u./ml) at various skin sites (Tables 5b and 5c).

3.13.2.1.2 Delayed hypersensitivity to phytohaemagglutinin

To assess non-specific DH reactions 100 µg PHA-P (Difco Laboratories, U.S.A.), in sterile PBS was injected at various skin sites in broilers and turkeys and skin thickness was measured (McCorkle, Olah and Glick, 1980). Attempts were made to study the nature of cellular responses after intradermal PHA-stimulation, in the skin and internal organs of turkeys, using histological, histochemical and immunofluorescent, and electron microscopic methods, as this is an area that has not yet been well defined (McCorkle, Simmons and Luginbuhl, 1982; McCorkle, Luginbuhl, Simmons, Morgan and Thaxton, 1983). Blood smears were examined from non-PHA-treated,

and PHA-treated control (0ppm) and OA-fed (4 ppm) turkeys for differential leucocyte counts.

3.13.2.1.3 Contact-hypersensitivity to dinitrochlorobenzene

Inflammatory skin reactions induced by DNCB (2, 4-dinitrochlorobenzene) were evaluated by sensitising the birds with topical applications of 20 µl DNCB in acetone: olive oil (4:1), three times a week for 2 weeks, on the left lateral thoracic skin (apterium) and frontal process (Willoughby, Walters and Spector, 1965). Two weeks after the final application of DNCB, the birds were challenged with 0.3 per cent DNCB (in vehicle) on the left as well as the right lateral thoracic skin sites (Awadhiya, Vegad and Kolte, 1982). The vehicle alone was applied to unsensitised sites as a control. The development of a skin reaction was scored 0 to 4+ as described by Chauhan and Verma (1983). In addition, other skin sites (Tables 5b and 5c) were also challenged in order to investigate the variation of the skin thickness at various times (Prescott, Wilkie, Hunter and Julian, 1982). The cellular reaction in skin tissue was studied in broiler chickens by histological and histochemical methods and by electron microscopy.

3.13.2.1.4 Arthus reaction and delayed hypersensitivity to bovine serum albumin

Broilers and turkeys were hyperimmunised with 1 per cent BSA (Sigma Chemical Company, U.S.A.) in PBS by a primary dose followed by 3 or 4 booster injections (first after 1 week and subsequently at 3 day intervals, Table 5a) and as controls, two birds from each group were also injected with PBS alone. Three weeks after the first injection the birds were challenged by intradermal injections (0.1 ml) of 1 per cent BSA at various skin sites which were then assessed for the development

of a direct Arthus reaction, especially in the lateral thoracic apterium (Luoma and Benedict, 1977). This assessment was made up to 48 or 72 hours afterwards and macroscopic lesions were scored from 0 to 4+ based on the intensity, severity and type of reaction (erythema, oedema, haemorrhage or necrosis) as well as the size of the reaction zone.

The thickness of the skin at other sites was also measured for DH response (Palladino, Grebenau and Thorbecke, 1978) in both broilers and turkeys. Contralateral sites were injected with 0.1 ml PBS as controls. The thickness of the frontal process (FP) and dorsal cloacal lip (DCL) at 0 hours was used as the baseline control.

3.13.21.5 Histology and histochemistry

Pieces of skin, liver, kidney and lymphoid organs from PHA-treated turkeys and of skins from DNCB-treated fowls were collected in formol saline for histology and histochemistry, and in ethanol for immunofluorescence. Skin and tissue sections were prepared for histological and immunofluorescent studies as described in sections 3.4 and 3.5. Skin was also embedded by the method of Frangioni and Borgioli (1979). Semithin, 1-2 µm, plastic sections were cut on an ultramicrotome (LKB, Pyramitome) using glass knives, and stained with H and E, May- Grünwald and Giemsa, toluidine blue, Best's carmine and the PAS reaction (Culling, 1966). Blood smears from turkeys were also stained by benzidine peroxidase and Sudan black (Caxton-Martins and Daimon, 1976).

For the histochemical demonstration of peroxidase positive granules using a method recently developed to differentiate fowl eosinophils from heterophils in tissues, formol saline fixed tissues from both broilers (DNCB tested) and turkeys (PHA tested) were processed by

the method of Maxwell (1984). Briefly, blocks of 1 x 1 x 5 mm tissues, following a 30 minutes wash in distilled water were incubated in two changes of peroxidase substrate (Hanker, Yates, Metz and Rostioni, 1977) for 3 hours at room temperature with agitation. The blocks were dehydrated, cleared, embedded in paraffin wax and sectioned. Sections were counterstained with H and E.

3.13.2.1.6 Electron microscopy

Pieces of skin were initially fixed in 5 per cent glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.2 and 850 mosm) for 1 hour at 4 °C, washed in 0.2 M Millonig's phosphate buffer (pH 7.2 and 420 mosm) overnight at 4 °C and post-fixed in 1 per cent osmium tetroxide in 0.1 M Millonig's phosphate buffer (pH 7.2 and 450 mosm) for 1 hour at 4 °C.

Liver, kidney and lymphoid organs (PHA-treated turkeys) were also processed and stained (section 3.8). The skin blocks, after fixation, were processed and embedded as described in section 3.8. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Pieces of skin from DNCB-treated fowls were also processed and stained for electron microscopy. Turkey white blood cells were processed by the method of Maxwell and Trejo (1970) for examination by electron microscopy.

3.13.2.2 Graft-versus-host (GVH) reaction

A GVH response, as assessed by splenomegaly in chick-embryos, was studied in OA-fed broiler fowls (Experiment III) at 8 and 10 weeks of age using the technique of Purchase, Chubb and Biggs (1968). The numbers of leucocytes and lymphocytes were determined in

0.1 ml blood from 5 birds from each of the 0 and 4 ppm OA groups, as in section 3.7. Blood (5 ml) was collected aseptically by cardiac puncture, into Alsever's solution, and was either used on the same day for chick-embryo inoculation or could be stored at 4°C for 1 week, without loss of activity. Before injection, the blood was centrifuged, the cells washed three times and resuspended to the original volume in sterile PBS. White-shelled allogenic 13-day-old-chick-embryos (Veterinary Laboratory, Lasswade and PRC), were candled and a window cut in the shell over a large vein of the chorio-allantoic membrane, after cleaning the shell surface with ethanol. The membrane, was cleared by the application of a drop of sterile liquid paraffin and 10^6 lymphocytes (adjusted with PBS to 0.1 ml of resuspended blood) were injected i.v. using a 30 G (gauge) needle. The window was sealed with sellotape and the embryos were incubated. For each blood sample, 8 to 10 embryos were inoculated. Eleven or ten embryos were kept as uninoculated controls. Embryos were candled daily and any deaths recorded. Six days after inoculation, the embryos were killed and their body weights recorded. Spleens and livers (minus gall bladders) from all the embryos were blotted dry and weighed.

The average increases in spleen and liver weights of the experimental (inoculated) groups over those of the control uninoculated group were expressed either as absolute values or as relative weight changes taking body weight into account. Mean ratio of spleen and liver weight was also calculated for each group.

3.14 Production Characteristics and Teratogenic Study

For this purpose Japanese quail (Experiment VI), maintained on 0, 4 and 8 ppm dietary OA from hatching (section 3.1.2), were utilised. At 6 weeks of age when

the birds came into production, there were 15, 13 and 13 females in the 0, 4 and 8 ppm OA groups respectively; correspondingly there were 20, 17 and 17 males. In all the three groups, weekly egg production was recorded until the end of the experiment at 11 weeks. The eggs laid during each 24 hour period were pooled for each group and were examined for gross abnormalities. The eggs were set for incubation in 3 batches.

The effects of OA on hatchability, fertility and embryonic development were studied in eggs from each group. Eggs were incubated for 5 days and then examined. The number of infertile eggs was recorded and cracked and infertile eggs were removed. Embryos dying during the first five days of incubation were considered as early embryonic deaths. The eggs were candled daily on the 7th to 12th days of incubation. Dead embryos were removed and examined. In addition to those dead, some living embryos were collected from each group on the 8th and 10th days of incubation and malformations of the head, neck, abdomen and limbs were recorded.

Embryonic mortality was recorded throughout the incubation period and live embryos from the control (0 ppm OA) group were culled for age-matched comparisons to be made.

At the end of incubation (17 days), hatched chicks were examined for morphological defects and clinical symptoms. An extra day was allowed for "pipped" quail chicks to hatch out. Chicks failing to hatch and dead-in-shell were removed from the shell and examined. Representative numbers of chicks and dead embryos were preserved in formol saline and all others were opened and examined under a dissecting microscope for cardiac abnormalities. These procedures were adopted in all the three batches.

At the end of the experiment when the adult quail were killed, tissues were collected for histology and for the demonstration of OA by immunofluorescence (section 3.5).

3.15 Statistical Analyses

All data were subjected to statistical analysis (Snedecor and Cochran, 1973) and were examined using the analysis of variance.

3.15.1 Growth response

For the analysis of body weight (Experiments I and V) assuming that all the birds had the same hatching weight, the growth curves were transformed into approximate straight lines using a square root transformation, on the basis of which a slope was calculated for each bird. The slopes were compared for the different treatments by sex combinations using analysis of variance (Rowell and Walters, 1976).

In Experiments II, III and IV, data on body weight were analysed by weighted analysis of variance examining the differences between different treatments and sexes within weeks. In these experiments, it was not possible to transform the data to give (approximately) linear growth as in Experiments I and V, because of the wide range of periods of observed growth due to intermittent killing of the birds (2 to 10 or even 16 weeks).

3.15.2 Organ weights

In Experiment I, the $\log_e$ of the organ weights as a percentage of body weight (relative organ weight) was subjected to analysis of variance. The estimated proportional value (EPV) for each organ in each treatment was calculated and differences were examined.

In subsequent experiments, however, absolute organ weights (instead of relative organ weight) were analysed by a weighted analysis of variance (Stevens, 1976), because treatment producing larger organ weight also produced more variation in organ weight. Treatment differences were also estimated by analysis of covariance (Shirley, 1977), taking body weight as a covariate (corrected organ weight), assuming a common relationship between body weight and organ weight for all treatments. Comparative changes in the organs of broilers and turkeys (Experiments III and IV) from week to week (with advancing age) were also examined by analysis of covariance.

3.15.3 Delayed hypersensitivity response

For DH responses, the increase in skin thickness was examined at each time interval by analysis of variance, to estimate the differences between diets and sensitisation procedures. Where appropriate the corresponding saline swelling was also included as a covariate.

3.15.4 Graft-versus-host reaction

Data on GVH were analysed by analysis of variance using Student's 't' test to examine the differences on the one hand between the uninoculated control group and the two inoculated groups (0 and 4 ppm OA) and secondly the differences between the two inoculated groups.

CHAPTER 4

RESULTS

RESULTS

Analyses of feed samples confirmed the absence of contaminating mycotoxins and the diets were considered satisfactory after microbiological examination. The control diets (0ppm) were negative for OA except in Experiments III and IV where minute amounts of ochratoxin A (7ng/g diet) could be detected. Expected levels of OA were confirmed in the prepared diets; levels as close as 3.98 ppm OA (3.98 µg/g) were detected in the analysis by immunoassay (ELISA) in a 4 ppm toxic diet.

4.1 Clinical Observations and Growth Response

4.1.1 Broilers

Young broiler chicks receiving OA diets developed clinical signs of dullness, huddling, decreased appetite, reduced feed intake, weakness, stunted development (Fig.1) and diarrhoea. No nervous signs were seen but in these birds the abdomen was often soft, flabby and distended (Fig.2). As the birds grew older, the size differences between OA fed-birds and controls became more marked (Fig.3). There was loss of carotenoid pigmentation as shown by the skin colouration of the shank (Fig.4) and this became more marked particularly as treatment-time increased, even at 2 ppm OA (Experiment II). The birds in the early stages of the experiment had a tendency to bleed and bruise even with only slight handling. When birds were being weighed, many tiny blood spots were sometimes found on the paper laid on the floor of the crate to catch droppings. The diarrhoeic faeces sometimes had a very high urate content.

Mortality was low in all the experiments. In Experiment I, only one bird in the 4 ppm OA group on 15th day, and in Experiment II four birds died between the second and fourth weeks. No deaths occurred in

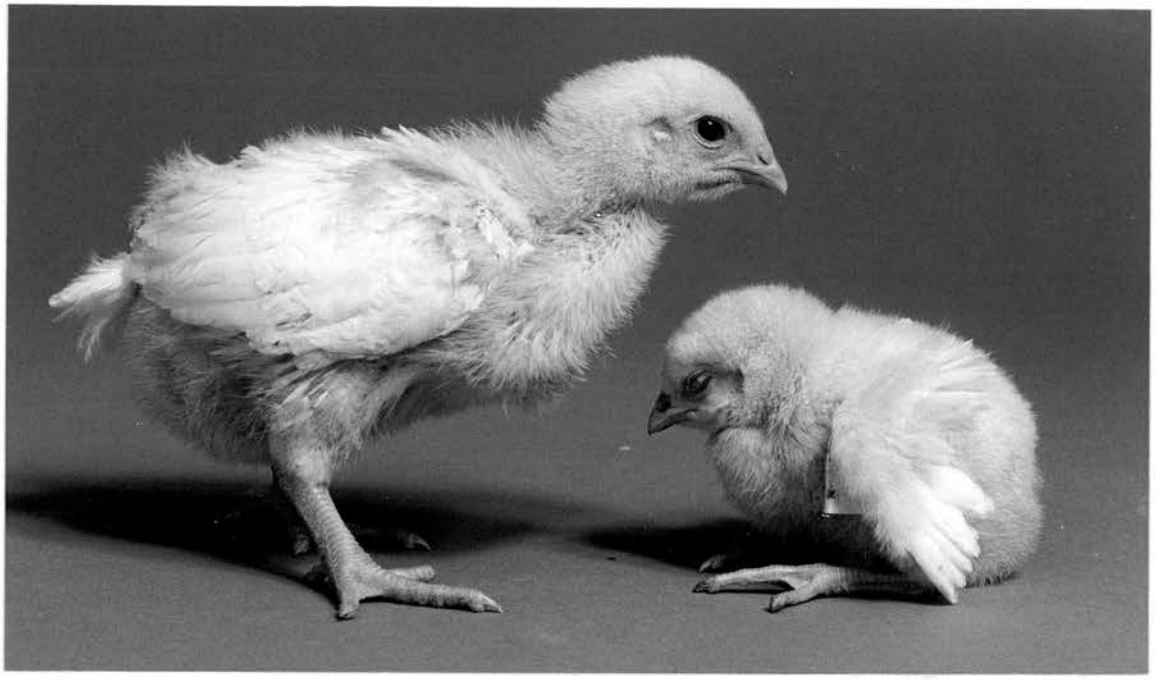


Fig.1. Depressed growth in a 4ppm-treated 1 w.o. fowl. On the left is a normal control bird.

Fig.2. A 3 w.o. fowl (4ppm OA) with soft, flabby and distended abdomen.

Fig.3. Depressed growth in a 5 w.o.fowl. On the left is a normal control bird.

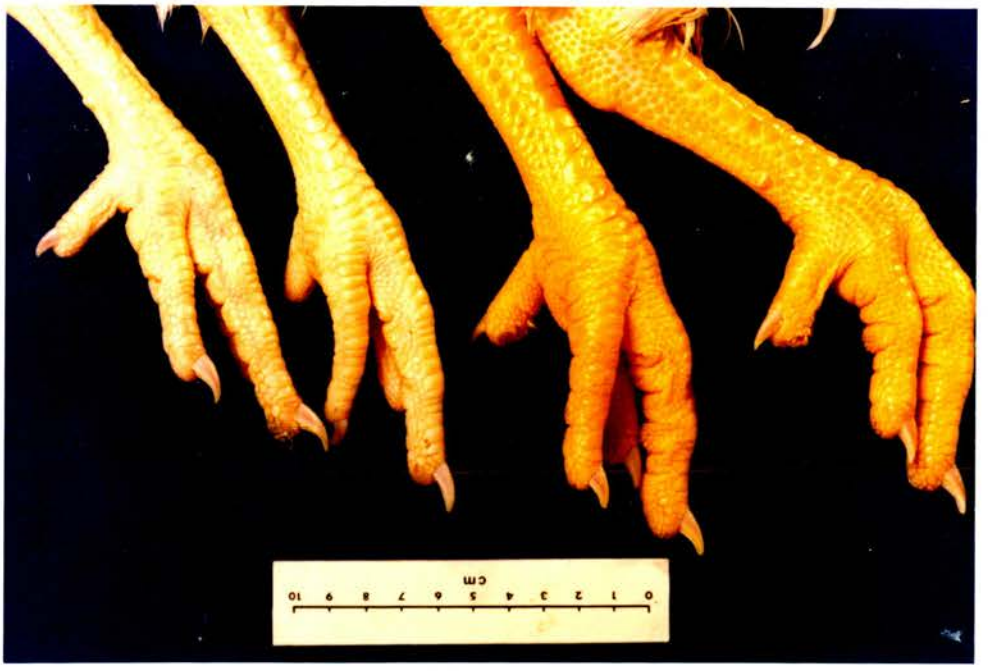
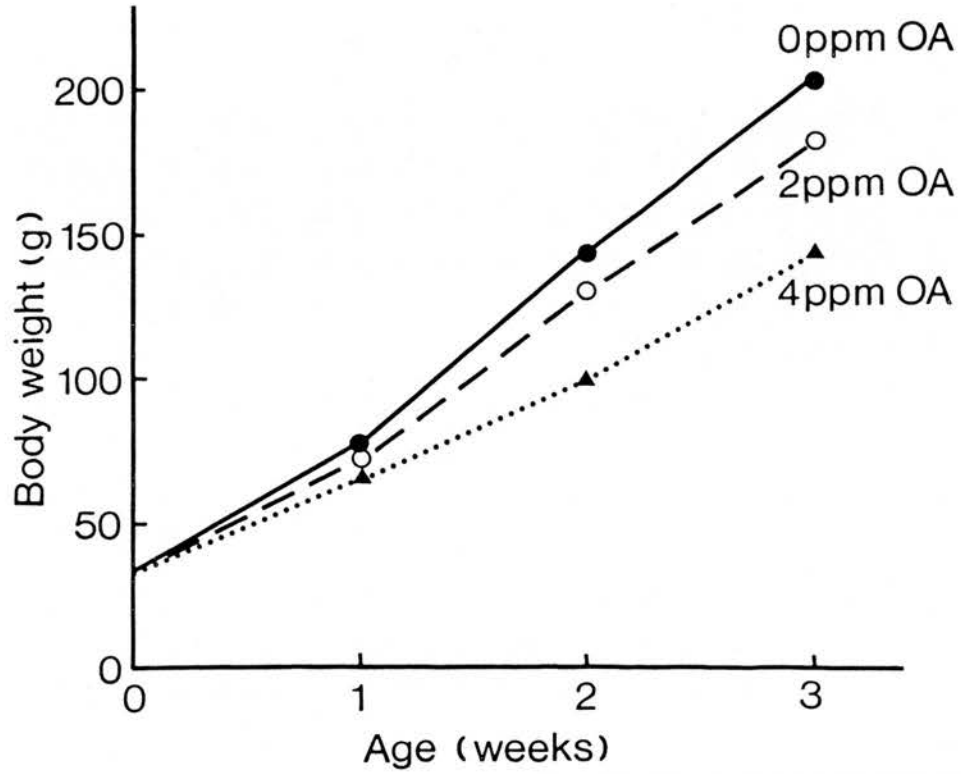


Fig.4. Loss of carotenoid pigmentation in the leg of a 6 w.o. fowl (2ppm OA). Normal pigmentation on the right (control).



OA Level In Diet	0 Week	1 Week	2 Weeks	3 Weeks	Average Slope (Log_e Weight)
0 ppm	34.59±2.94*	75.32±13.08	143.36±18.39	201.32±21.71	0.100^{a}
2 ppm	34.10±2.07	72.90±10.43	131.05±20.50	181.10±23.43	0.087^{b}
4 ppm	32.62±2.26	66.87±10.57	99.50±29.50	142.75±45.52	0.073^{c}

a, b, c Values in a column with a different supercript differ significantly ($P<0.05$) from each other.

* Mean ± S.D.

Fig.5. Effect of OA on growth response of young broiler chicks.

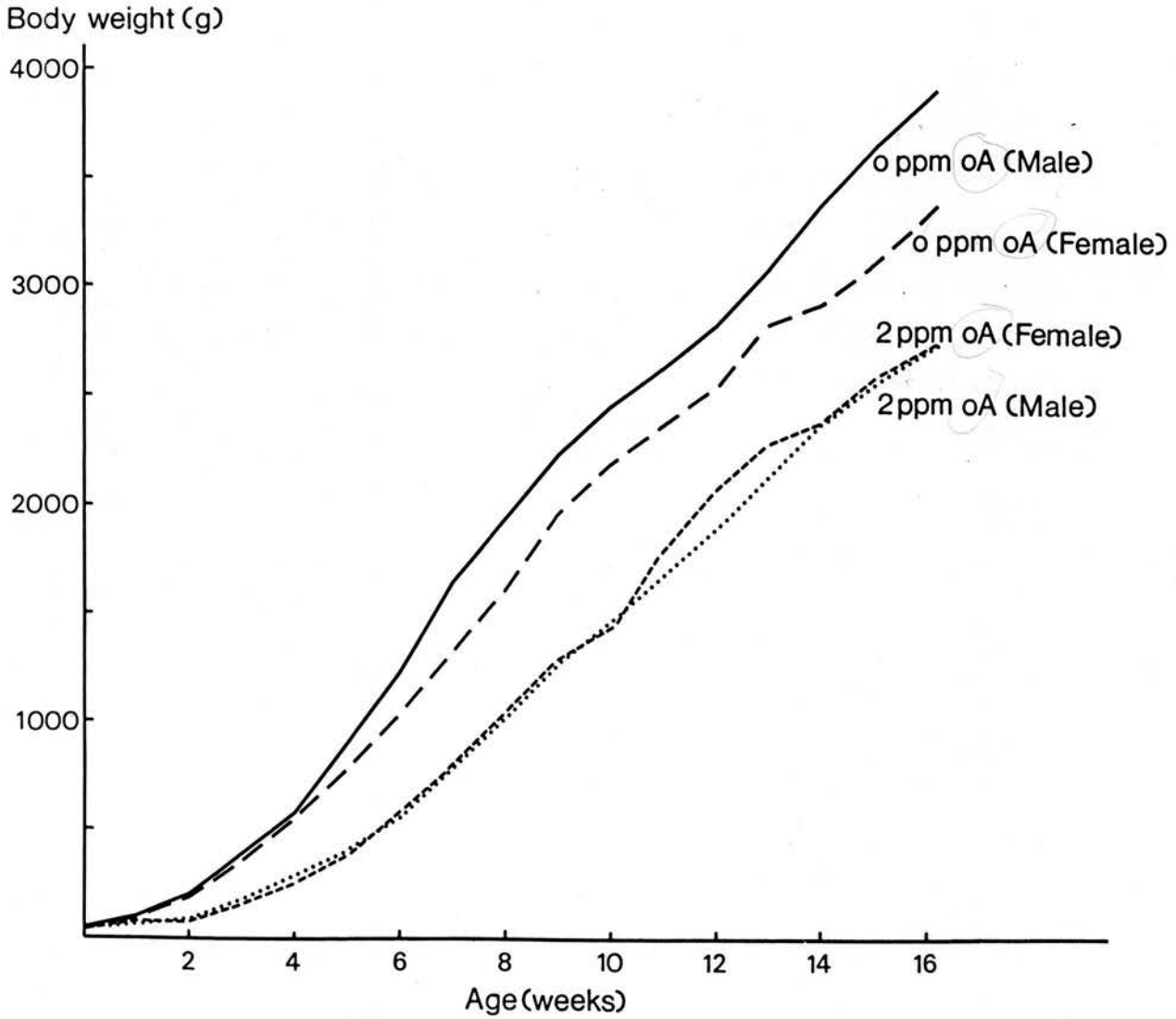


Fig.6. Effect of OA on growth response of male and female broiler chickens.

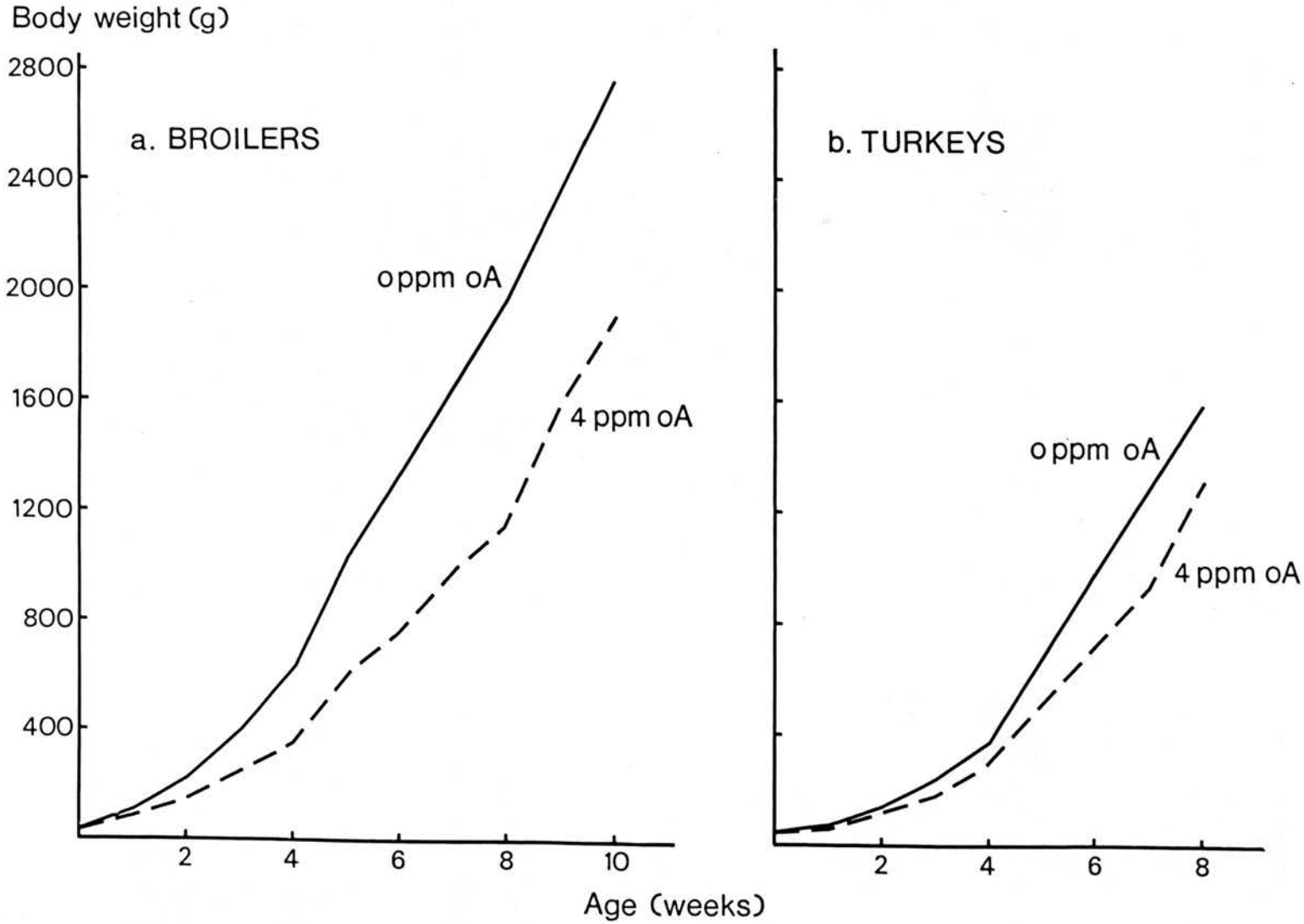


Fig.7. Growth response in (a) broilers and (b) turkeys.

Experiment III.

The effects of OA on growth response in the three experiments are shown in Figs. 5-7a . In OA-treated birds, depression of growth could be detected two weeks after OA-treatment started and the effect became more severe as age increased. Regression analysis of the data (Experiment I, Fig.5) revealed a significant dose-related depression in the body weight. Weighted analyses of variance (Experiment II, Fig.6 ; Experiment III, Fig.7a) indicated a steady divergence in weight week by week between OA-treated and control birds starting from 2 week-old up to 10 week-old (Experiment III, $F_{10,117}$ of 23.23) or 16 week-old (Experiment II, $F_{16,536}$ of 46.2). Weight differences between OA and control birds were highly significant ($P<0.001$). No differences between the sexes were noted in the reduced growth rate of OA-fed birds except in long-term treatment (Experiment II, Fig.6), when males were more severely affected than the females (For comparison see Fig.9).

4.1.2 Turkeys

Clinical signs were similar to those observed in broilers but the loss of carotenoid pigmentation was not obvious. The affected poults were emaciated and stunted (Fig.8) with signs of occasional diarrhoea. One bird died at 3 weeks after OA-treatment started (Experiment V). There were no deaths in Experiment IV; however, two birds died of anaphylactic shock from the 0ppm (control) group during intravenous BSA-immunisation.

There was a significant depression in the growth ($p<0.05$) of OA-fed birds with increasing age (Experiment IV, Fig.7b; Experiment V, Fig. 9) in both males and females. Growth depression (Fig.9) in 4ppm OA-treated

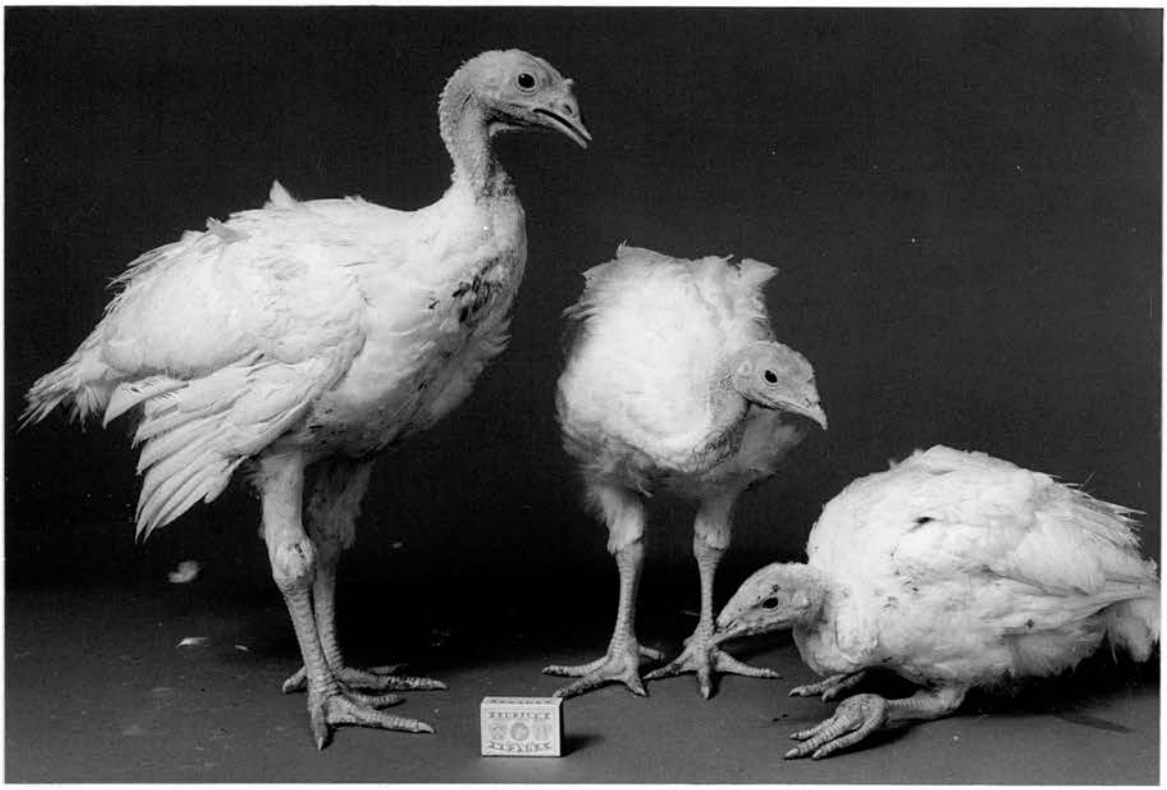


Fig.8. Stunted growth (4ppm OA) in two 7 w.o. turkeys. On the left is a normal control bird.

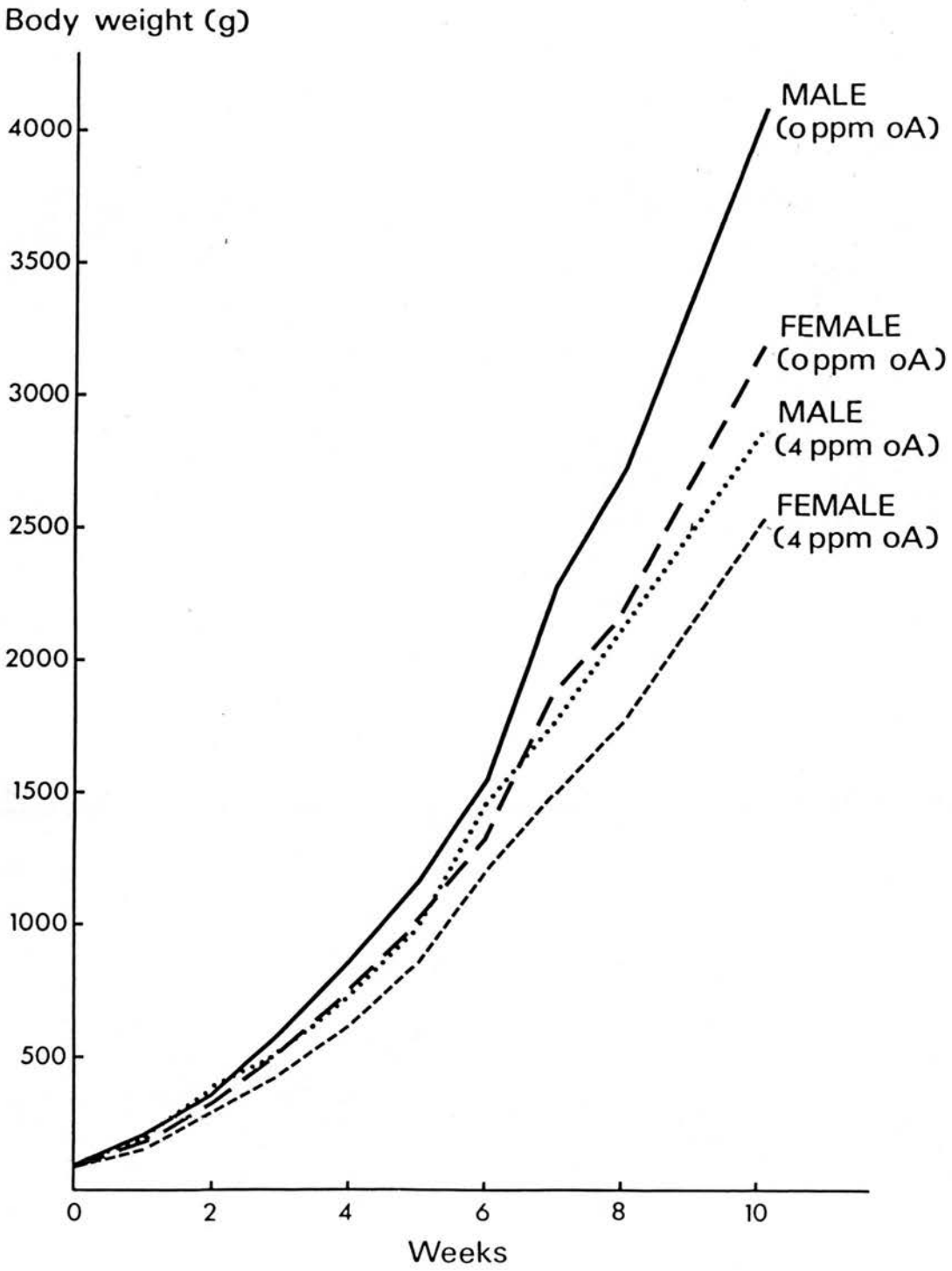


Fig.9. Effect of OA on growth response of male and female turkeys.

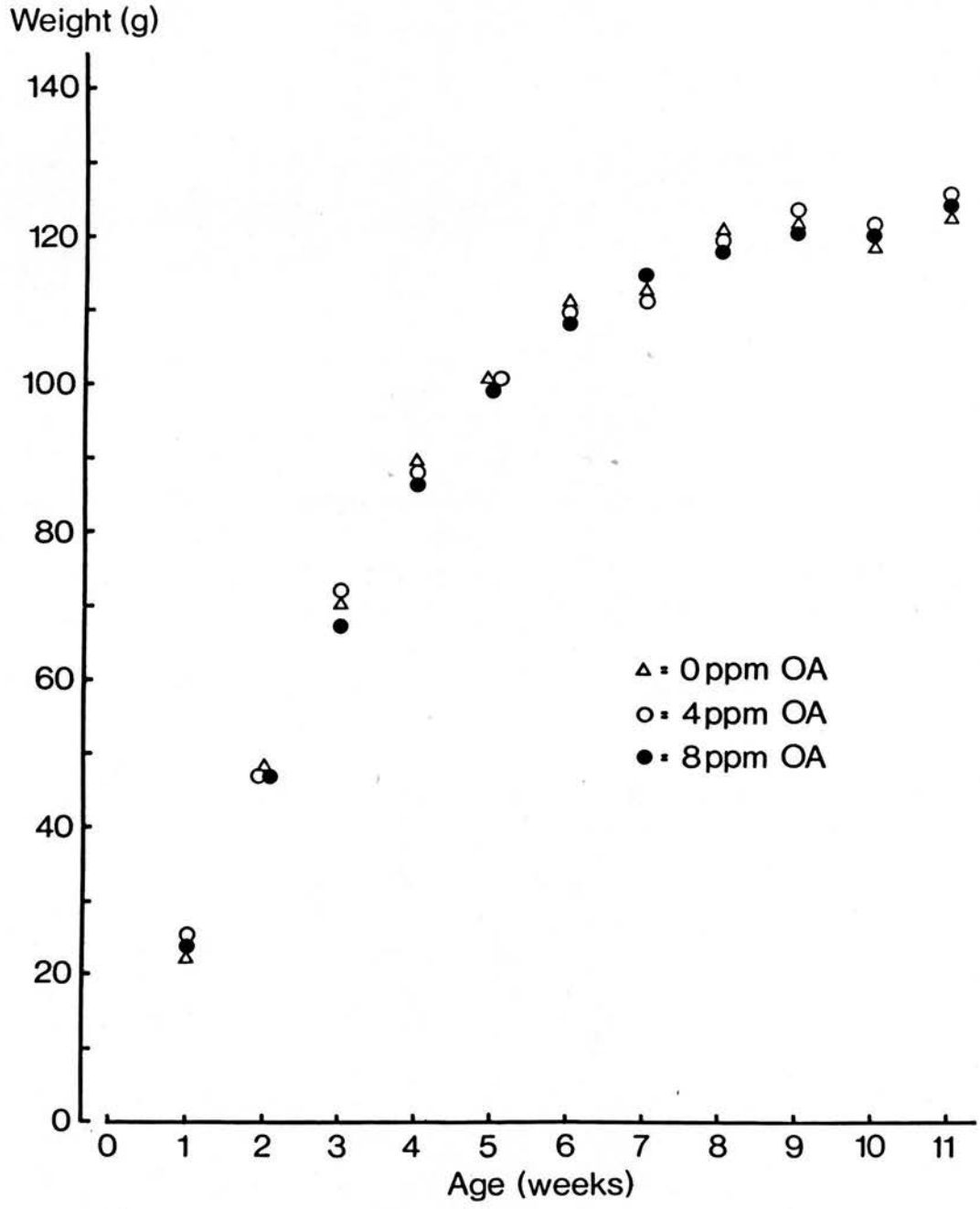


Fig.10. Growth response in control and OA-fed quail.

males (as measured by comparison with 0ppm OA (control) males) was similar to that in OA-treated females (compared with 0ppm OA (control) females). The growth of turkey poults reared on litter (Experiment V) was better than that of the brooder-raised turkey poults (Experiment V). In both experimental trials, the earliest a significant depression of growth could be detected was at 3 weeks after feeding with the OA diet had begun. There was a consistent divergence week by week in the depression in weight gain similar to, although less marked than, that in broilers (Experiment IV, $F_{8,110}$ of 10.78). No difference between the sexes was seen in the growth response in turkeys.

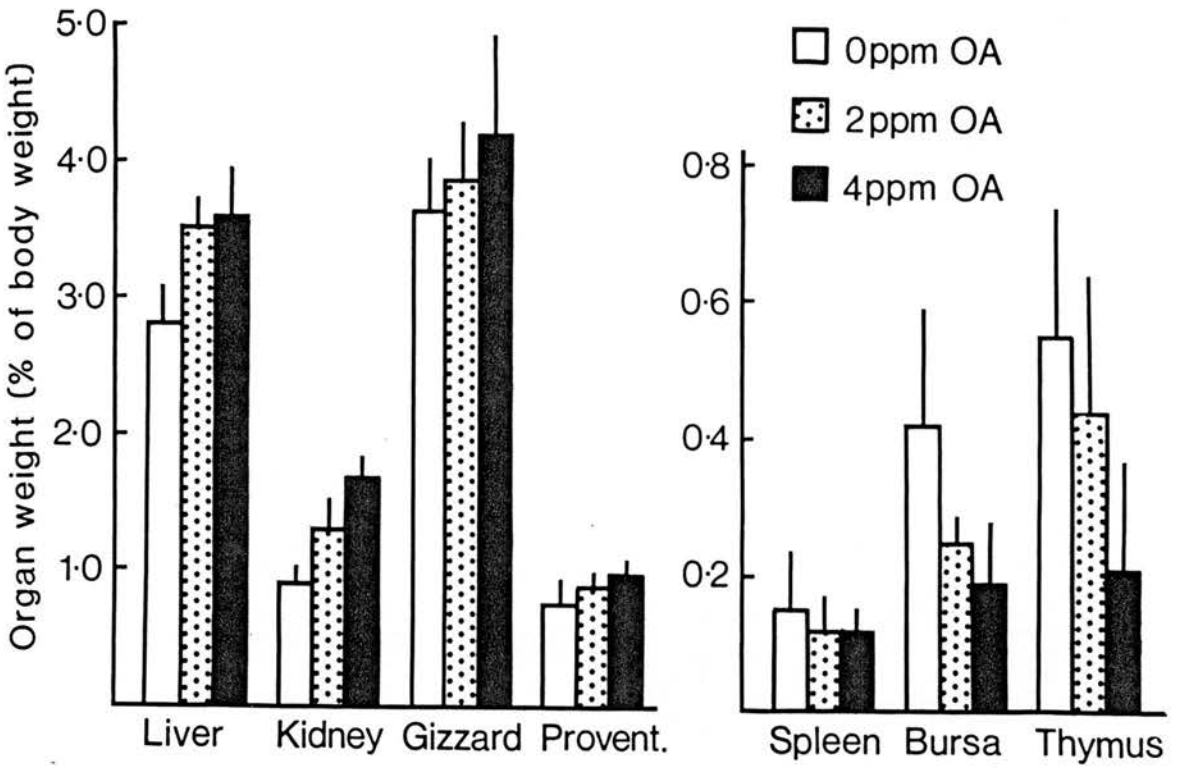
4.1.3 Quail

Clinical signs in quail were less marked, though occasional diarrhoea and huddling could be seen in the 8ppm OA group. The weekly growth rate was not affected in quail either at 4 or 8 ppm OA feeding (Fig.10) nor was there any difference between the sexes in growth.

4.2 Organ Weight

The effects of OA on organ weights in broilers and turkeys are shown in Fig.11 and Tables 6-8. Only data from those organs which showed changes in the weights due to OA are presented with the data on broilers and turkeys being presented together.

In 3-week-old broilers (Experiment I, Fig.11) the liver, kidney and proventriculus were increased in size, with the kidney showing enlargement of about one and a half times at 2 ppm and of almost two times at 4 ppm OA, as judged from the estimated proportional value, EPV (Fig.11) On the other hand, the thymus and bursa of Fabricius were significantly decreased in size, the effect



OA LEVEL IN DIET	LIVER		KIDNEY		GIZZARD		PROVENTRICULUS		SPLEEN		BURSA		THYMUS	
	WEIGHT	EPV	WEIGHT	EPV	WEIGHT	EPV	WEIGHT	EPV	WEIGHT	EPV	WEIGHT	EPV	WEIGHT	EPV
0 ppm	2.81±0.31*	1.00^{a}	0.92±0.10	1.00^{a}	3.65±0.39	1.00^{a}	0.76±0.19	1.00^{a}	0.15±0.09	1.00^{a}	0.42±0.17	1.00^{a}	0.55±0.19	1.00^{a}
2 ppm	3.52±0.22	1.25^{b}	1.32±0.23	1.40^{b}	3.88±0.43	1.06^{a}	0.89±0.11	1.17^{b}	0.12±0.05	0.82^{a}	0.25±0.04	0.61^{b}	0.44±0.20	0.75^{a}
4 ppm	3.60±0.36	1.31^{b}	1.69±0.16	1.86^{c}	4.20±0.72	1.18^{a}	1.00±0.07	1.35^{c}	0.12±0.03	0.92^{a}	0.19±0.09	0.47^{b}	0.21±0.15	0.33^{b}

a,b,c Values in a column with different superscripts differ significantly (P<0.05)
EPV Estimated Proportional Value
* Mean ± S.D.

Fig.11. Effect of OA on the relative organ weight of broiler chicks.

Table 6. Effect of OA on weight of lymphoid organs in broilers (Experiment III) and turkeys (Experiment IV)

Organ	Group	AGE (weeks) 2	4	6	8	10	F. value*
A. BROILERS							(F_4, 11)
Body wt.	NC	226.10	470.80	1189.00	2034.35	2750.00	
	OA	135.89	234.35	619.50	1083.25	1886.67	
Spleen	NC	0.28	0.62	3.02	5.79	6.38	62.75
	OA	0.16	0.29	1.32	2.21	4.03	
	ED[1]	0.16 (0.07)	0.38 (0.12)	- 0.02 (0.26)	- 0.64 (0.38)	0.25 (0.36)	3.69
Bursa of Fabricius	NC	0.52	1.34	2.78	5.11	6.39	12.27
	OA	0.28	0.53	1.08	1.35	3.57	
	ED[1]	- 0.05 (0.21)	- 0.31 (0.35)	- 0.45 (0.60)	- 1.74 (0.88)	- 0.99 (0.83)	1.01
Thymus	NC	1.21	2.71	6.07	6.87	12.61	3.76
	OA	0.27	0.67	1.37	1.96	6.43	
	ED[1]	- 0.75 (0.62)	- 1.55 (1.00)	- 3.28 (1.66)	- 2.45 (2.41)	- 4.20 (2.42)	0.82
B. TURKEYS							
Body wt.	NC	123.25	266.20	961.60	1582.60		(F_3, 11)
	OA	140.60	264.15	587.50	1335.26		
Spleen	NC	0.07	0.14	0.89	1.94		7.30
	OA	0.07	0.13	0.50	1.11		
	ED[1]	- 0.01 (0.05)	- 0.01 (0.07)	- 0.14 (0.19)	- 0.61 (0.18)		3.39
Bursa of Fabricius	NC	0.25	0.73	1.70	2.46		18.35
	OA	0.21	0.43	0.84	1.50		
	ED[1]	- 0.07 (0.06)	- 0.29 (0.10)	- 0.23 (0.16)	- 0.56 (0.14)		3.87
Thymus	NC	0.27	0.65	1.50	2.40		10.74
	OA	0.19	0.37	0.56	1.27		
	ED[1]	- 0.10 (0.09)	- 0.28 (0.13)	- 0.38 (0.21)	- 0.75 (0.20)		3.10
C. BROILER-TURKEY							(F_3, 19)
Spleen	ED[2]	0.00 (0.13)	0.06 (0.19)	1.10 (0.39)	1.79 (0.54)		5.80
Bursa of Fabricius	ED[2]	0.00 (0.15)	0.09 (0.23)	0.49 (0.33)	1.50 (0.42)		4.33
Thymus	ED[2]	0.69 (0.41)	1.38 (0.63)	3.22 (0.88)	2.21 (1.07)		2.55

NC (Normal Control) 0ppm OA; OA (ochratoxin A) 4ppm OA

ED[1] Estimated difference of the mean organ weights (body weight included as a covariate) between OA and NC birds (and their standard errors).

ED[2] Estimated difference of the depression of the mean organ weights (body weight included as a covariate) between NC and OA birds, in broilers compared with turkeys (and their standard errors).

* Difference in weight (divergence) between 1 and 4ppm OA groups week by week, was tested using 'F' test.

Table 7. Effect of OA on organ weights(g) in 4 week old turkey poults

Treatment	Body weight	Liver	Kidney	Spleen	Bursa of Fabricius	Thymus
I. Absolute organ weight						
NC Group	848	20.3	6.6	0.63	1.10	1.10
OA Group	588	18.3	6.8	0.43	0.60	0.50
E.D. (S.E.) OA-NC	-260(80.2)	-2.0(3.4)	0.2(1.0)	-0.20(0.12)	-0.50(0.2)	-0.60(0.14)
95% C.I.	(-450, -70)	(-10, 6)	(-2.2, 2.4)	(-0.48, 0.08)	(-0.97, -0.03)	(-0.93, -0.27)
II. Corrected organ weight						
E.D. (S.E.)		4.3(4.7)	2.0(1.4)	0.04(0.15)	-0.04(0.24)	-0.3(0.19)
95% C.I.		(-7, 16)	(-1.4, 5.4)	(-0.33, 0.41)	(-0.6, 0.6)	(-0.8, 0.2)
Regression slope (S.E.)		0.024(0.014)	0.007(0.004)	0.0008(0.0004)	0.002(0.0007)	0.001(0.0006)

Significant differences are underlined.

NC (Normal control) - 0 ppm OA; OA (ochratoxin A) - 4 ppm OA.

E.D. (S.E.) - Estimated differences of the mean weight between OA and NC birds (and their standard errors).

being most marked in the thymus which was reduced by two thirds at 4 ppm OA. The changes were dose-related. An increase in the size of the gizzard and a decrease in the size of the spleen were noticed, though these changes were not statistically significant. The crop and Harderian gland did not show any significant changes. The weight of the heart showed inconsistent results. No differences between sexes were noticed in the organ weights.

The growth and development of lymphoid organs in particular were depressed in broilers and turkeys starting from two weeks (Experiments III and IV) and these changes became more severe as age increased (Table 6). The consistent and marked reduction in the sizes of the thymus, bursa of Fabricius and spleen of broilers and turkeys were evident in both absolute and corrected organ weight analyses. When the effects of OA on the lymphoid organs in broilers and turkeys were compared (Table 6, section C), the depression was found to be more severe in broilers than in turkeys and this became more marked with increasing age (up to 8 weeks studied).

Other organs of broilers and turkeys (Experiments III and IV) either were not affected or showed inconsistent results. The kidney in broilers and both the liver and kidney in turkeys showed a tendency towards enlargement, though these changes were not significant. Changes in the organs of the upper digestive tract (crop, proventriculus and gizzard) and the heart were variable.

The data on organ weights in turkeys (Experiment V) at 4 and 10 weeks of age are shown in Tables 7 and 8 respectively. The effects of sensitisation of birds to Mycobacterium avium (and PPD) and BSA are shown in Table 8, which illustrates the differences, especially where the effect of sensitisation varies, between the 0ppm and 4ppm OA groups. As in previous experiments, the

Table 8. Effect of OA on organ weight (g) in 10 week old turkey poults.

Treatment	Sensitization	Body weight	Liver	Kidney	Spleen	Bursa of Fabricius	Thymus
I. Absolute organ weight							
NC Group	BSA	3938.36					3.70
	MA (PPD)	3278.63					2.87
	Combined		73.04	20.34	2.72	2.99	
OA Group	BSA	2503.56					1.85
	MA (PPD)	2852.67					2.15
	Combined		67.18	19.61	2.70	2.08	
	E.D. (S.E.) OA-NC	-1606(245)					-1.91(0.26)
	95% C.I.	(-2105, -1107)					(-2.44, -1.39)
II. Corrected organ weight							
E.D. (S.E.)	BSA		24.44(5.42)		1.35(0.31)		
	MA (PPD)		1.51(3.75)		-0.06(0.22)		
	Combined			2.44(0.86)		-0.27(0.20)	-0.67(0.22)
95% C.I.	BSA		(13.23, 35.63)		(0.71, 1.99)		
	MA (PPD)		(-6.23, 9.25)		(-0.51, 0.39)		
	Combined			(0.66, 4.21)		(-0.68, 0.14)	(-1.12, -0.22)
Regression slope (S.E.)			0.018(0.003)	0.0038(0.0006)	0.0008(0.0001)	0.0007(0.0001)	0.0007(0.0001)

Significant differences are underlined.

NC (Normal control) - 0 ppm OA; OA (Ochratoxin A) - 4 ppm OA.

E.D. (S.E.) - Estimated differences of the mean weights between OA and NC birds (and their standard errors).

BSA - Bovine serum albumin.

MA (PPD) - Mycobacterium avium (and purified protein derivative).

weights of the thymus and bursa of Fabricius, as assessed both by absolute and by corrected organ weight analyses, were significantly reduced at both 4 and 10 weeks in the OA-fed groups. No significant differences were noted in the weights of other organs at 4 weeks. However, using the corrected organ weight analysis (analysis of covariance), a significant enlargement could be detected in the livers and spleens from OA-treated birds after BSA stimulation (Table 8). The kidneys showed a tendency to increase in weight at 4 weeks in OA-treated birds and were signifcantly enlarged at 10 weeks. The heart, crop, proventriculus and gizzard gave variable results at both 4 and 10 weeks of age.

4.3 Haematological Changes

Table 9 shows the effect of OA on total blood cell counts in broilers at 4 weeks after OA-treatment. There were no significant alterations in total cell, RBC, WBC and thrombocyte counts at 4 ppm OA levels. Similarly differential leucocyte counts were not affected in broilers (Table 10a). However, there was a slight increase in lymphocytes with a corresponding non-significant decrease in heterophils at both 3 and 4 weeks of age in the 4 ppm OA group. The blood profile approached normality in broilers maintained on a 2 ppm OA diet for 12 weeks (Table 10a).

There were no significant changes in blood cell counts in turkeys at 4 or 10 weeks of age when fed 4 ppm OA from the time of hatching (Table 10b).

However, there was a significant increase in lymphocytes in quail with a corresponding decrease in heterophils after 11 weeks on the OA diets, particularly at the 8 ppm level (Table 10c).

Table 9. Effect of OA on total blood cell counts/cmm in broilers (Experiment III)(mean ± SEM)[a]

Treatment	Total cell count($\times 10^6$)	Parameters studied		Thrombocyte count
		RBC count ($\times 10^6$)	WBC count	
0ppm OA	2.00±0.12	1.97±0.12	12,551± 1,667	22,941± 5,428
4ppm OA	2.18±0.07	2.14±0.07	12,425± 2,456	22,663± 3,943

[a]No significant differences are apparent between the control (0ppm) and OA groups.

Table 10a. Effect of OA on differential leucocyte counts (per cent) in broilers (mean±SEM)[a]

Treatment	No. of birds examined	Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Experiment I	(3 weeks)					
0ppmOA	10	24.7 ±2.17	2.0 ±0.45	6.8 ±0.77	59.0 ±2.90	7.5 ±1.87
2ppmOA	10	18.4 ±2.48	1.4 ±0.54	6.6 ±1.13	64.7 ±2.80	8.8 ±1.13
4ppmOA	8	21.3 ±3.90	1.9 ±0.44	6.0 ±0.91	64.1 ±5.71	6.8 ±1.53
Experiment II	(12 weeks)					
0ppmOA	9	24.3 ±2.59	1.9 ±0.68	4.1 ±0.72	63.5 ±3.71	6.1 ±1.1
2ppmOA	7	24.1 ±5.69	2.4 ±0.87	3.1 ±0.74	63.1 ±6.28	7.1 ±1.06
Experiment III	(4 weeks)					
0ppmOA	5	22.6 ±3.54	2.2 ±1.60	4.6 ±0.75	63.6 ±2.29	7.0 ±1.14
4ppmOA	5	18.0 ±4.60	1.6 ±0.68	7.6 ±1.60	66.6 ±1.03	6.4 ±3.36

[a]No significant differences are apparent between the control (0ppm) and OA groups.

Table 10b. Effect of OA on differential leucocyte counts (per cent) in turkeys (mean ± SEM)[a]

Treatment	No. of birds examined	Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Experiment IV	(4 weeks)					
0ppmOA	5	46.4 ±3.08	1.4 ±0.60	5.0 ±1.38	43.8 ±2.31	3.4 ±0.81
4ppmOA	5	50.2 ±3.60	1.2 ±0.80	3.6 ±1.44	39.4 ±2.44	5.6 ±1.03
Experiment V	(10 weeks)					
0ppmOA	8	51.6 ±3.75	2.6 ±0.60	3.0 ±0.76	24.1 ±3.06	18.6 ±1.65
4ppmOA	12	45.4 ±2.93	3.5 ±0.73	5.1 ±0.98	35.9 ±2.31	10.08 ±1.41

[a]No significant differences are apparent between the control (0ppm) and OA group.

Table 10c. Effect of OA on differential leucocyte counts (per cent) in 11 week-old quail (mean ± SEM)

Treatment	No. of birds examined	Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes
0ppmOA	8	34.0 ±2.46[a]	4.1 ±1.16[a]	0.5 ±0.38[a]	59.1 ±3.03[a]	2.3 ±056[a]
4ppmOA	4	30.3 ±6.91[a]	4.8 ±0.84[a]	0.3 ±0.25[a]	60.8 ±6.05[a]	4.3 ±1.38[a]
8ppmOA	4	19.5 ±2.84[b]	3.5 ±1.19[a]	1.3 ±0.95[a]	72.5 ±2.40[b]	3.3 ±2.29[a]

[a,b] Values in a column with different superscripts differ significantly ($P < 0.02$).

4.4 Blood Pyruvate Carboxylase Activity and Biotin Levels

The effects of OA on blood PC activity and plasma biotin levels in broilers and turkeys at 3 weeks of age are presented in Table 11.

In broilers (Experiment II and III), there was a tendency towards an increase in the blood PC activity at both 2 and 4 ppm OA levels as compared with controls (0 ppm OA). Correspondingly, plasma biotin levels were also increased. The dietary biotin content (112 µg/kg) was moderately low in these experiments.

In turkeys (Experiment V) fed a diet very low in biotin (55 ug/kg), blood PC activity and plasma biotin levels were closely related and showed marked ($P < 0.05$) increases in OA-treated birds. However, in Experiment IV when the turkeys were fed a diet adequate in biotin (218 µg/kg), the 4 ppm OA level in the diet did not induce any significant change in blood PC activity, though the pooled plasma biotin level appeared to be depressed (Table 11).

4.5 Plasma Testosterone and Progesterone Levels

The results of a preliminary study on the plasma levels of steroid hormones (testosterone and progesterone) conducted in broilers after being fed OA diets for 6 weeks are presented in Table 12.

OA did not cause any significant changes in the level of these hormones in the plasma in broilers of either sexes.

Table 11. Blood pyruvate carboxylase (PC) activity and biotin levels in 3 week-old broilers and turkeys

	Broilers			
Parameters	Experiment II		Experiment III	
	0ppm OA	2ppm OA	0ppm OA	4ppm OA
No. of birds	16	16	8	8
PC activity in RBCs*	432 ±39.0[a]	470 ±50.0[a]	273.49 ±21.67[a]	300.70 ±27,83[a]
Biotin level in pooled plasma**	1.322	2.407	2.262	1.620
Available biotin in diet (µg/kg)	112	112	112	112
	Turkeys			
Parameters	Experiment IV		Experiment V	
	0ppm OA	4ppm OA	0ppm OA	4ppm OA
No. of birds	8	8	16	16
PC activity in RBCs	142.56 ±26.96[a]	136.00 ±21.47[a]	37.79 ±6.2[a]	66.49 ±10.0[b]
Biotin level in pooled plasma	1.620	0.810	1.322	2.407
Available biotin in diet (µg/kg)	218	218	55	55

* PC activity expressed as n mol $^{14}CO_2$ incorporated /ml blood/hour at 38 °C (mean ± SEM)

** ng/litre

a,b values with different superscripts differ significantly ($P < 0.05$) from each other.

Table 12. Plasma testosterone and progesterone levels (pg/100µl) in OA-fed broilers (mean ± SEM)[a]

Treatment	No.tested (male, female)	Testesterone level		Progesterone level	
		male	female	male	female
0ppm OA	5,5	45.96±3.11	34.65±2.87	16.20±0.58	16.43±0.97
4ppm OA	7,4	40.23±1.86	39.47±1.07	17.57±0.80	16.60±1.27

[a]No significant differences are apparent between the control (0ppm) and OA groups.

4.6 Gross Pathological Changes

The OA-treated broiler chickens were emaciated and their bones were thin, soft and pliable particularly at 2 and 3 weeks of age. The kidney was enlarged and pale and contained urate deposits particularly in those birds which had died (Fig.12). The liver was also enlarged. The bile was paler and less viscous than normal. There was a slight catarrhal enteritis in some birds. The lining of the gizzard was often dry. The spleen and bursa appeared to be reduced in size. At 16 weeks of age, the development of the ovary and testes was retarded as compared with controls. Gross lesions were not detected in other organs.

In turkeys and quail, no specific gross lesions could be detected except for a slight enlargement of the kidney and liver in OA-treated birds.

4.7 Histopathological Changes

4.7.1 Broilers

Almost similar changes were seen in different organs at 2, 3, 4 and 10 weeks of age. The changes were studied in greatest detail at 3 weeks of age and only selected tissue sections were examined at other ages.

4.7.1.1 Kidney

In sections of kidney from OA-fed birds, many of the proximal convoluted tubules (PCT) and some of the distal convoluted tubules (DCT) were enlarged, distended and hypertrophied (Figs.13a and 13b). The majority of the enlarged tubules, many of which were misshapen, contained translucent, homogeneous, round, hyaline globules (Fig.13b). These globules were PAS-negative

Fig.12. Fowl 3 w.o. kidney. Note enlarged, swollen and pale kidneys in a 4ppm OA-treated bird (bottom). Normal control kidney (mahogany colour) is on the top.

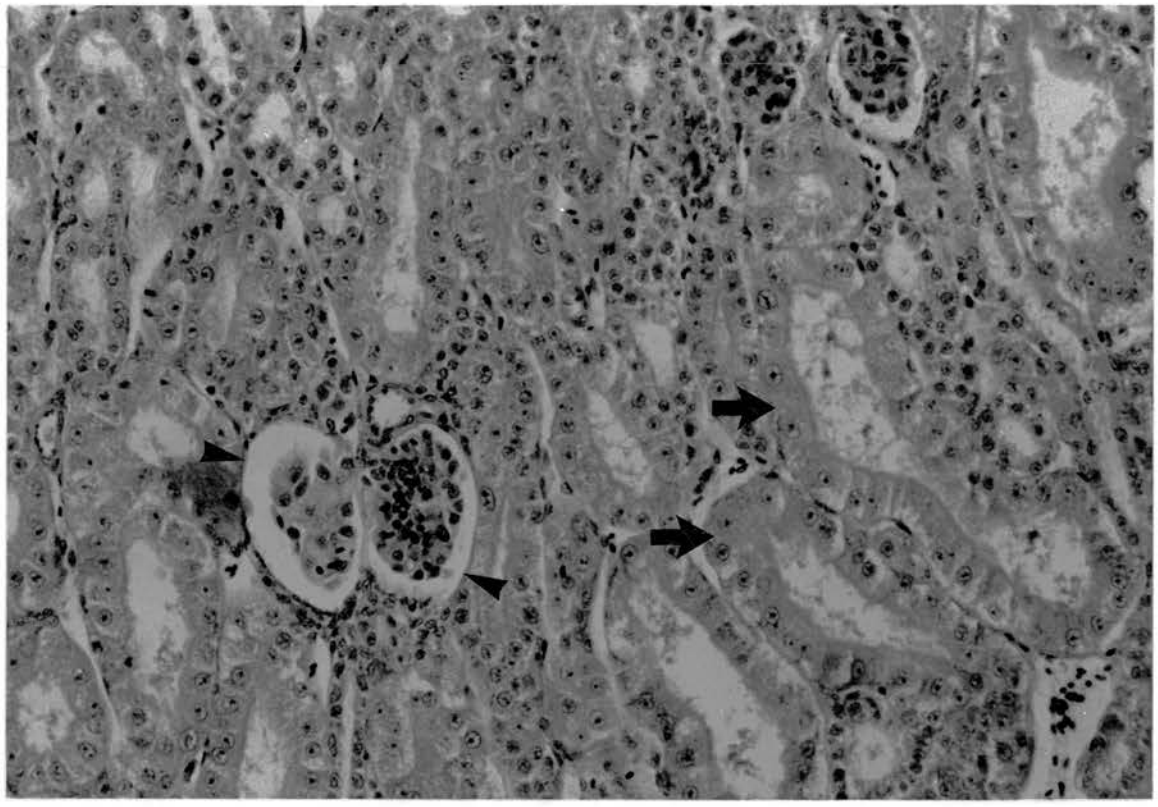


Fig.13a. Fowl. 3 w.o. (4ppm OA) kidney. Distension, enlargement and hypertrophy of PCT (arrow) and enlarged Bowman's capsule (arrowhead). H & E x 400.

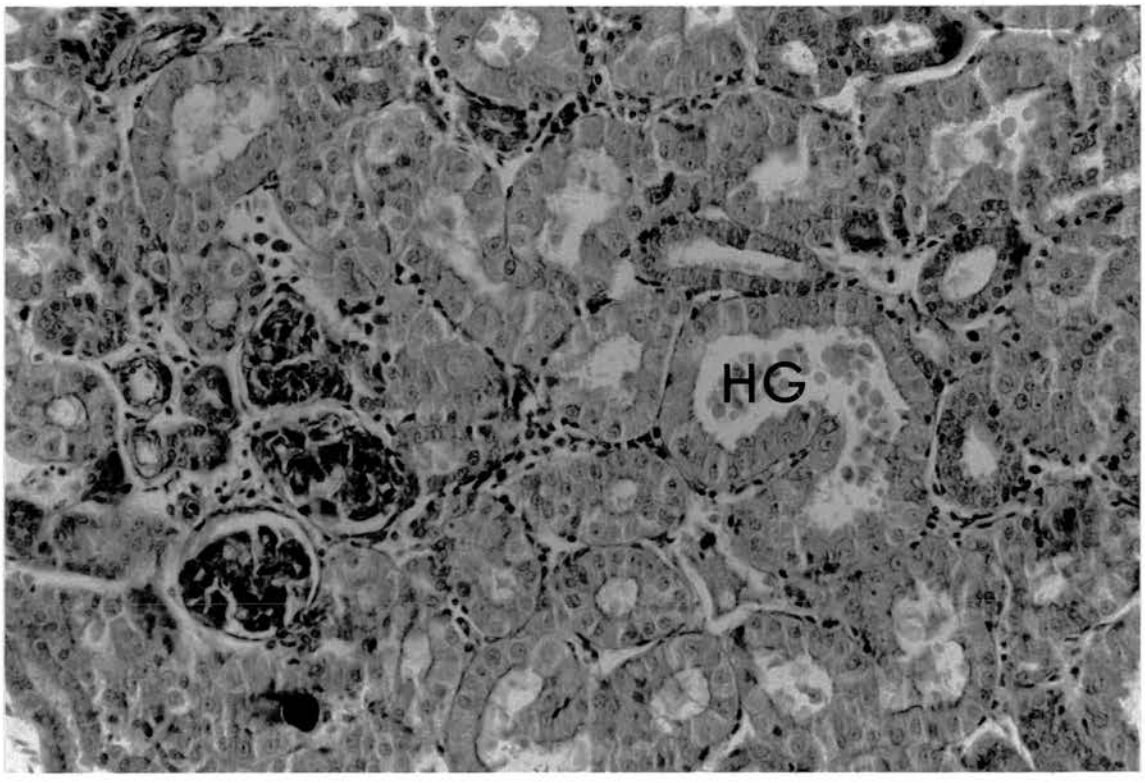


Fig.13b. PCT changes as in Fig.13a and the presence of hyaline globules (HG) in the lumen. H & E x 400.

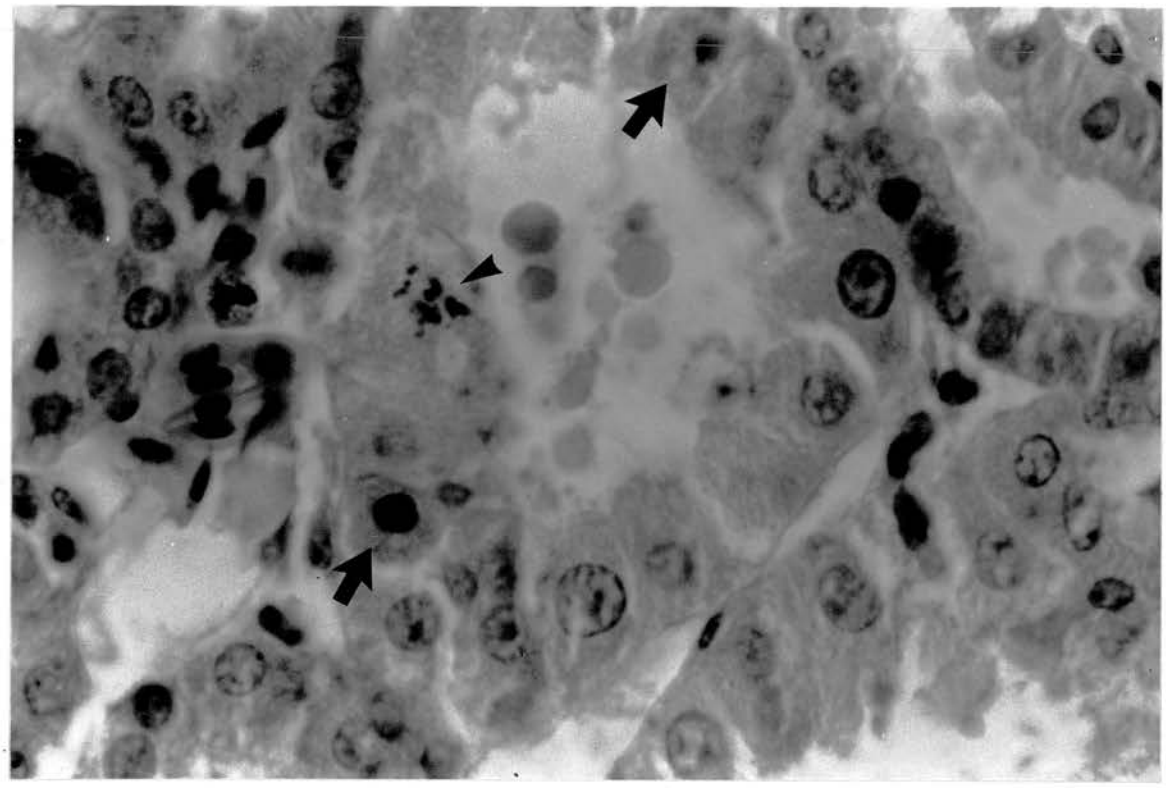


Fig.13c. Fowl. 3 w.o. (4ppm OA). Kidney. PCT. Individual cell necrosis. Note the presence of pyknotic (arrow) and karyorrhectic (arrowhead) nuclei in the tubular epithelium and hyaline globules in the lumen. H & E x 1580.

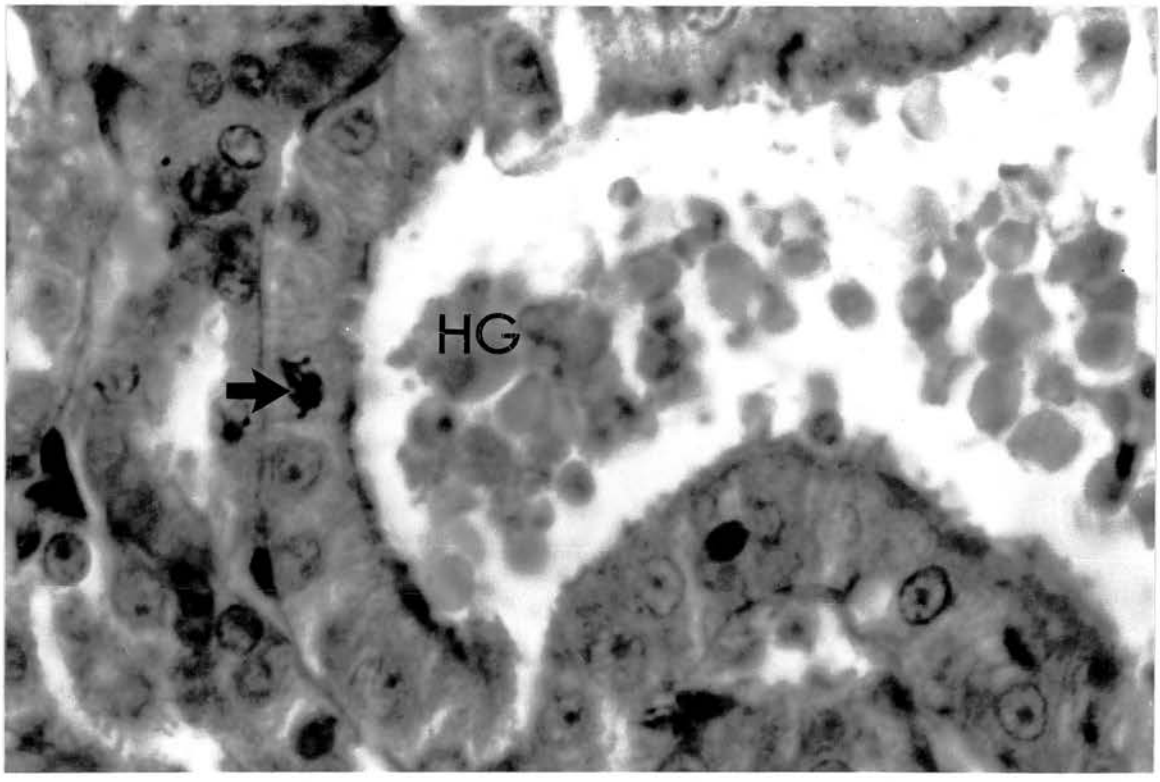


Fig.13d. An enlarged and hypertrophied tubule (Fig.13b) with a mitotic figure (arrow) in a cell and many hyaline globules (HG) in the lumen. Brush border is intact. PAS x 1580.

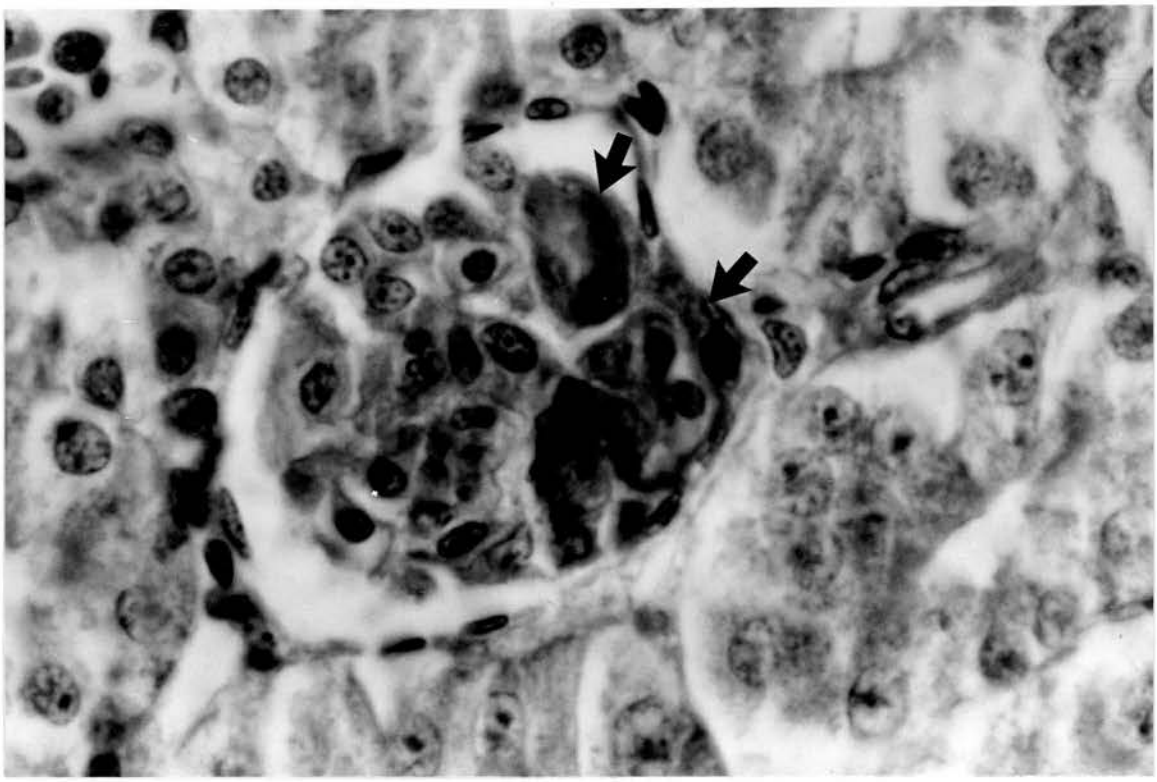


Fig.13e. Fowl. 3 w.o. (4ppm OA). Kidney glomerulus showing thickening of the glomerular basement membrane in the capillary loop (arrow). PAS x 1580.

and varied from a few to many. Nodular infiltrations of lymphoid cells were common. In some areas, the PCT were compressed and dense, with an increased number of cells per tubule. Very often at 4ppm OA, there was necrosis of individual cells in the PCT (Fig.13c), though other degenerative changes, except for ballooning of some cells, were not usually observed. Many mitotic figures (Fig.13d) were also seen in kidneys from OA-treated birds. The brush border was usually intact. Neither oedema nor inflammatory reactions were detected. In some birds, haemorrhages were seen in the parenchyma.

There was thickening of the glomerular basement membrane (GBM) in either one or more capillary loops; this was most easily seen in PAS-treated sections (Fig.13e). The glomerular basement membrane was thickened in 22, 29 and 2.5 per cent of glomeruli examined (491, 391 and 412) from birds given 2, 4 or 0ppm OA, respectively (Experiment I).

4.7.1.2 Liver

Changes varied from slight degeneration to vacuolation of the hepatic cells (Fig.14a). Sometimes a perivascular inflammatory (heterophilic) reaction and nodular lymphoid infiltration were seen. Some congestion and haemorrhage were also evident. Glycogen was increased throughout the liver tissue (Fig.14b), although this was more marked at the periphery of lobules. The increase appeared to be dose-related. In some birds, the central vein appeared to be broken and many hepatic cells were seen in the lumen of the blood vessel (Fig.14c).

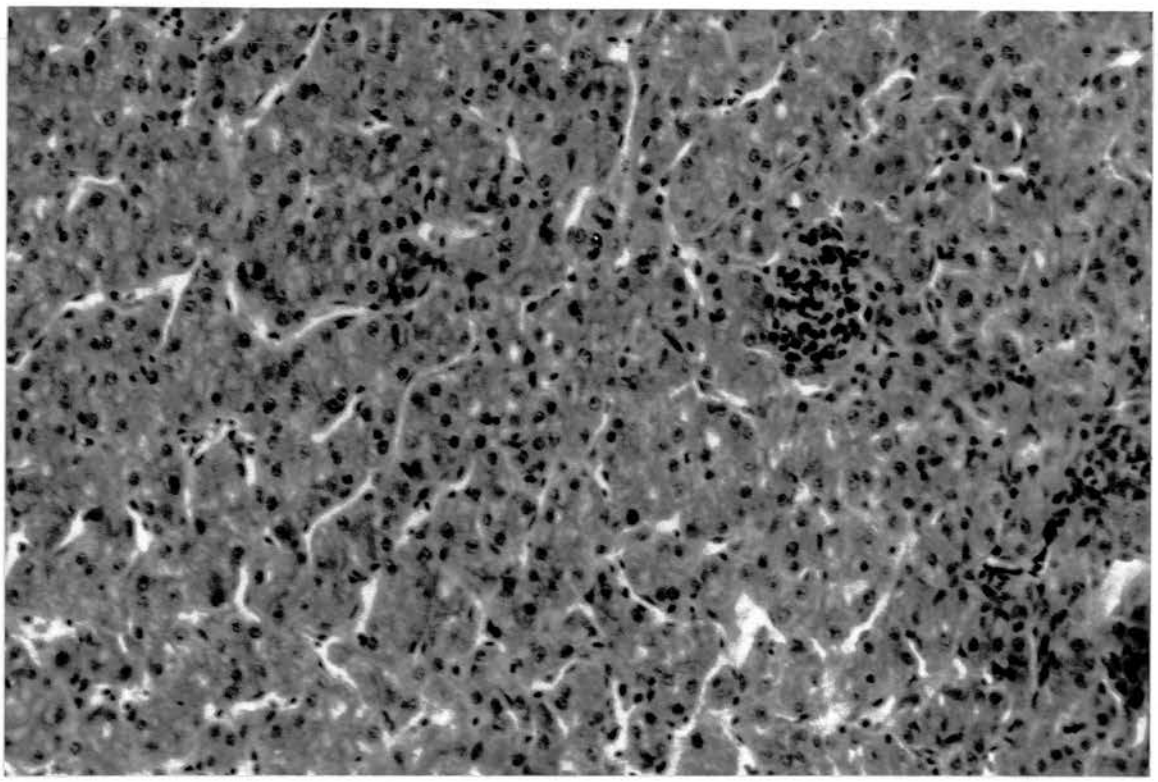


Fig.14a. Fowl. 3 w.o. (2ppm OA). Liver. Note the vacuolated hepatic cells and also the presence of a lymphoid nodule. H & E x 400.

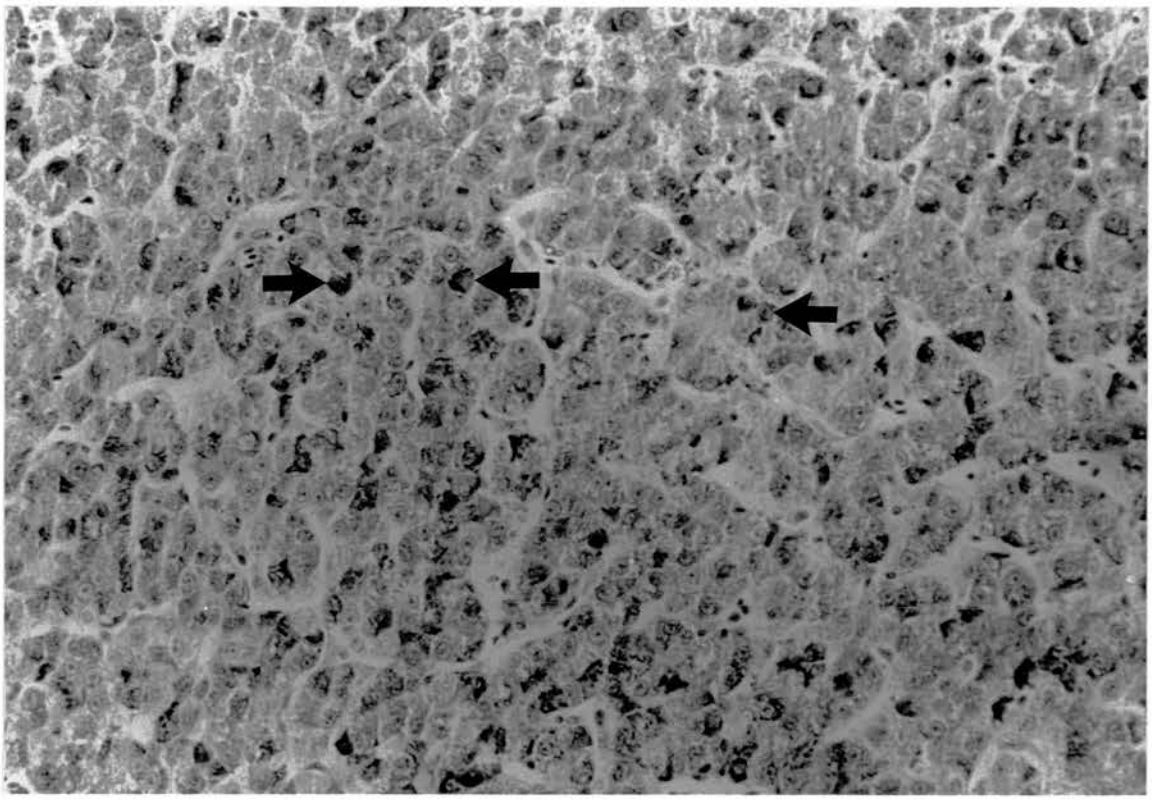


Fig.14b. Fowl. 3 w.o. (4ppm OA). Liver showing the accumulation of glycogen (arrow) in the hepatic cells. Best's Carmine x 400.

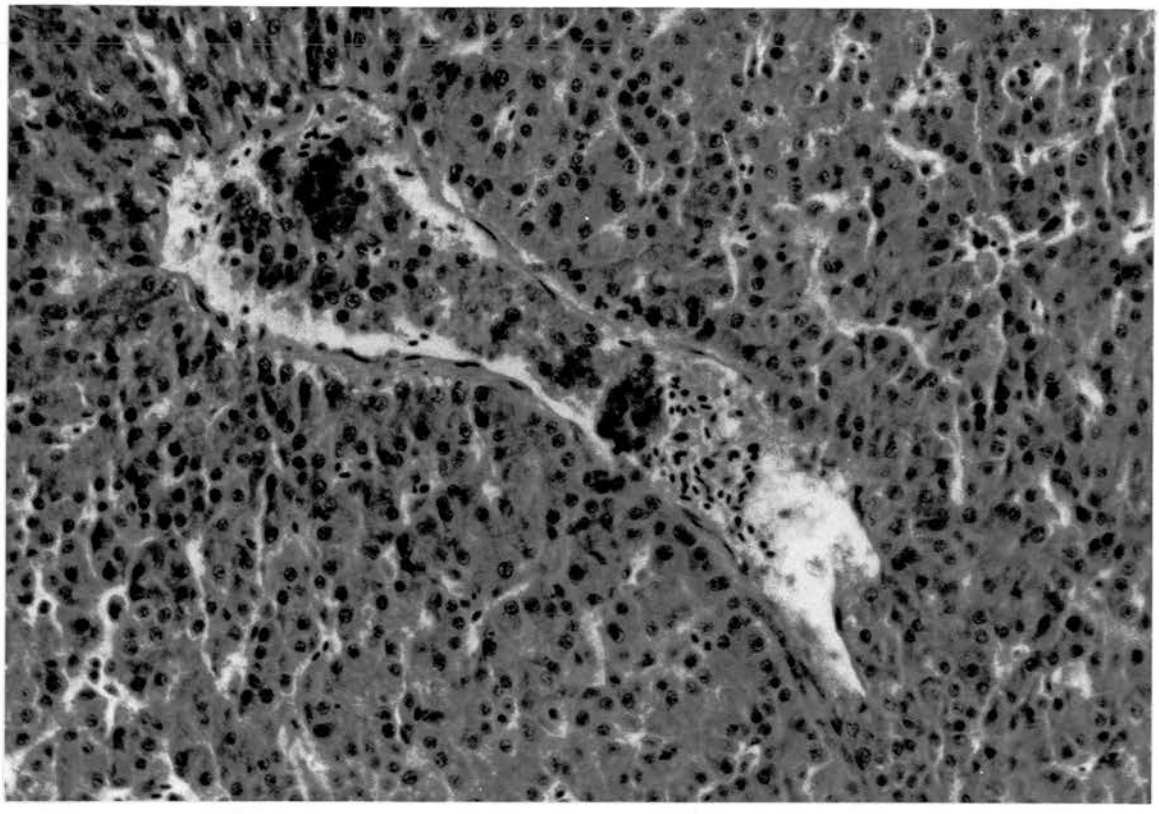


Fig.14c. Fowl. 3 w.o. (2ppm OA). Liver showing a break in the continuity of a blood vessel and the presence of hepatocytes in the lumen. H & E x 400.

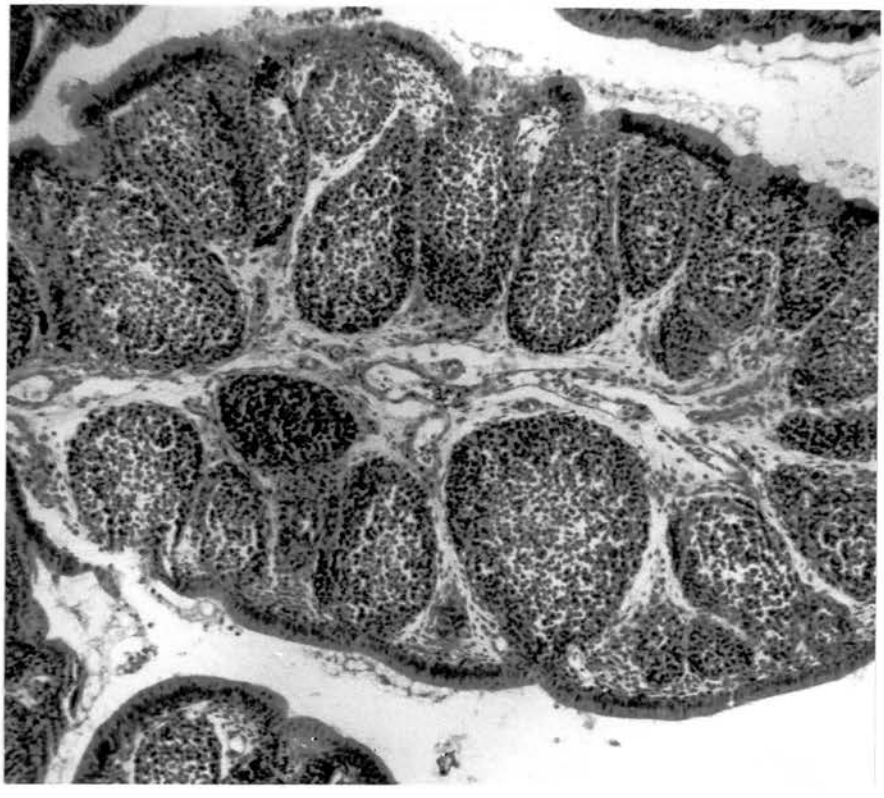


Fig.15a. Fowl. 3 w.o. (4ppm OA). Bursa of Fabricius showing general lymphoid cell depletion and reduction in the size of follicles. The epithelium is unaffected. H & E x 154.

4.7.1.3 Bursa of Fabricius

There was severe lymphoid depletion from the follicles (Figs.15a,15b and 15c), which appeared small and rounded and were often surrounded by fibrous connective tissue (Fig.15b).

The increased cellularity of the subepithelial and inter-follicular areas was due to heterophils and mononuclear cells, including plasma cells and lymphocytes. Haemorrhage had occurred in some cases. However, the epithelium appeared to be intact and unaffected by the administration of OA (Fig.15a).

4.7.1.4 Thymus

There was depletion of lymphoid cells from the thymus. This was more apparent in the cortex than in the medulla (Figs.16b and 16c) and often occurred together with some congestion, haemorrhage and heterophilic infiltration (Fig.16d). The clear-cut distinction between the cortex and medulla as seen in controls (Fig.16a) was often indistinct in OA-treated birds.

4.7.1.5 Spleen

There was a marked reduction in the numbers of lymphocytes in the white pulp (Figs.17a and 17b). In some areas the distinction between red and white pulp had become obscure. Germinal centres were reduced both in number and in size in sections from OA-treated birds (Fig.17c).

4.7.1.6 Harderian gland

The Harderian gland from control fowls contained a

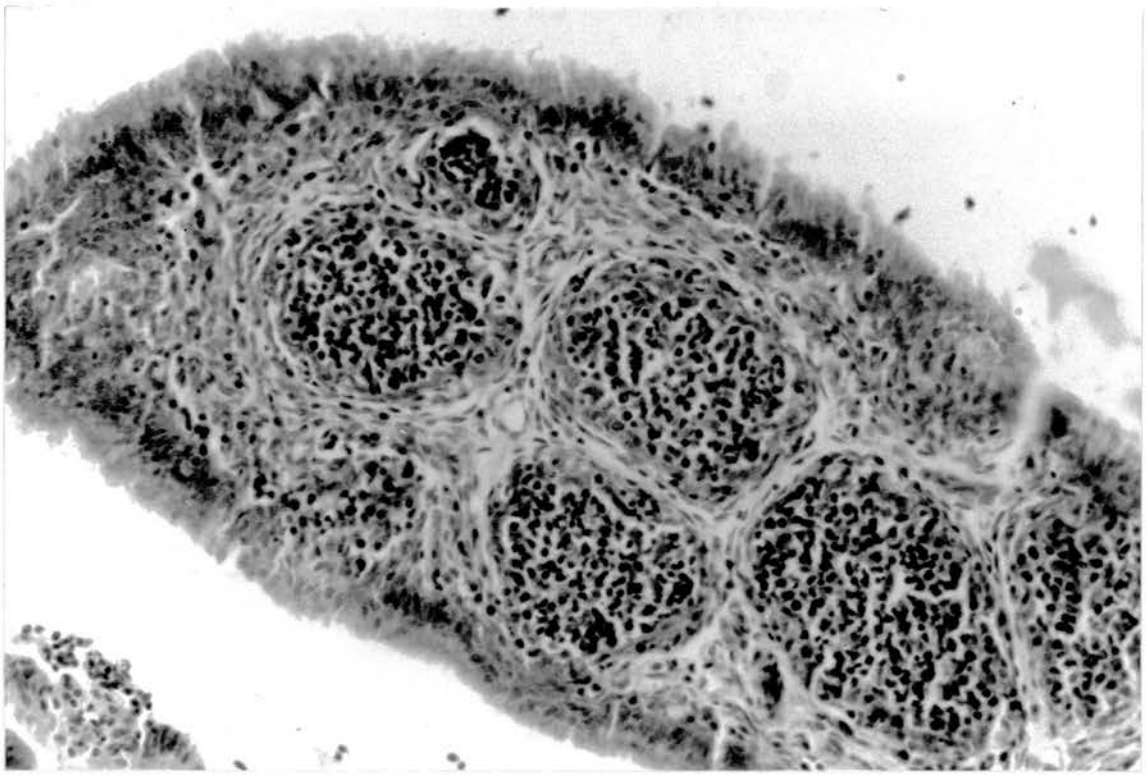


Fig.15b. Fowl. 3 w.o. (4ppm OA). Bursa. Small,round,depleted lymphoid follicles surrounded by fibrous connective tissue. Indistinct cortico-medullary demarcation and increased interfollicular cellularity. H & E x 400.

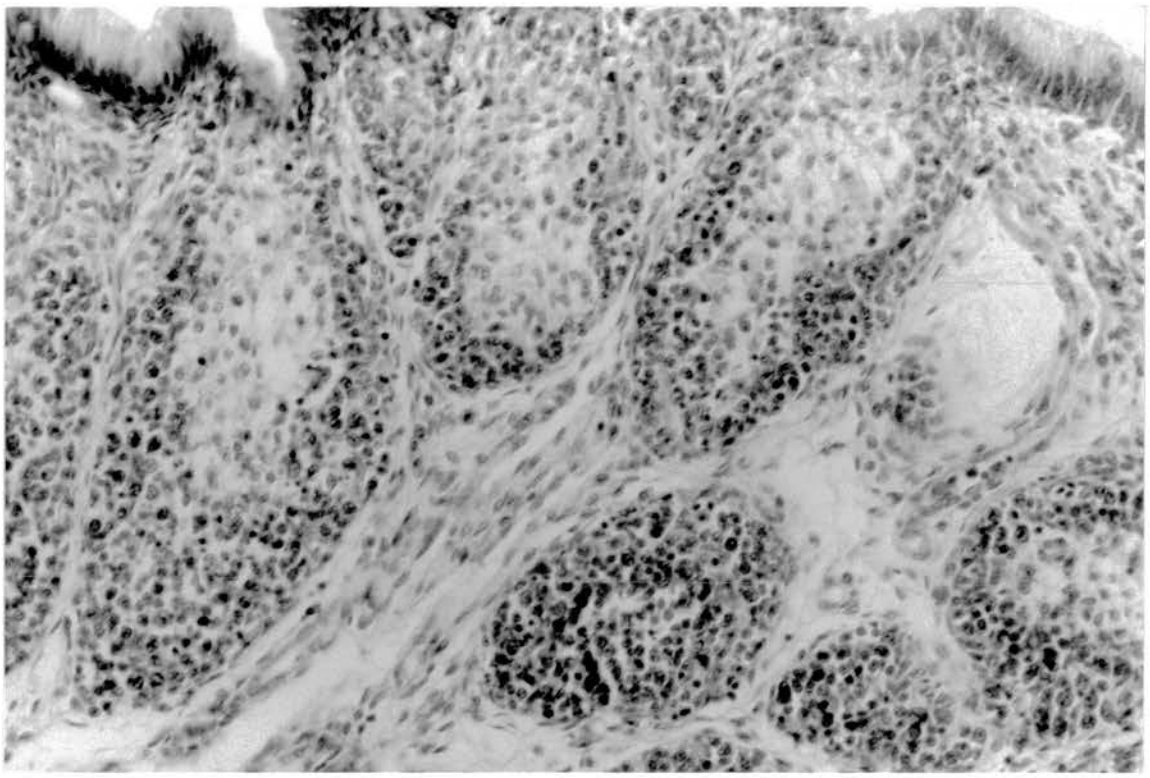


Fig.15c. Fowl. 2 w.o. (2ppm OA). Bursa showing severe depletion of lymphoid follicles. H & E x 400.

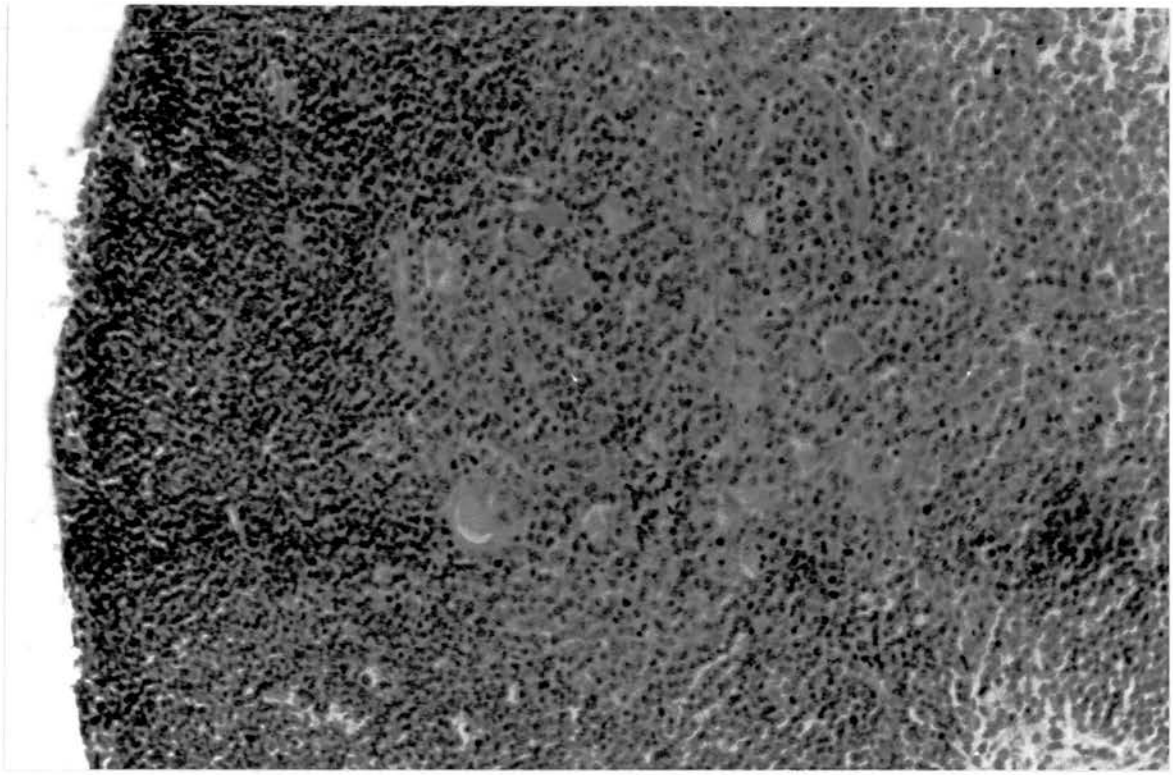


Fig.16a. Fowl. 3 w.o. (Control). Thymus. Note thickly populated cortex distinct from thinly populated medulla. H & E x 400.

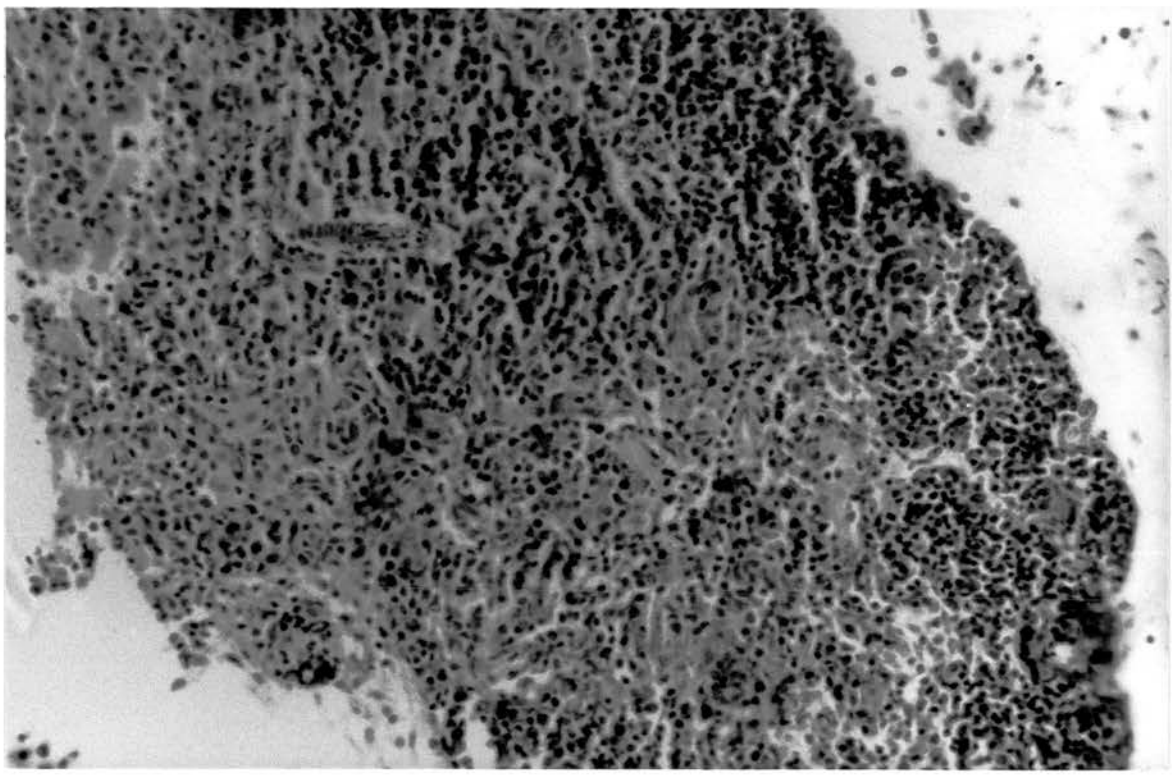


Fig.16b. Fowl. 3 w.o. (4ppm OA). Thymus. Note severely depleted cortex. Cortex and medulla are indistinguishable. H & E x 400.

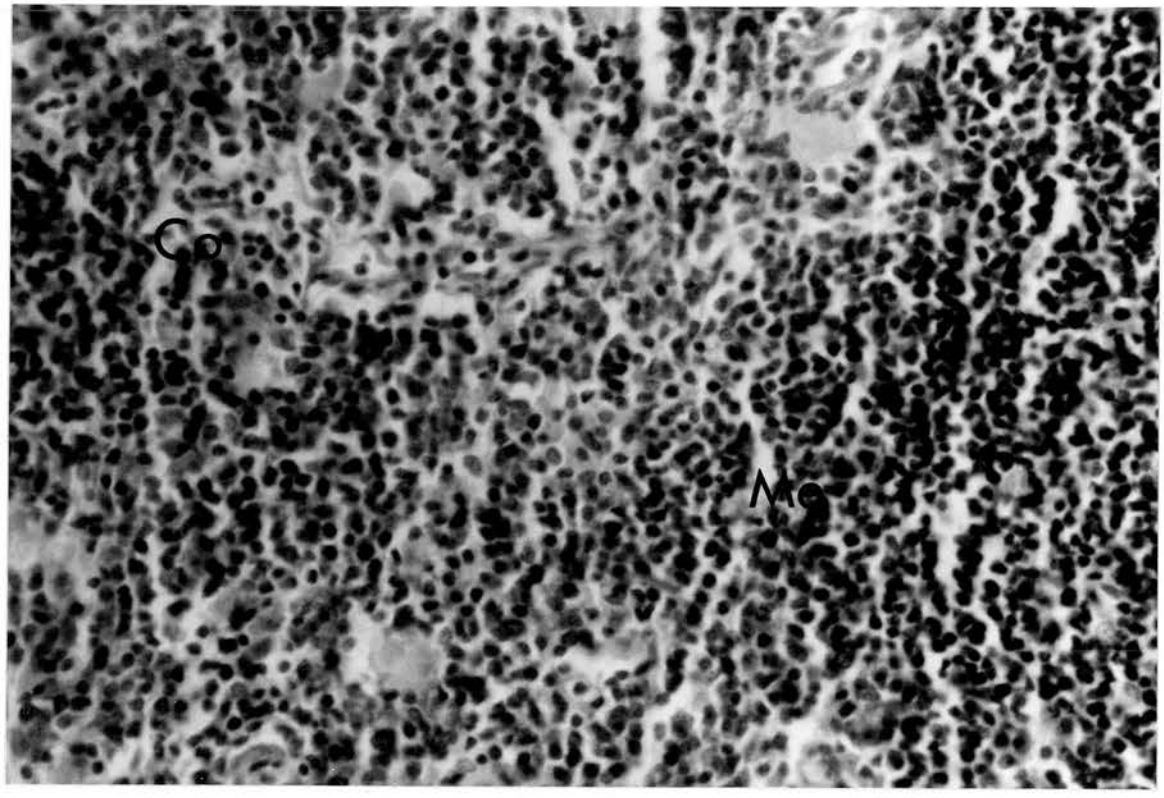


Fig.16c. Fowl. 3 wo.o. (2ppm OA). Thymus showing general depletion of lymphoid cells. Cortex (Co) and medulla (Me) are not well demarcated. H & E x 640.

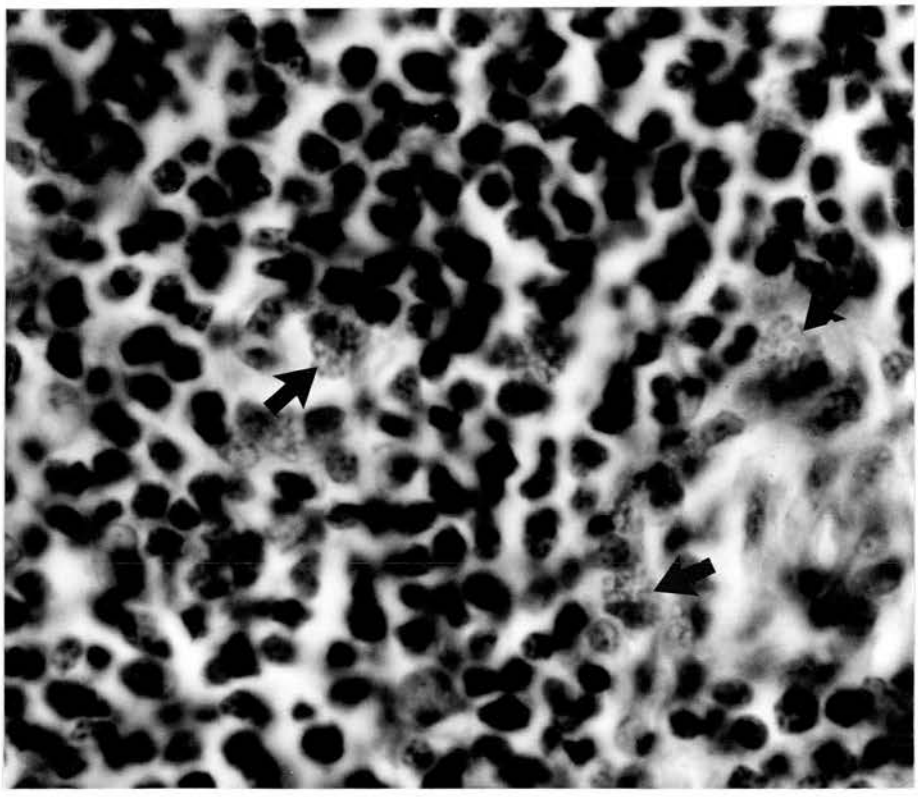


Fig.16d. Fowl. 3 w.o. (4ppm OA). Thymus. Note the presence of many heterophils in the cortex. H & E x 1580.

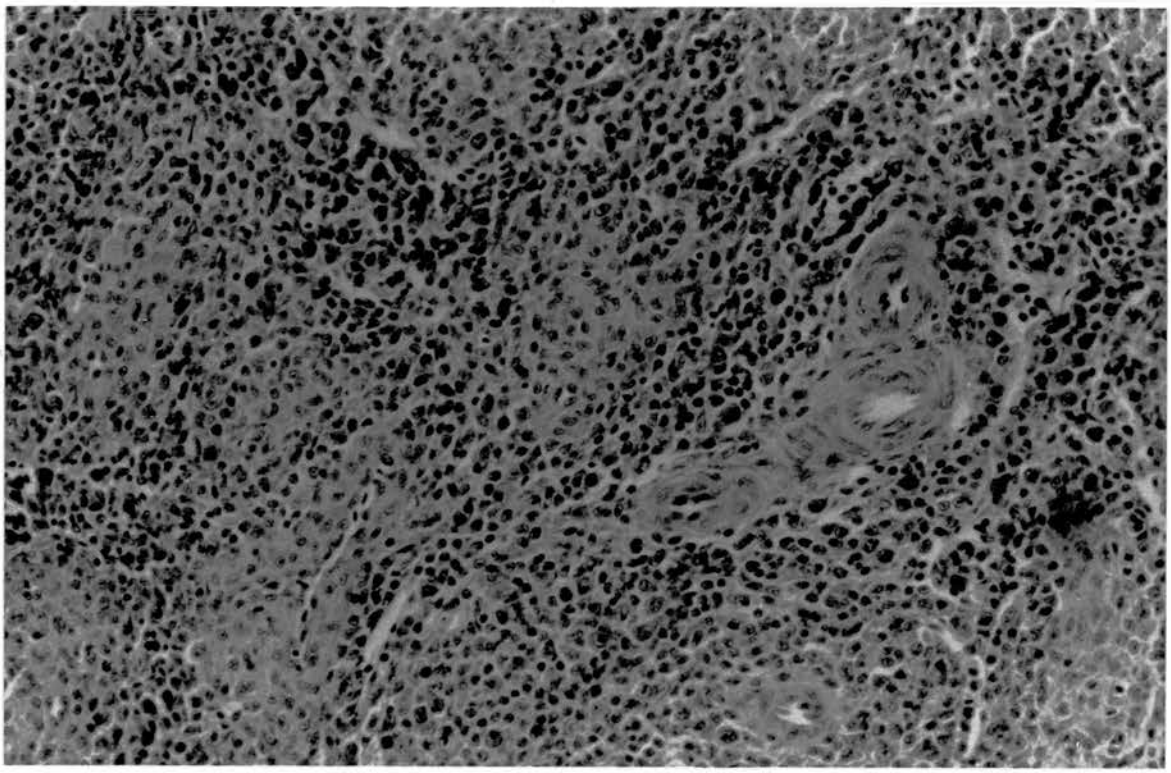


Fig.17a. Fowl. 3 w.o. (4ppm OA). Spleen. Note the depletion of lymphoid cells from the white pulp. H & E x 400.

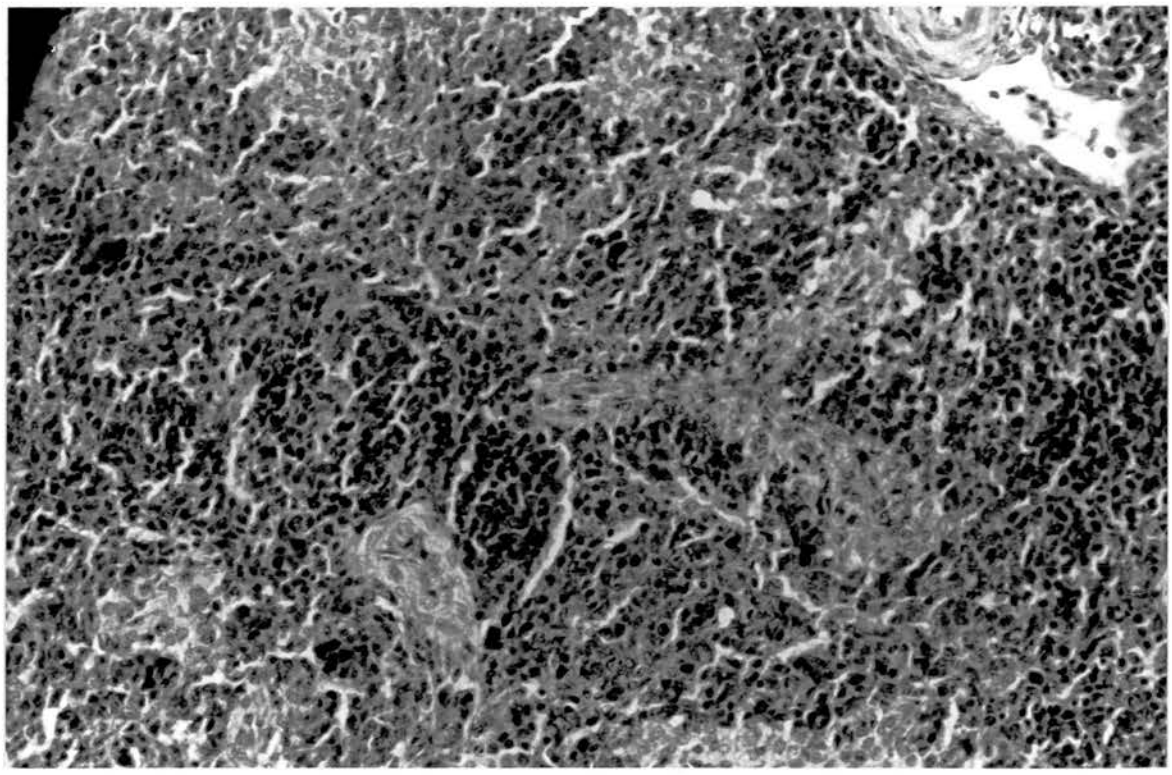


Fig.17b. Fowl. 3 w.o. (4ppm OA). Spleen showing depleted and disorganised white pulp area. H & E x 400.

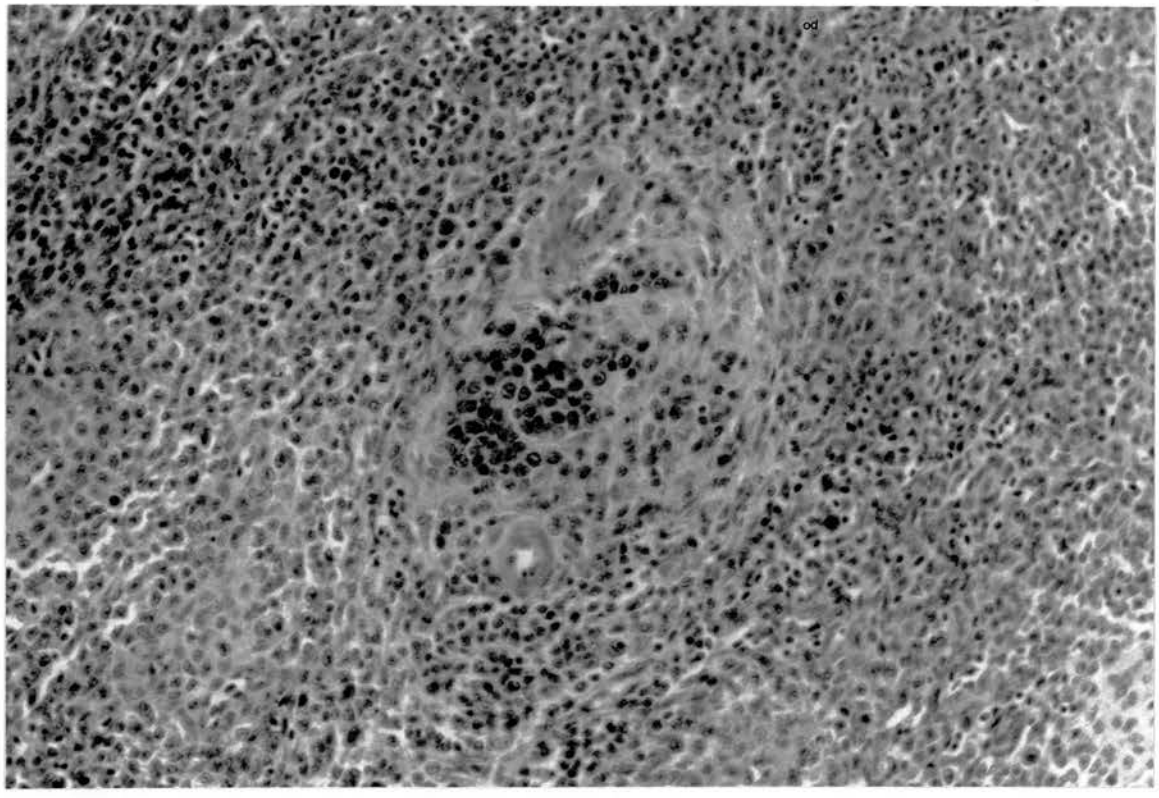


Fig.17c. Fowl. 3 w.o. (2ppm OA). Spleen showing lymphoid depletion in a germinal centre near a blood vessel and indistinguishable white pulp. H & E x 400.

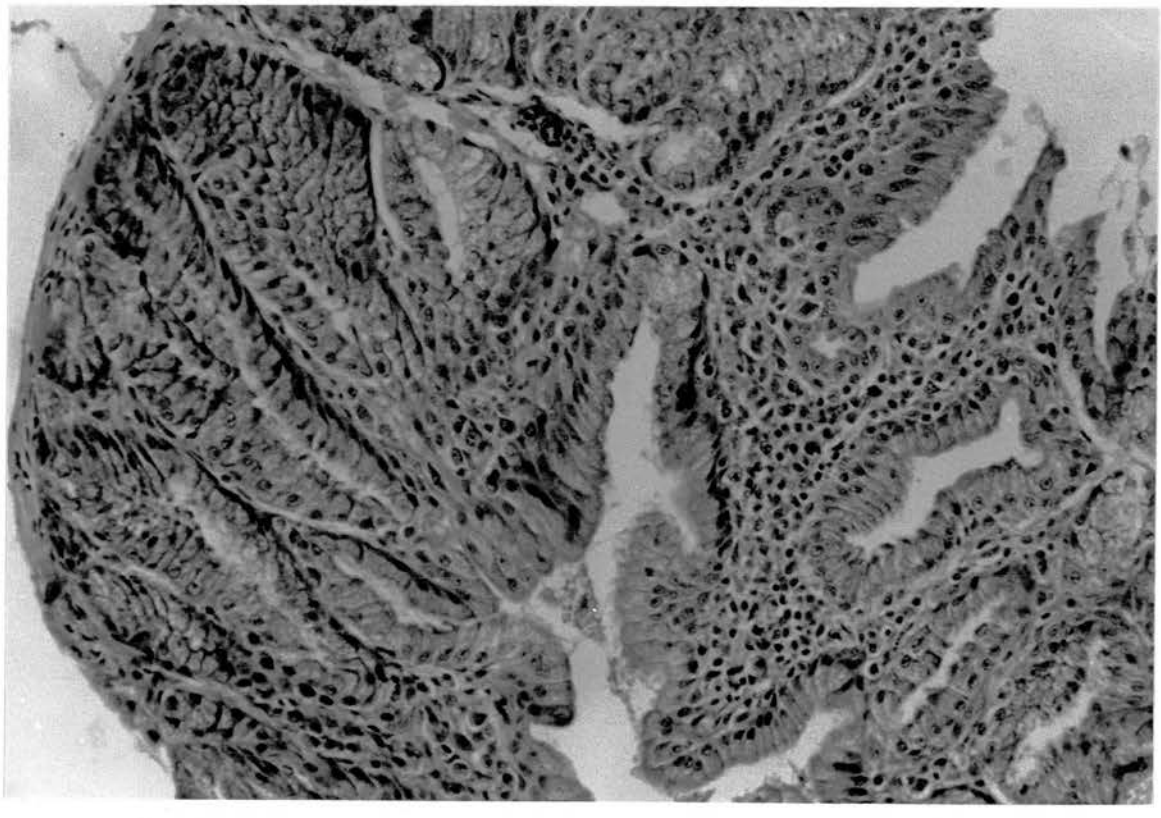


Fig.18. Fowl. 3 w.o. (control). Harderian gland. Note the presence of a large number of plasma cells in the subepithelial region. H & E x 400.

large number of plasma cells in the interstitium of the gland between both primary and secondary tubules (Fig.18). In sections of Harder's gland from OA-fed birds the plasma cell population was reduced and there was congestion, haemorrhage and sometimes an infiltration of heterophils. Frequently the epithelial lining of the secondary tubules was broken and plasma cells, epithelial cell debris and erythrocytes were present in the lumen (see Fig.90).

4.7.1.7 Intestinal lymphoid organs

In the Peyer's patches and caecal tonsils, there was some depletion of lymphoid cells from both the diffuse lymphoid tissue and from the germinal centres which were also reduced in number. Heterophilic infiltration was commonly seen in the lamina propria (Fig.19a), muscularis mucosa, submucosa and even in the muscular layers (Fig.19b). Heterophils were also seen in the intestinal glands and crypts. In some cases degeneration of the villi and glands was apparent as also were haemorrhages.

4.7.2 Turkeys

Similar changes but of decreased intensity were seen in various organs in turkeys.

In the kidney, there was dilatation of the proximal convoluted tubules, congestion, haemorrhage, lymphoid and heterophilic infiltrations and a thickening of the glomerular basement membrane. The lesions did not differ greatly at 10 weeks of age from those at 4 weeks of age.

Liver changes included vacuolation of the cytoplasm, congestion, haemorrhage and the presence of glycogen. The glycogen was found in increased concentrations at 10 weeks

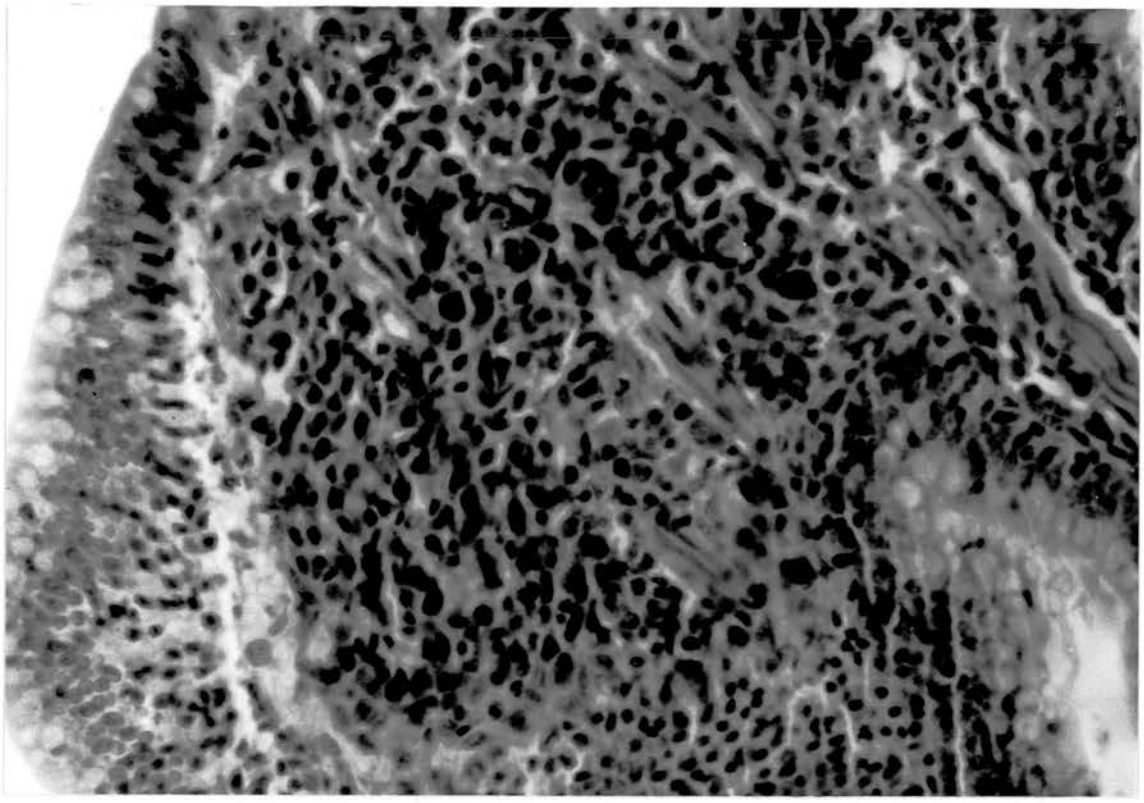


Fig.19a. Fowl. 3 w.o. (2ppm OA). Peyer's patch showing haemorrhage and heterophilic infiltration in the lamina propria. H & E x 640.

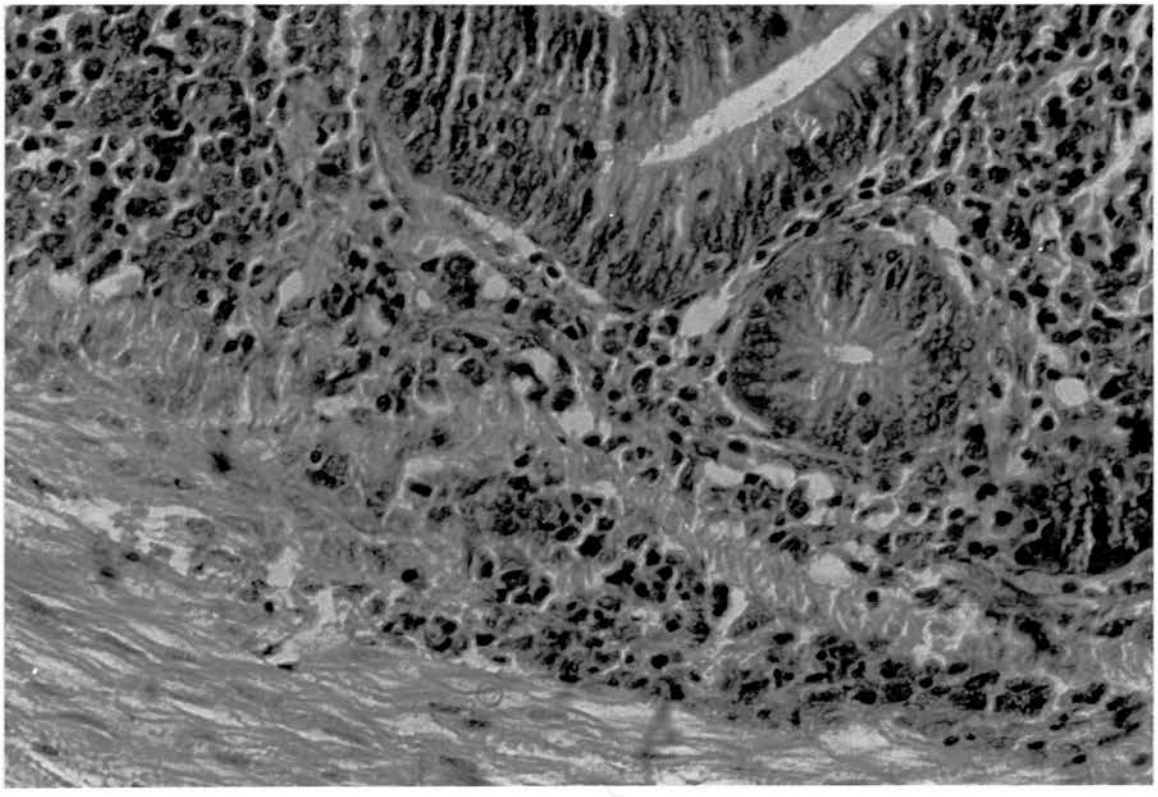


Fig.19b. Fowl. 3 w.o. (4ppm OA). Caecal tonsil. Note the presence of a large number of heterophils in the lamina propria, muscularis mucosa and submucosa. H & E x 640.

of age in comparison with the amount at 4 weeks of age.

In the bursa of Fabricius there was congestion, a moderate depletion of lymphoid cells from the follicles and some mitotic changes. Many pyknotic cells could be seen in the follicles. The epithelium was not affected.

The thymus had a depleted lymphoid population in both the cortex and medulla. Some heterophils were also seen in the medulla. In some areas the lymphoid depletion was so marked that only patches of lymphoid aggregates were seen giving the appearance of a germinal centre. Hassal's corpuscles were seen disintegrating in some cases.

The spleen appeared to show inconsistent changes, though lymphoid depletion could be noticed. Subcapsular haemorrhages were sometimes seen. The numbers of germinal centres were reduced in OA-treated turkeys.

No consistent changes were detected in the Harderian gland, though the plasma cell population appeared to be slightly reduced.

Peyer's patches and caecal tonsils had fewer germinal centres and depletion of lymphoid cells both in the diffuse lymphoid tissue and germinal centres. Congestion, haemorrhage and heterophilic infiltration could be seen occasionally. Heterophils were seen in and around the glands and in the lamina propria.

4.7.3 Quail

Quail tissues were examined after 11 weeks of OA feeding. Histopathological changes in various organs varied from mild to moderate.

The kidney occasionally showed dilatation of the proximal convoluted tubule, thickening of the glomerular basement membrane in some glomeruli, congestion and some lymphoid infiltration, particularly at 8ppm dietary OA level.

Liver cells were occasionally enlarged, more eosinophilic and had more fat vacuoles compared with controls. The bile ducts were prominent and in some areas a slight biliary hyperplasia was seen. Occasionally the blood vessels were congested. In PAS-stained sections increased glycogen was found in a dose-related fashion, both in small round granular and in large particle forms.

In the bursa of Fabricius, thymus and spleen, there was a slight lymphoid depletion. In the spleen, the lymphoid follicles (periarteriolar adenoid sheaths) were very close to each other. In Peyer's patches and caecal tonsils a distinct depletion of lymphoid elements could be seen in the germinal centres and diffuse lymphoid tissue.

4.8 Ultrastructural Changes

Sequential ultrastructural changes in the kidneys, livers and the small intestines, particularly Peyer's patches were studied in broilers and turkeys, from 2 weeks after 4ppm OA-feeding up to 8 or 10 weeks at 2 week intervals. In addition, studies were made in broilers, at 3 weeks after feeding OA at 2 and 4ppm levels.

4.8.1 Broilers

4.8.1.1 Kidney

2 Weeks

Proximal convoluted tubules(PCT). PCT from untreated control birds were characterised by a brush border of microvilli with their associated structures lining the lumen and by epithelial cells containing nuclei and cytoplasmic organelles, such as densely packed mitochondria, smooth and rough endoplasmic reticulum, peroxisomes (microbodies), lysosomes etc. A few lipid droplets (ld) were also occasionally seen in the cytoplasm (Fig.21a).

In kidneys from OA-treated birds changes were seen in various constituents of the PCT cells, particularly the mitochondria.

Mitochondria. Light and dark mitochondria were seen in the majority of PCT cells. Dark mitochondria were relatively normal but the light ones were swollen occupying most of the cell. Light mitochondria were degenerate with few cristae (Fig.20a and 20b). Some mitochondria also showed a ring-shaped transformation. Distal convoluted (DCT) and collecting tubules (CT) were not greatly altered, though occasionally a few collecting tubule cells contained one or two ring-form mitochondria.

Glomerulus. In untreated fowls, the centre of the glomerular tuft contained a mass of mesangial cells around which the capillary loops were arranged. The glomerular capillary consisted of epithelial foot processes, glomerular basement membrane (GBM) and endothelial cell. The cytoplasm of the endothelial cells was continuous with the GBM throughout the internal surface of the capillary loops in the form of an uninterrupted delicate, thin, even membrane containing some fibrillary components in the dense central core (Fig.26a).

Many glomeruli in the OA-treated fowls had thickened GBM in the capillary loops.

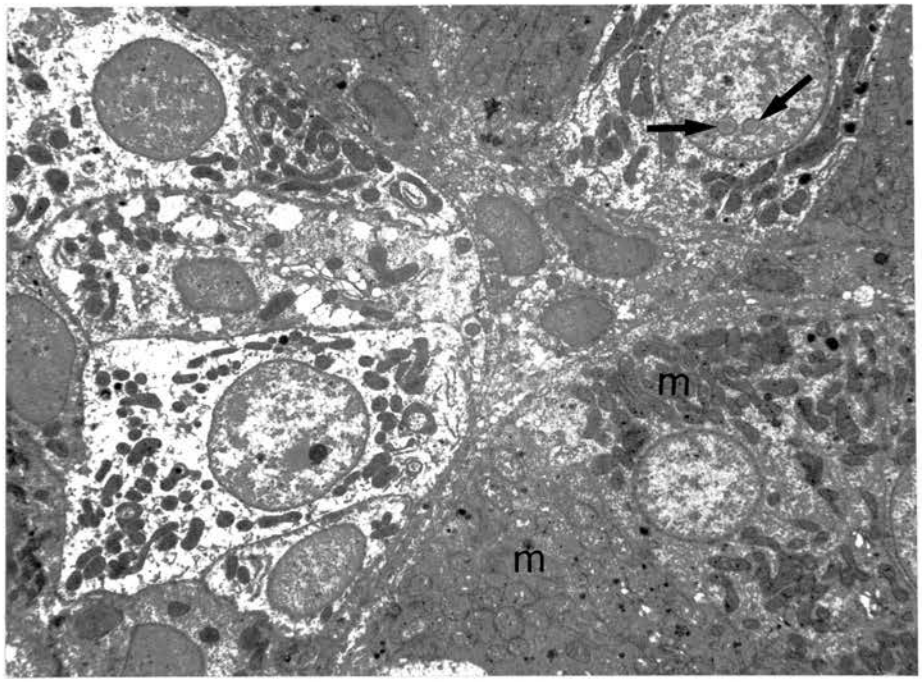


Fig.20a. Fowl.2w.o.(4ppm OA). Kidney.Proximal convoluted tubule(PCT) epithelial cell showing light and dark mitochondria(m).Dark mitochondria are relatively normal; light ones are swollen,rounded up and occupy most of the space. In the CT epithelial cell some ring forms of mitochondria are seen. Intranuclear lipid droplets (arrow) are also present in one of the cells. x 4374.

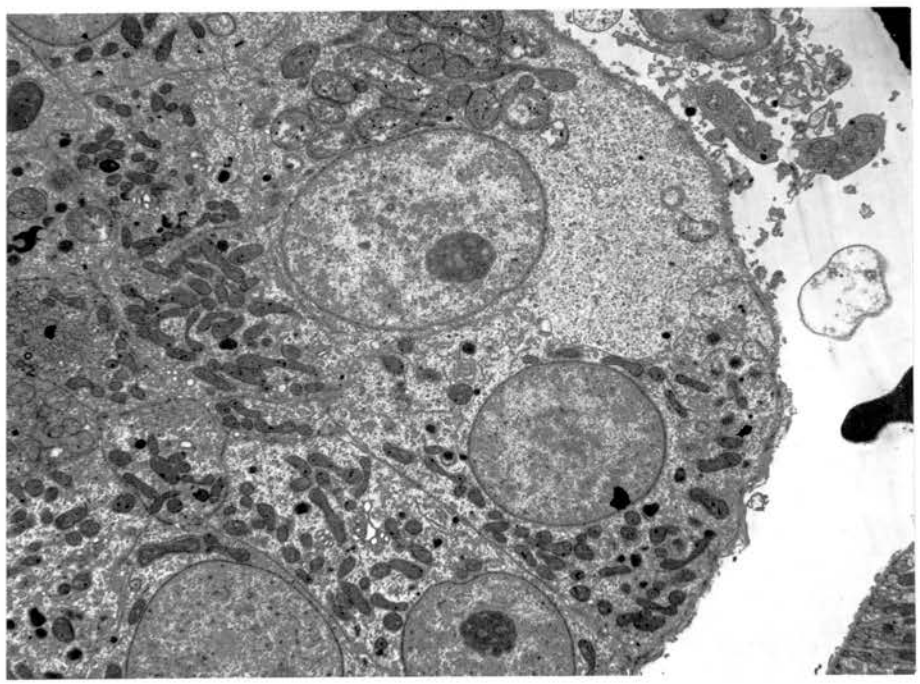


Fig.20b. Fowl. 2 w.o. (4ppm OA). Kidney. PCT epithelial cell showing degeneration and loss of cristae and increase in number of dense granules in the swollen mitochondria.x 4374.

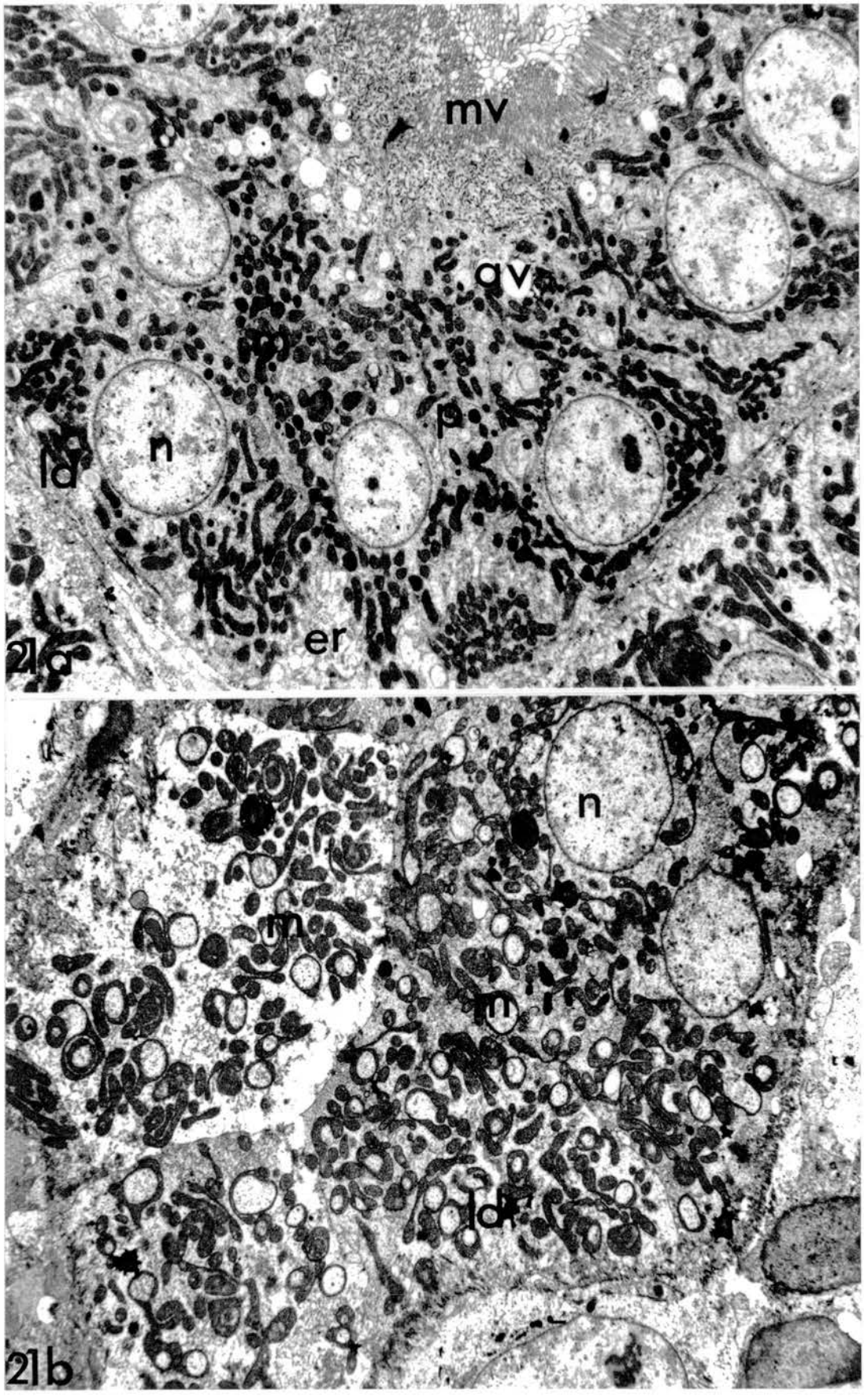


Fig.21. Kidney. Proximal convoluted tubules(PCT).(a)PCT from an untreated control fowl showing the normal structure x 4374.(b)PCT from an OA-treated(2ppm) fowl(3w.o.) showing U-,C-,cup,signet-ring and doughnut-mitochondria and lipid droplets.x 4374. mv microvilli; n nucleus; m mitochondria; av apical vacuoles; ld lipid droplets; er endoplasmic reticulum; p peroxisomes.

3 weeks

PCT. Mitochondria. The majority of the mitochondria were abnormal in shape. They were strikingly pleomorphic , being often enlarged, distended or elongated. Many of the elongated forms were bent upon themselves taking on a "U" or "ring" form (Fig.21b). Fusion of the membranes was often observed where the ends of the mitochondria met (Figs.22d and 22e). The spaces (vacuoles) within such ring forms were lined by a double membrane and appeared either empty or contained cytoplasmic material, peroxisomes or ld. They were placed either centrally (Fig.22a) or eccentrically (Figs.21b, 22b and 22f) so making the mitochondrion appear uneven in thickness. Such mitochondria had normal matrices but often tightly-packed cristae. In extreme cases, one ring form could be found within another so causing concentricity or a chondriosphere (Fig.21b) and mitochondria were recognisable within membrane-bound cytolysosomes (Figs.22e and 22f).

The matrices of the mitochondria contained electron-dense mitochondrial granules which were more prominent and numerous in the affected PCT (Figs.23a and 23b). These granules varied from 45 to 117 nm in size (they measured 30 to 50 nm in sections from normal birds) and could be observed anywhere in the matrix (Fig.23b). They also occurred in the ring-form mitochondria (Fig.22c).

The numbers of mitochondria appeared to be reduced in many affected PCTs (Figs.25a and 25b).

Peroxisomes. Peroxisomes were increased in number and size in ochratoxicosis. Their size varied from 0.13 to 0.9 um (normal: 0.1 to 0.5 um) and were round, oval or elongated and bound by a single membrane (Fig.23a). Their granular matrix contained a crystalline nucleoid and lacked periodicity (Fig.23a inset).

Fig.22a-f. Pleomorphic mitochondria in PCT epithelial cells from an OA-treated (4ppm) fowl (3 w.o.).

22a. Annular mitochondrion of uniform thickness. x 51840.

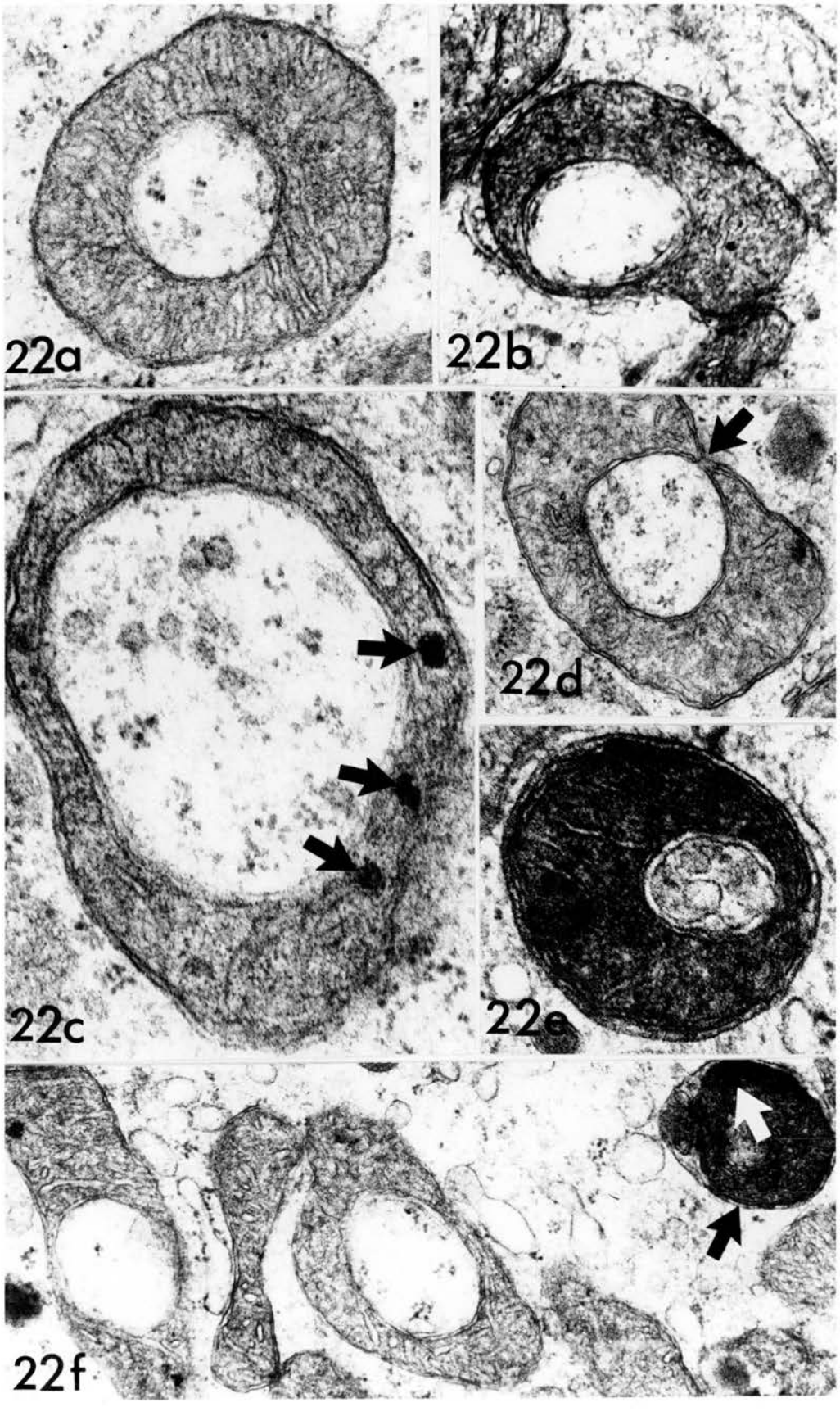
22b. A 'signet-ring' mitochondrion of uneven thickness. x 38880.

22c. A 'signet-ring' mitochondrion containing three large mitochondrial dense granules (arrows) with large central hole. x 82080.

22d. Fusion of the tips of a curved mitochondrion (arrow) forming a doughnut shape. x 38080.

22e. A double-membrane-bound doughnut mitochondrion (cytolysosome) enclosed within a double-membraned structure and thereby revealing four closely-associated membranes. x 82080.

22f. Ring-form mitochondria with 'holes' centrally or eccentrically placed. Note the presence of a cytolysosome (arrow) enclosing a doughnut mitochondrion in the process of fusion with two prominent dense granules (white arrow). x 38880.


22a
22b
22c
22d
22e
22f

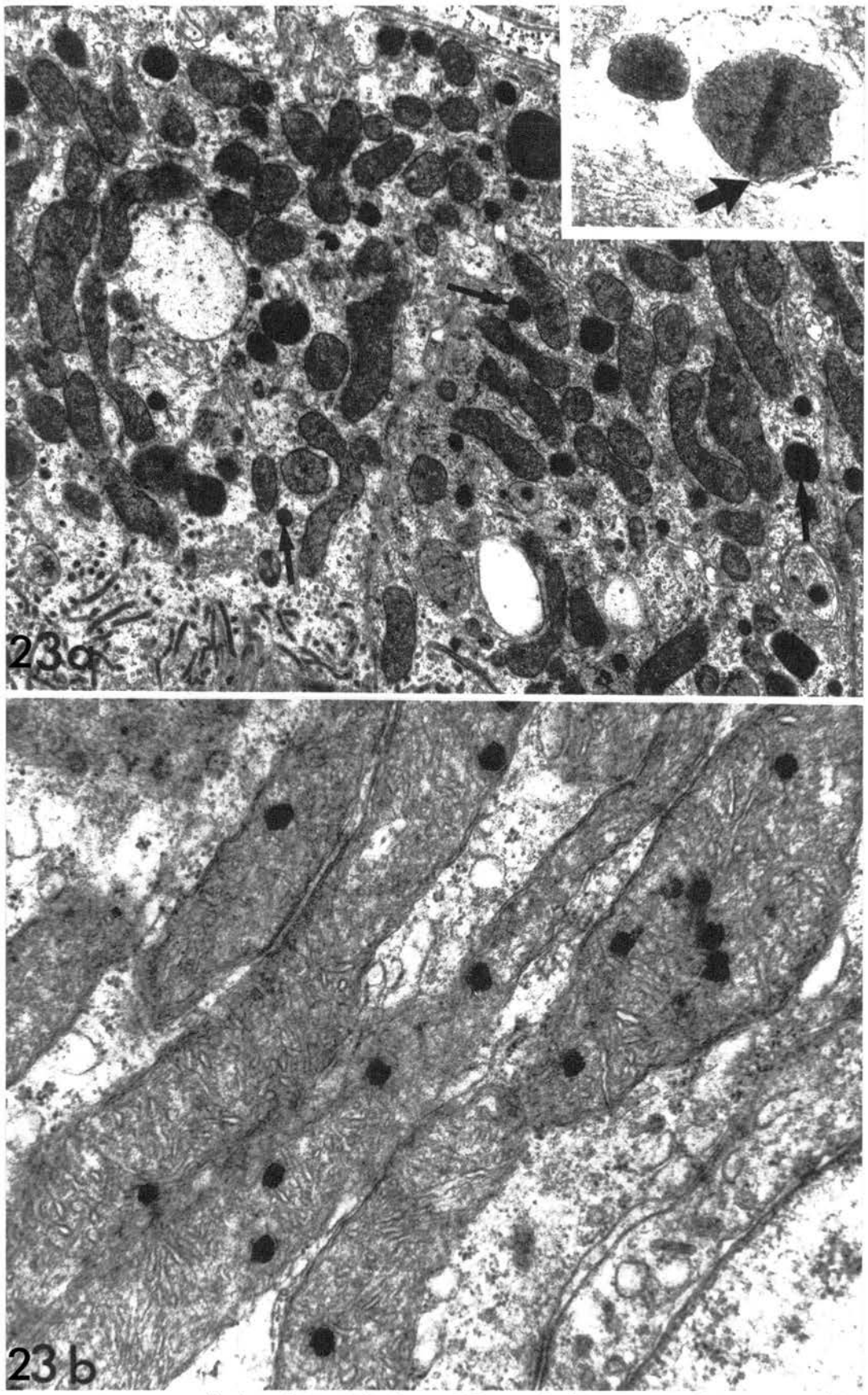


Fig.23. (a&b) Kidney. (a)Epithelial cells of the PCT from an OA-treated fowl.Note the presence of large numbers of peroxisomes (arrow) and prominent mitochondrial dense granules.x 11664. The inset shows a peroxisome with a crystalline rod-like nucleoid (arrow).x 38880.(b) Elongated mitochondria from PCT cells.Note the presence of numerous large electron-dense mitochondrial granules. x 38880.

Lysosomes. The lysosomes of affected PCT were normal in appearance, number and size which ranged from 0.93 to 1.7 um.

Endoplasmic reticulum. The smooth endoplasmic reticulum (SER) appeared dilated and contained round electron-dense bodies of variable size (Fig.24).

Cytoplasmic lipid droplets (ld). Numerous ld ranging in size from 0.3 to 2.0 um were seen in the cytoplasm. They were usually more numerous towards the basal part of the cells, round, oval or irregular in shape and they had a homogeneous structure with a moderately electron-dense and characteristic striated (wavy) appearance (Figs.24 and 25a). They were enclosed by an irregular dense osmiophilic border with a distinct interface. Some ld were also found in the basal processes and in the interstitium.

Golgi bodies and other cytoplasmic organelles did not appear to be affected in ochratoxicosis.

Nucleus. Nucleoli were prominent in some of the PCT epithelial cells from OA-treated birds. Some of the nuclei contained lipid inclusions (ld) measuring from 0.03 to 0.76 um in diameter. Their shape varied from round or oval to irregular with a dense osmiophilic border (Fig.25b). Usually ld were seen at the periphery of the nucleus in close proximity to the nuclear membrane (Fig.25b inset).

Distal convoluted tubules (DCT). Some DCT were also affected showing a decrease in the number of mitochondria, vacuolation of the cytoplasm and some ld. Collecting tubules, collecting ducts and medullary loops showed no apparent deviation from the normal.

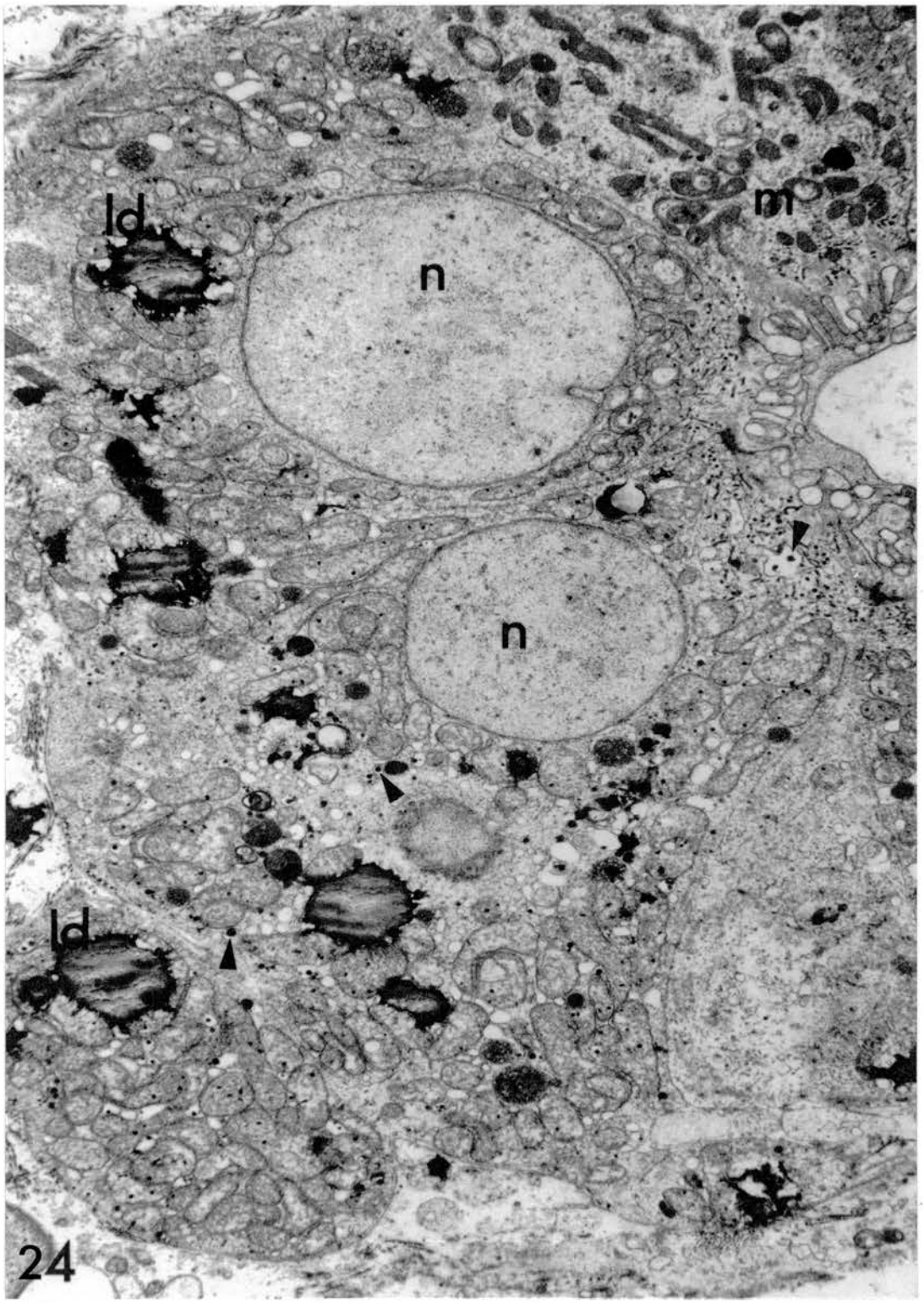


Fig.24. Kidney PCT epithelial cells from an OA-treated fowl showing numerous cytoplasmic lipid droplets (ld) some of which mask the organelles. Note the crenated, irregular, dense osmiophilic border of the ld. A large number of electron-dense small round bodies are in the dilated SER profiles (arrowheads). Doughnut mitochondria (m) are also present adjacent to the nucleus (n). x 8208.

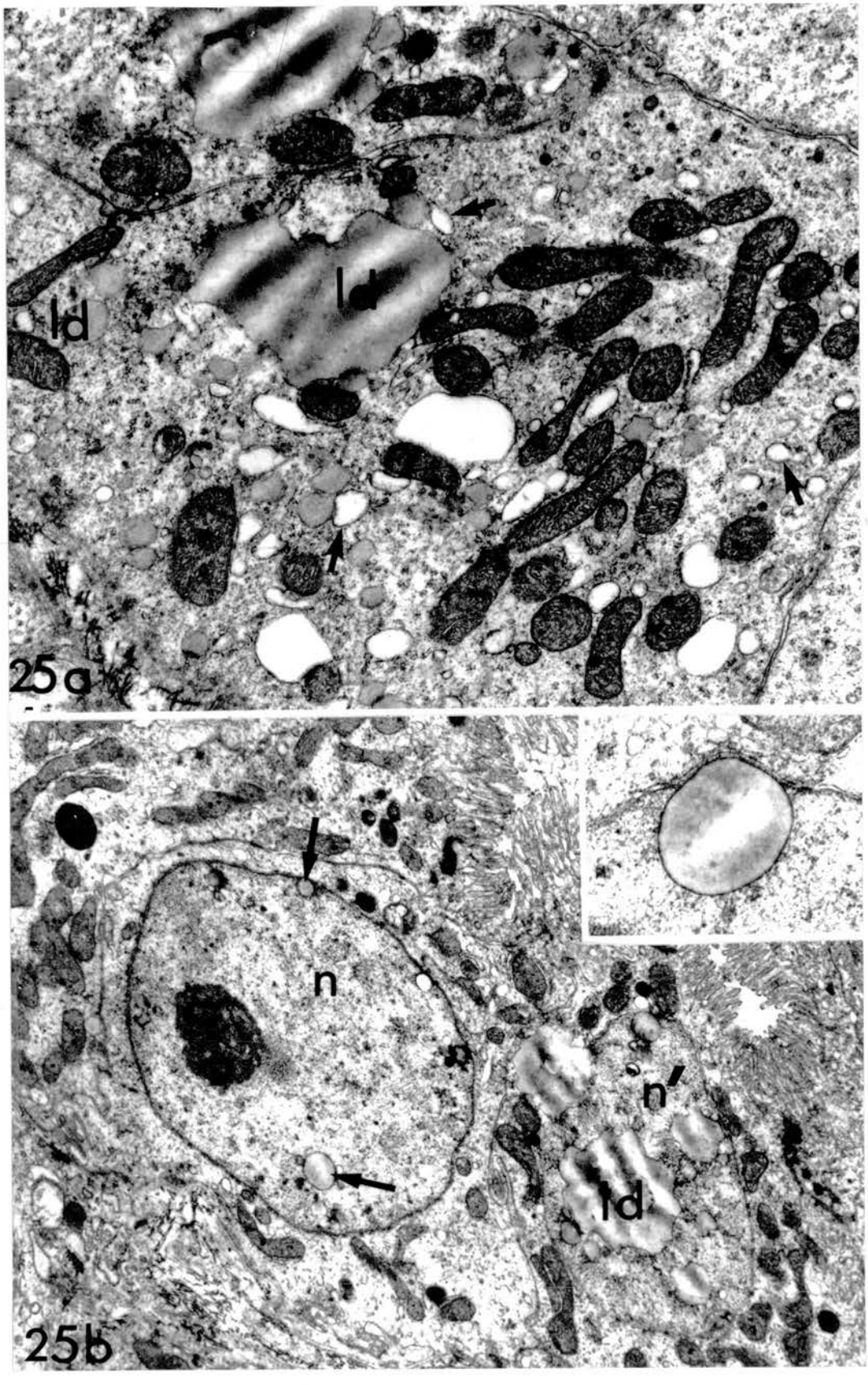


Fig.25. (a&b) Kidney.(a)An epithelial cell(PCT) from an OA-treated fowl showing characteristic small and large sized ld. Note the dilated ER (arrows) containing small ld. x 18792. (b)PCT cells with several round lipid inclusions at the periphery of the larger nucleus (n). The smaller nucleus (n')contains irregular ld (up to 0.5µm).Note the scarce mitochondrial population.x 9396. The inset shows a large ld (o.76µm),adjacent to the nuclear membrane. x 25056.

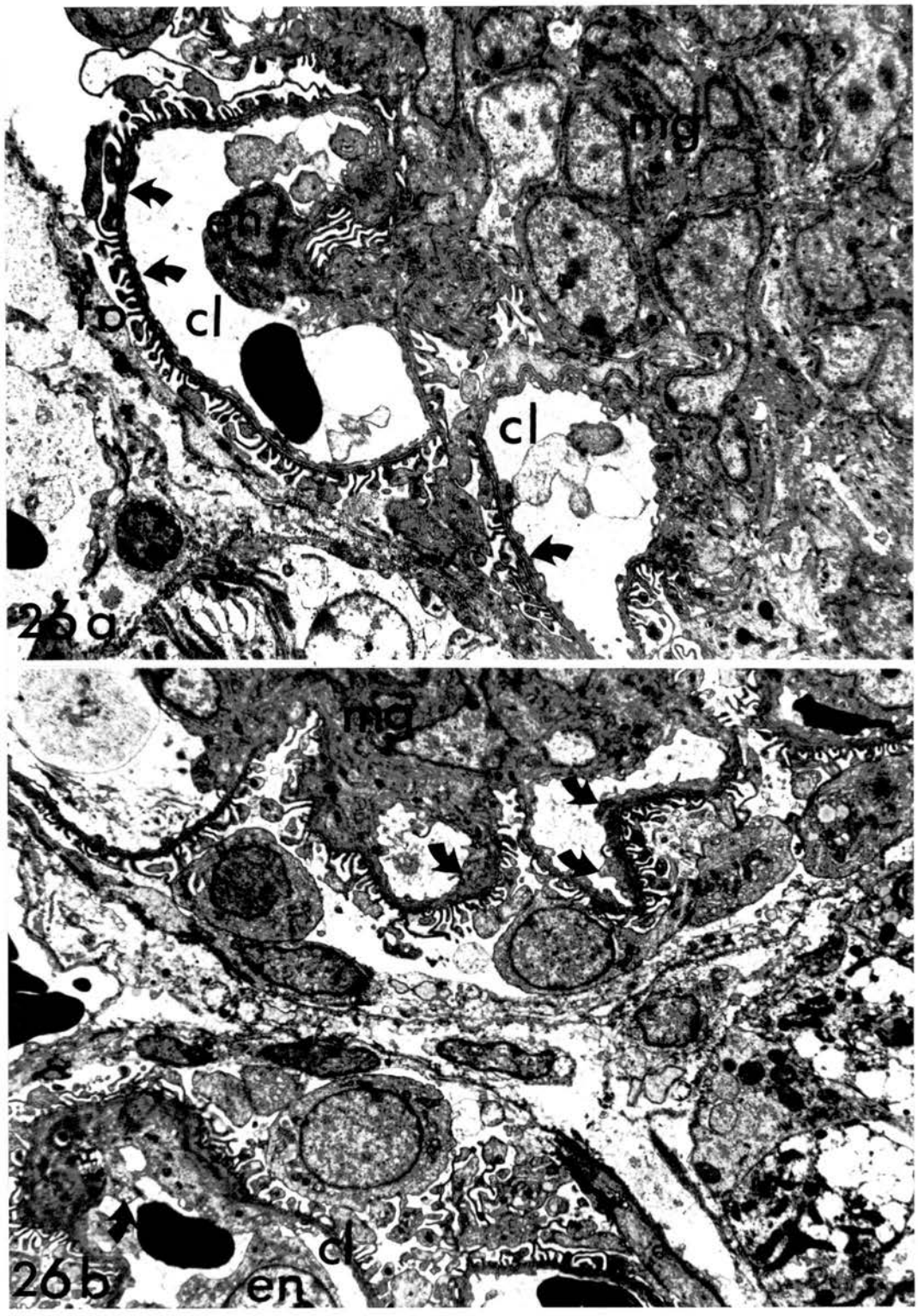


Fig.26. (a&b). Kidney.Glomeruli showing glomerular basement membrane (GBM).(a)Control fowl. Note the GBM as a thin even membrane (arrows) throughout the capillary loops. x 4374. fp foot processes; cl capillary lumen; en endothelial cell; mg mesangium. (b) OA-fed (4ppm) fowl.Note localised thickening of the basal lamina of the GBM (arrows) and the obliteration of a capillary lumen (cl) by thickened GBM and swollen endothelial cell (en). x 4374.

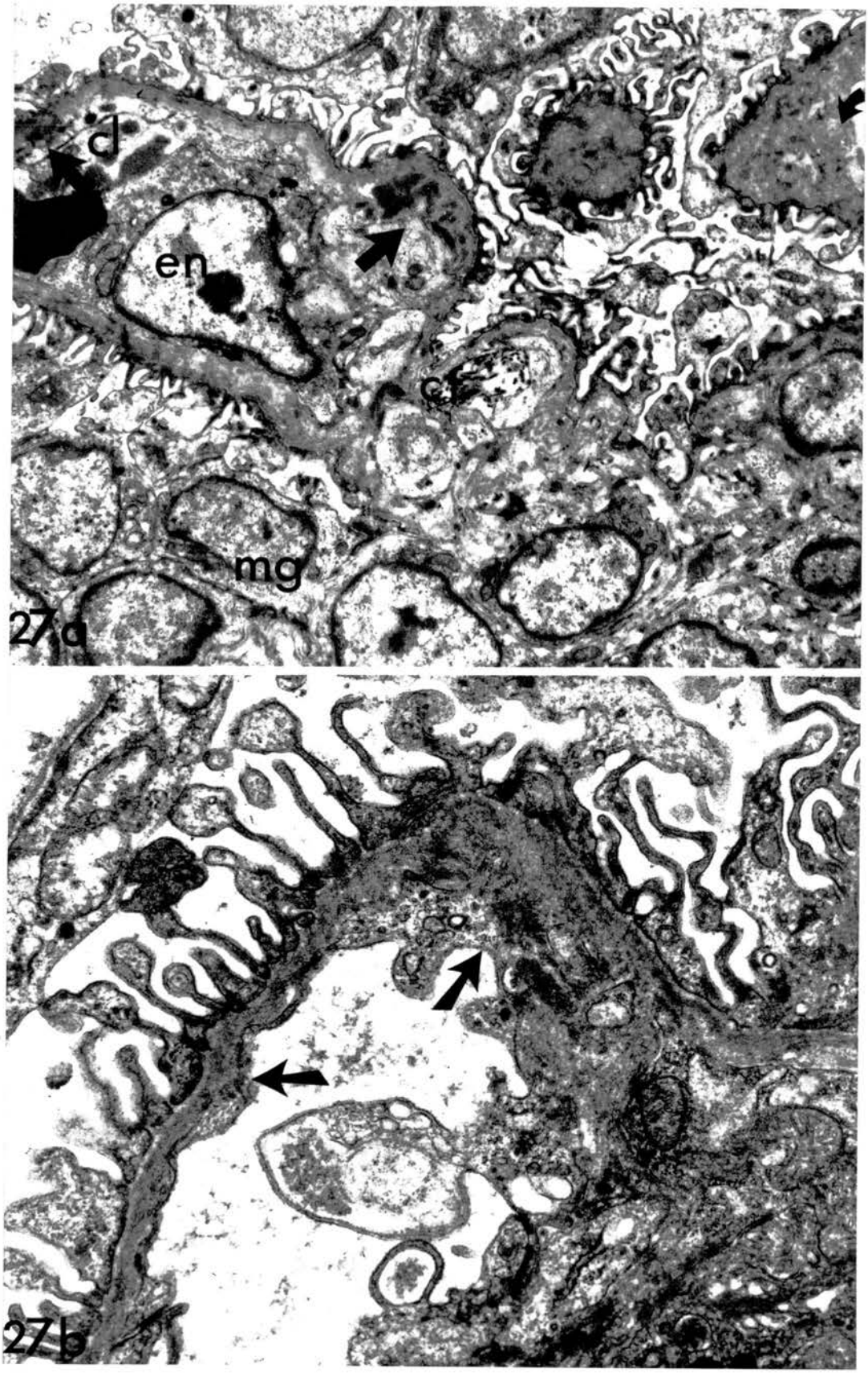


Fig.27. (a&B) Higher magnification of the thickened GBM from an OA-treated fowl. (a) Thickened GBM (arrows) showing extensive degeneration and collagen fibres (cf). The endothelial cell (en) is swollen and the capillary lumen (cl) is reduced. x 7614. (b) GBM showing thickened dense core, extensive degeneration (arrow), and collagen fibres. x 18792.

Glomerulus. In kidneys from OA-treated fowls, the GBM was unevenly thickened either locally or throughout the capillary loop (Fig.26b). There was extensive degeneration of the GBM. Aggregations of collagen fibres were often seen in the thickened region of the dense central core (Figs.27a and 27b). The endothelial cells were swollen and these together with the thickened GBM sometimes caused almost complete obliteration of the capillary lumen (Figs.26b and 27a).

4 weeks.

PCT. Mitochondrial ring forms were more frequent than those present at 2 weeks. The smooth endoplasmic reticulum (SER) was dilated. Lysosomes increased in number and size. Some of the large lysosomes were granular but many contained lipid droplets of variable sizes in their phagosomes. Infolding of the plasma membrane was also seen (Fig.28). Other changes were similar (or more marked) to those at 3 weeks.

DCT. Some of the cells showed dilated ER and irregular nuclei.

CT. The mitochondria were slightly smaller in many cells.

Glomerulus. GBM thickening was similar or even more marked in some cases.

6 weeks

PCT. Changes progressed in severity. Ring-shaped mitochondria were seen in many PCT. Some of the cells had swollen and degenerated mitochondria which had lost their cristae (Fig.29). Many cells had very few mitochondria and the presence of remnants of degenerated mitochondria were noticed (Fig.30). An increase in the

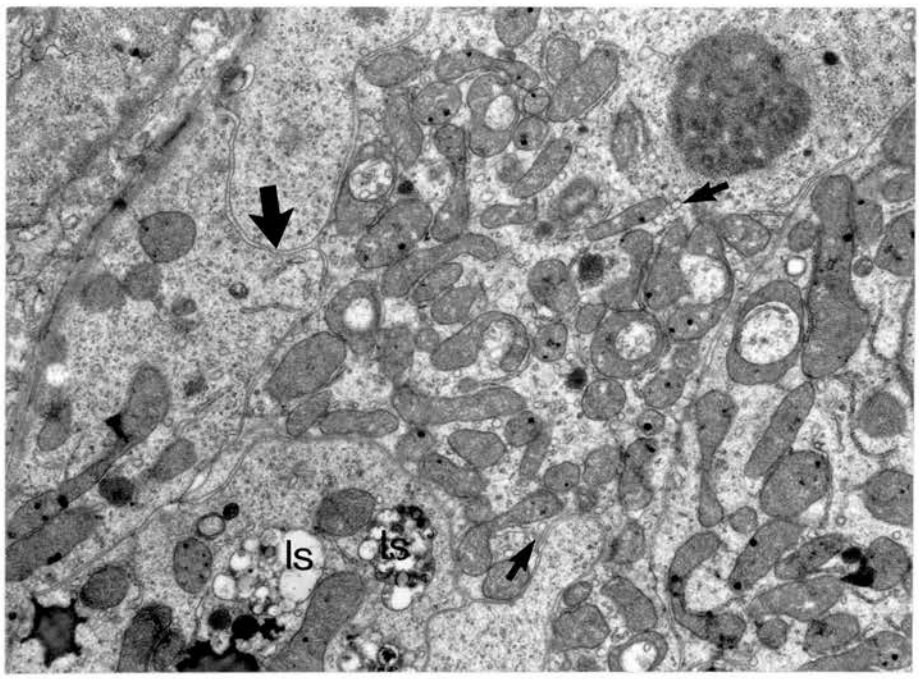


Fig.28. Fowl.4w.o.(4ppm OA). Kidney.PCT. Note mitochondrial ring forms,dilatation of SER (small arrow) and presence of many large granular lipid-containing lysosomes (ls). Infolding of plasma membrane is also evident (large arrow). x 11664.

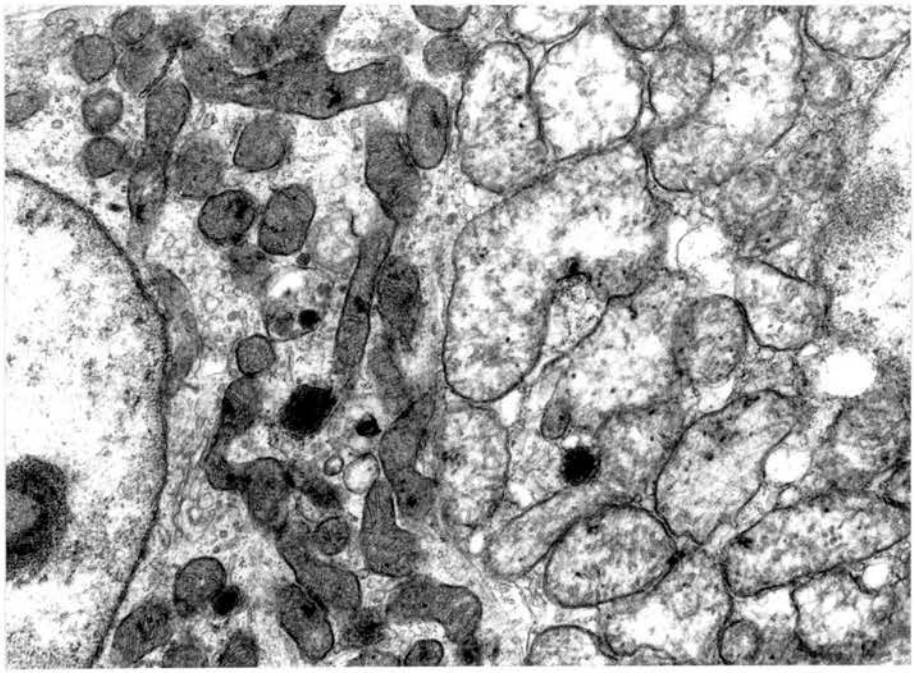


Fig.29. Fowl. 6 w.o. (4ppm OA). Kidney. PCT. Mitochondria showing swelling and enlargement with degenerating and indistinct cristae. Some normal mitochondria are also present. x 18792.

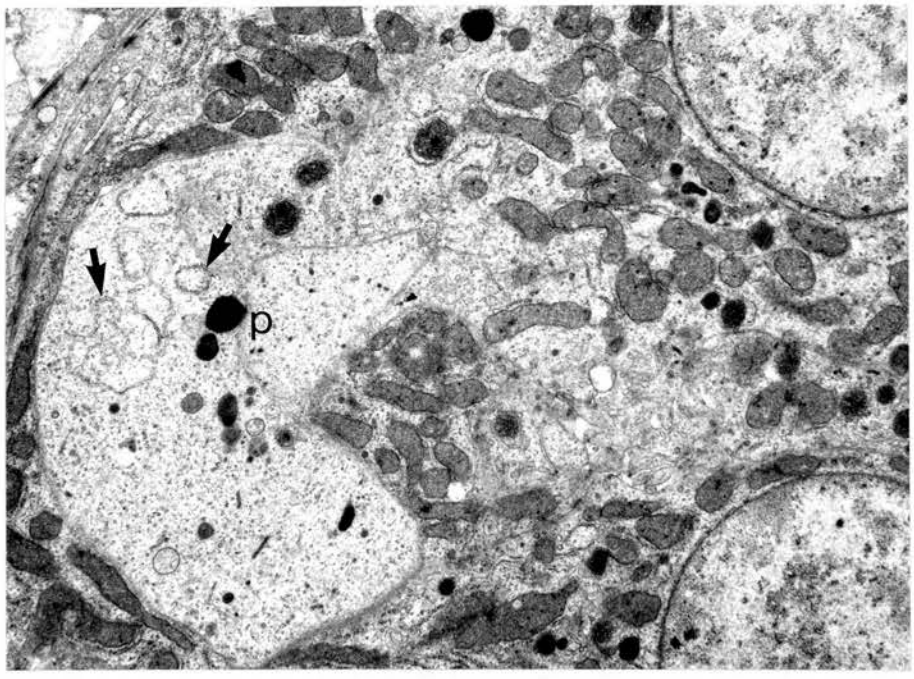


Fig.30. Fowl. 6 w.o. (4ppm OA). Kidney. PCT. Epithelial cell showing paucity of mitochondria in an empty looking cell. Remnants of double layered mitochondrial membrane (arrow) are seen. The adjacent cell is less affected but increase in number of peroxisomes (p) is apparent. x 9396.

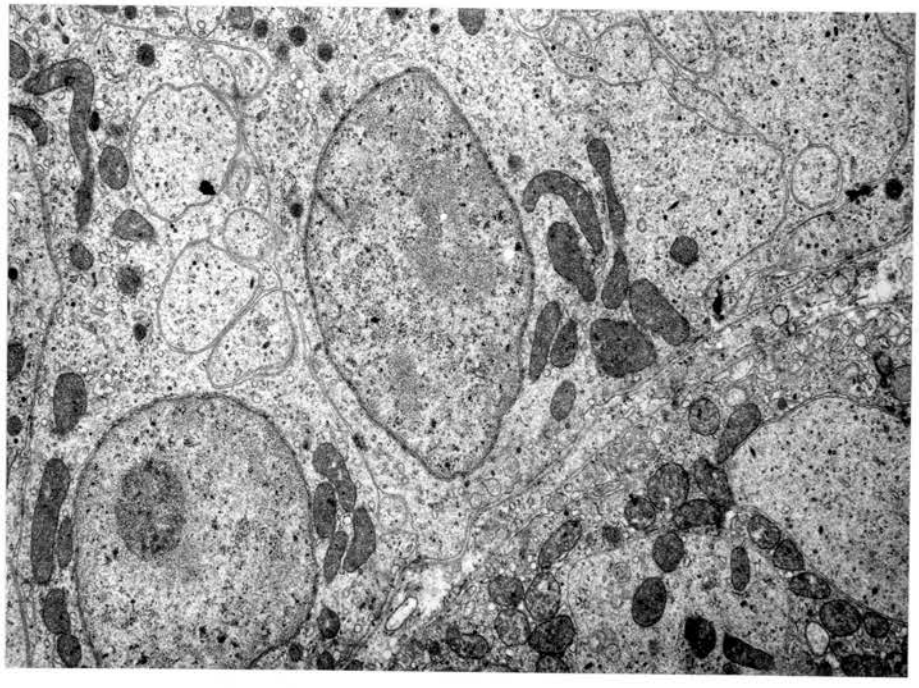


Fig.31. Fowl. 8 w.o. (4ppm OA). Kidney. PCT. Scarce mitochondrial population and infolding of plasma membranes in the epithelial cell. Smooth endoplasmic reticulum is dilated. x 9396.

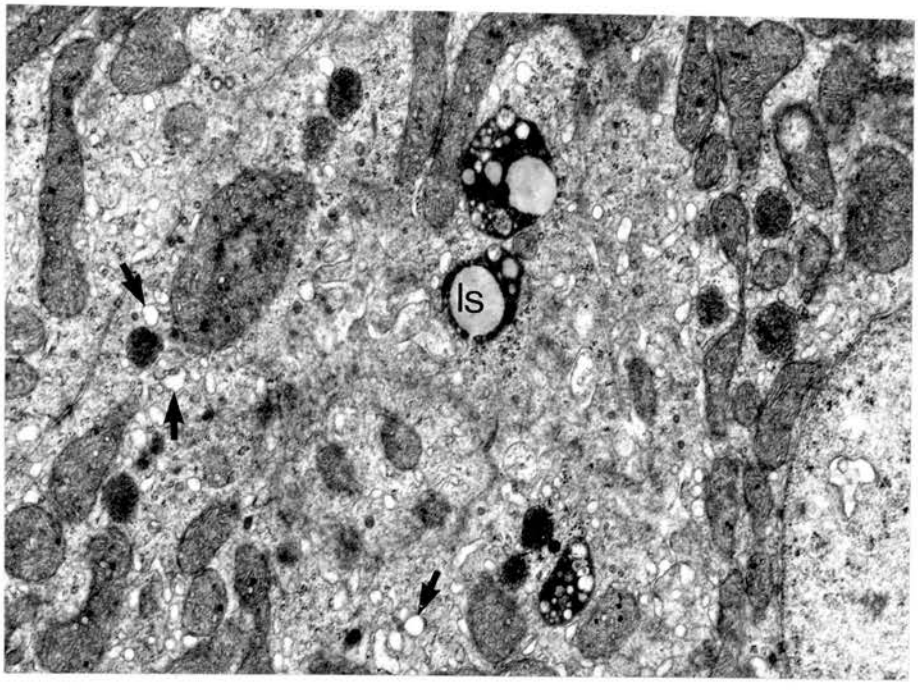


Fig.32. Fowl. 8 w.o. (4ppm OA) Kidney. PCT. Epithelial cell showing mitochondria in the process of degeneration, dilated ER (arrow) and presence of many lipid-containing lysosomes (ls). x 18792.

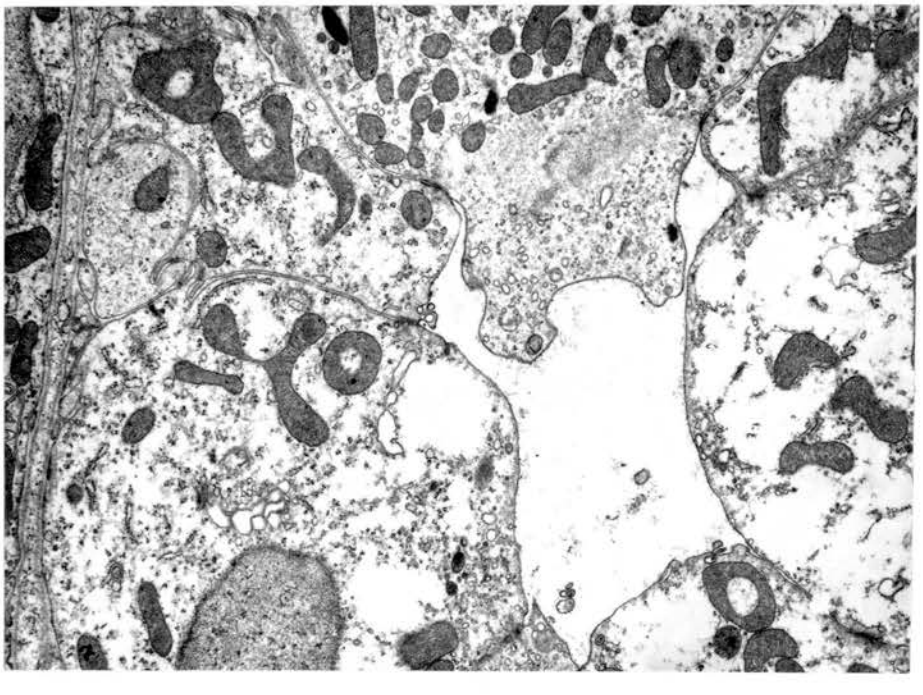


Fig.33. Fowl. 8 w.o. (4ppm OA). Collecting tubule. Epithelial cells. Note sparse mitochondria, a few showing ring-transformation and dilated SER. Infolding of plasma membrane is also present. x 11664.

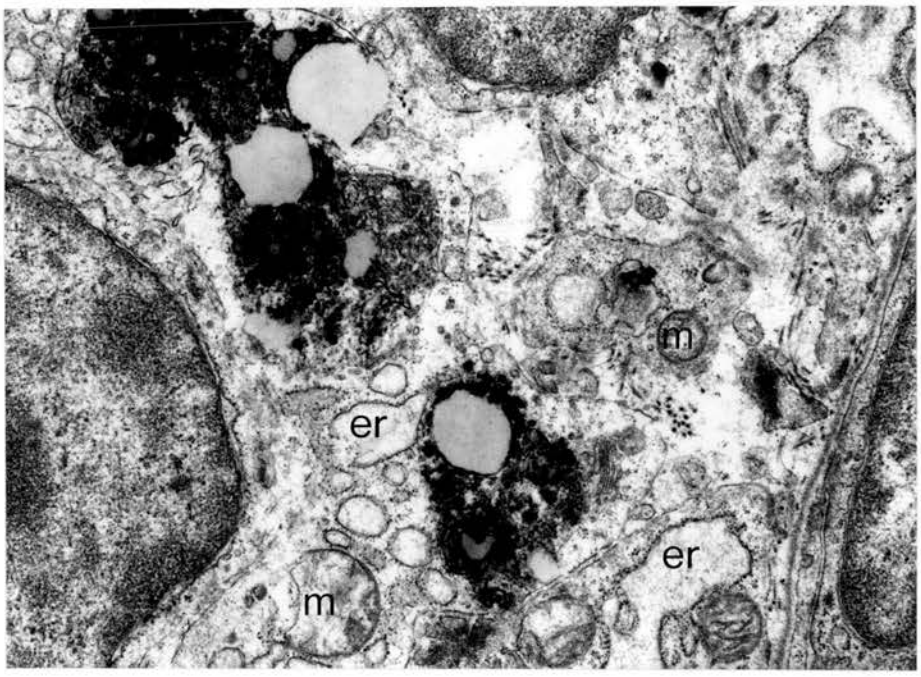


Fig.34. Fowl 10 w.o. (4ppm OA). Kidney. PCT epithelial cell showing a few damaged mitochondria (m), a very much dilated rough endoplasmic reticulum (er) and large lipid-containing lysosomes. x 23004.

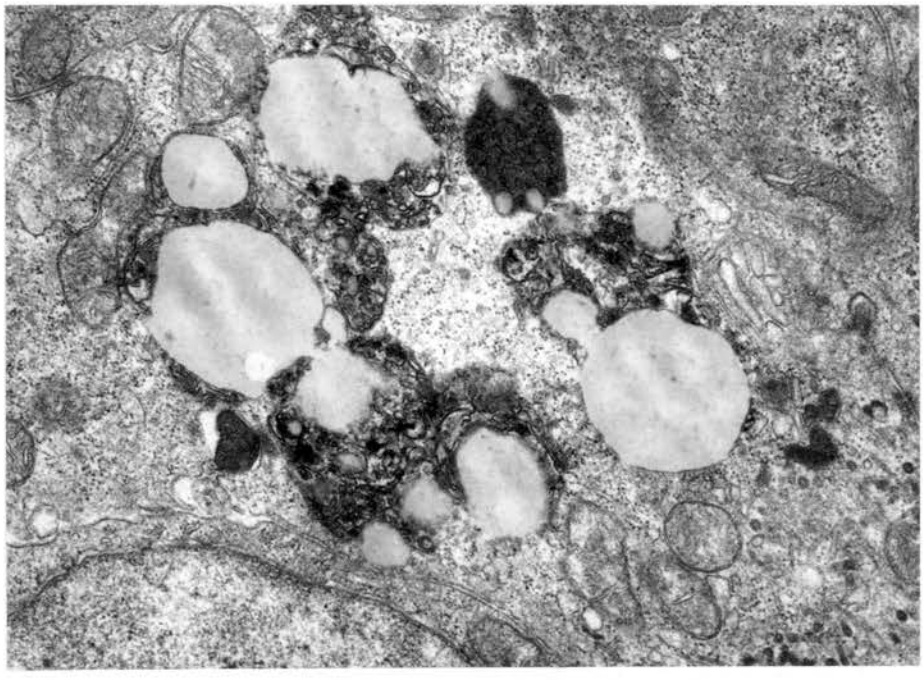


Fig.35. Fowl. 10 w.o. (4ppm OA). Kidney. PCT. Note large lysosomal bodies containing cytoplasmic organelles and very large lipid droplets. Remnants of a few mitochondria are also present. x 30610.

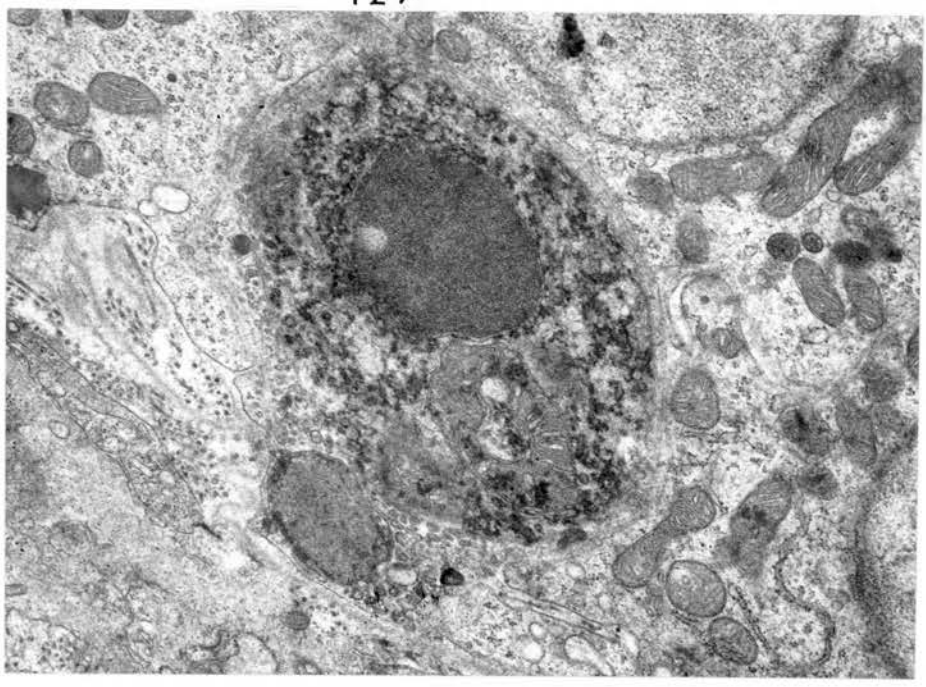


Fig.36. Fowl. 10 w.o. (4ppm OA). Kidney. PCT. A large lysosome containing a few still recognisable mitochondria and much cellular debris. x 23004.

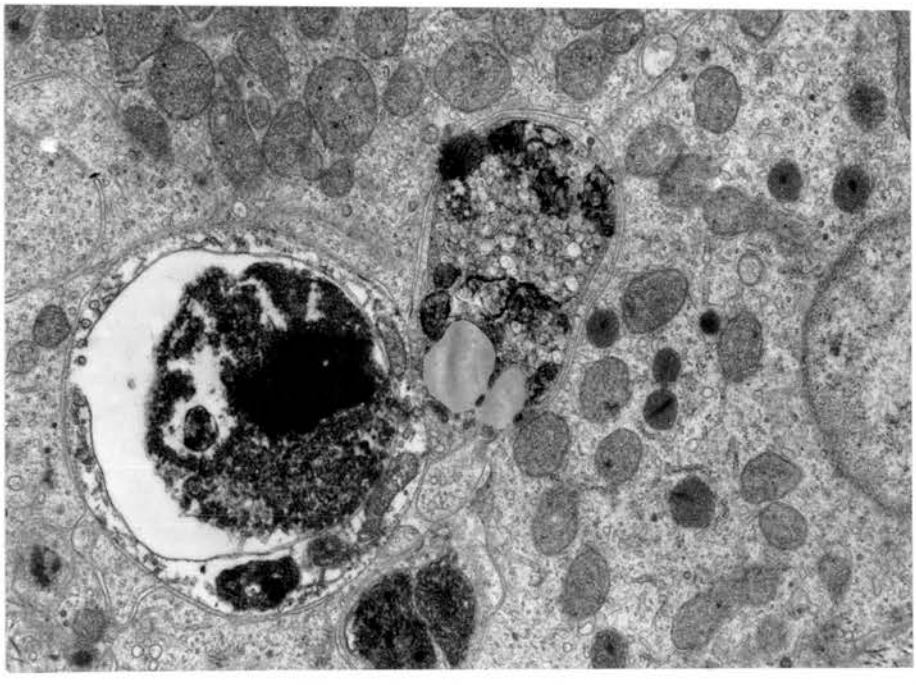


Fig.37. Fowl. 10 w.o. (4ppm OA). Kidney. PCT. Lysosome containing lipid droplets. Note the presence of digested and disintegrated material in the large phagosome of a big lysosme. x 16200.

number of peroxisomes was noted in many cells. Other changes were similar to those at 4 weeks.

8 weeks

PCT. PCT showed severe mitochondrial ring-transformation, an increase in the number of peroxisomes, dilated SER and infolding of the plasma membrane (Fig.31). Mitochondrial damage was very conspicuous as also was the presence of large lysosomal bodies containing lipid droplets and cellular debris (Fig.32). Many cells showed a severe loss of mitochondria from the cytoplasm (Figs.31 and 32). Changes in other parts of the tubules were similar to those at 6 weeks of age. Collecting tubules showed sparse mitochondrial population and dilatation of smooth endoplasmic reticulum (Fig.33). GBM thickening was a common feature.

10 weeks

PCT. Ring-shaped mitochondria were very few in number, the mitochondrial population being drastically reduced. Peroxisomes were increased in number and the rough endoplasmic reticulum was very much dilated. There was evidence of pronounced lysosomal activity (Fig.34), the lysosomes being greatly enlarged and containing large lipid droplets and cytoplasmic organelles (Fig.35). A few degenerating but still recognisable mitochondria, which were engulfed by these lysosomes (Fig.36) could be seen. Some lysosomes contained disintegrating and digested material in a large phagosome (Fig.37). In the collecting tubules, dilated SER and small mitochondria were seen. GBM thickening was also noticed.

4.8.1.2 Liver

2 and 3 weeks. Small amounts of glycogen scattered unevenly in the cytoplasm in the form of electron-dense granules measuring about 20 nm in diameter was a

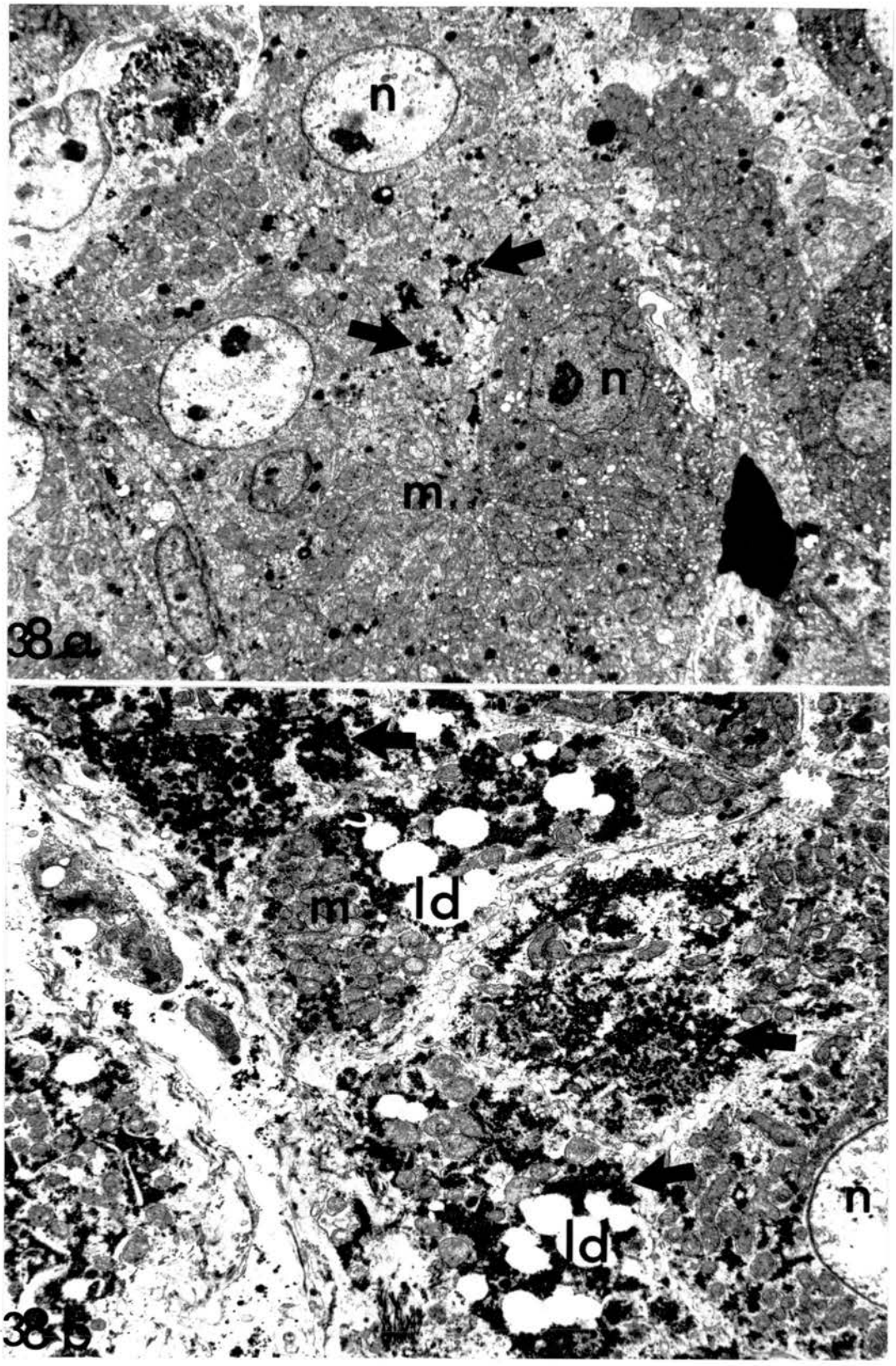


Fig.38. (a&b) Liver. (a) Control fowl. Note the presence of small aggregates of electron-dense glycogen granules (arrows) in the cytoplasm. x 4374. n nucleus; m mitochondria. (b)OA-treated (4ppm). Note large aggregates of glycogen granules (arrows) and vacuoles previously occupied by lipid droplets. The mitochondria (m) are pushed aside in some cells. x 6156. n nucleus.

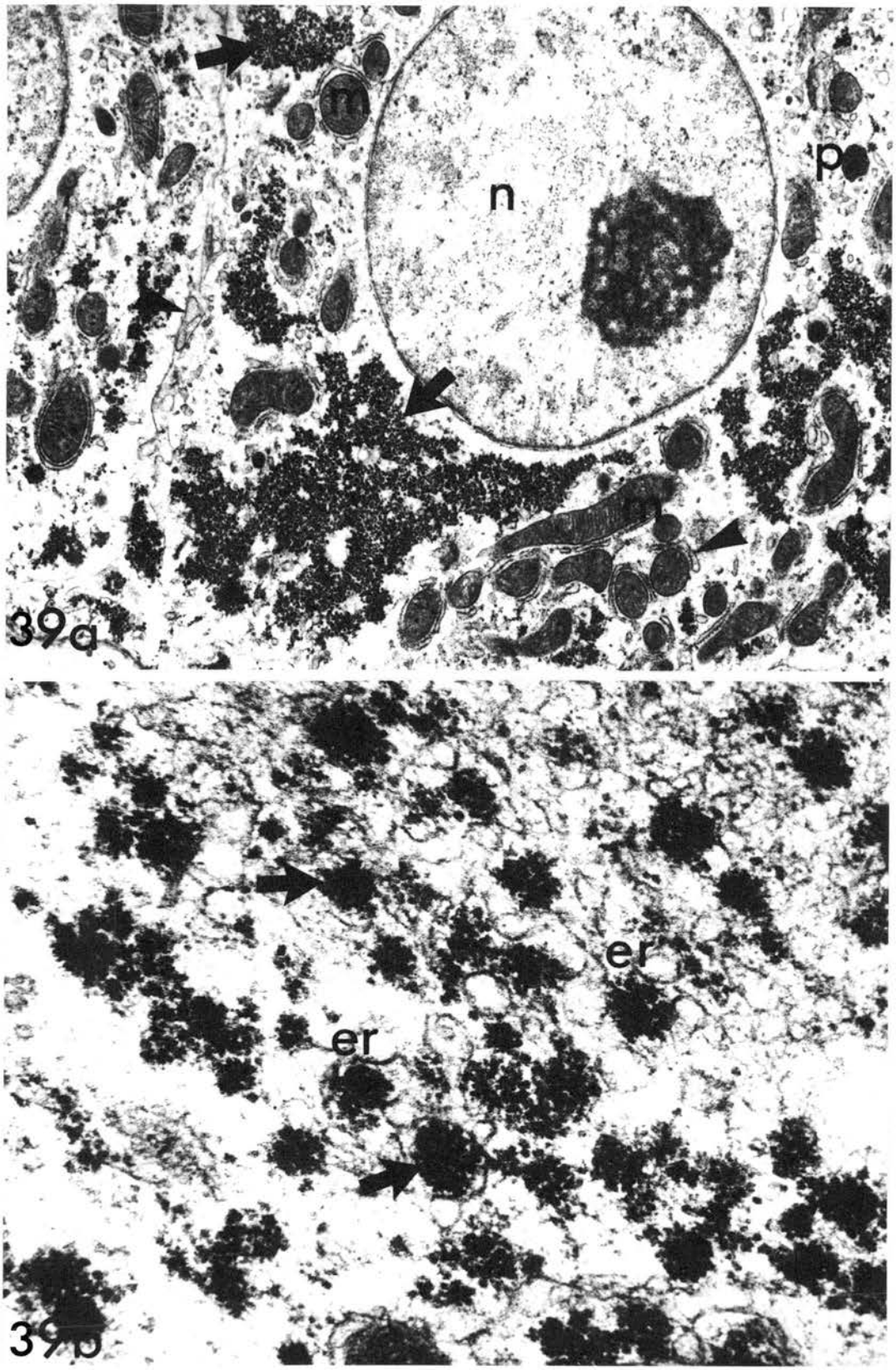


Fig.39. (a&b) Liver from OA-treated fowls. (a) Glycogen, as monoparticulate granules (arrows), is increased. The mitochondria (m), ER (arrowheads) and peroxisomes (p) appear to be largely unaffected. x 11664. (b) Aggregates of glycogen rosettes (arrows) in the cytoplasm in close association with the smooth endoplasmic reticulum (er). x 61560.

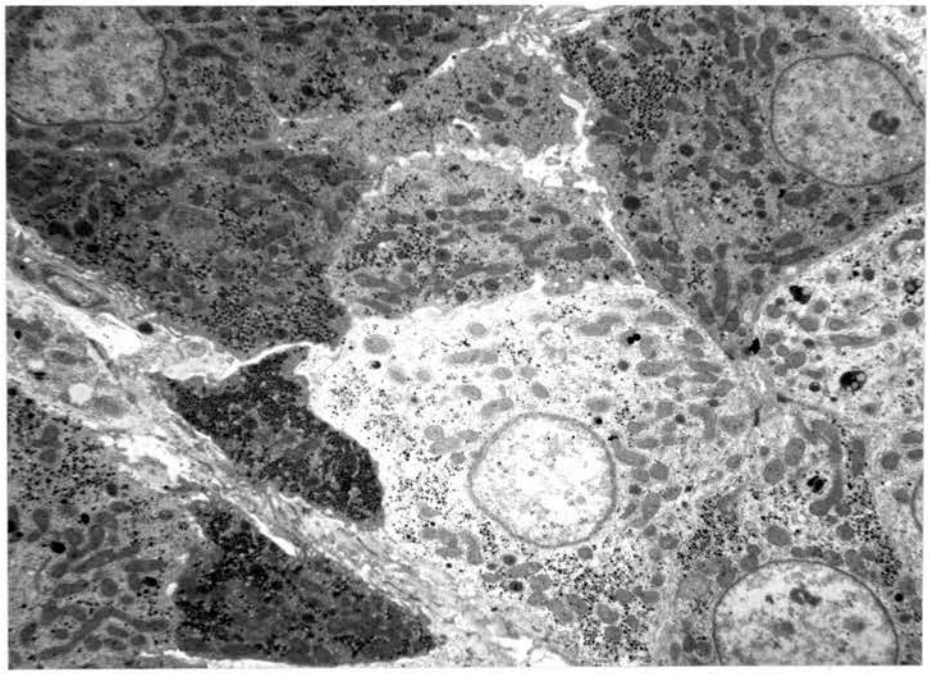


Fig.40. Fowl. 6 w.o. (4ppm OA). Liver. Hepatocytes showing glycogen granules in varying concentration. Dark cells contain more glycogen than the light cells. In one cell, slightly enlarged lysosomes are also present. x 5022.

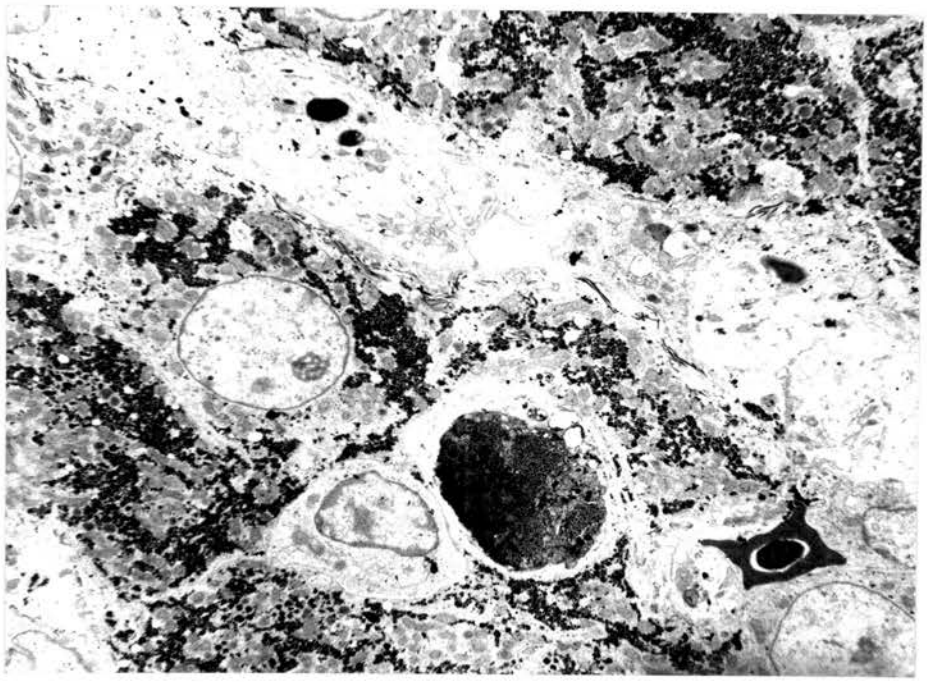


Fig.41. Fowl. 8 w.o. (4ppm OA). Liver. Increased glycogen accumulation in hepatocytes. A large lysosomal body containing glycogen is present. x 4374.

characteristic feature of the hepatocytes from normal control birds (Fig.38a). In liver sections from OA-treated birds, the glycogen content was considerably increased (Fig.38b) and not infrequently the hepatocytes were entirely studded with glycogen. The glycogen appeared as large aggregations of electron-dense granules, either in a monoparticulate form, with individual particles ranging in size from 15 to 30 nm in diameter (Fig.39a) or in the form of rosettes in close association with the SER (Fig.39b). Their size and concentration varied widely. Sometimes these aggregates seemed to mask the cell organelles. Many electron-dense peroxisomes, ranging from 0.15 to 0.42 um in diameter were also seen in the cytoplasm of affected cells. Small lipid droplets were seen as clear round or oval vacuoles in the cells (Fig.38b). Lipid droplets and glycogen aggregates sometimes appeared to push the mitochondria and other cell organelles to the periphery of the hepatocytes. However, the nucleus, mitochondrial architecture, endoplasmic reticulum, peroxisomes and lysosomes appeared to be largely unaffected (Fig.39a). Birds fed 4 ppm OA had a higher glycogen content in the liver cells than those fed 2 ppm OA.

4 and 6 weeks. Glycogen accumulation increased in the hepatocytes in varying degrees, the peripheral areas showing higher concentrations. Two populations of dark and light cells were seen. Usually the dark cells contained more glycogen (Fig.40). In many cells, lysosomal activity appeared to have increased. In some hepatocytes larger nucleoli than normal were noticed in the nuclei. Other organelles were not affected.

8 weeks. A large accumulation of glycogen was noticed throughout, particularly in the dark cells. When examined under low power, a tubular arrangement was

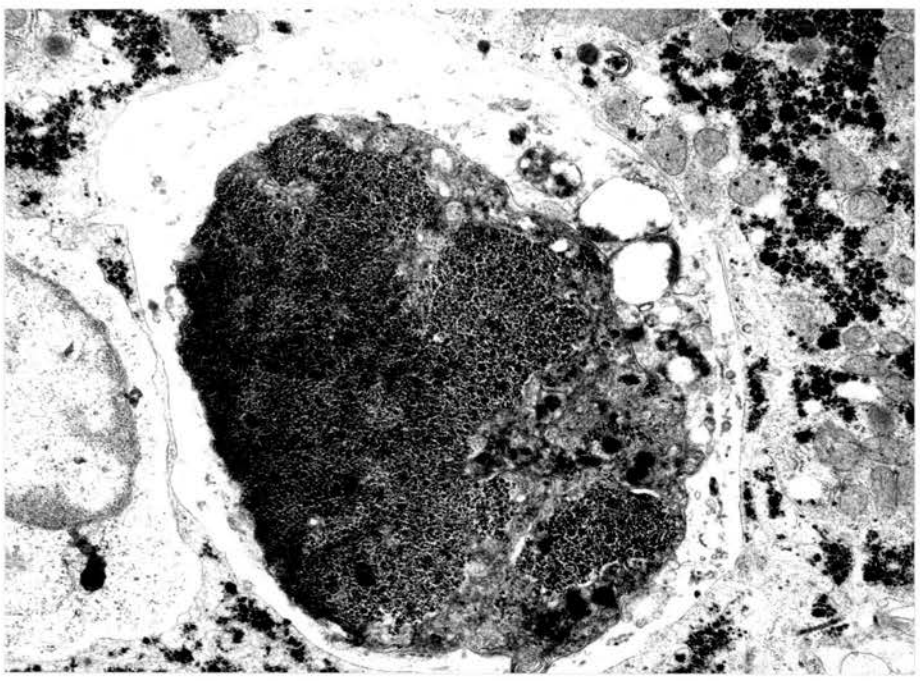


Fig.42. High power view of the lysosomal body in Fig.41. Note vacuolation in the cytoplasm and large aggregates of glycogen particles adjacent to cellular debris. x 16200.

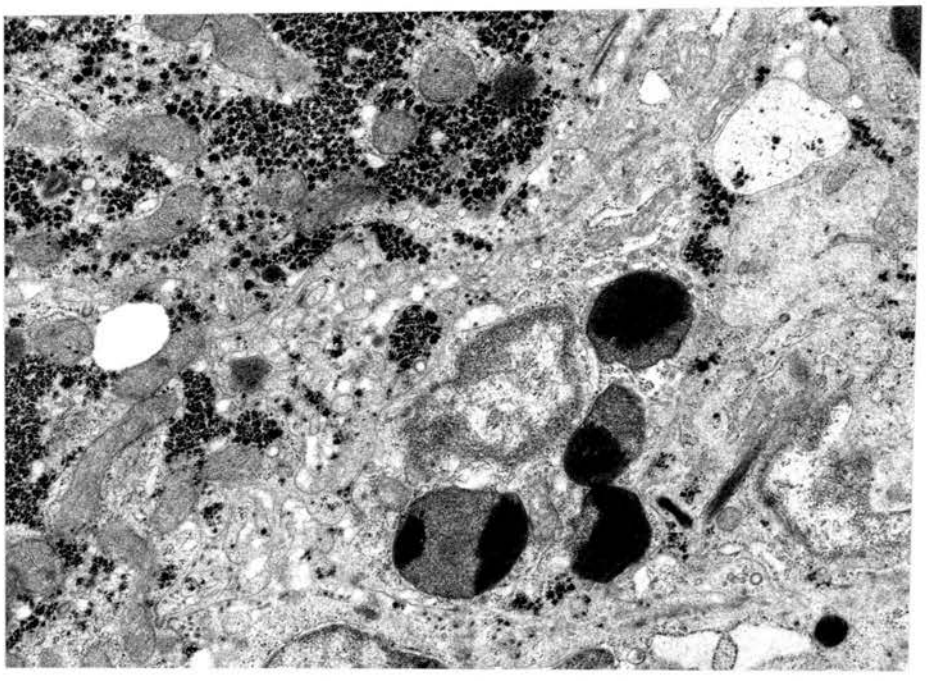


Fig.43. Fowl. 10 w.o. (4ppm OA). Liver. A Kupffer cell containing large granules of light and dark electron-density (lysosomes). Note unaffected mitochondria in the hepatocyte with glycogen accumulation. x 18792.

evident due to the presence of glycogen-studded hepatocytes around the sinusoids. In some cells, lysosomes containing a large amount of glycogen and cellular debris were increased in number and were sometimes so enlarged as to occupy the whole cell (Figs.41 and 42). There was evidence of increased Kupffer cell activity.

10 weeks. In addition to excessive glycogen and increased lysosomal activity in the hepatocytes, Kupffer cells were seen containing large granules in their cytoplasm having both lightly and intensely electron-dense characteristics (Fig.43). Some glycogen was also present in the cytoplasm.

4.8.1.3 Bursa of Fabricius, thymus and spleen

These lymphoid organs were examined at 3 weeks of age. In general, there was a degeneration of lymphoid cells and many pyknotic cells were seen in OA-treated birds. The lymphocytes had vacuolated cytoplasms in all the organs. Macrophage activity was increased with pyknotic cells and debris being seen in their phagosomes.

4.8.1.4 Peyer's patch

Many degenerating and dying lymphoid cells were seen both in the diffuse lymphoid tissues (DLT) and the germinal centres (GC) after feeding OA for 2 weeks (Fig.44). The changes progressed and at 3 weeks of age the germinal centres became severely vacuolated. Many indented lymphoid cells with blast characteristics were also seen showing vacuolation in cytoplasm (Fig.45). Some macrophages and a few plasma cells with damaged endoplasmic reticulum were also seen. Intraepithelial lymphocytes, globule leucocytes and mast cells could be seen. At 4 weeks,

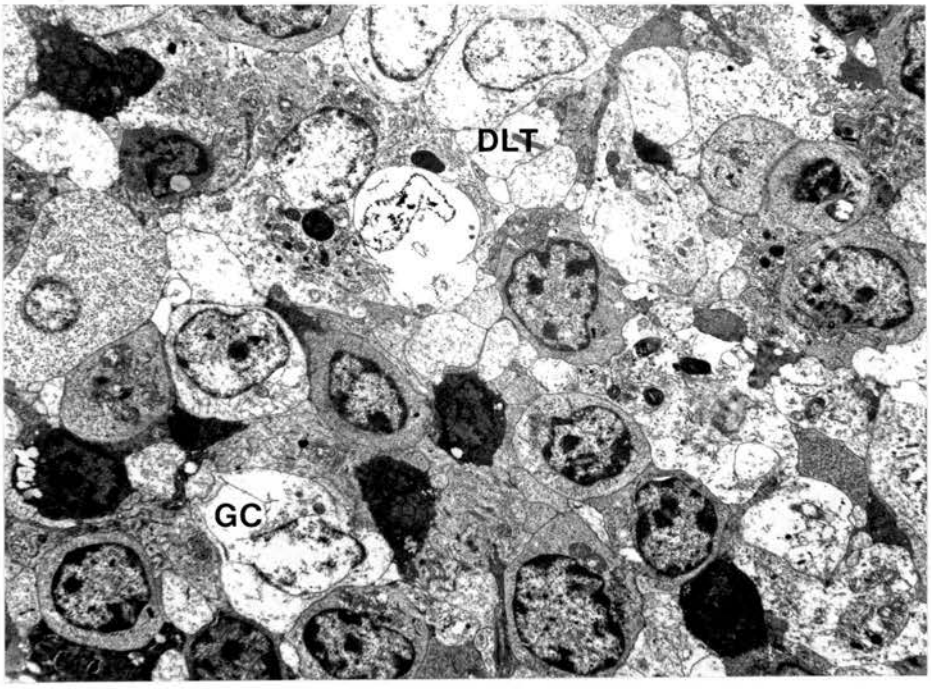


Fig.44. Fowl. 2 w.o. (4ppm OA) Peyer's patch (PP). Degenerating and disintegrating lymphoid cells in diffuse lymphoid tissue (DLT) and germinal centre (GC) giving a vacuolated appearance. x 4374.

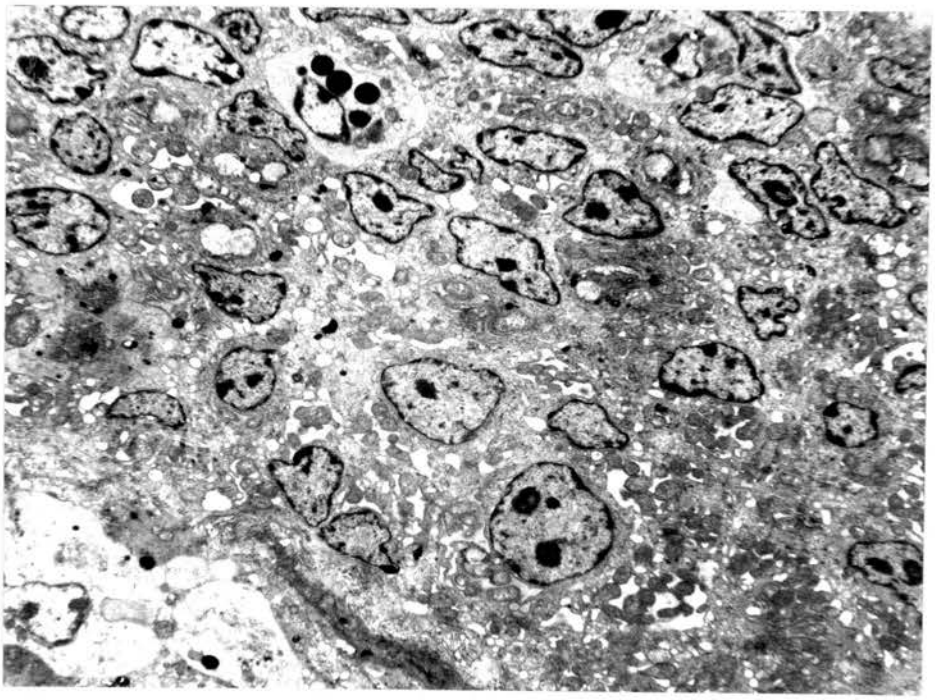


Fig.45. Fowl. 3 w.o. (4ppm OA). PP. GC showing vacuolation. Many lymphoblasts with prominent nucleoli are also present. Adjacent DLT is degenerated. x 4374.

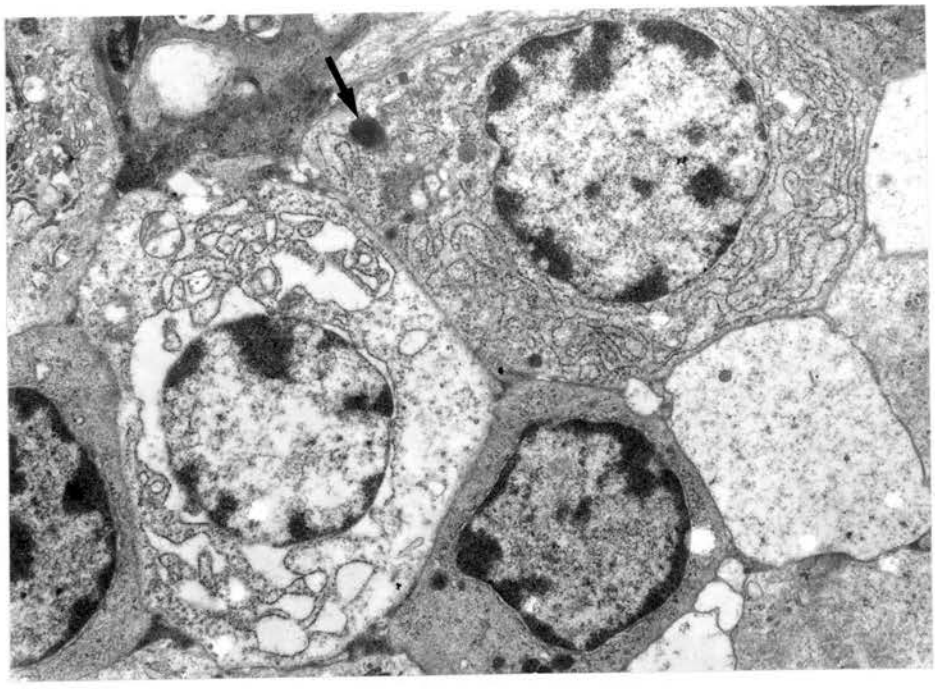


Fig.46. Fowl. 4 w.o. (4ppm OA). PP. Plasma cell showing degenerative changes in the cytoplasm. Mitochondria are disintegrating and endoplasmic reticulum is dilated and degenerating. One of the plasma cells contains elctron-dense round Russell body (arrow) in the cytoplasm. x 11664.

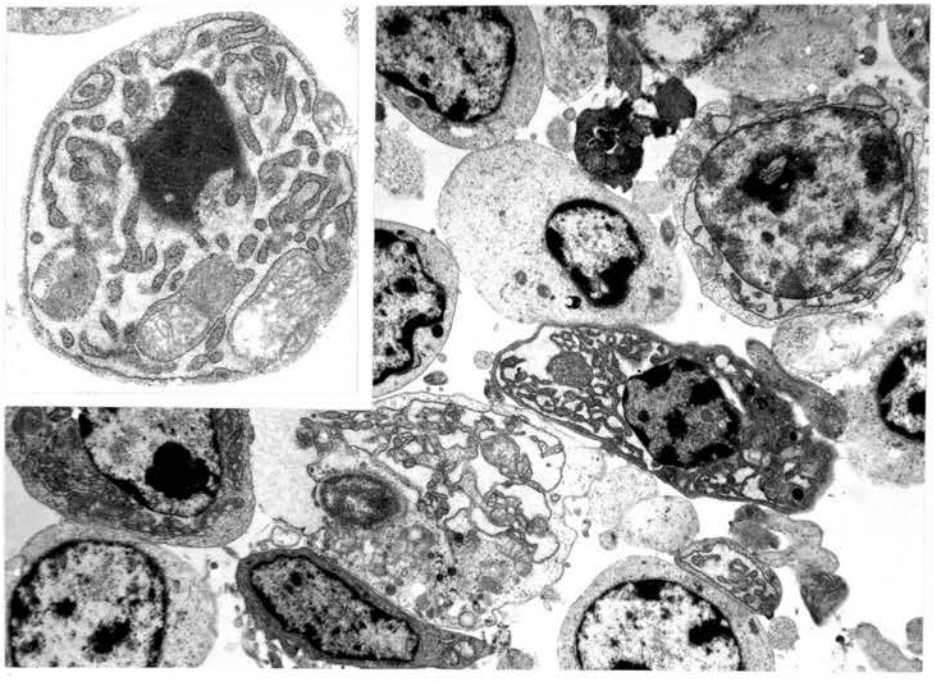


Fig.47. Fowl. 6 w.o. (4ppm OA). PP. DLT area showing degeneratin, plasma cells, lymphocytes and lymphoblasts. Inset shows a degenerating plasma cell with pyknotic nucleus and degenerating mitochondria. x 12528.

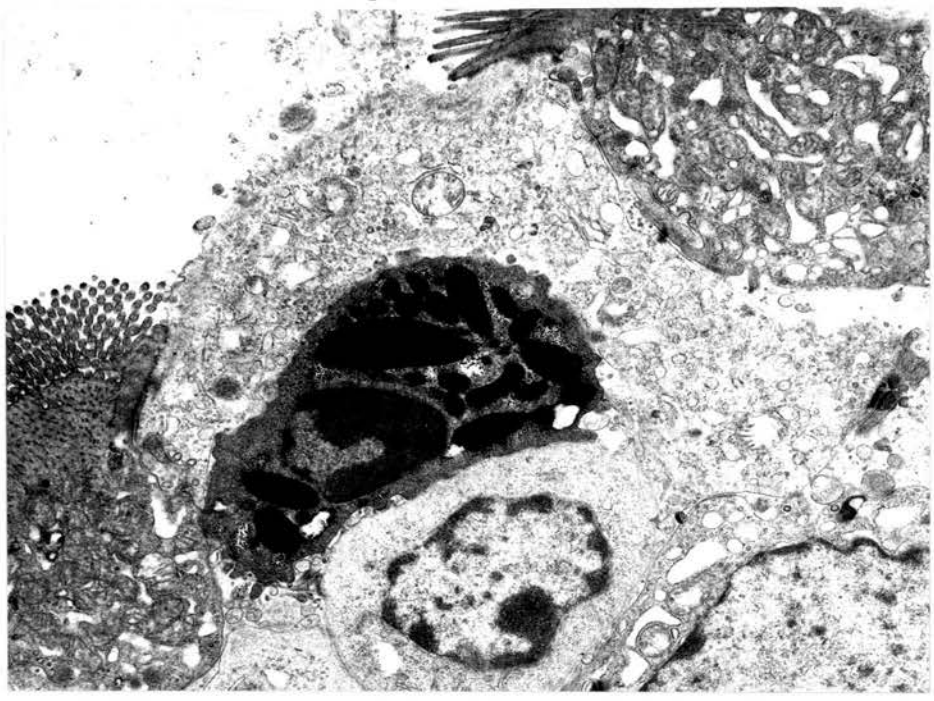


Fig.48. Fowl. 8 w.o. (4ppm OA).PP. A degenerating epithelial cell containing a heterophil rich in glycogen and a lymphocyte. Loss of microvilli, degeneration of mitochondria and dilatation of endoplasmic reticulum are also evident. x 11664.

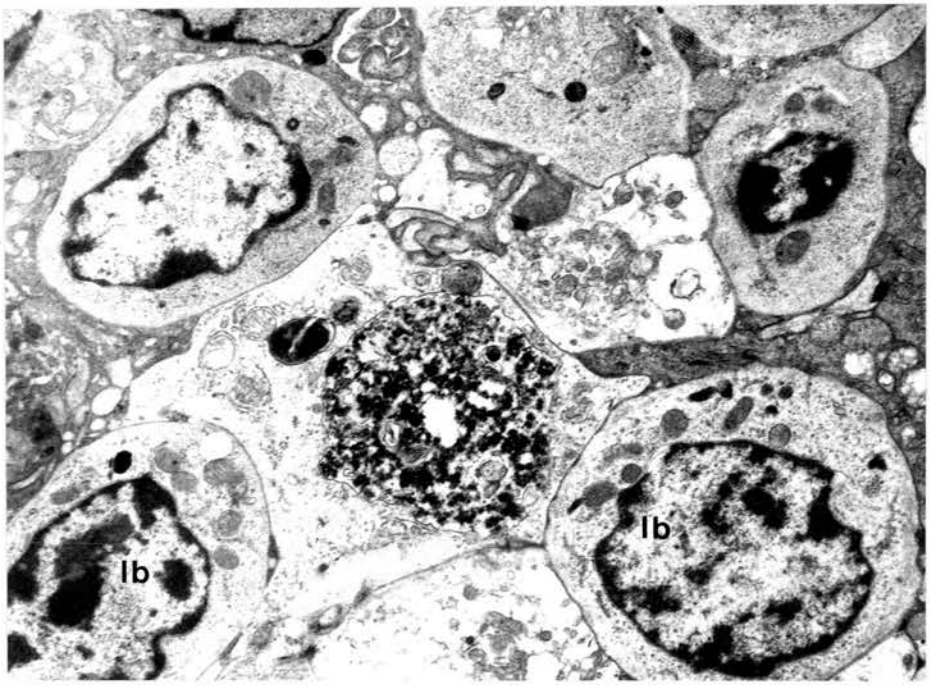


Fig.49. Fowl. 10 w.o. (4ppm OA). PP. Lysosome containing degenerating cell and debris is shown in the DLT. Lymphoblasts (lb) and some degenerating cells are also present. x 9396.

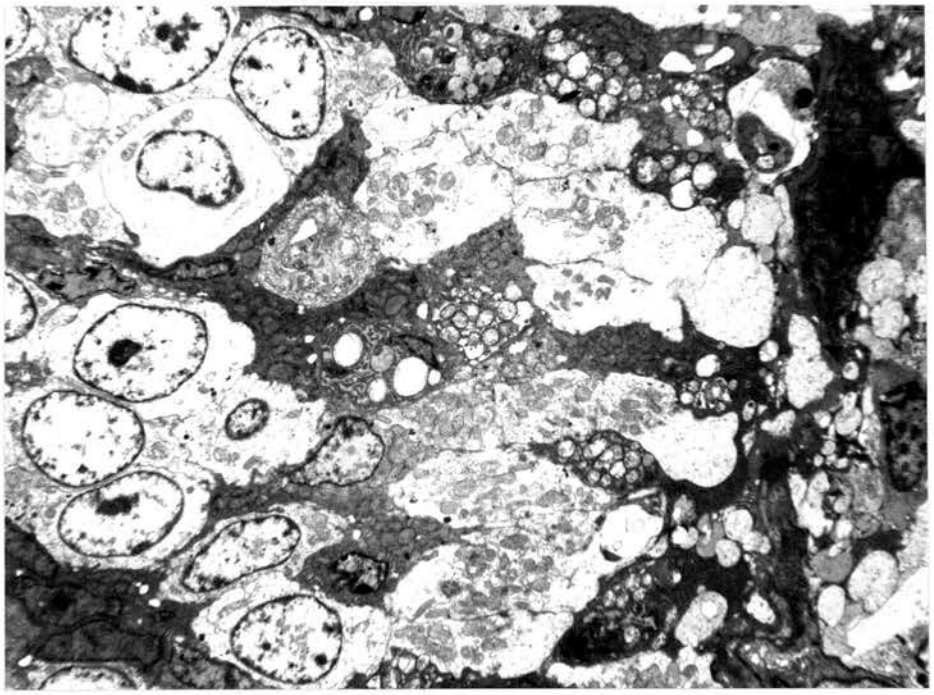


Fig.50. Fowl. 4 w.o. (4ppm OA). Caecal tonsil. Note vacuolation, mitochondrial damage and degeneration of lymphoid cells. x 4374.

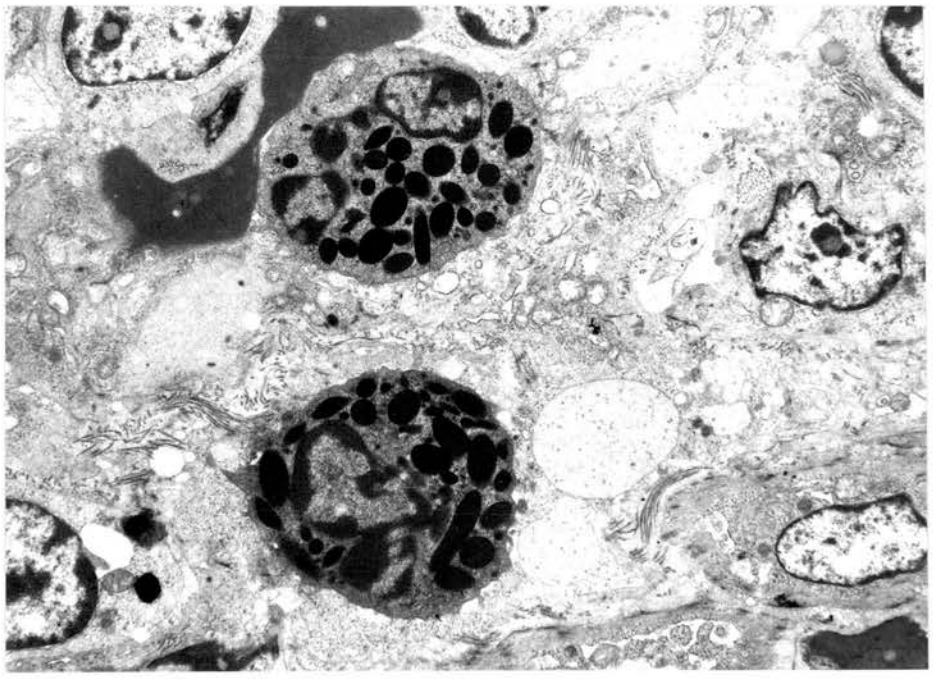


Fig.51. Fowl. 4 w.o. (4ppm OA). Caecal tonsil showing the presence of two heterophils and early degenerative changes in lymphoid cells. x 7614.

the changes were similar but plasma cell damage (Fig.46) was more frequent. At 6 weeks of age as well as lymphoid cell necrosis plasma cell degeneration became more severe (Fig.47). At 8 weeks of age intraepithelial lymphocytes, heterophils (Fig.48) and globule leucocytes were seen. The epithelial cells showed a loss of microvilli, dilatation of the endoplasmic reticulum and degeneration of mitochondria. Changes were similar at 10 weeks of age. In addition lysosomal activity was marked (Fig.49). Macrophages were also present.

4.8.1.5 Caecal tonsils

Changes were similar in the caecal tonsils which showed marked vacuolation and degeneration of lymphoid cells (Fig.50) and plasma cells in the diffuse lymphoid tissue and germinal centres. Mitochondrial degeneration was a constant feature. Many heterophils were also seen adjacent to degenerating lymphocytes and lymphoblasts (Fig.51), in the deeper part of DLT.

4.8.2 Turkeys

4.8.2.1 Kidneys

Changes in the PCT at 2 weeks of age were similar to those seen in broilers, though less marked. The glomerulus had thickening of the GBM together with extensive degeneration (Fig.52). At 4 weeks of age, mitochondrial changes in the PCT ranged from swelling and degeneration to ring-formation (Fig.53). Lipid droplets were increased. The distal convoluted tubule (DCT) had irregular muclei, dilated endoplasmic reticulum and a light thin elongated and dark mitochondrial population (Fig.54). The mitochondrial and glomerular changes increased in degree at 6 and 8 weeks of age. The peroxisomes increased in number and

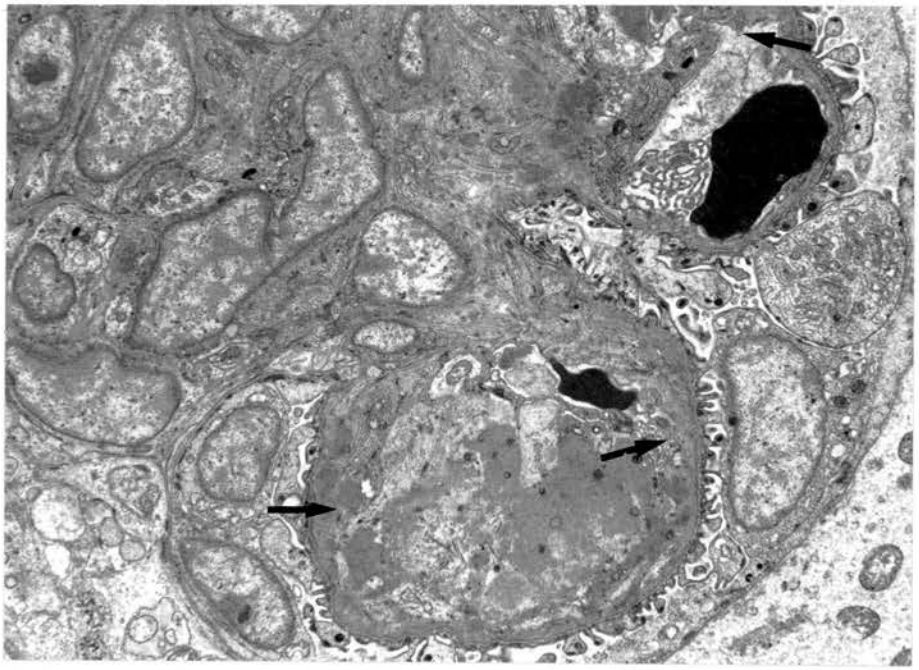


Fig.52. Turkey. 2 w.o. (4ppm OA). Kidney. Glomerulus. Note extensive degeneration and thickening of glomerular basement membrane (arrows). Lumen of the large capillary loop is obliterated. x 6136.

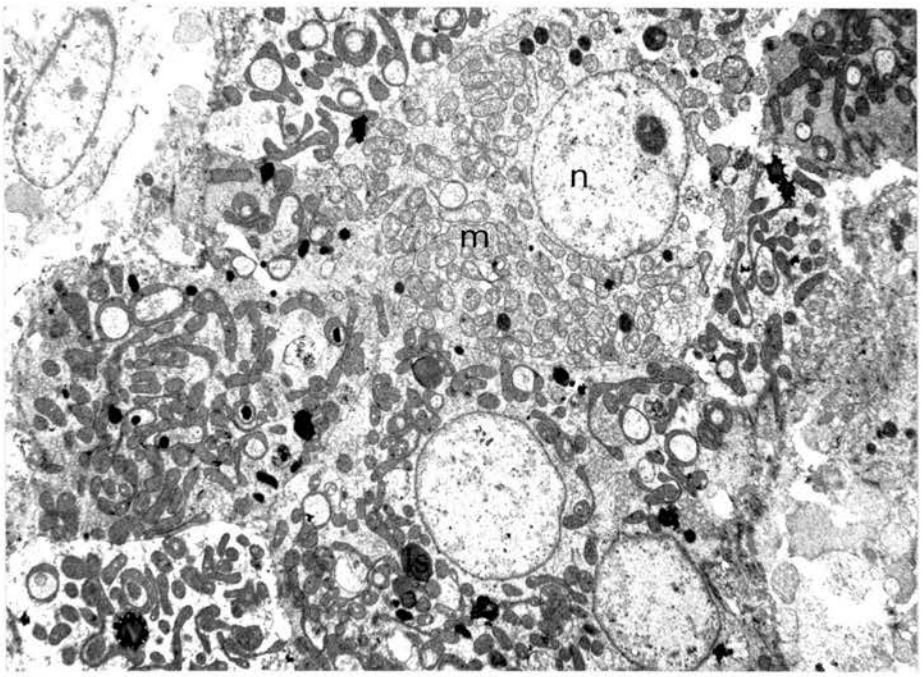


Fig.53. Turkey. 2 w.o. (4ppm OA). Kidney. PCT. Mitochondria (m) show swelling, degeneration, increase in lipid droplets and severe ring-transformation. Some lysosomes (ls) are also present. x 4374.

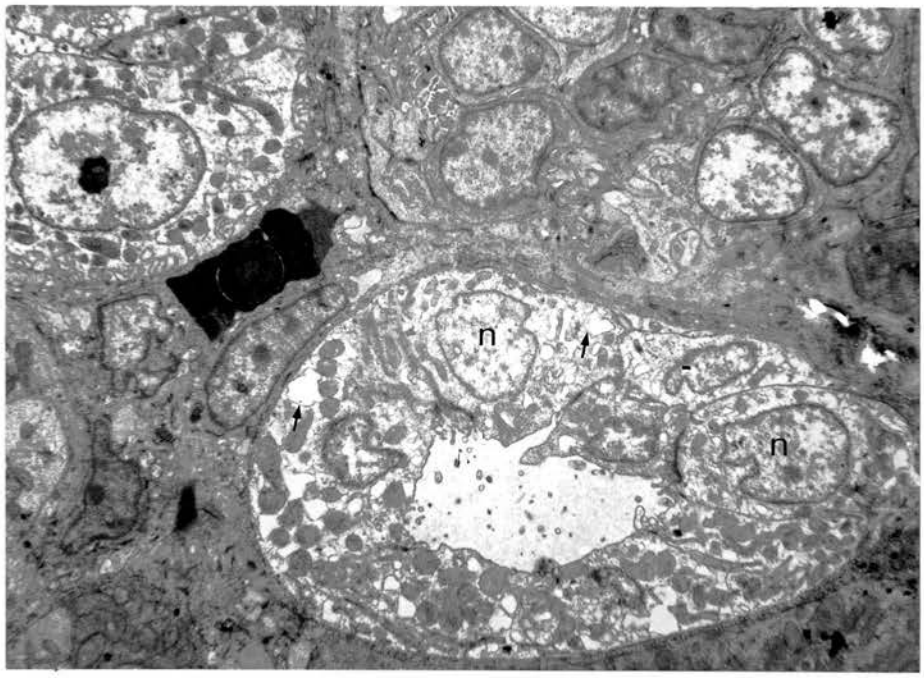


Fig.54. Turkey. 4 w.o. (4ppm OA). Kidney. DCT. Epithelial cells showing irregular nuclei, dilated endoplasmic reticulum (arrows) and light thin elongated and dark round mitochondria. x 4374.

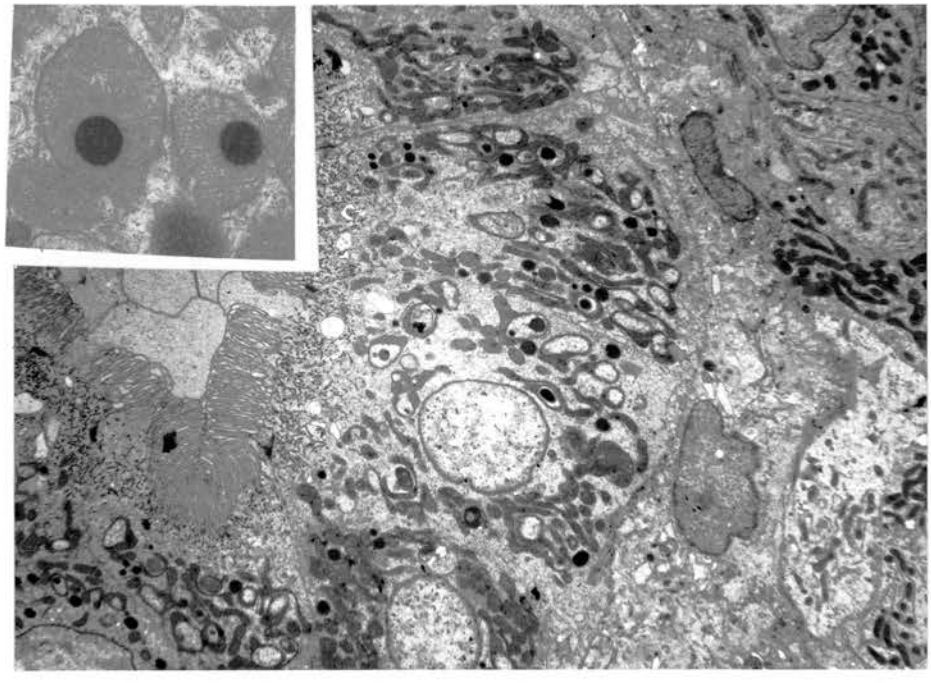


Fig.55. Turkey. 8 w.o. (4ppm OA) Kidney. PCT. Typical ring-shaped and misshapen mitochondria many of which enclose peroxisomes. x 4374. The inset shows the presence of peroxisomes on the mitochondria. x 30610.

size and many were seen either in ring form mitochondria (Fig.55) or even on the mitochondria themselves (Fig.55 inset). The mitochondrial population was reduced in many cells. The collecting tubules were also affected at 8 weeks of age. Variations of the mitochondrial population could be seen both in size and in electron density. The endoplasmic reticulum was indistinct and plasma membrane infolding was evident in some cells (Fig.56).

4.8.2.2 Liver

An accumulation of glycogen was seen in the hepatocytes in birds at 2 and 4 weeks of age although this was less marked than that seen in broilers. Evidence of increased lysosomal activity was seen in many hepatocytes at 4 weeks (Fig.57). At 6 and 8 weeks of age glycogen accumulation became more marked (Figs.58 and 59). The mitochondria and endoplasmic reticulum were usually unaffected but sometimes the mitochondrial cristae could be indistinct (Fig.58). Haemorrhages and Kupffer cell activity were seen. Lysosomal activity was evident.

4.8.2.3 Bursa of Fabricius, thymus and spleen

Ultrastructural changes were studied in these lymphoid organs at 2 and 4 weeks after OA-feeding.

As in broilers, the bursa of Fabricius showed widespread vacuolation, degeneration and pyknosis of the lymphoid cells and evidence of mitochondrial damage (Fig.60) and marked lysosomal activity (Fig.61). Many macrophages were seen phagocytosing pyknotic lymphoid cells and cellular debris. Plasma cells with mitochondrial degeneration often contained Russell bodies (Fig.62).

The thymus showed varying degrees of degenerative

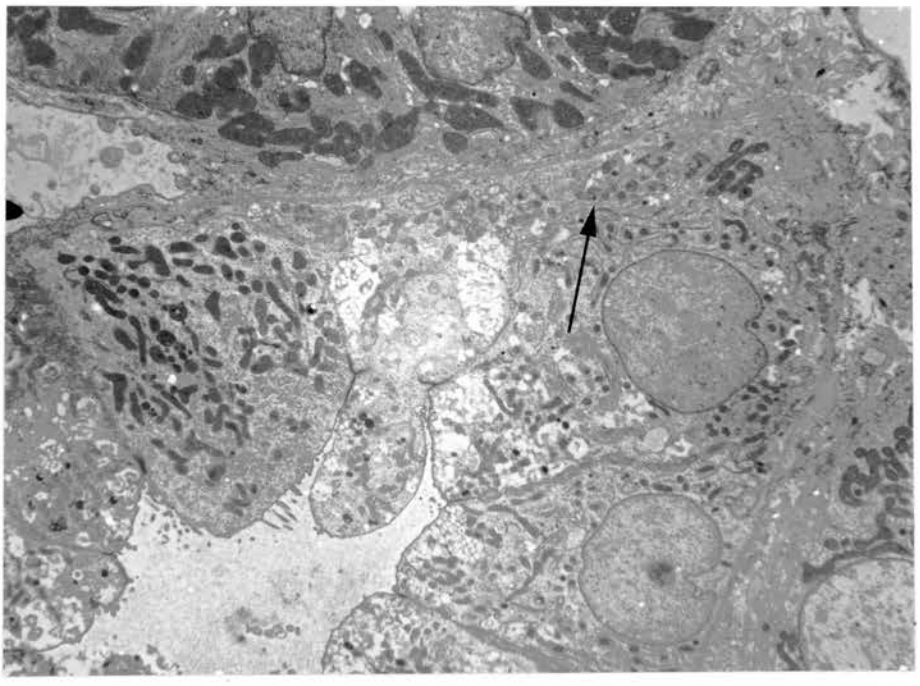


Fig.56. Turkey. 8 w.o. (4ppm OA). Kidney. Collecting tubule showing variations in mitochiondrial population (small, medium, large) and infolding of plasma membrane (arrow). x 4374.

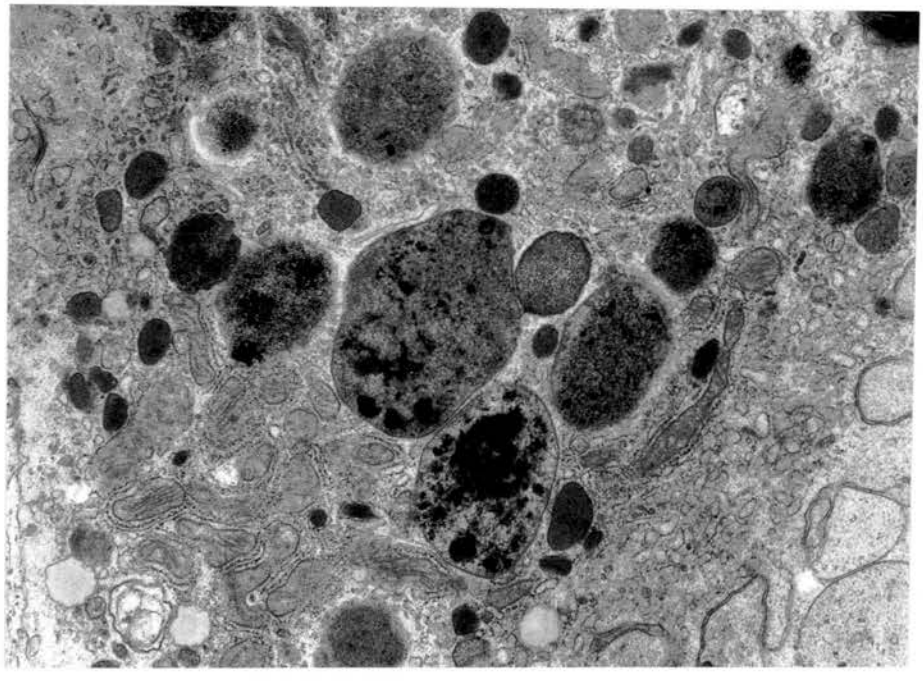


Fig.57. Turkey. 4 w.o. (4ppm OA). Liver. A hepatocyte showing increased number of lysosomes of varying sizes. Smooth endoplasmic reticulum is slightly dilated. x 18792.

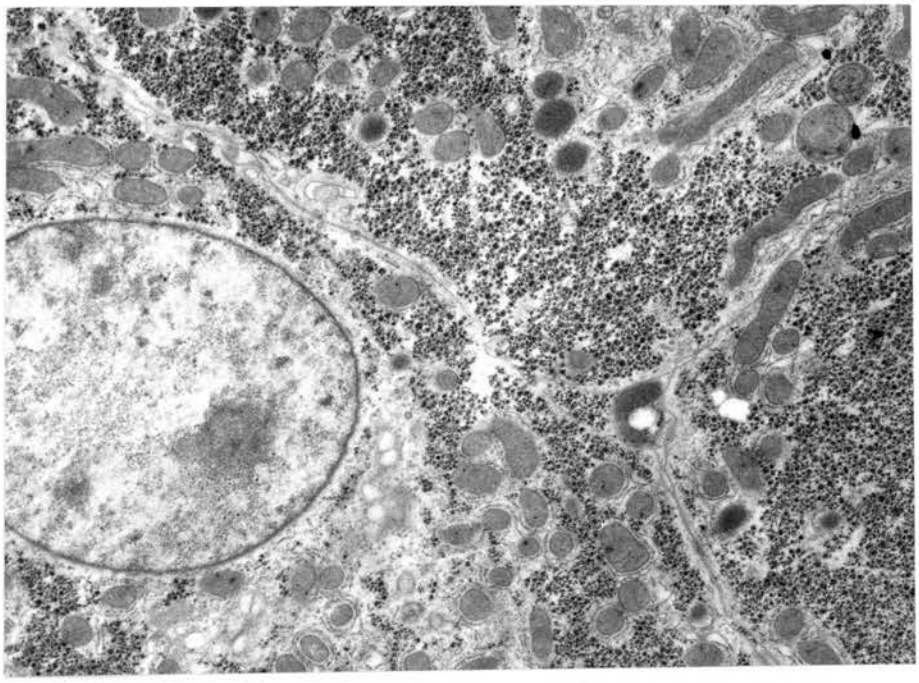


Fig.58. Turkey. 6 w.o.(4ppm OA). Liver. Glycogen granules are scattered in the cytoplasm. Mitochondrial cristae are indistinct. x 11664.

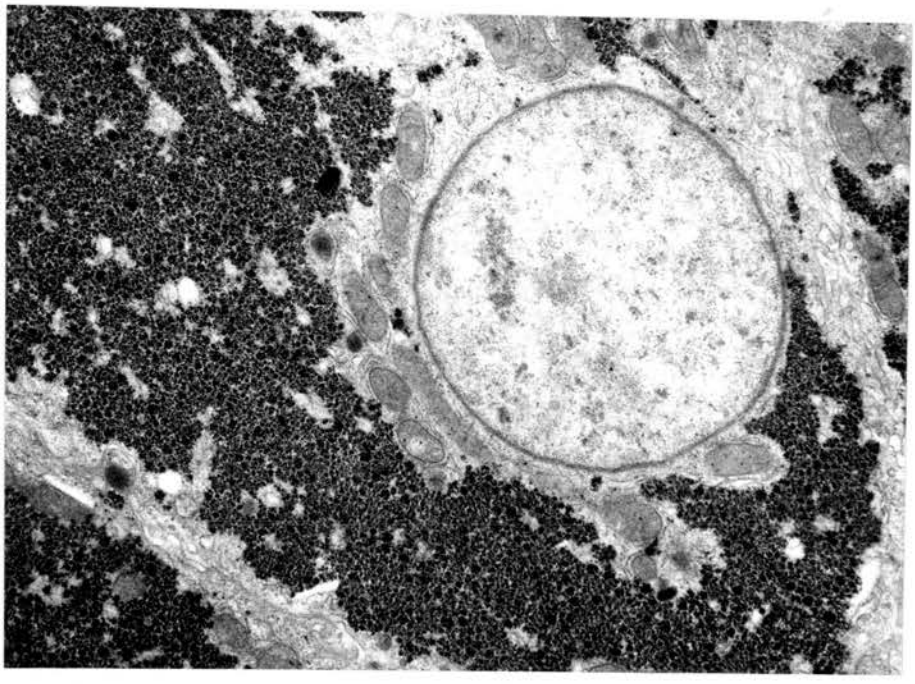


Fig.59. Turkey. 6 w.o. (4ppm OA). Liver. Glycogen granules in the hepatocytes. Other organelles are relatively normal. x 11664.

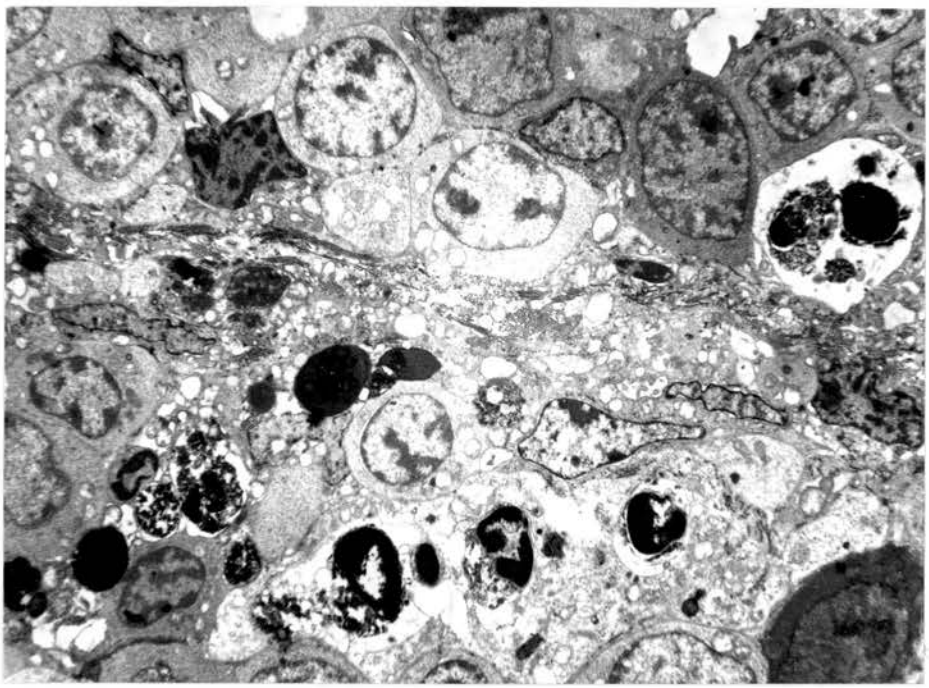


Fig.60. Turkey. 2 w.o. (4ppm OA). Bursa of Fabricius. Lymphoid follicles showing mitochondrial degeneration, vacuolation in the cytoplasm of lymphocytes and pyknotic and degenerating lymphoid cells. Increased number of lysosomes (ls) are present. x 4374.

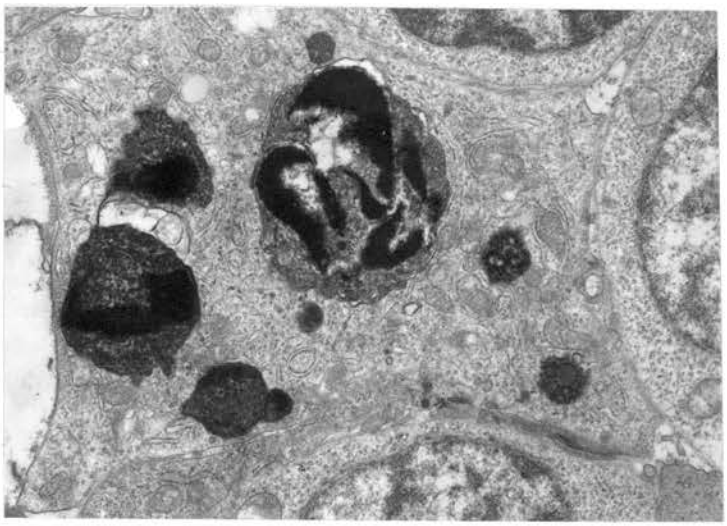


Fig.61. Turkey. 2 w.o. (4ppm OA). Bursa showing increased lysosomal activity. In a phagosome many pyknotic cells are seen. x 16200.

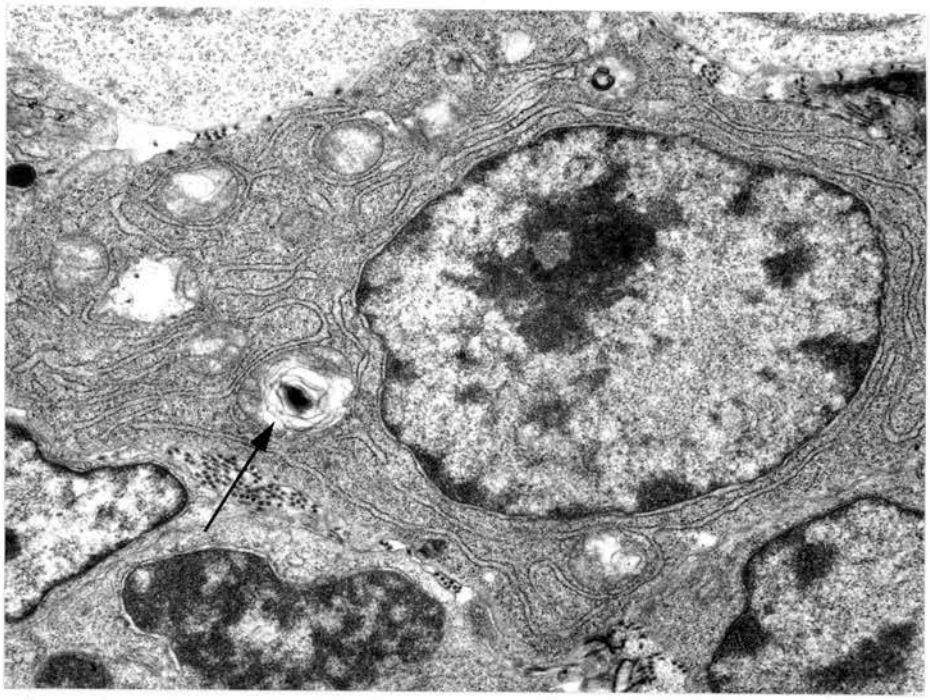


Fig.62. Turkey. 2 w.o. (4ppm OA). Bursa. A plasma cell showing degenerating mitochondria and an electron-dense Russell body (arrow). x 16200.

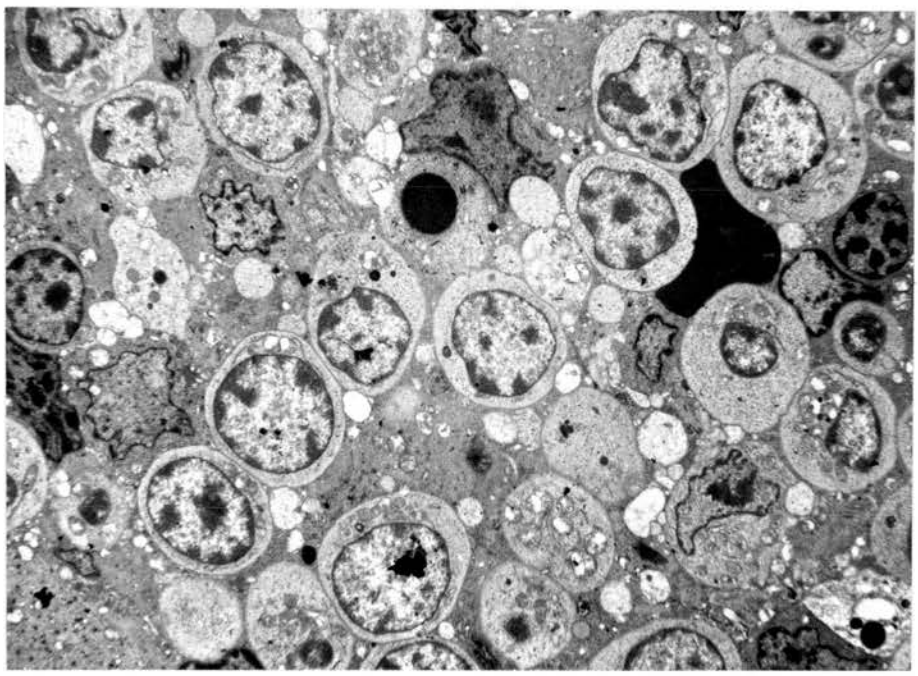


Fig.63. Turkey. 2 w.o. (4ppm OA). Thymus. Lymphocytes showing vacuolation, and degeneration of mitochondria. Many pyknotic cells are also present. x 4374.

changes, marked vacuolation and mitochondrial damage in the lymphoid cells (Fig.63). In some areas these changes were so marked that it was difficult to identify normal lymphocytes (Fig.64). Macrophage and heterophil activity was present (Fig.65).

In the spleen, the changes were similar but less marked. Vacuolation of lymphocytes was less frequent. Many lymphoblasts were seen, some of which had irregular nuclei. Some plasmablasts were seen containing Russell bodies and there was an infiltration of heterophils. A few mast cells were present (Fig.66).

4.8.2.4 Peyer's patch

At 2 and 4 weeks of age, degenerative changes were seen in the lymphoid cells of DLT and GC. Macrophage activity was evident and some heterophils with long extending cytoplasmic processes were seen. Immature and mature mast cells though fewer in number than in controls were seen at the base of the glands. At 6 weeks, marked degenerative changes were present both in lymphocytes as well as in plasma cells (Fig.67). At this time also large macrophages containing degenerating cells in the phagosomes were evident. At 8 weeks, lymphoid degeneration together with mitochondrial damage and vacuolation was more severe (Figs.68 and 69). Plasma cell degeneration became more marked as shown by dilated endoplasmic reticulum, degenerating mitochondria, vacuolation and sometimes the presence of Russell bodies or even complete disintegration (Figs.69 and 70). Lysosomal activity was also marked near degenerating plasma cells (Fig.71). Lymphoid populations were reduced in DLT and GC.

4.8.2.5 Caecal tonsil

As with the Peyer's patch, vacuolation in the

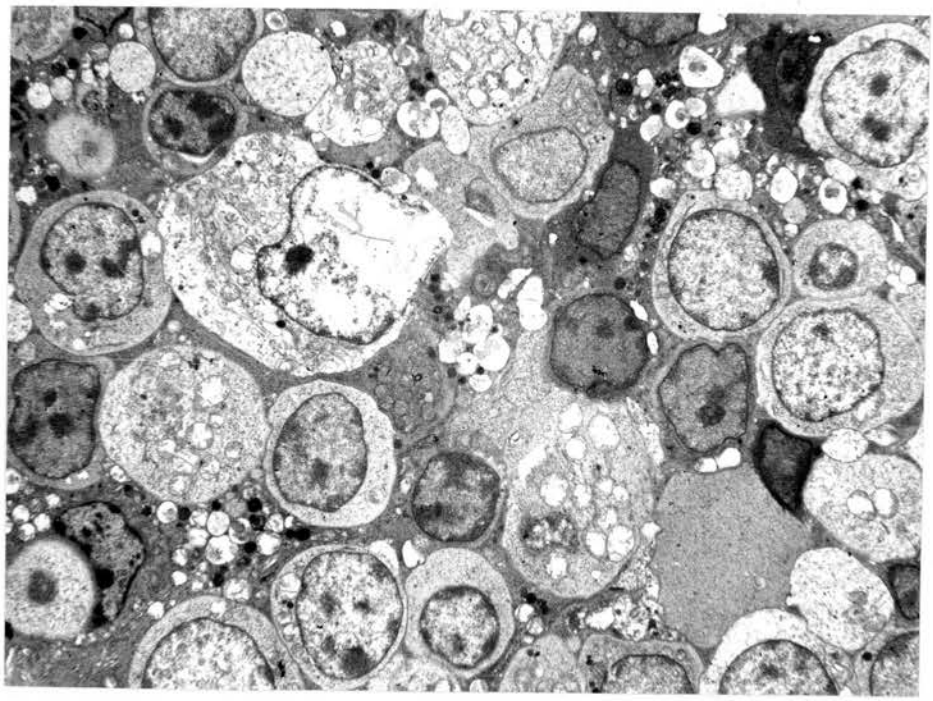


Fig.64. Turkey. 2 w.o. (4ppm OA). Thymus. Degenerating and dying lymphoid cells and marked mitochondrial damage in most of the cells. A large lymphocyte is showing vacuolation and damage in the nucleus.x 4374.

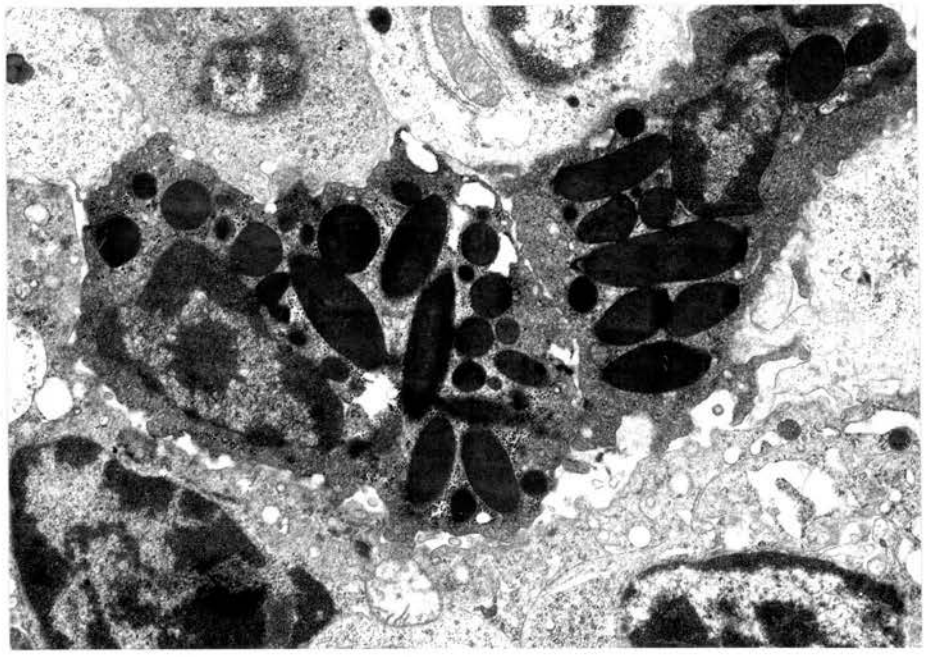


Fig.65. Turkey.4w.o. (4ppm OA). Thymus. Note the presence of heterophils in the medullary region. x 16200.

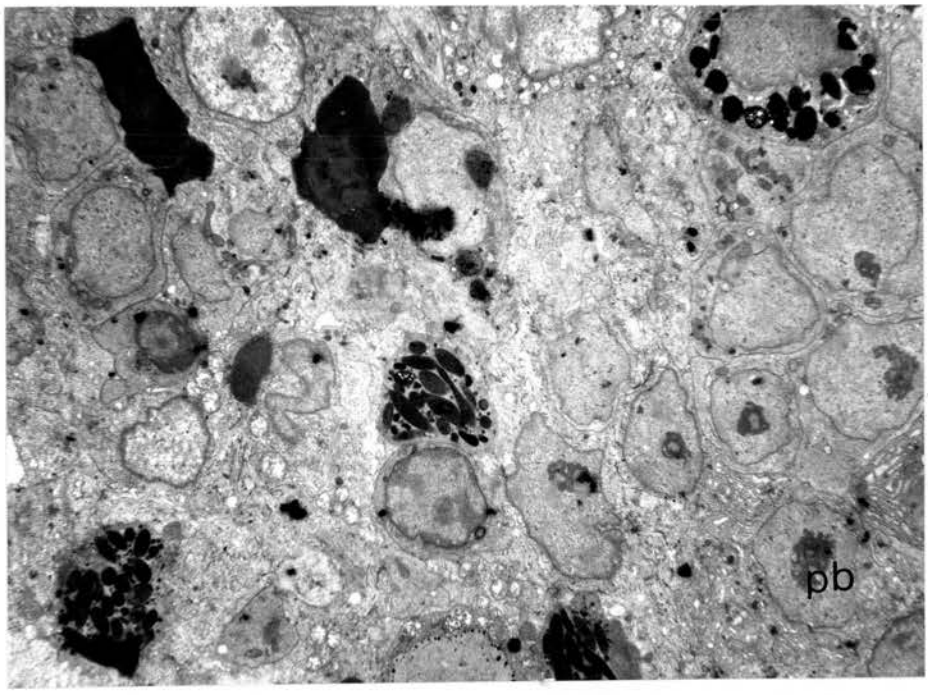


Fig.66. Turkey. 2 w.o. (4ppm OA). Spleen. Lymphoblasts with prominent nucleoli, degenerating lymphoid cells, a few plasmablasts (pb) and heterophils are present. x 4374.

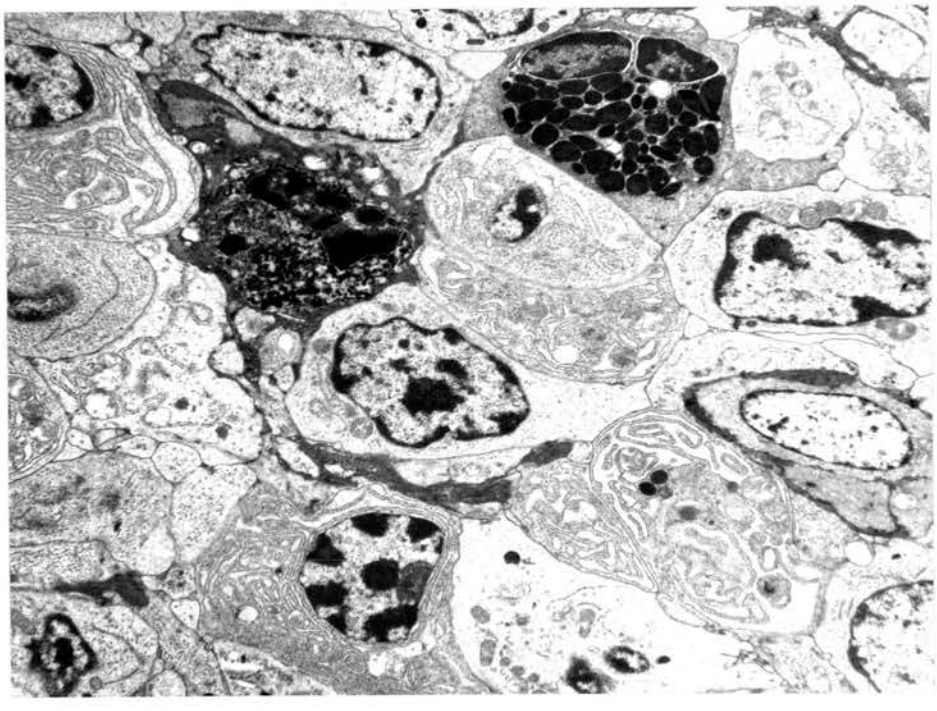


Fig.67. Turkey. 6 w.o. (4ppm OA). Peyer's patch. DLT region showing a dark macrophage, degenerating lymphocytes and plasma cells in various stages of disintegration. A heterophil is also present. x 5022.

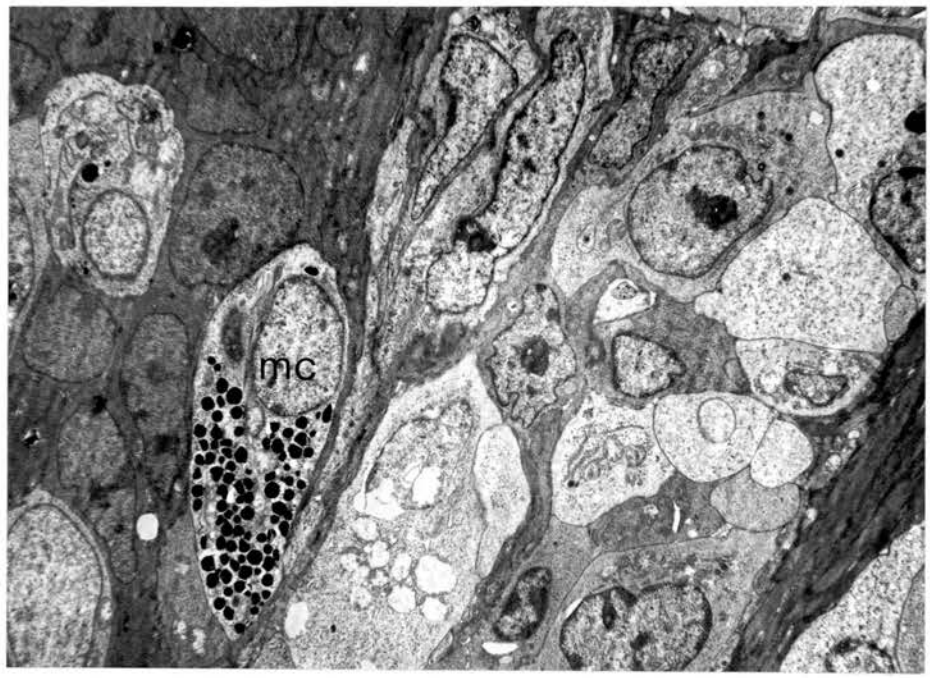


Fig.68. Turkey. 8 w.o. (4ppm OA).Peyer's patch. DLT region showing many empty dead cells and some degenerating and dying cells. A mast cell (mc) is also to be seen at the base of the gland. x 4374.

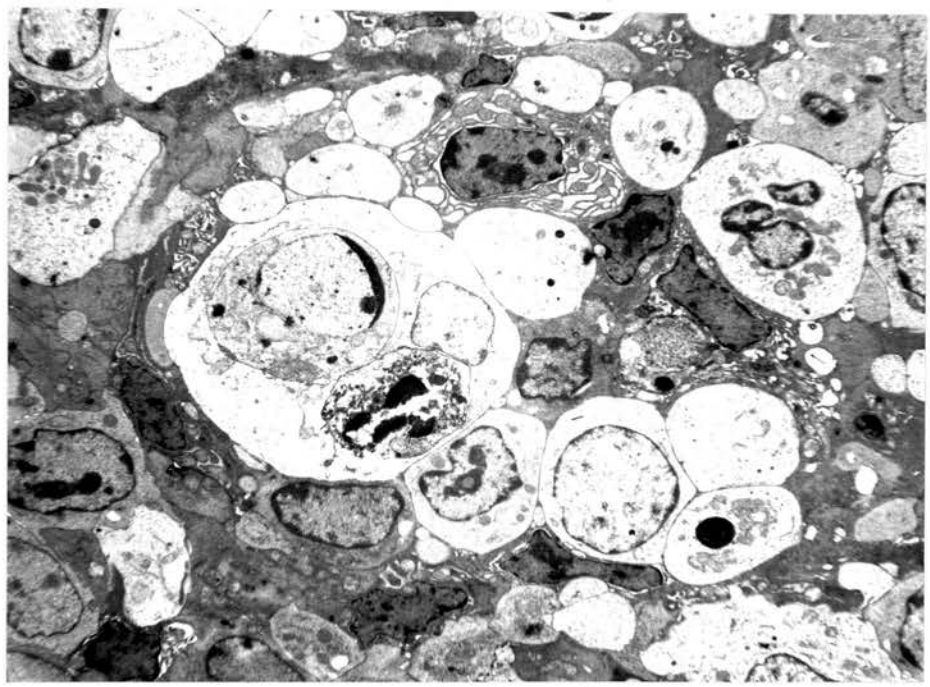


Fig.69. Turkey. 8 w.o. (4ppm OA). Peyer's patch. DLT region. Most of the lymphoid cells and plasma cells are disintegrating. A large degenerating macrophage is also present. x 4374.

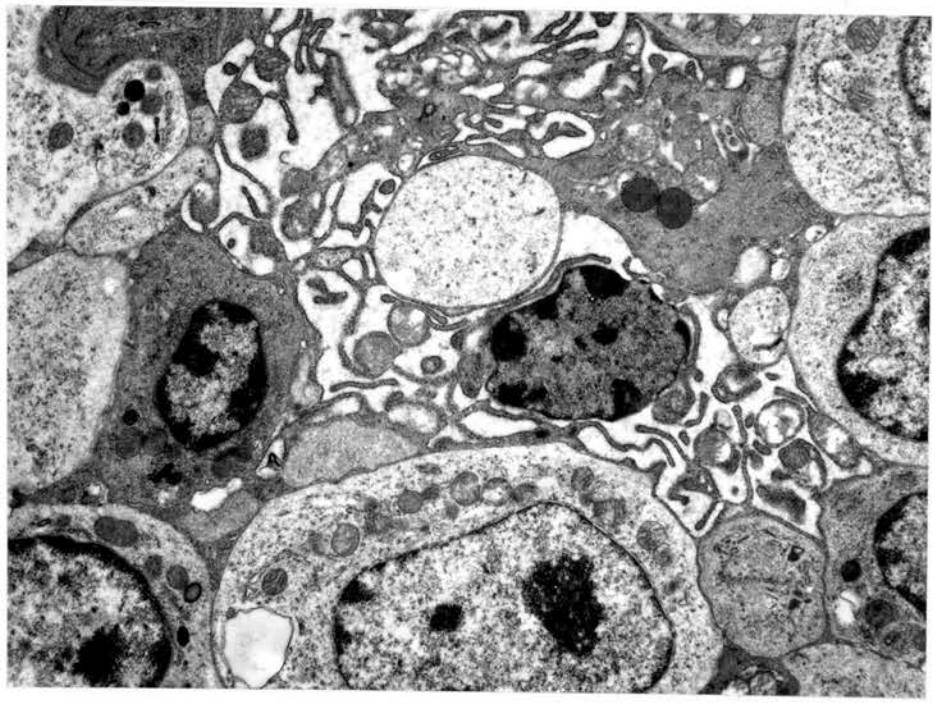


Fig.70. Turkey. 8 w.o. (4ppm OA). Peyer's patch. Plasma cells showing degeneration of mitochondria and loss of endoplasmic reticulum. The lymphoblast is showing early degenerative changes and the presence of a large vacuole. x 9396.

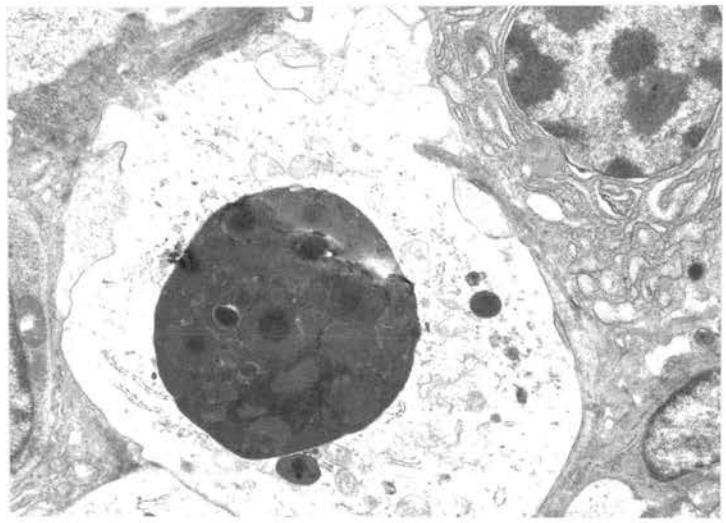


Fig.71. Turkey. 8 w.o. (4ppm OA). Peyer's patch. Note the presence of a large lysosome in a dead cell adjacent to a plasma cell. x 7776.

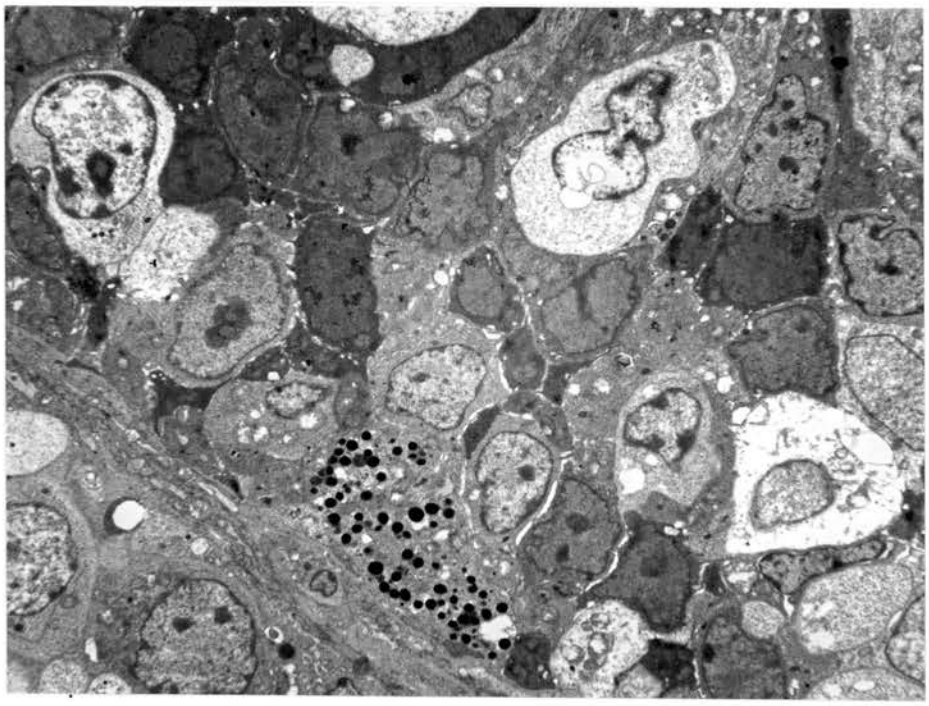


Fig.72. Turkey. 2 w.o. (4ppm OA). Caecal tonsil. A gland showing a mast cell at the base, degenerating lymphoid cells and vacuolation in whole area. x 4374.

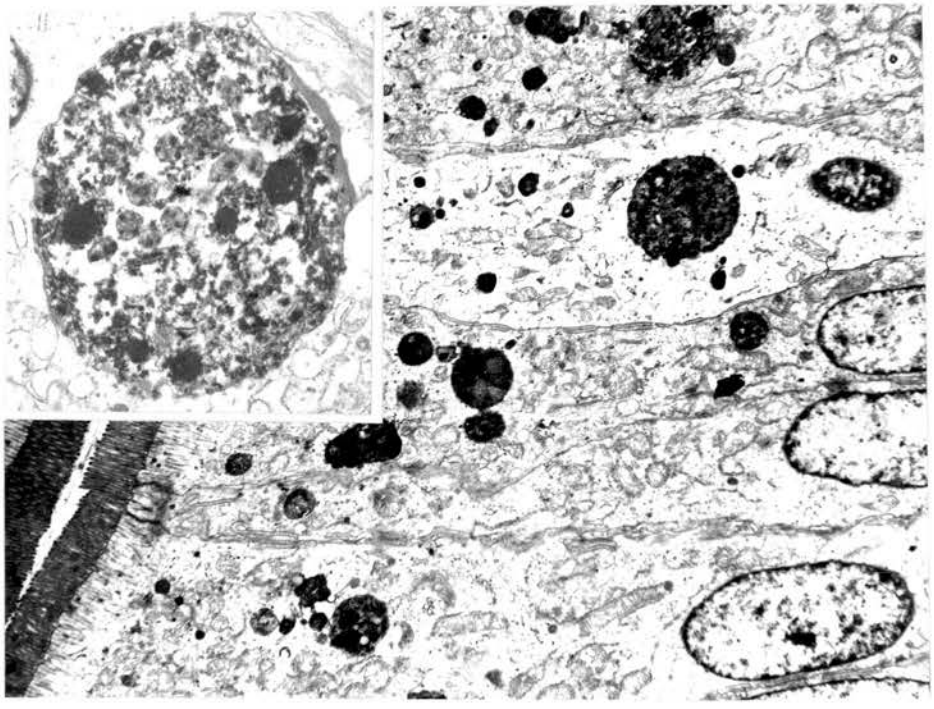


Fig.73. Turkey. 4 w.o.(4ppm OA).Caecal tonsil.Epithelial cells showing degeneration of mitochondria and other cytoplasmic organelles and increased number of lysosomes. x 4374. The inset shows a high power view of a lysosome degenerating mitochondria and dilated endoplasmic reticulum. x 7776.

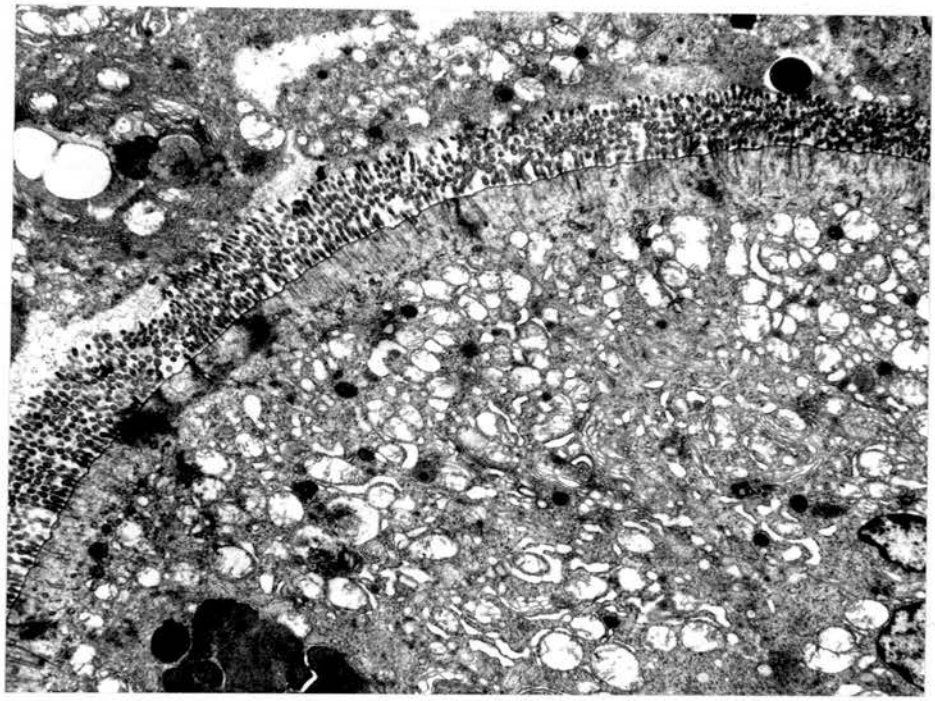


Fig.74. Turkey. 4 w.o. (4ppm OA). Caecal tonsil. Epithelial cells showing degnerating mitochondria and marked vacuolation. x 6156.

lymphoid cells was seen in DLT and GC. Mast cells could be seen at the base of the gland and some degenerating cells were also present (Fig.72) at 2 weeks of age. At 4 weeks of age, epithelial cells also showed degenerative changes in the cytoplasm together with increased lysosomal activity (Fig.73). The epithelium gave a vacuolated appearance due to degenerating mitochondria (Fig.74). Plasma cell and lymphocyte degeneration was increased as also was macrophage activity.

4.9 Bone Changes

4.9.1 Bone and toe ash content

Ash contents of tarso-metatarsal bones and middle toes from 0 and 4 ppm OA-treated groups of broilers and turkeys are presented in Tables 13a and 13b respectively. There were no significant differences between the two groups in the ash contents of the bones and the middle toes either in broilers (from 4 to 10 weeks of age) or in turkeys (from 4 to 8 weeks of age). There was, however, a slight reduction in the ash content of the tarso-metatarsal bones, particularly in the OA-treated broilers (43.86 per cent) as compared with controls (48.93 per cent). The weights of the defatted tarso-metatarsal bones and unextracted middle toes were consistently lower in the OA-treated groups (Table 13a).

4.9.2 Patho-morphological changes

The pelvic limb bones (femur, tibio-tarsus, tarso-metatarsus and digits) were smaller and appeared to be reduced (Fig.75). Radiological examination did not reveal any significant abnormalities.

Histopathological examination of the femur, tibio-tarsal and tarso-metatarsal bones showed marked changes, particularly in the diaphyses, in the OA-treated birds.

Table 13a. Effect of OA on the bone and toe ash content (per cent) in broilers (mean ± SEM)[a]

Age (weeks)	group	Torsometatarsus bone		Middle toe	
		weight (g)	ash	weight (g)	ash
4	0ppmOA	1.680 ± 0.106	46.46 ± 0.235	0.703 ± 0.065	12.98 ±0.138
	4ppmOA	0.938 ± 0.085	40.21 ± 0.850	0.429 ± 0.031	11.88 ± 0.87
6	0ppmOA	2.545 ±0.306	48.46 ±0.231	1.717 ± 0.147	14.65 ± 0.17
	4ppmOA	1.448 ±0.224	41.72 ± 1.548	1.084 ± 0.042	13.73 ± 1.52
8	0ppmOA	3.812 ±0.735	48.39 ± 0.73	3.018 ± 0.729	12.80 ±0.063
	4ppmOA	2.515 ± 0.022	42.81 ±0.884	1.721 ±0.186	12.22 ±1.083
10	0ppmOA	4.577 ± 0.339	52.41 ±0.235	4.915 ±0.506	12.02 ± 0.87
	4ppmOA	3.730 ± 0.137	50.70 ±1.013	3.371 ±0.221	11.46 ±0.891

[a]No significant differences were noticed between 0 and 4ppm OA groups ($P < 0.1$).

Table 13b. Effect of OA on bone and toe ash content (per cent) in turkeys (mean ± SEM)[a]

Age (weeks)	group	Torsometatarsus bone		Middle toe	
		weight (g)	ash	weight (g)	ash
4	0ppmOA	0.993 ± 0.115	45.26 ± 0.201	0.60 ± 0.101	13.99 ± 0.048
	4ppmOA	0.689 ± 0.095	44.54 ± 1.147	0.418 ± 0.091	13.50 ± 0.072
6	0ppmOA	1.717 ± 0.330	45.07 ± 0.08	1.076 ± 0.223	14.80 ± 0.050
	4ppmOA	1.035 ± 0.021	44.03 ± 0.287	0.623 ± 0.012	14.50 ± 0.085
8	0ppmOA	3.274 ± 0.526	48.27 ± 0.402	1.810 ± 0.314	17.70 ± 0.192
	4ppmOA	2.450 ± 0.408	45.60 ± 0.645	1.439 ± 0.213	16.39 ± 0.277

[a]No significant differences were noticed between 0 and 4 ppm OA groups ($P < 0.1$).

Fig.75. Turkey. 4 w.o. Pelvic limb bones. Note the underdeveloped bones in 4 ppm OA-treated bird (right). Control is on the left.

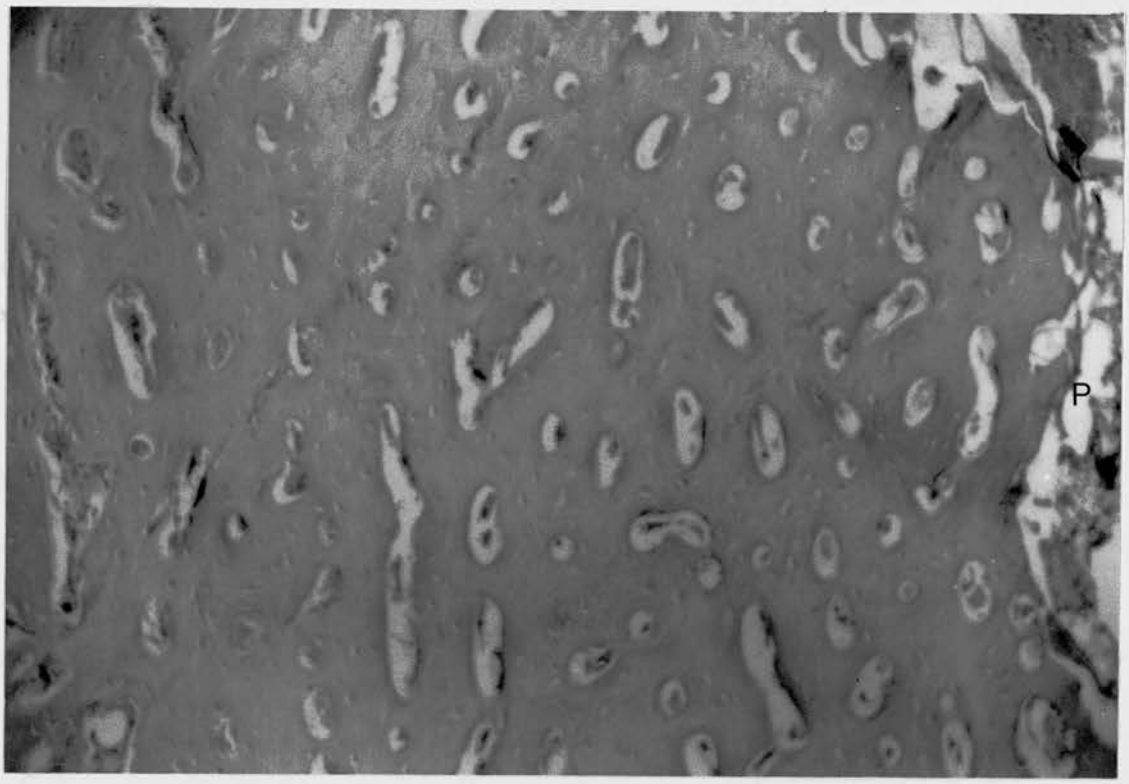


Fig.76a. Turkey. 4 w.o. (control). Femur. Normal cortical bone from approximately mid-diaphysis demonstrating normal appositional bone deposition beneath periosteum (P). SOFG x 120.

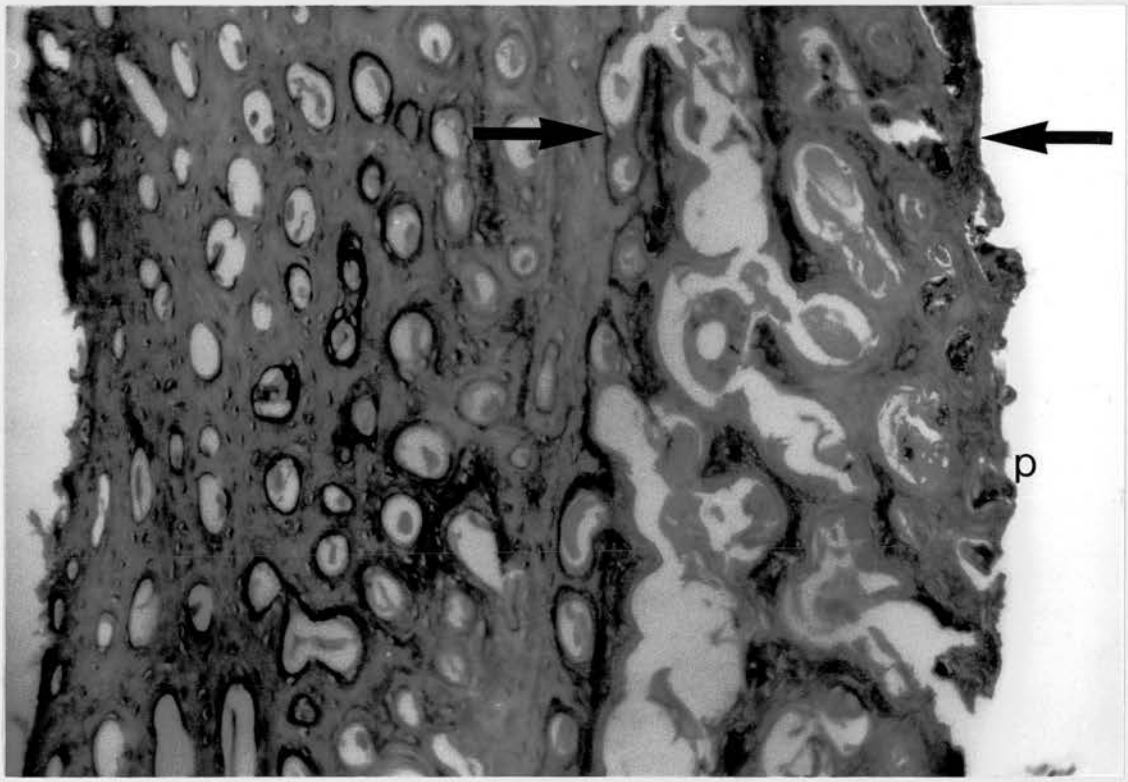


Fig.76b. Turkey. 4 w.o. (4ppm OA) Femur. Abnormal cortical bone development (mid-diaphysis) demonstrating abnormal (scant) subperiosteal new bone apposition (between arrows). TB x 120.

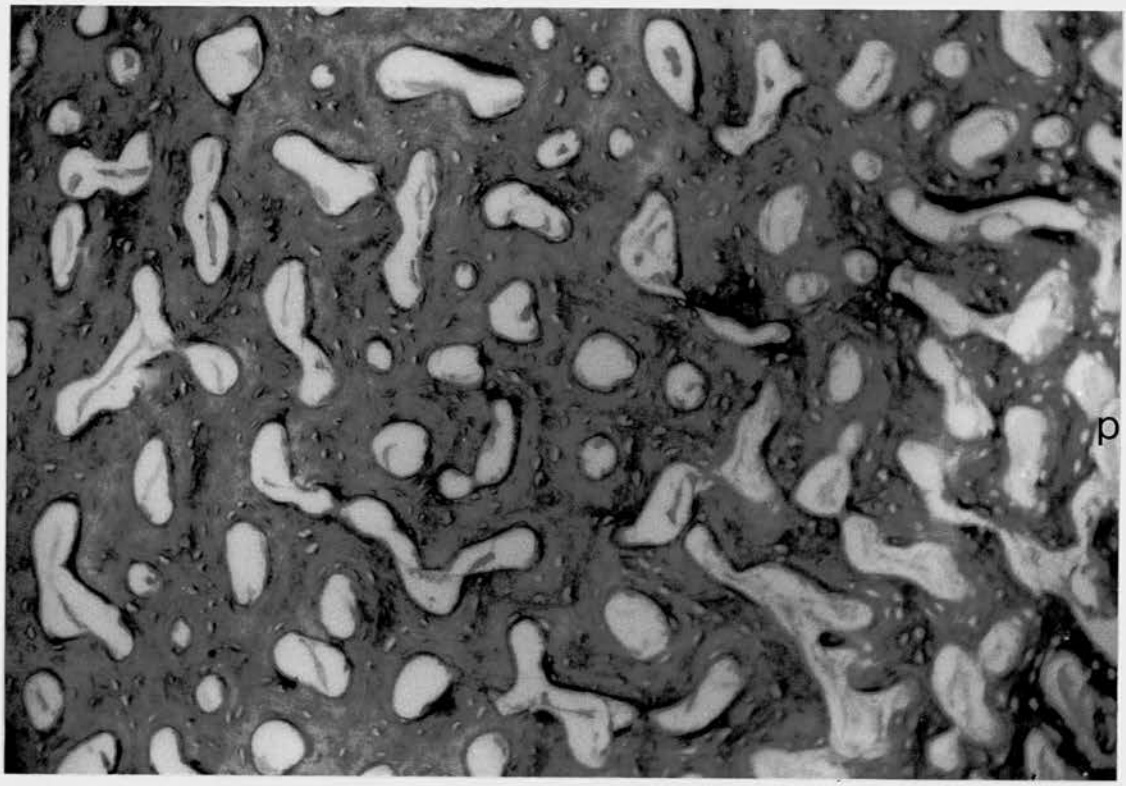


Fig.77a. Turkey. 4 w.o.(Control). Tibio-tarsus. Normal cortical bone from approximately mid-diaphysis demonstrating normal appositional bone deposition close to periosteal surface. TB x 120.

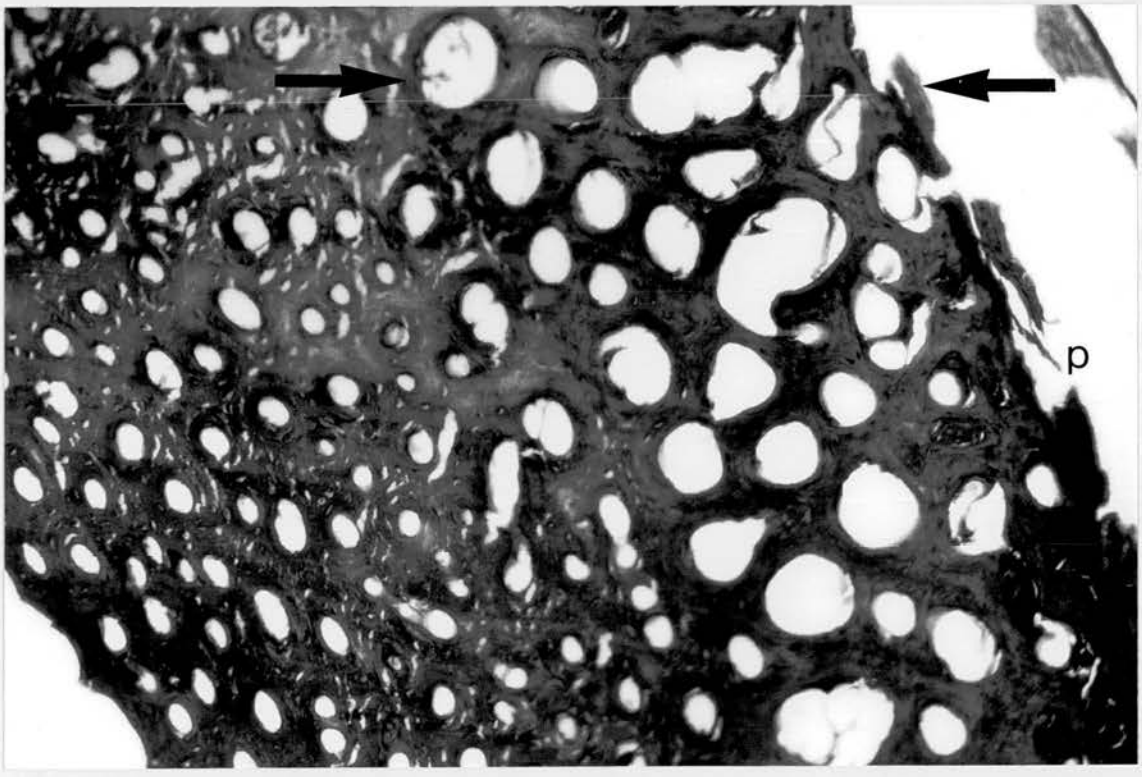


Fig.77b. Turkey. 4 w.o. (4ppm OA). Tibio-tarsus. Abnormal cortical bone development (mid-diaphysis) demonstrating abnormal (scant) subperiosteal new bone apposition (between arrows). SOFG x 120.

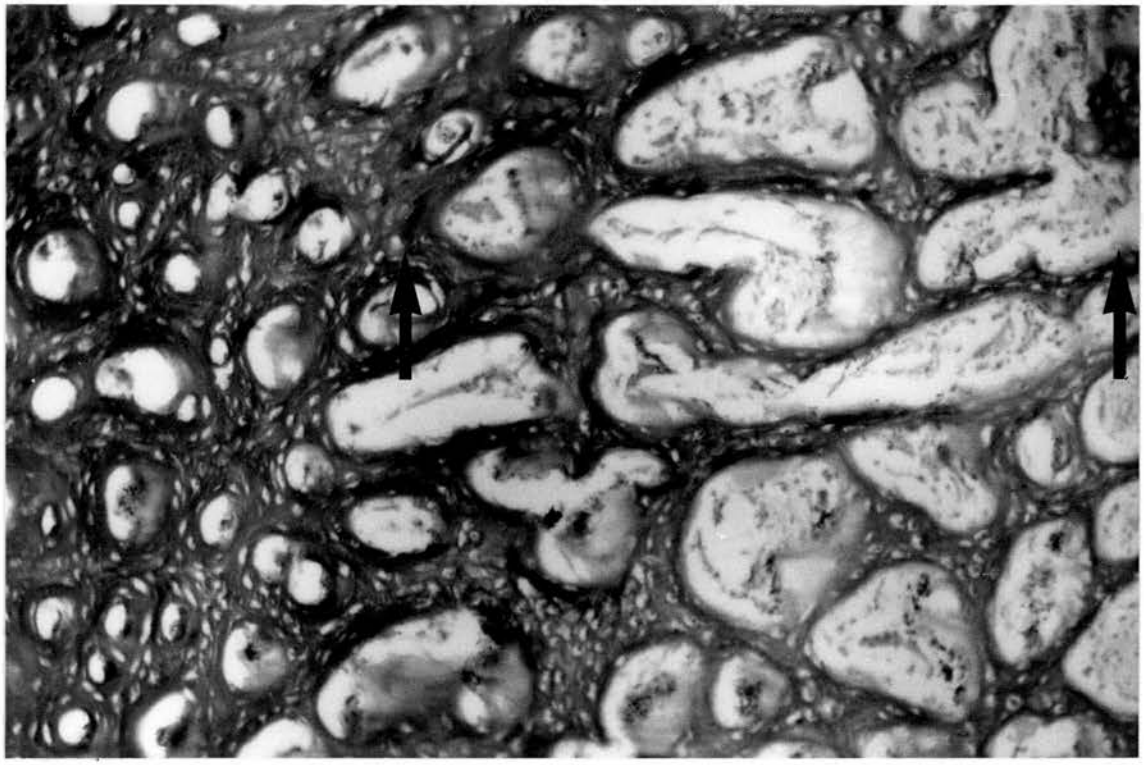


Fig.78. Turkey. 4 w.o. (4ppm OA). Tarso-metatarsus. Abnormal cortical bone development (mid-diaphysis) showing abnormal (scant) subperiosteal new bone apposition (between arrows). H & E x 120.

Periosteally derived lamellae were tenuous, with thin seam of bone deposition. Haversian bone which was deposited prior to ochratoxin ingestion was relatively normal. Similar changes were seen in femurs (Fig.76b), tibio-tarsi (Fig.77b) and tarso-metatarsi (Fig.78) from OA-treated birds but not in those from controls (Figs.76a and 77a).

4.10 Serological and Immunological Changes

4.10.1 Immunoglobulin levels

4.10.1.1 Serum immunoglobulins

A good radial immunodiffusion was obtained at 36 hours of incubation of petridishes (Fig.79). The effect of OA on immunoglobulin levels in the serum of broiler chicks (Experiment I) is presented in Fig.80. Immunoglobulin concentrations were lower in sera from OA fed birds than in those from controls. IgA levels were 66.35 ($\pm$2.77) per cent and 62.04 ($\pm$5.80) per cent of the normal concentration in birds fed 2 and 4 ppm OA respectively. IgG levels in birds of these two groups were lowered to 63.77 ($\pm$4.88) per cent and 62.29 ($\pm$7.75) per cent and IgM values were 64.88 ($\pm$3.98)per cent and 56.91 ($\pm$3.69) per cent of normal values.

Serum immunoglobulin levels in turkeys (Experiment V), determined by radial immunodiffusion using antisera against chicken IgA, IgG and IgM, after 4 weeks of 4 ppm OA-treatment are depicted in Fig.81. No reaction was observed for IgA. Serum IgG levels were not altered by OA-treatment (98.83 $\pm$ 8.09 per cent of normal concentration) in turkeys. However, a significant ($p < 0.1$) drop in the IgM levels was noticed (84.38 $\pm$ 3.98 per cent of normal value) in OA-treated birds.

The PRIST IgE technique using antisera to human IgE

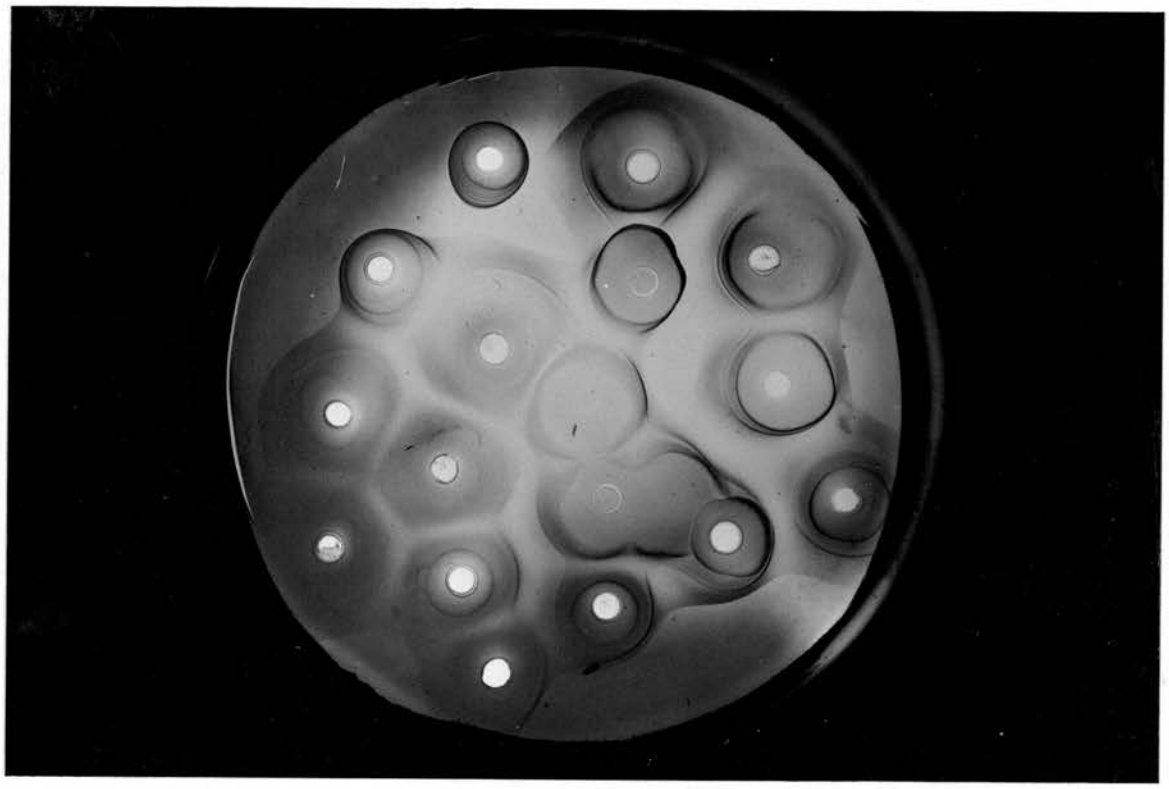


Fig.79. Radial immunodiffusion. Fowl IgG showing precipitin reaction at 36 hours.

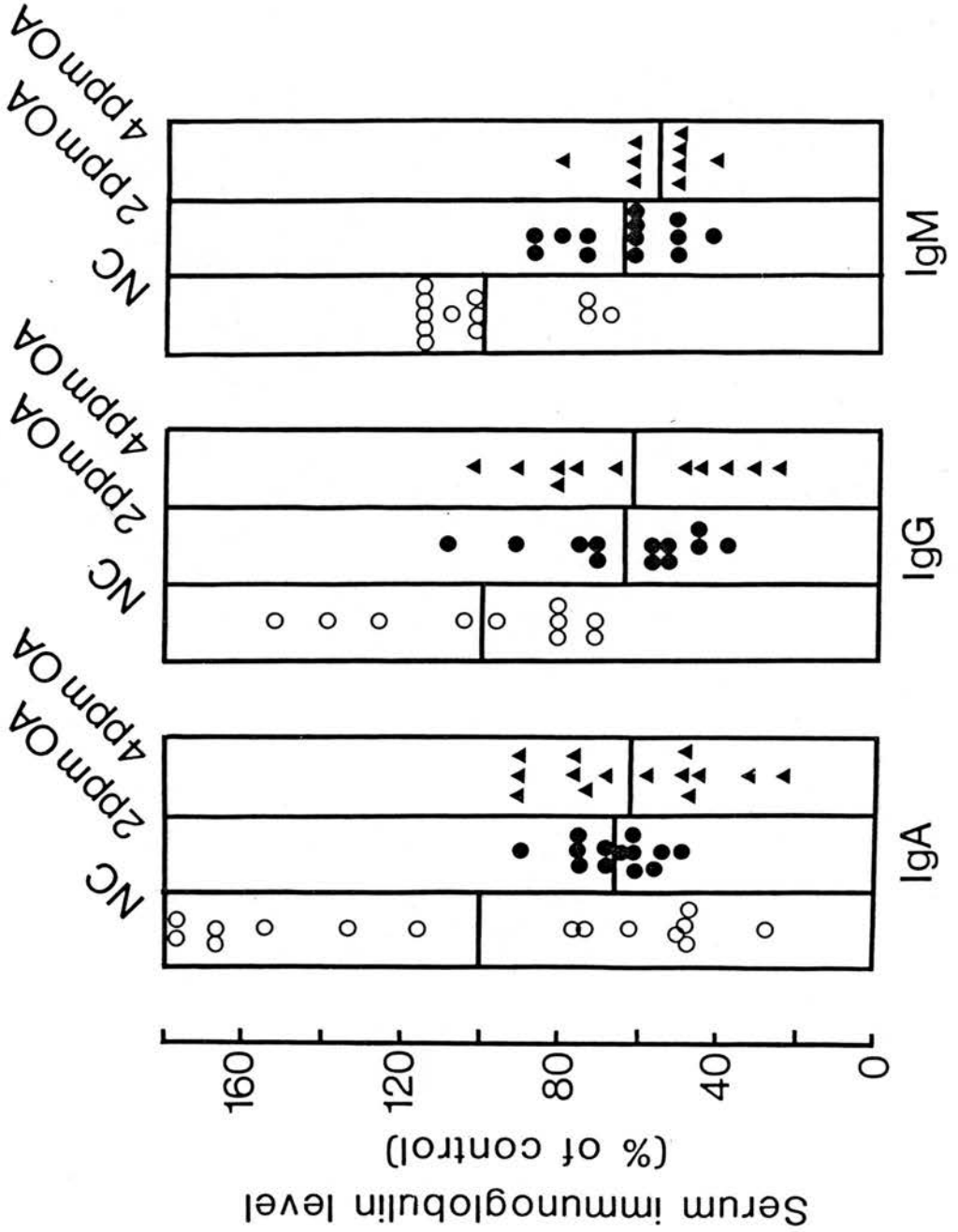


Fig.80. Effect of feeding OA on the serum immunoglobulin levels in broiler chicks.

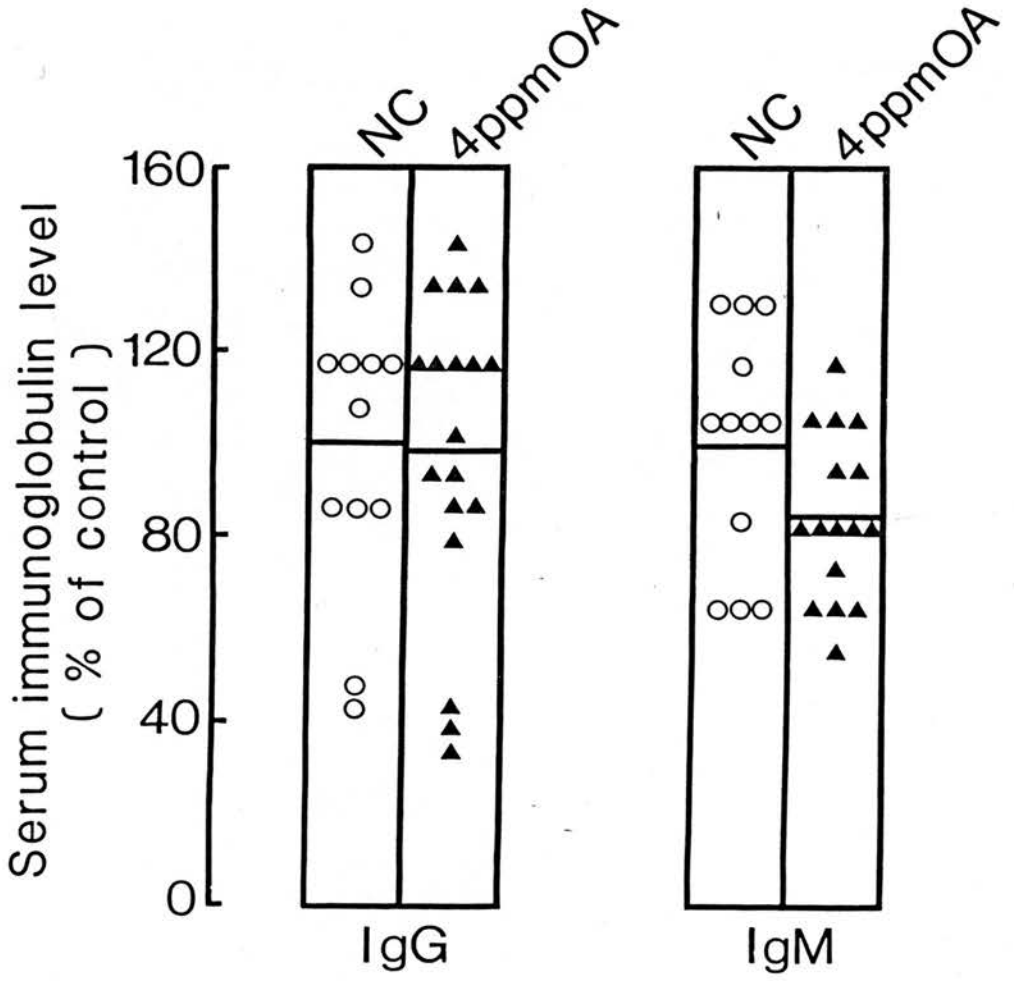


Fig.81. Effect of feeding OA on the serum immunoglobulin levels in turkeys.

showed no differences in IgE levels in sera from OA-treated broilers and turkeys compared with those from controls. Very low levels of IgE, ranging from 400 to 695 counts in birds from Experiment I (broilers), 518 to 546 counts from Experiment II (broilers) and 384 to 461 counts from Experiment V (turkeys), per minute of 125 I labelled activity, were measured in all the sera tested. It was not possible to calculate the exact concentrations of IgE per litre of serum because of the very low counts.

4.10.1.2 Tissue immunoglobulins

Immunofluorescence was used to study the concentration, localisation and intensity of IgA, IgG and IgM in various tissues of broilers and turkeys. The technique was also employed to examine the tissue response of these birds to injections of bovine serum albumen (BSA) antigen.

In general, similar responses, though of varying intensity and extent, were noticed at 2, 3, 4, 6, 8 and 10 weeks of age in broilers and up to 8 weeks of age in turkeys. The immunoglobulin concentrations were reduced in all the lymphoid organs studied in OA-treated birds as compared with controls. This reduction was more marked in broilers than in turkeys. Specific antisera to turkey immunoglobulins were not available but labelled antisera to fowl immunoglobulins (IgA, IgG and IgM) gave good results in demonstrating immunoglobulins in turkey tissues.

Differences in area and intensity of fluorescence were usually noted in tissues from OA-fed birds treated for the immunofluorescent demonstration of IgA, IgG and IgM when compared with those from controls.

4.10.1.2.1 Broilers

Bursa of Fabricius

In sections of bursa of Fabricius from control birds fluorescence was mainly confined to the medulla of the follicles for all three immunoglobulins (Fig.82), whereas in sections from OA-treated birds cells with cytoplasmic fluorescence were found in the subepithelial regions (Fig.83) and interfollicular areas; these cells tended to be large and few in number. The reaction was similar for all three immunoglobulins though in bursae from both control and OA-treated birds fewer cells were positive for IgM than for IgG or IgA. The difference in fluorescence between control and treated-birds were most marked at 4 ppm OA (Experiment I).

Thymus

Cells with cytoplasmic fluorescence for all three immunoglobulines were relatively reduced in numbers in sections from OA-treated birds (Fig.84). Hassall's corpuscles fluoresced non-specifically more frequently in sections from control birds.

Spleen

Immunofluorescent staining for immunoglobulins was very variable in sections of spleen from OA-fed birds and fluorescing cells appeared to be fewer in number (Fig.85) than in sections from control birds. Germinal centres were usually also reduced both in number and size in the spleens from treated birds. Fluorescing cells were noticed rarely in the germinal centres (Fig.87) whereas a few positive cells were always noticed in the germinal centres in control birds (Fig.86). In some cases, mulberry-like cells were seen fluorescing in the spleens of OA-treated birds (Fig.88). Anti-BSA-immunoglobulins

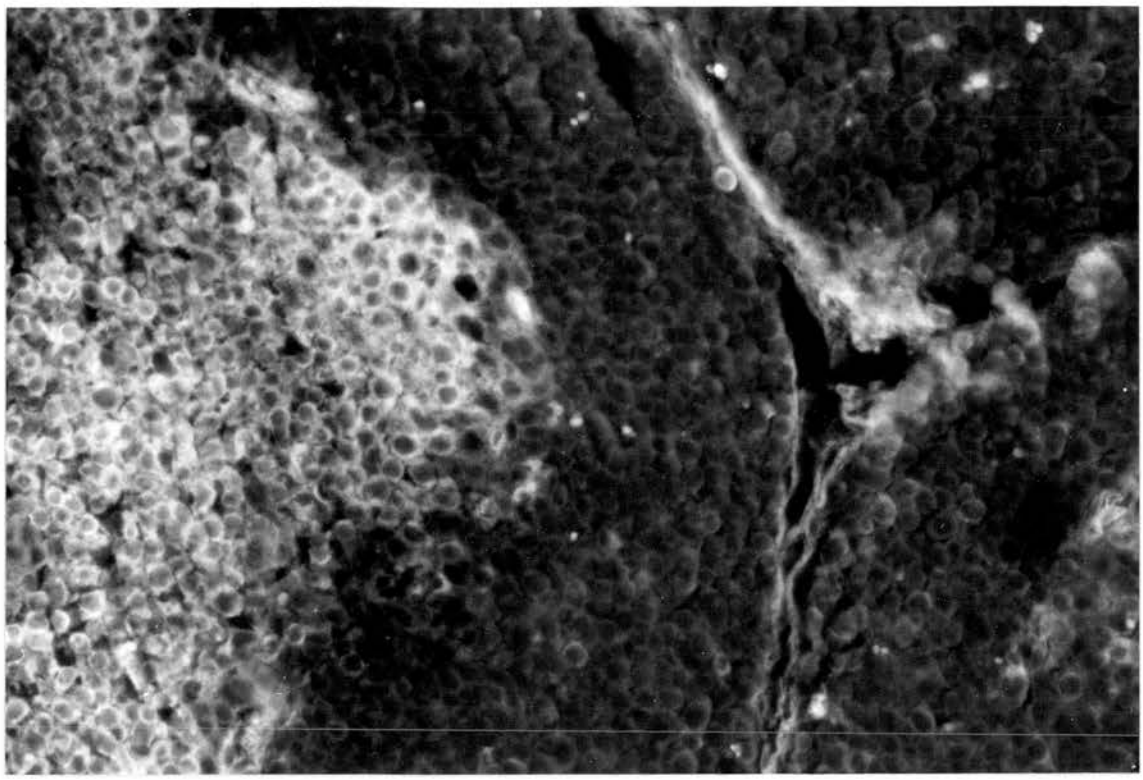


Fig.82. Fowl. 3 w.o.(Control). Bursa of Fabricius showing fluorescing lymphocytes in the medullary region of a thickly populated lymphoid follicle. Anti-IgG x 640.

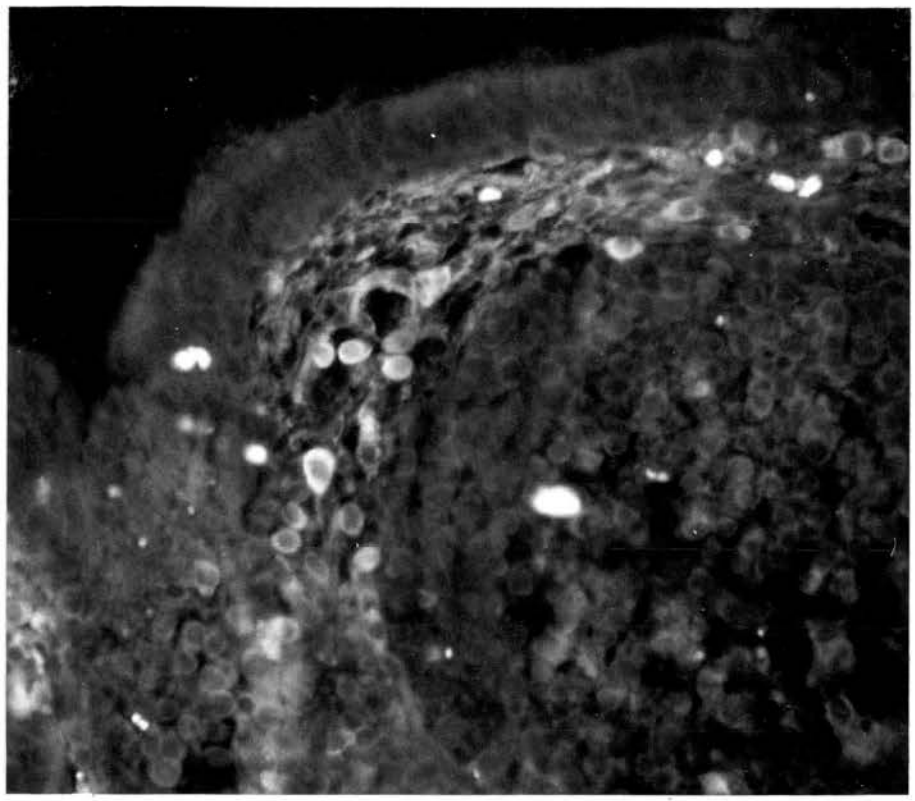


Fig.83. Fowl. 3 w.o. (4ppm OA). Bursa showing fluorescing lymphocytes in the subepithelial region. Lymphoid follicles are negative and depleted. Anti-IgG x 640.

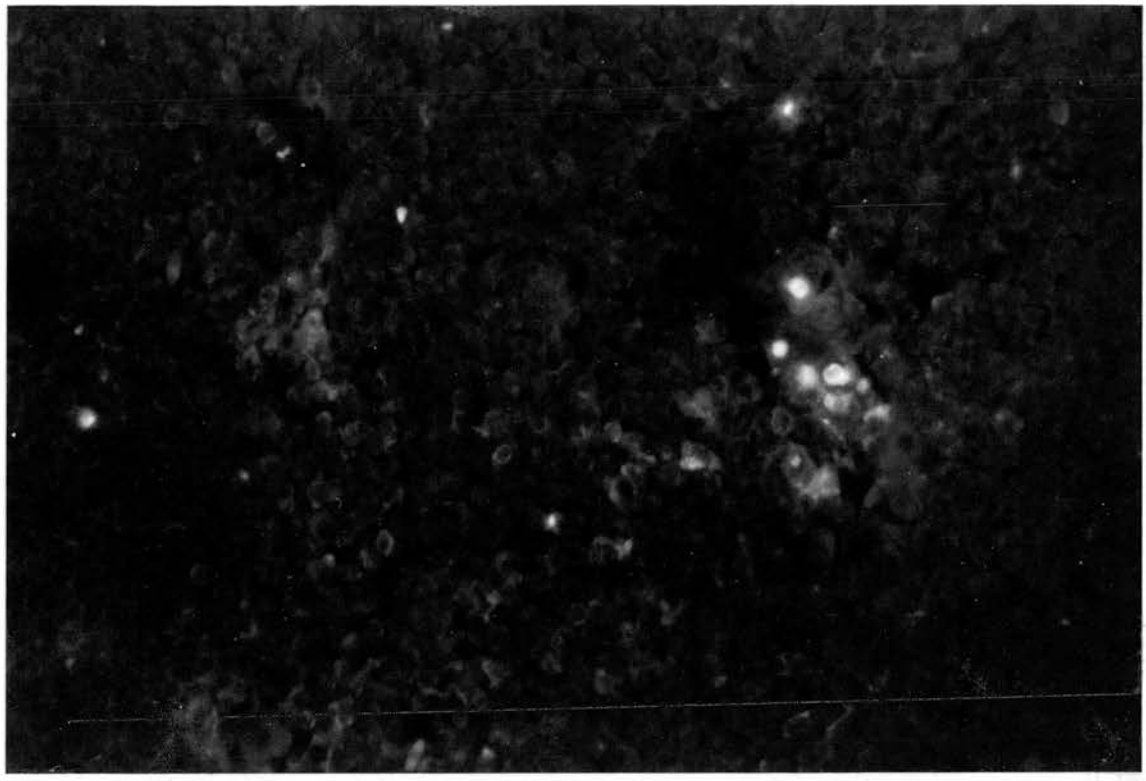


Fig.84. Fowl. 4 w.o. (4ppm OA). Thymus showing few positive cells. Anti-IgM x 640.

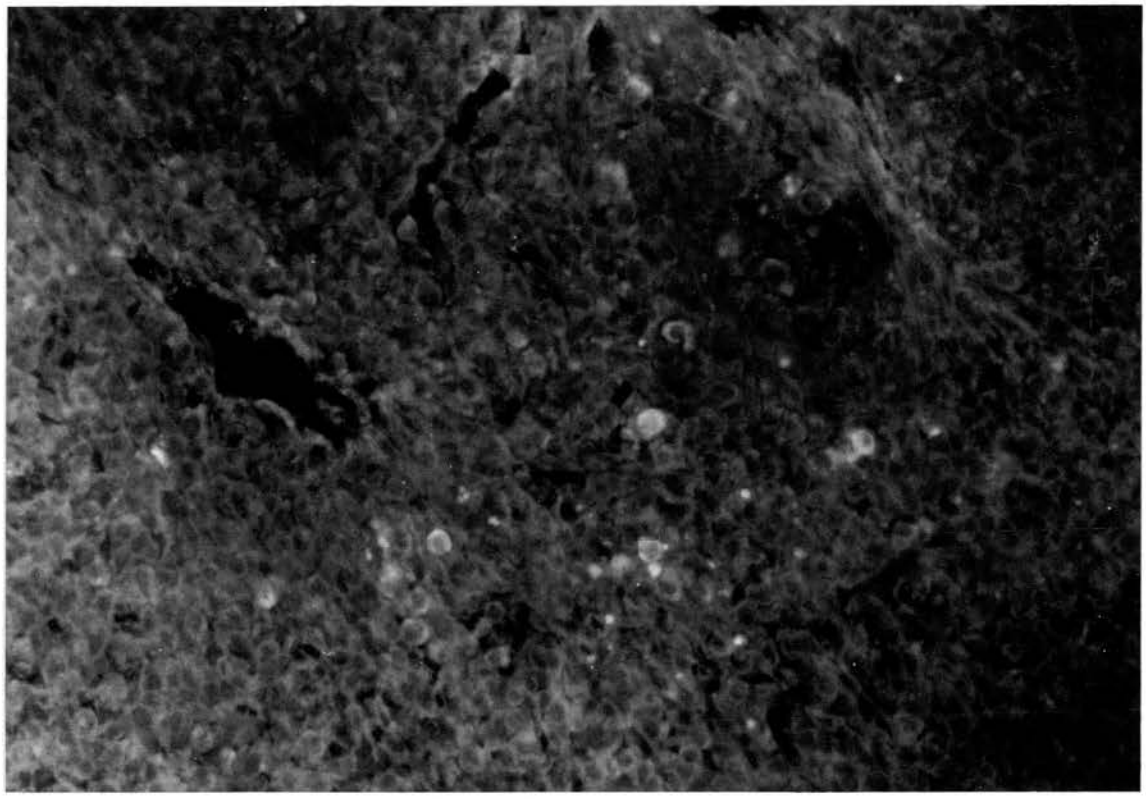


Fig.85. Fowl. 10 w.o. (2ppm OA). Spleen showing only a few strongly positive cells. Anti-IgA x 640.

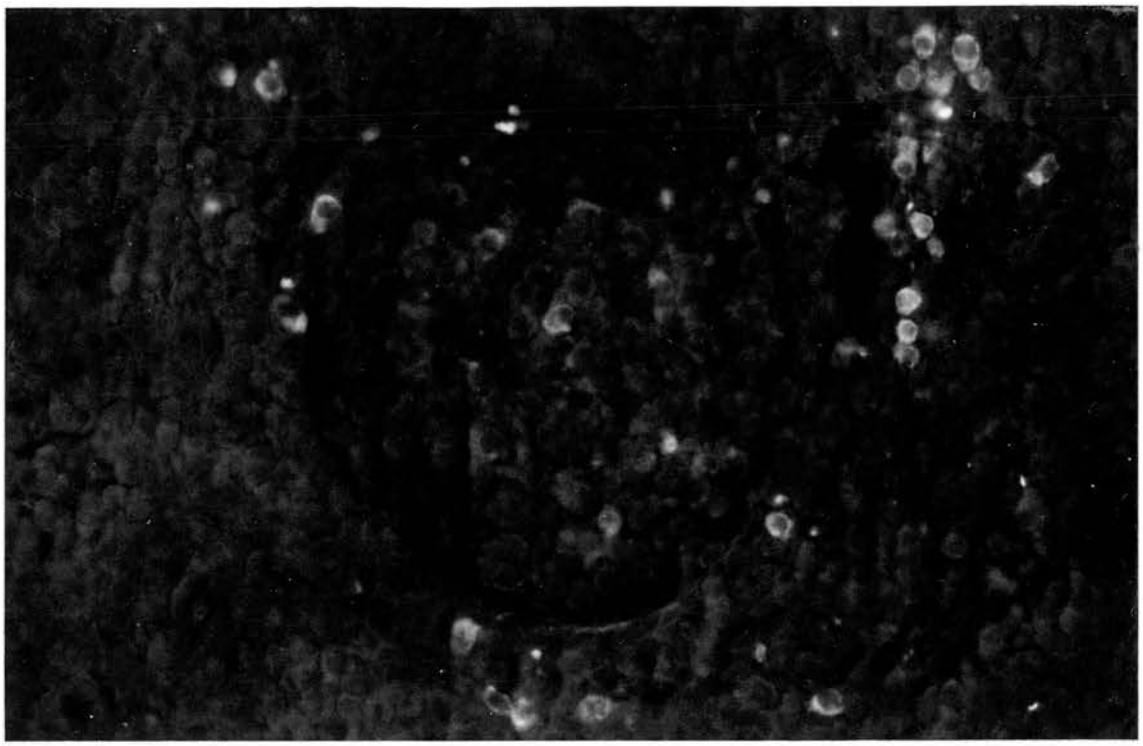


Fig.86. Fowl. 4 w.o. (Control). Spleen. Germinal centre shows some positive lymphocytes in addition to many positive cells surrounding it. Anti-IgM x 640.

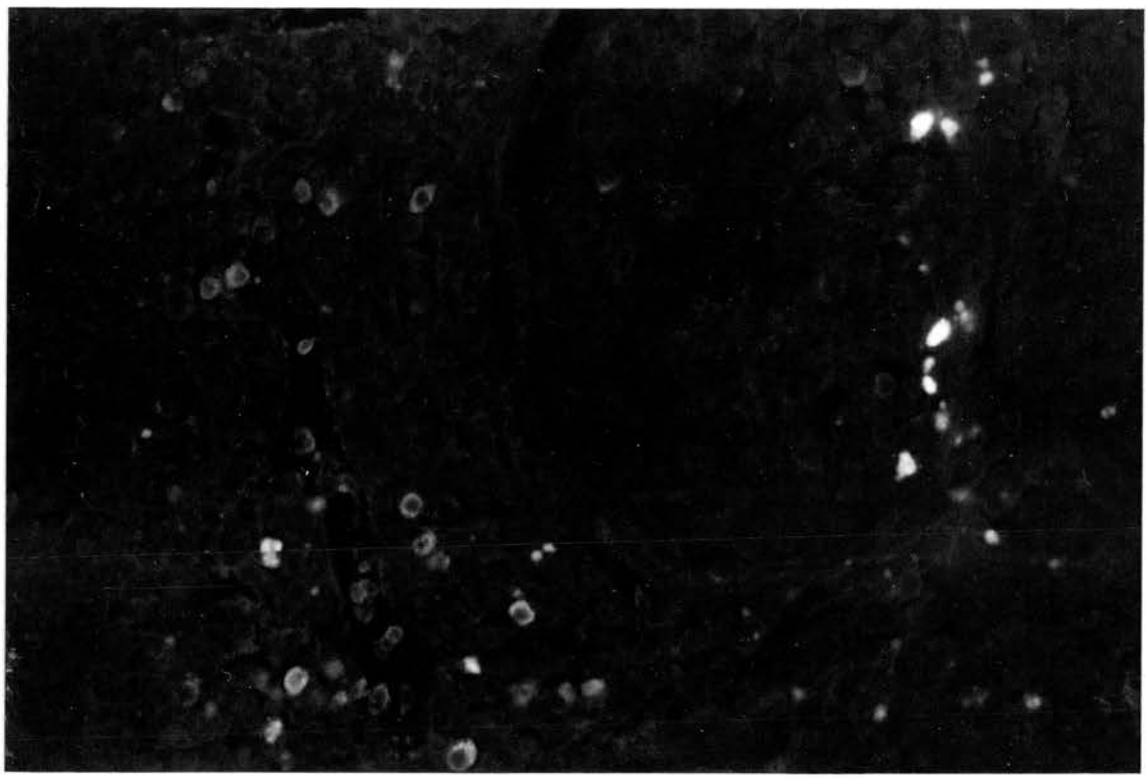


Fig.87. Fowl. 4 w.o. (4ppm OA). Spleen. Germinal centre is negative and a few brightly positive cells are seen nearby. Anti-IgM x 640.

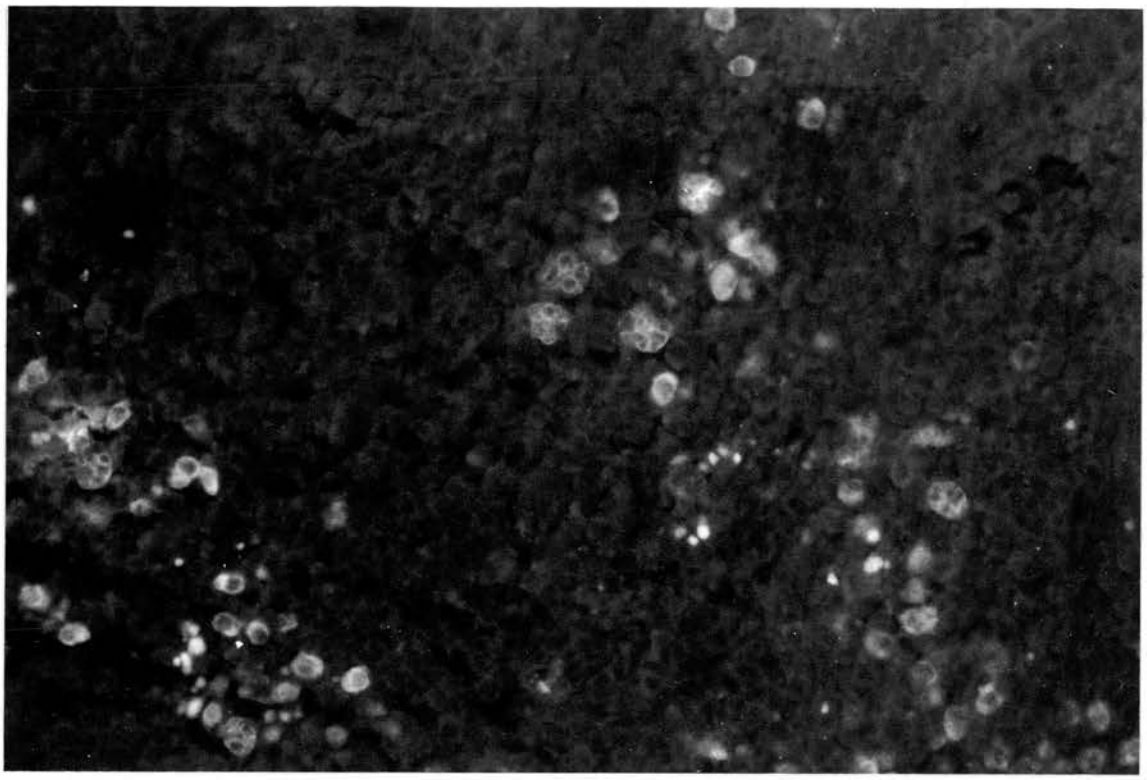


Fig.88. Fowl 4 w.o. (4ppm OA). Spleen. A few lymphocytes and many mulberry-like cells show positive fluorescence. Anti-IgM x 640.

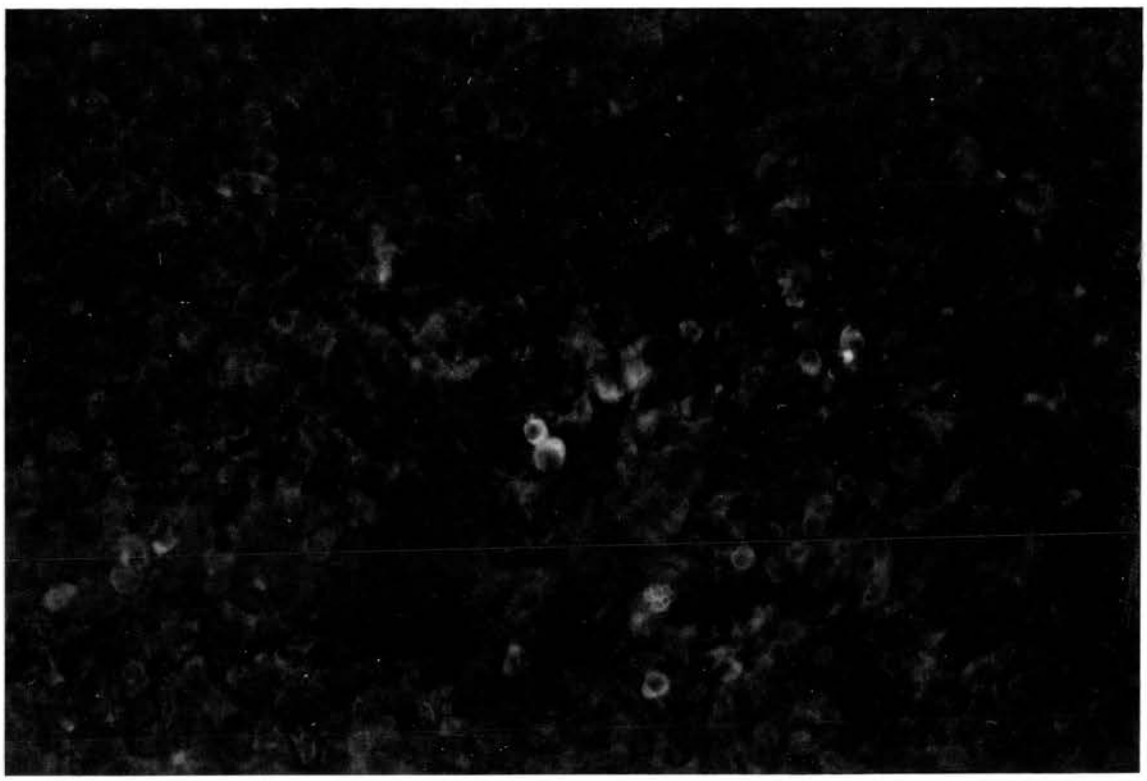


Fig.89. Fowl.(Control, BSA-injected) showing a few positive lymphocytes. Anti-BSA x 640.

were noticed in the spleen of control birds (Fig.89) but rarely in the OA-treated birds.

Harderian gland

In sections of Harderian gland from OA-fed birds, plasma cells fluorescing for all three immunoglobulins were markedly reduced in numbers. Fluorescent plasma cells were seen in the lumina of the secondary tubules, which often had a break in the integrity of their epithelium (Fig.90).

Intestinal lymphoid organs

In caecal tonsils and Peyer's patches from OA-treated fowls, cells with cytoplasmic fluorescence for IgG and IgM were larger and fewer in number than in sections from control birds. Very few positive cells were found in the germinal centres (Fig.91). A similar reaction for IgA was also seen but the mucous lining and often the goblet cells fluoresced a bright apple green. In control birds, many positive cells (IgA) could be seen in the intestine (Fig.92).

Liver

In the liver immunoglobulin-containing cells were seen only occasionally (Fig.93) and there appeared to be no difference between sections from control and OA-treated birds. Sometimes, the bile ducts also appeared to be stained (Fig.94).

Kidney

In sections of kidney from OA-treated and control fowls, no differences were seen in immunoglobulin localisation though there was often an infiltration of immunoglobulin-positive, especially IgG, cells (Fig.95)

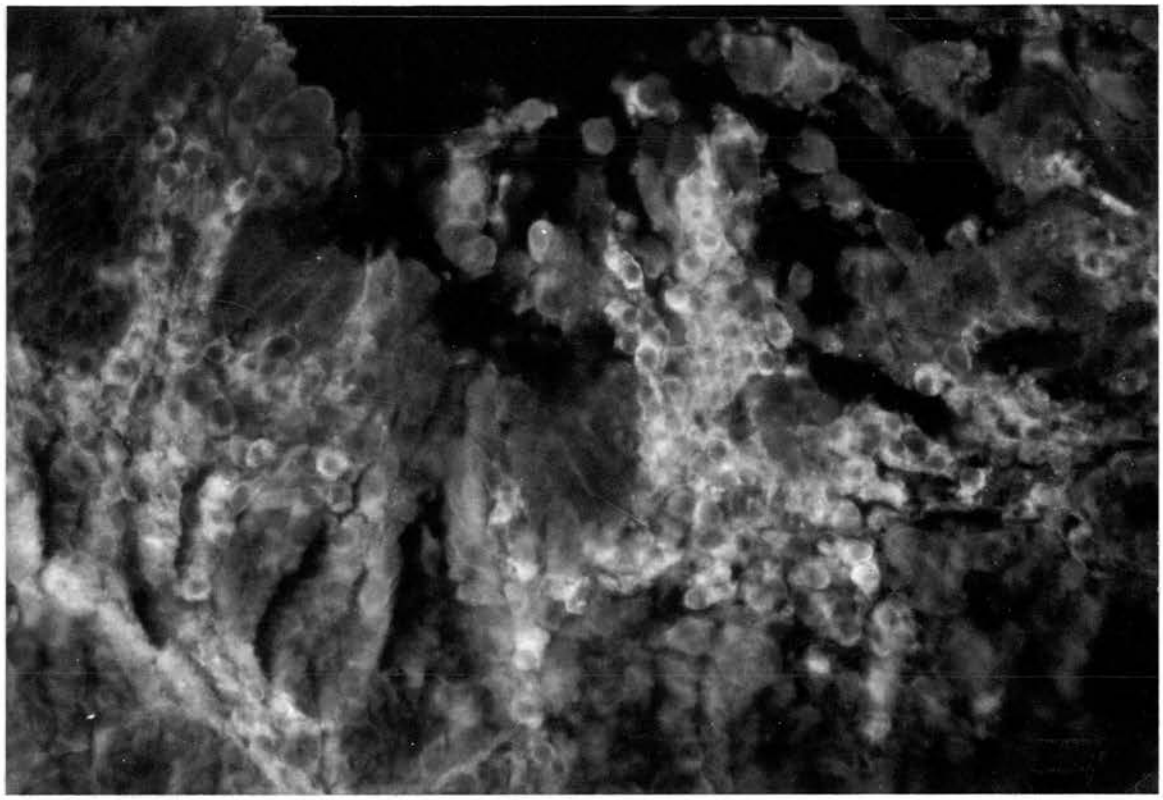


Fig.90. Fowl. 3 w.o. (4ppm OA). Harderian gland showing breakdown of the duct epithelium and release of positive plasma cells into the lumen. Anti-IgM x 640.

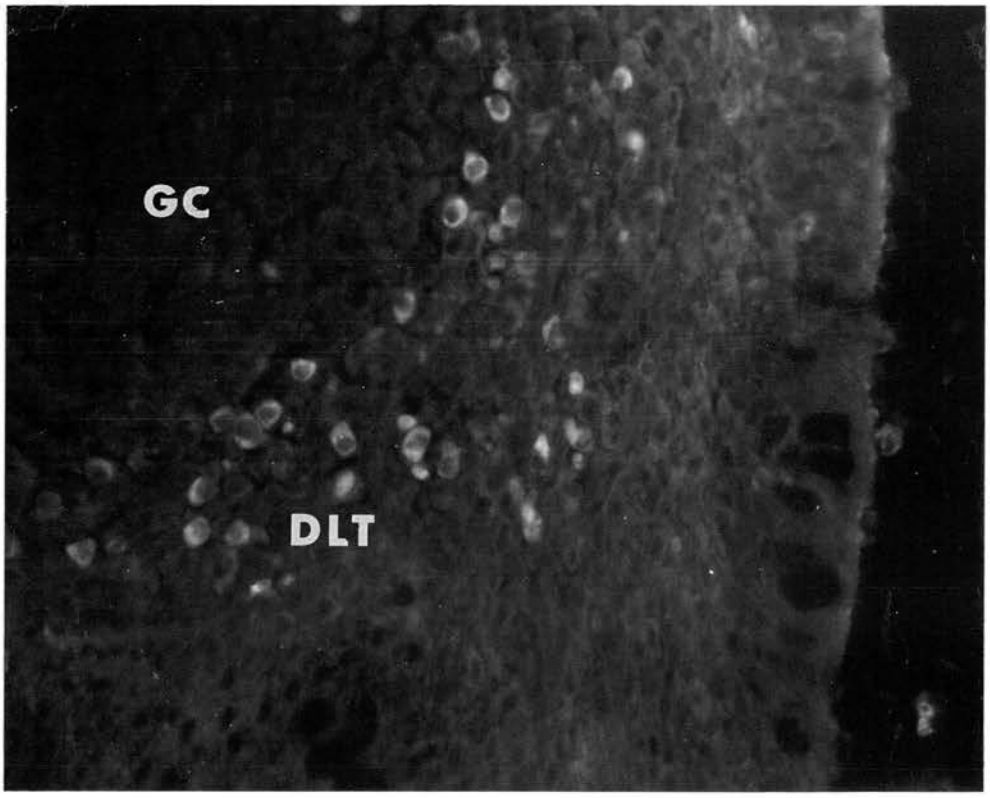


Fig.91. Fowl. 3 w.o. (2ppm OA). Caecal tonsil showing a few isolated positive cells in the diffuse lymphoid tissue (DLT). The germinal centre (GC) has no positive cells. Anti-IgM x 640.

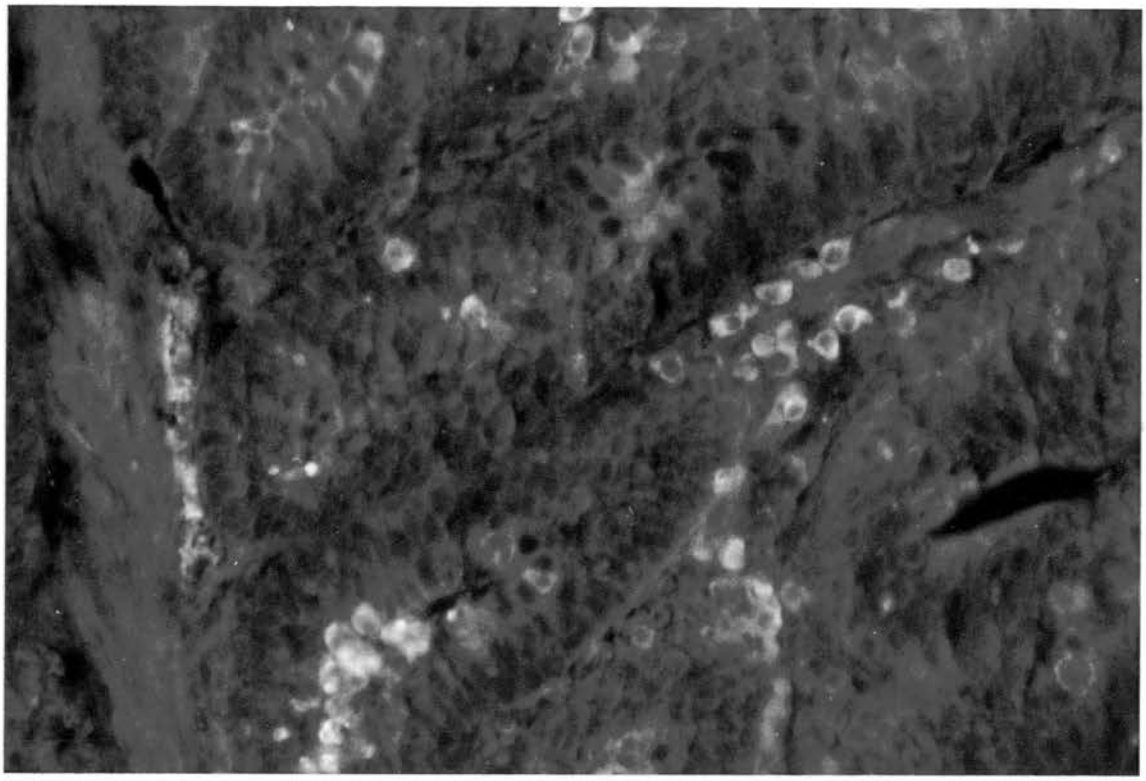


Fig.92. Fowl. 4 w.o. (Control). Duodenum showing many large positive cells around the glands. Anti-IgA x 640.

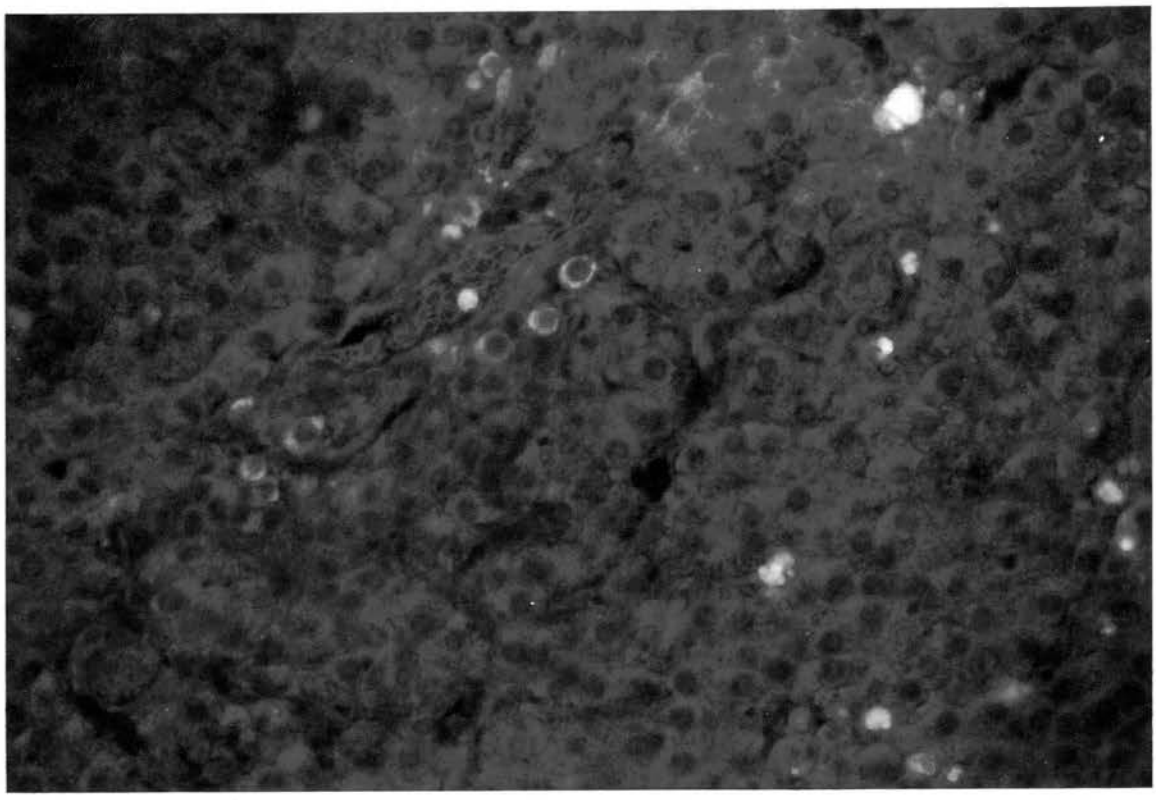


Fig.93. Fowl. 2 w.o. (2ppm OA). Liver. Note the presence of a few positive cells in the hepatic parenchyma. Anti-IgA x 640.

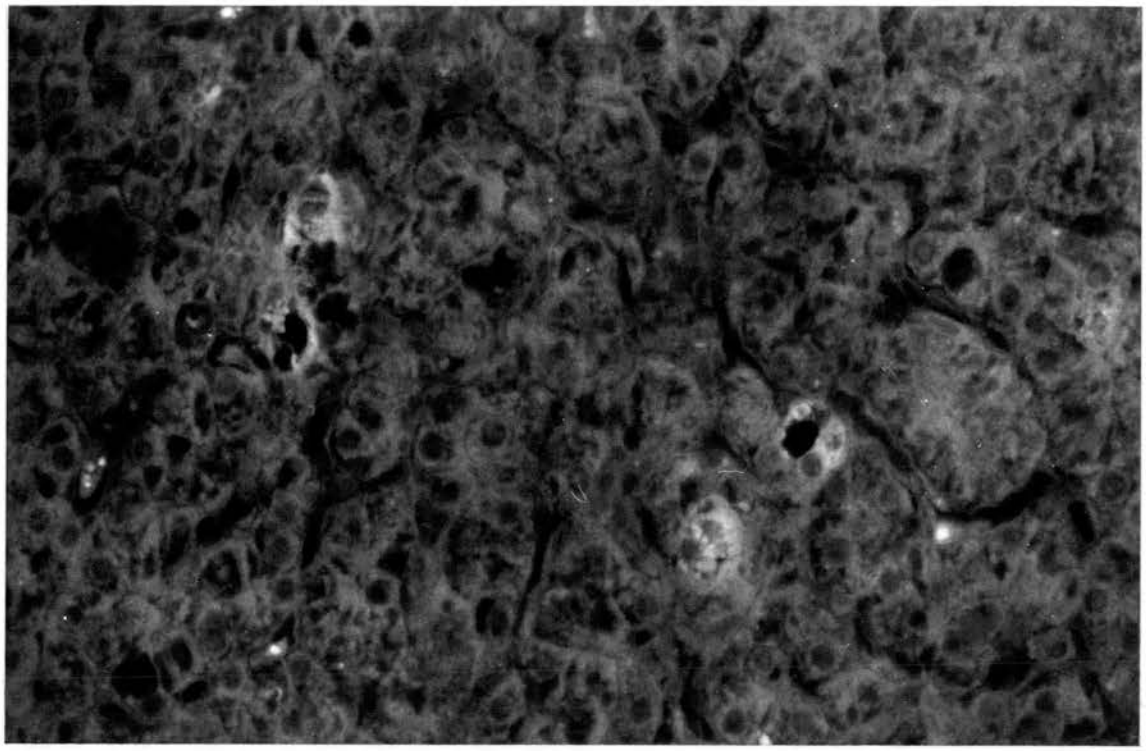


Fig.94. Fowl. 4 w.o. (4ppm OA). Liver. Bile ducts are positively stained. Anti-IgA x 640.

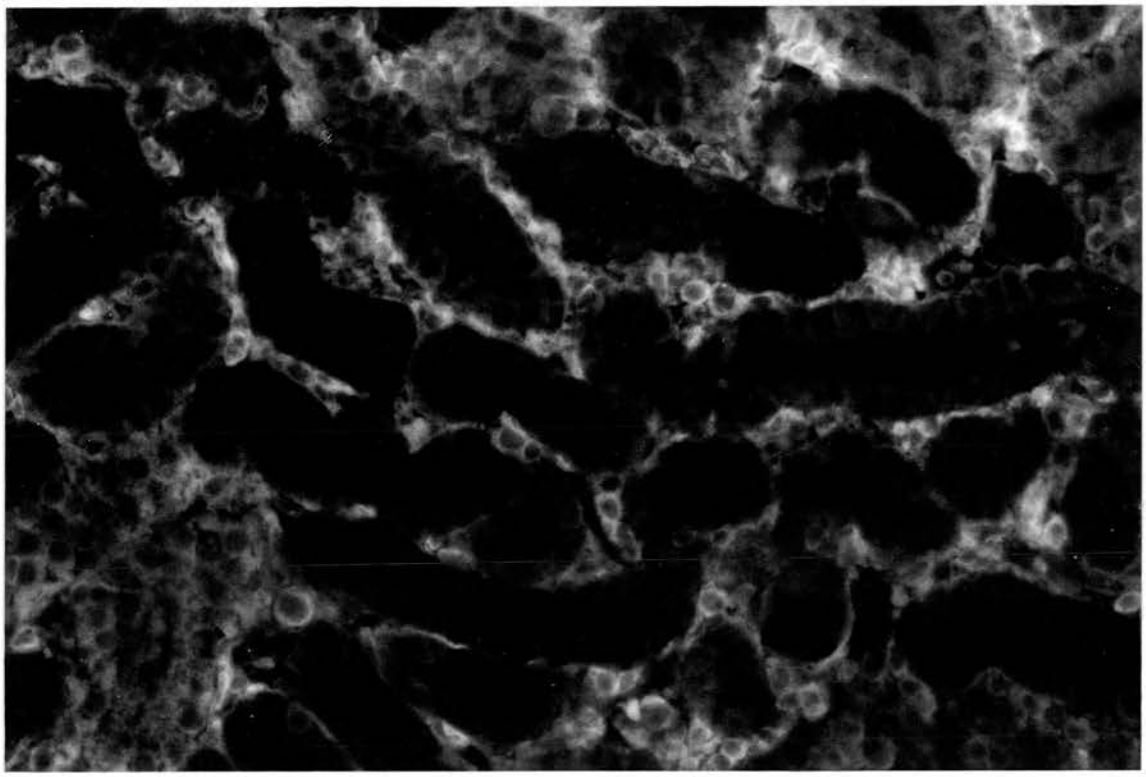


Fig.95. Fowl. 6 w.o. (2ppm OA). Kidney. Note the presence of fluorescing lymphocytes in the parenchyma. Anti-IgG x 640.

into kidneys from OA-fed birds. However, staining of IgG in the glomeruli was seen more frequently and more strongly in sections from OA-fed birds.

4.10.1.2.2 Turkeys

The reaction was similar, though less marked and less consistent compared with broilers. A similar type of response but with variable intensity was observed in various organs in OA-treated turkeys at 2, 4, 6 and 8 weeks of age.

Bursa of Fabricius

As in broilers, fluorescing cells were usually confined to the medulla in control turkeys, though occasional positive cells could be seen in the lumen of the bursa in close proximity to the epithelial surface (Fig.96). In OA-treated birds, the number of positive cells was drastically reduced and in depleted lymphoid follicles there were very few positive cells. As in broilers, the subepithelial region showed a few moderately positive cells (Fig.97). In sections of bursa from control turkeys immunised with BSA there were often clumps of cells with membrane and cytoplasmic fluorescence in the follicles (Fig.98) but rarely in sections from immunised OA-treated birds.

Thymus

Reduced numbers of positive cells were seen in the thymus from OA-treated birds. In addition, Hassall's corpuscles were seen disintegrating and frequently were stained (Fig.99).

Spleen

Reactions in the spleen were not very consistent.

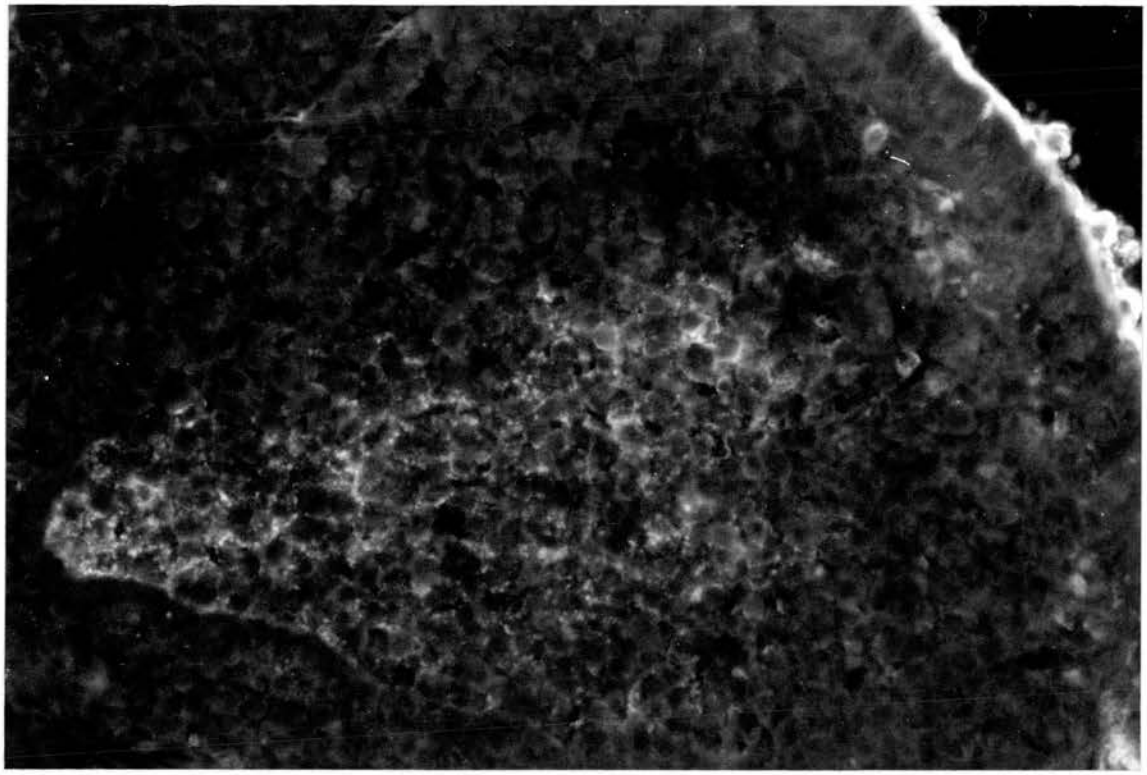


Fig.96. Turkey 4 w.o. (Control). Bursa of Fabricius. Moderate medullary fluorescence and some positive cells in the lumen at the epithelial surface. Anti-IgM x 640.

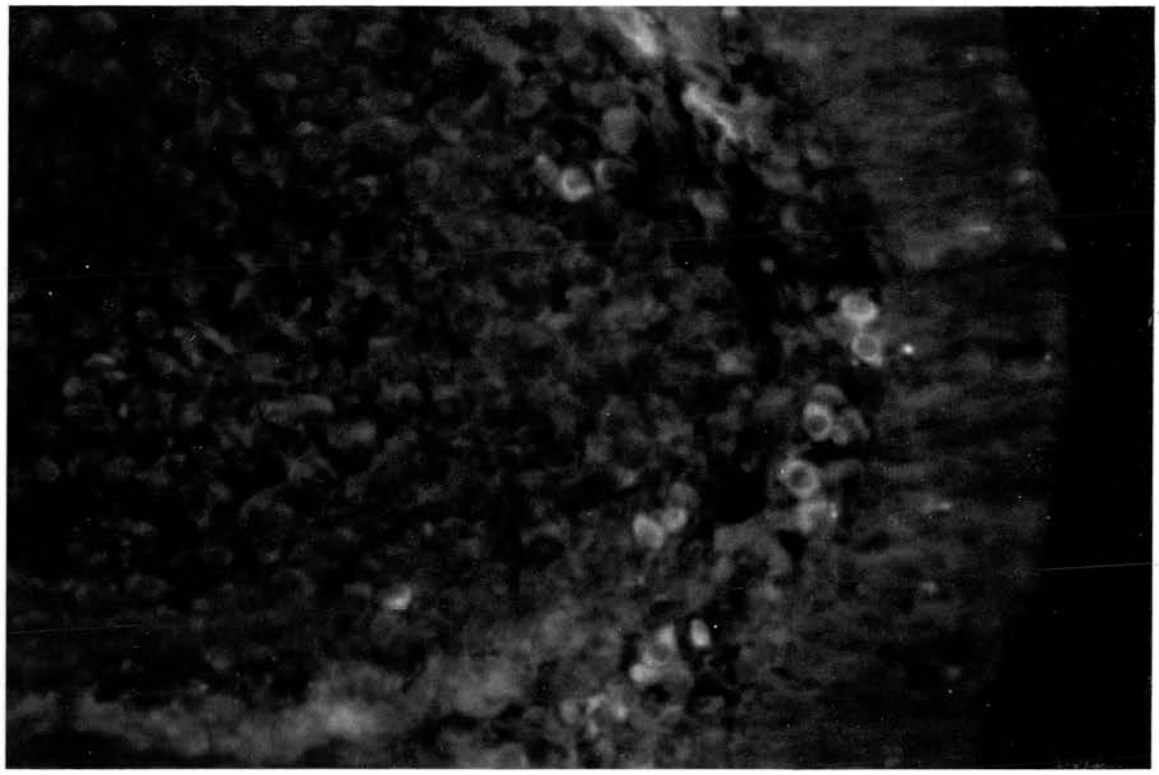


Fig.97. Turkey. 4 w.o. (4ppm OA). Bursa showing the absence of positive cells in the depleted medullary region of the follicle and some positive cells in the subepithelial region. Anti-IgM x 750.

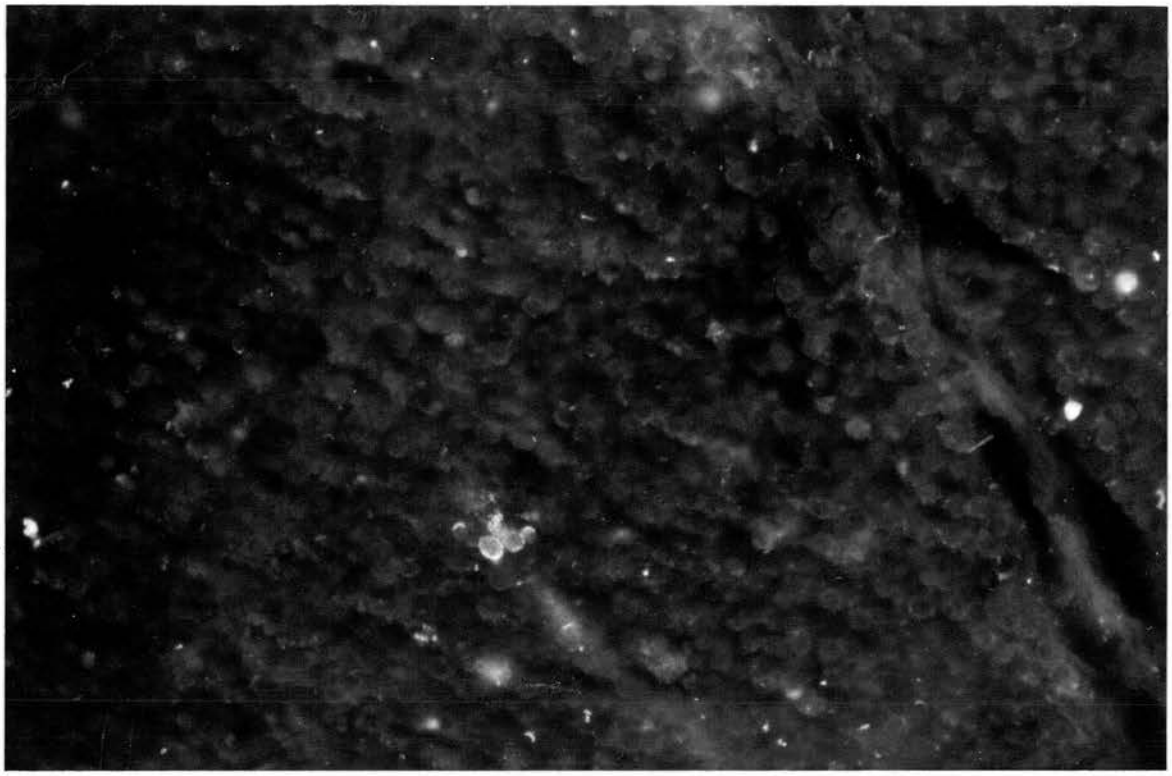


Fig.98. Turkey (Control, BSA-injected). Bursa showing a small clump of positive cells in the bursal follicle. Anti-BSA x 640.

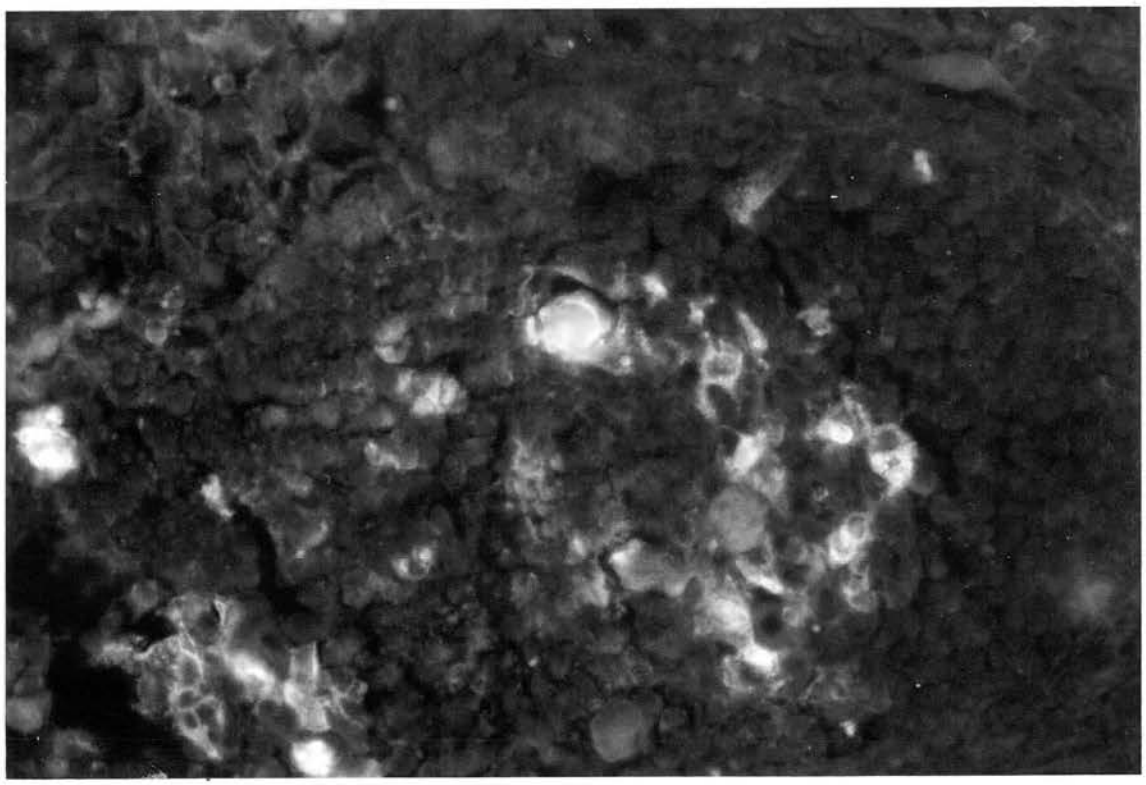


Fig.99. Turkey. 4 w.o. (4ppm OA). Thymus showing disorganised Hassall's corpuscles positively stained. Fluorescing lymphocytes are very few in number. Anti-IgA x 640.

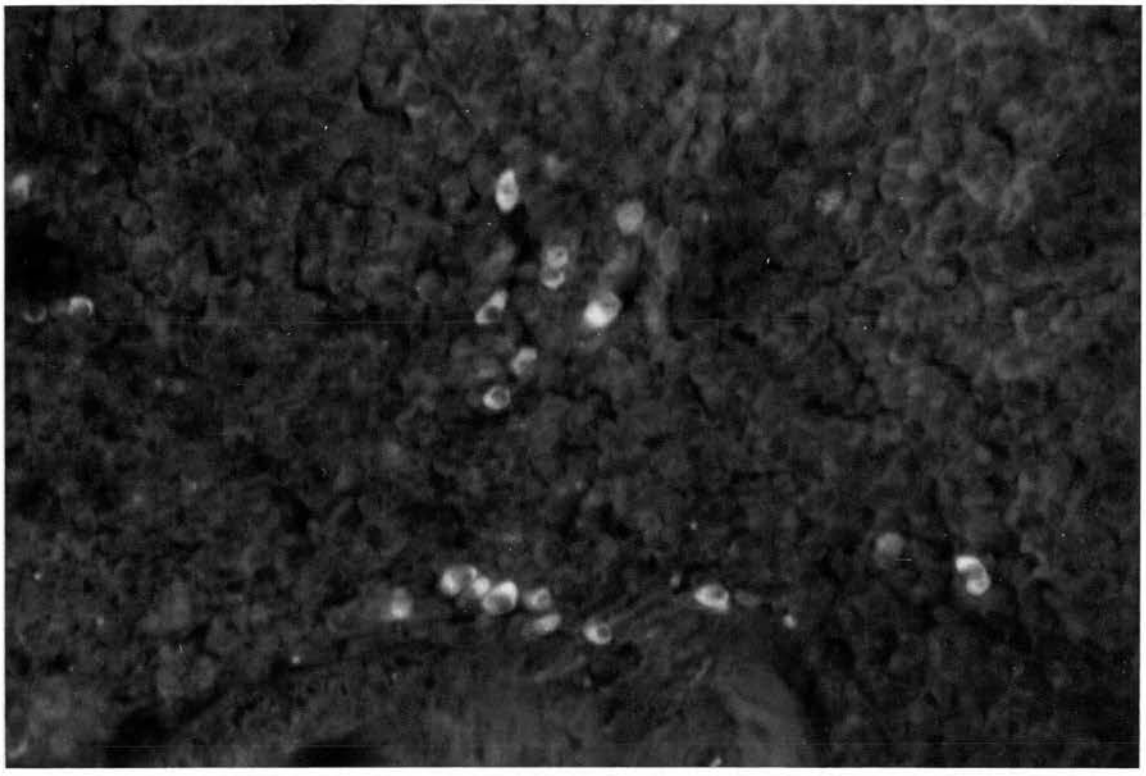


Fig.100. Turkey. 4 w.o. (Control). Spleen showing many strongly positive cells. Anti-IgA x 640.

However, immunoglobulin positive cells were generally reduced in concentration and intensity (Fig.102) in OA-treated birds as compared with controls (Figs.100 and 101).

Harderian gland

No consistent difference could be determined between control and OA-treated birds, although numbers of fluorescing cells appeared to be reduced in the glands of OA-treated birds.

Intestinal lymphoid organs

As in broilers there was depletion of lymphoid cells from both the diffuse lymphoid tissues and the germinal centres in the caecal tonsils and in the Peyer's patches. In all these areas only a few positive cells were detectable (Fig.103).

Liver

Occasionally positive cells could be seen in the hepatic parenchyma of both control and OA-treated birds.

Kidney

Rarely fluorescing lymphoid cells were seen in the renal parenchyma, though many glomeruli appeared to be positive (Fig.104) in OA-treated birds. The glomerular basement membrane had a linear fluorescence in some of the glomeruli (Fig.104). Sometimes, brush border and luminal surface of the proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) also showed positive staining particularly with IgA (Fig.105). Occasionally, the epithelial cells of the PCT were stained with anti-BSA immunoglobulins in both control and OA-treated birds (Fig.106).

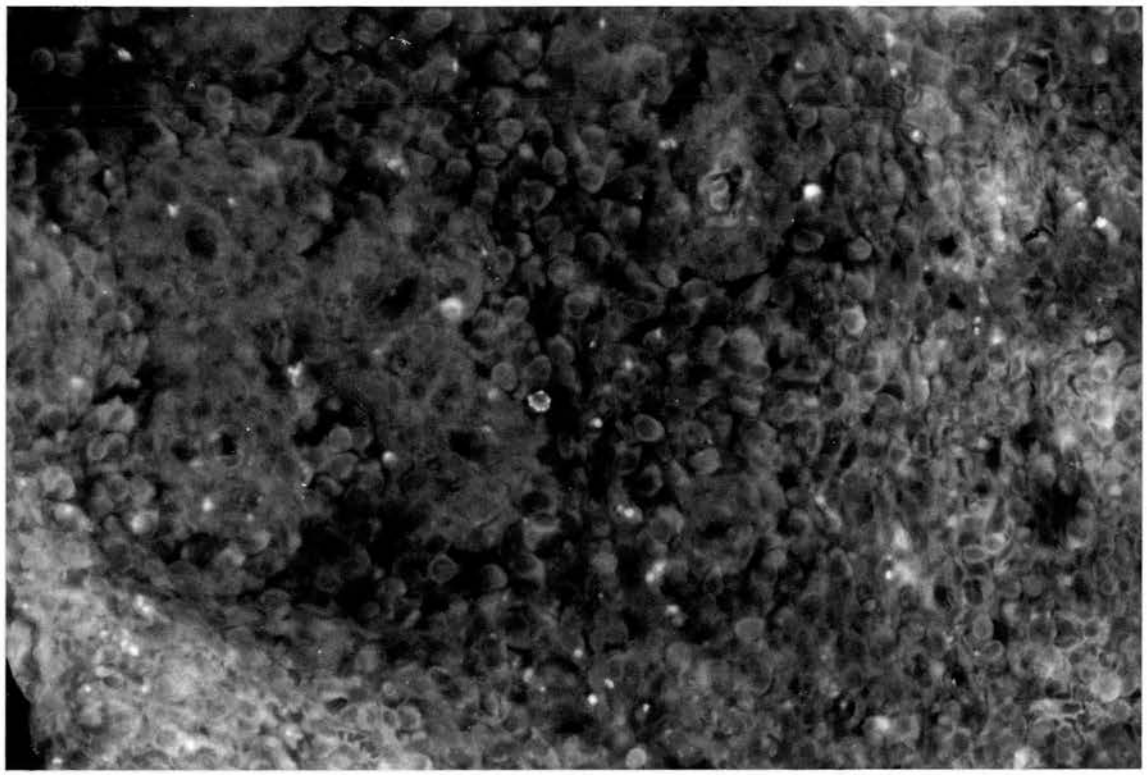


Fig.101. Turkey. 10 w.o. (Control, BSA-injected). Spleen. Note the presence of many positive cells. A cell in the centre is showing membrane-fluorescence. Anti-BSA x 640.

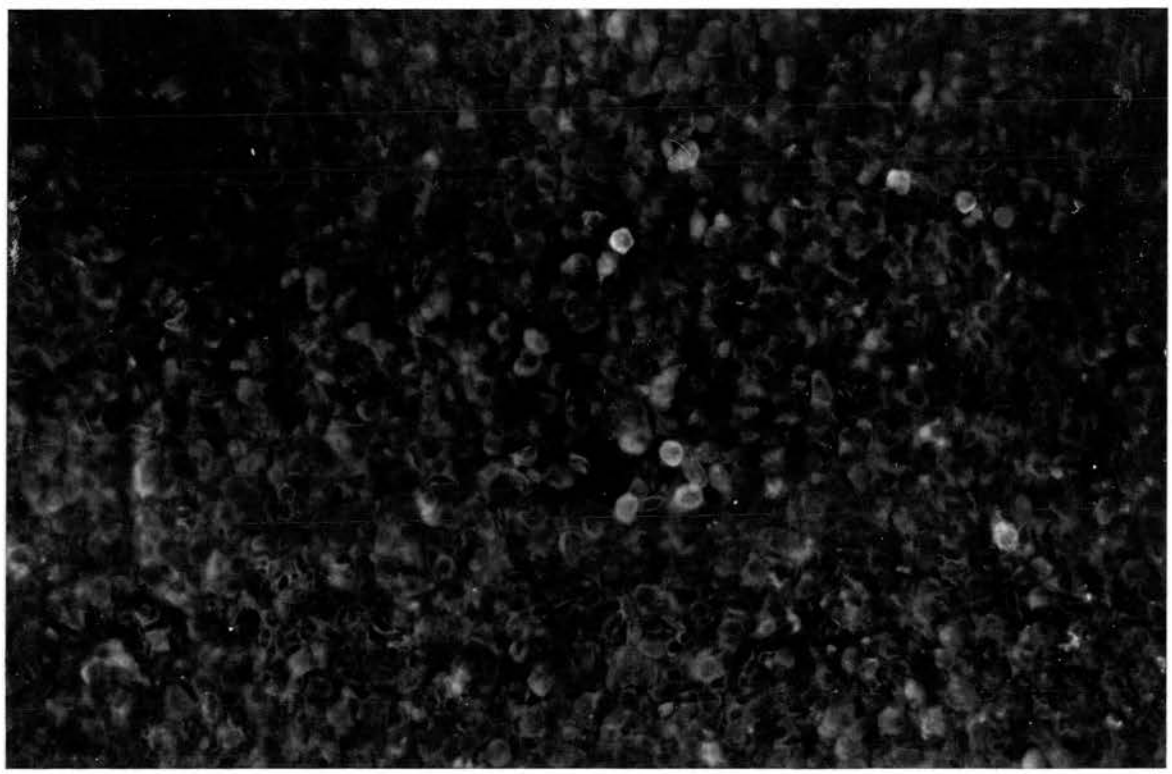


Fig.102. Turkey. 10 w.o. (4ppm OA, BSA-injected). Spleen. Note the strongly positive staining of only a few lymphocytes. Anti-BSA x 640.

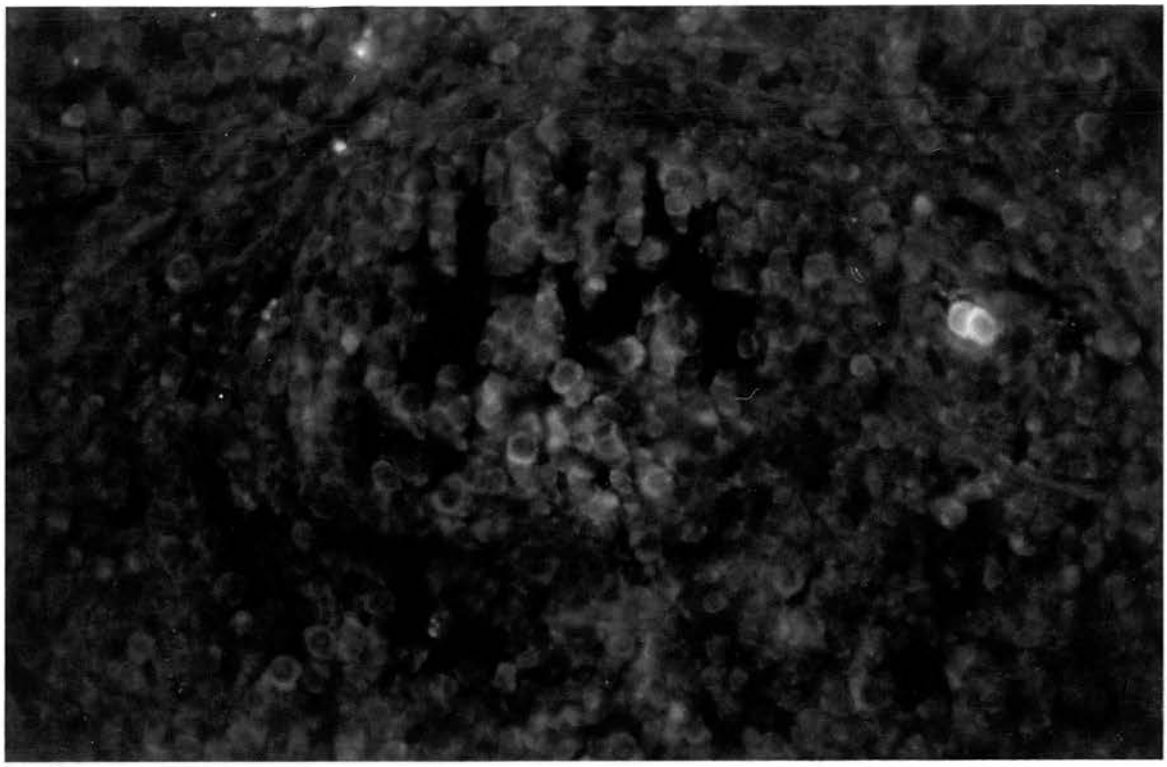


Fig.103. Turkey. 10 w.o. (4ppm OA). Peyer's patch showing a depleted germinal centre and the presence of only a few strongly positive cells nearby. Anti-IgA x 640.

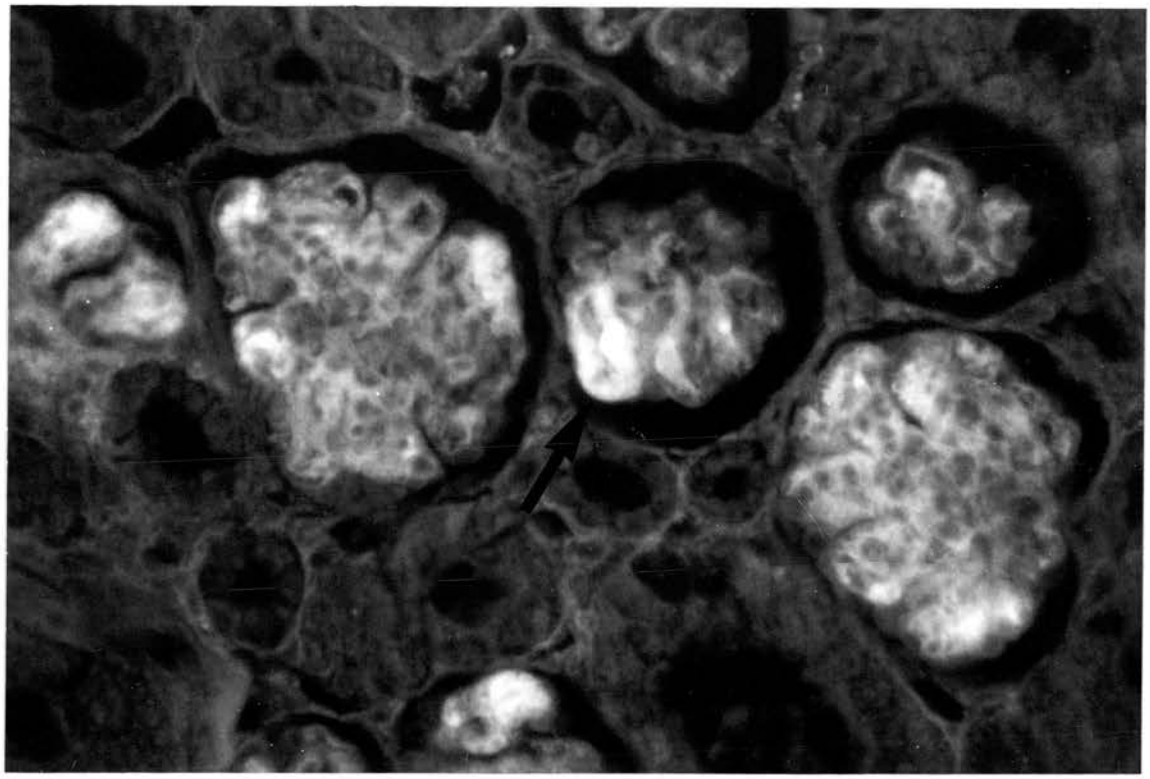


Fig.104. Turkey. 4 w.o. (4ppm OA). Kidney. Note the positive fluorescence of glomeruli. GBM is thickened and stained (arrow). Anti-IgG x 640.

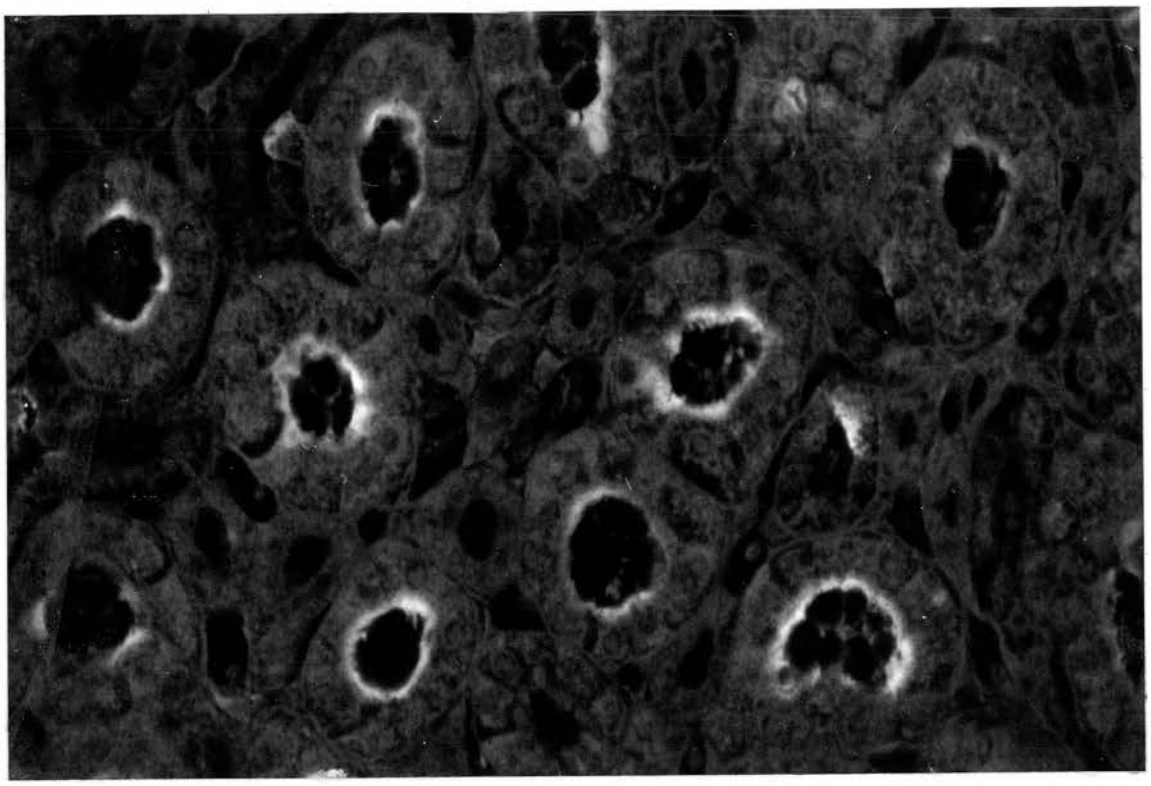


Fig.105. Turkey. 2 w.o. (4ppm OA) Kidney showing staining of the luminal surface of the kidney tubules. Anti-IgA x 640.

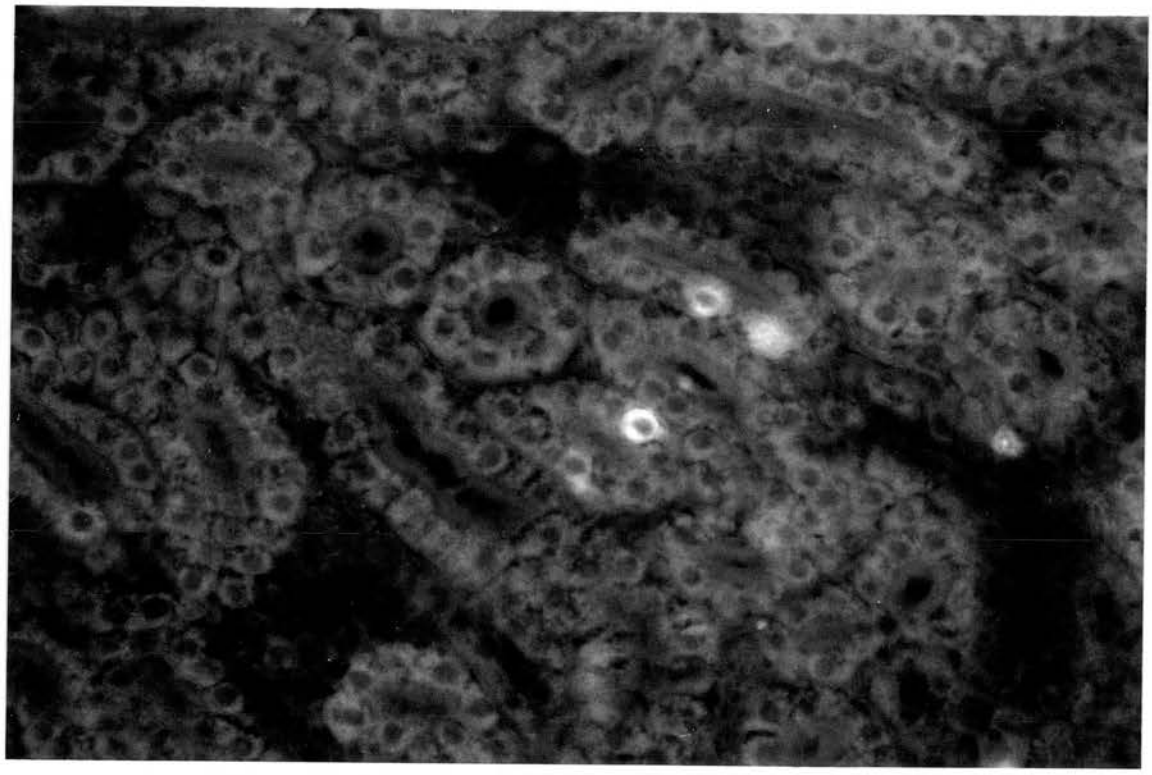


Fig.106. Turkey (Control, BSA-injected). Kidney showing positive staining of some epithelial cells of the proximal convoluted tubules. Anti-BSA x 640.

4.10.2 Cell mediated immune response

4.10.2.1 Broilers

For the evaluation of delayed hypersensitivity reactions in broilers, the post-auricular apterium appeared to be the best site for injection, observation of macroscopic lesions, measurement and also for the study of cellular reactions. Another site which gave a good picture of macroscopic lesions was the lateral thoracic apterium, though it was difficult to measure the increase in its thickness. From other skin sites variable results were obtained depending upon the test-antigens. The wing web was easier to inject, but feathers posed a problem for measurement and the cellular reactions were also variable. The comb was difficult to inject. The dorsal cloacal lip was difficult to inject, sometimes bruised the skin, unpleasant to the bird and posed a problem to obtain an accurate measurement. The wattle also appeared to be a satisfactory site in adult birds.

A good intradermal injection was indicated when there was cutaneous blanching and the formation of a vesicle.

4.10.2.1.1 Delayed hypersensitivity to avian tuberculin, PPD

Classical DH response to avian tuberculin, PPD and mean increase in the thickness of the skin at various sites at different time intervals in 0 and 2 ppm OA groups are presented in Fig. 107. At 4-6 hours, test sites were no different from normal areas. Changes were apparent at 24 hours, and became more pronounced by 48 hours in both M.avium-and avian PPD-sensitised broilers. Test sites were thickened, soft and warm to touch. The most marked effects were seen at the post-auricular

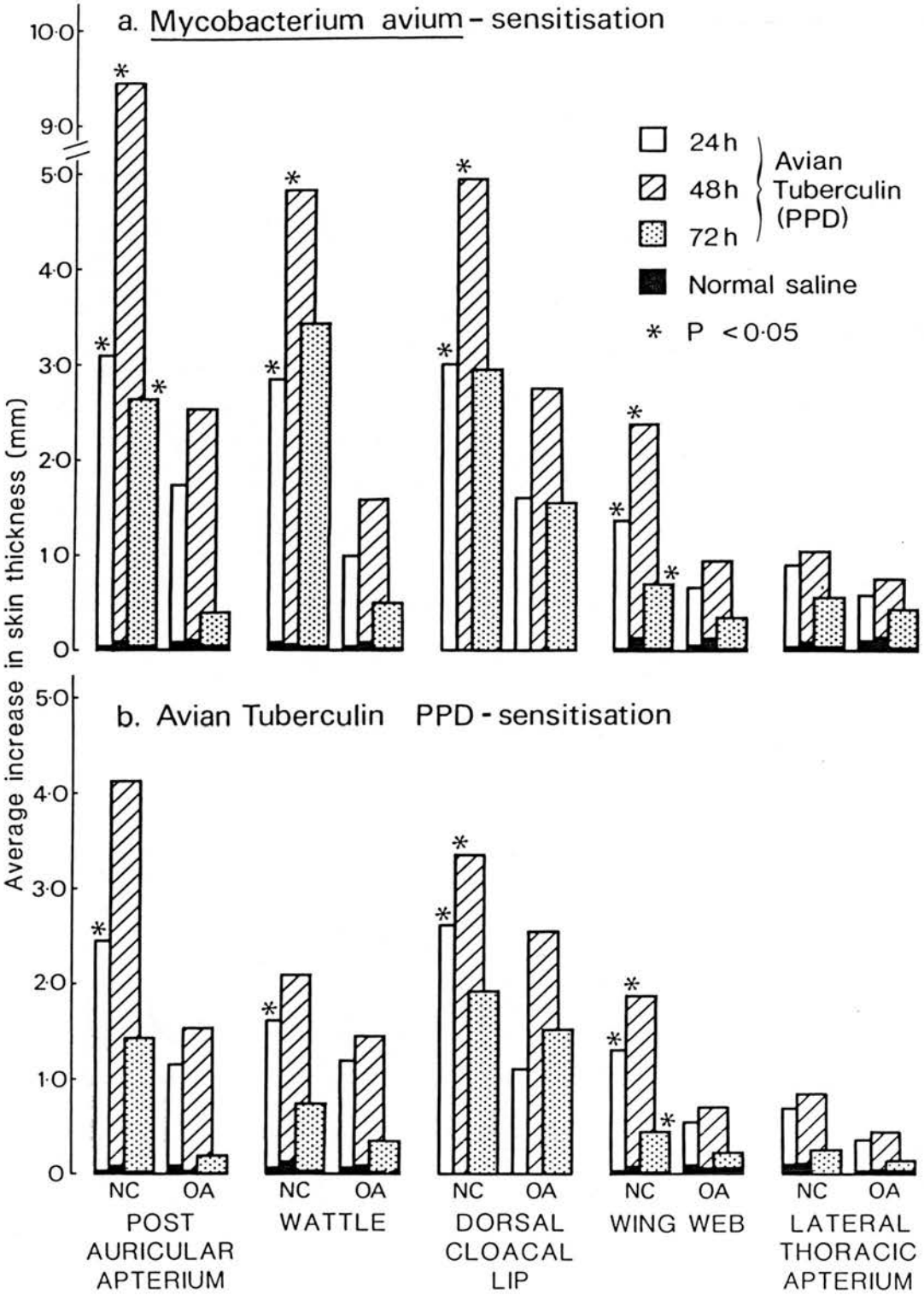


Fig. 107. Average increases in skin thickness (mm) in sensitised broilers in response to avian tuberculin, PPD.

apterium (PAA), followed by the wattle, dorsal cloacal lip (DCL), wing web and lateral thoracic skin or apterium (LTS). In PAA and wattle, the reaction extended beyond the test injection sites, resulting in a very large swelling around the ear lobe with the wattle becoming enlarged and pendulous. At all the skin sites tested, the reaction as well as skin thickness was markedly depressed in the OA-treated groups. Sensitisation by killed M. avium resulted in a more marked DH response than sensitisation by avian tuberculin, PPD. Liquid paraffin injections did not cause any significant cutaneous reaction, though Freund's complete adjuvant (FCA) injections resulted in a slight increase in skin thickness at 24 hours, which later subsided. Control injections of saline caused a negligible reaction.

Results of the statistical analyses of the data are presented in Table 14. In PAA and Wattle (Table 14, part a), M. avium - sensitised birds showed a greater and significant suppression in thickness both at 24 and 48 hours, as compared to avian tuberculin, PPD-sensitised birds. Estimated differences (E.D.) and their 95 per cent confidence intervals (C.I.) showed that OA-treated birds always had a smaller increase in skin thickness compared with controls. The differences between control and OA groups at all the skin sites tested except the lateral thoracic apterium (Table 14, part b) were significant ($P < 0.05$). Linear regression slope for saline swelling showed no interaction with OA-treatment.

Histopathological examination of the test-skin sites showed a typical granulomatous reaction, characterised by a mononuclear cellular reaction together with some heterophilic infiltration. No eosinophils were noticed. The type of cellular reaction in the OA-group was essentially similar to that of the control (0ppm OA) broilers but the reaction was less intense and less marked.

Table 14. Analyses of increases in skin thickness (mm) in sensitised broilers in response to avian tuberculin, PPD (a) Post-auricular apterium (PAA) and Wattle[a]

Test sites	Treatment	OA - NC		
		24hr	48hr	72hr
I. PAA MA primed	E.D.(S.E.) 95% C.I.	- -	-6.65(1.39) (-9.79,-3.51)	- -
PPD Primed	E.D.(S.E.) 95% C.I.	- -	-0.59(1.55) (-4.20,2.82)	- -
combined (MA+PPD)	E.D.(S.E.) 95% C.I.	-1.46(0.59) (-2.79,-0.13)		-1.61(0.44) (-2.69,-0.53
MA-PPD	E.D.(S.E.) 95% C.I.	0.67(0.55) (-0.57,1.91)		0.35(0.50) (-0.87,1.57)
II. Wattle MA primed	E.D.(S.E.) 95% C.I.	- -	-3.28(0.69) (-4.84,-1.72)	-3.0(0.22) (-3.54,-2.46)
PPD primed	E.D.(S.E.) 95% C.I.	- -	-0.53(0.76) (-2.25,1.19)	-0.40(0.18) (-0.84,0.04)
combined (MA+PPD)	E.D.(S.E.) 95% C.I.	-0.99(0.30) (-1.67,-0.31)	- -	- -
MA-PPD	E.D.(S.E.) 95% C.I.	0.38(0.28) (-0.25,1.01)	- -	- -

Table 14. continued
(b) Lateral thoracic apterium (LTS), wing web (WW) and dorsal cloacal lip (DCL)

Test sites	Treatment	OA - NC		
		24hr	48hr	72hr
I. LTS Combined (MA+PPD)	E.D.(S.E.) 95% C.I.	-0.31(0.22) (-0.81,0.19)	-0.39(0.23) (-0.91,0.13)	-0.21(0.16) (0.60,0.18)
MA-PPD	E.D.(S.E.) 95% C.I.	0.25(0.22) (-0.25,0.75)	0.27(0.22) (-0.27,0.77)	0.18(0.19) (-0.29,0.65)
II. WW Combined (MA+PPD)	E.D.(S.E.) 95% C.I.	-0.75(0.19) (-1.18,-0.32)	-1.12(0.22) (-1.62,-0.62)	-0.32(0.082) (-2.33,1.67)
MA-PPD	E.D.(S.E.) 95% C.I.	0.09(0.16) (-0.27,0.45)	0.17(0.22) (-0.33,0.67)	0.20(0.087) (-0.01,0.41)
III. DCL Combined (MA+PPD)	E.D. (S.E.) 95% C.I.	-1.45(0.52) (-2.63,-0.27)	-1.45(0.63) (-2.88,-0.03)	-0.78(0.54) (-2.10,0.54)
MA-PPD	E.D.(S.E.) 95% C.I.	0.45(0.52) (-0.73,1.63)	0.86(0.63) (-0.57,2.29)	0.53(0.55) (-0.82,1.88)

[a] Significant differences are underlined.

4.10.2.1.2 Delayed hypersensitivity to PHA-P

At 4-6 hours after PHA-P test injections, some reaction (swelling and softness) was noticed, particularly in PAA and wattle (Fig.108). Good reaction was noticed in DCL (Fig.109a) also. The thickness of the various skin sites tested was greatest 24 hours after injection (Fig.110). There was a significant difference between the increased skin thickness of control (0ppm) and OA-treated (2ppm) broilers (Figs.108 and 109b). OA treatment caused a marked depression in the PHA-induced DH response (Fig.110 and Table 15); the difference being 6.3mm in PAA at 24 hours, compared with the controls. PBS injection resulted in very slight and negligible swelling (Fig.108). In skin sections, it was possible to see an intense cellular reaction, composed of mononuclear cells (lymphoid cells, macrophages), granulocytes (heterophils and peroxidase positive eosinophils) and occasional mast cells, which was less marked in the skin of OA-treated broilers.

4.10.2.1.3 Arthus reaction and DH to BSA

Arthus reactions. The development of the direct Arthus reaction was evaluated by the diameter, intensity and persistence of macroscopic lesions at both the lateral thoracic apterium (LTS) and dorsal cloacal lip (DCL), up to 48 hours. The scoring of lesions in the Arthus reaction is summarised in Table 16. In control (0ppm OA) broilers, macroscopic lesions in the LTS (petechiae and slight oedema) became apparent 4-6 hours after the test injection and became more pronounced and severe at 24 hours. Erythema, confluent haemorrhages and haemorrhagic discolouration of the skin, oedema and development of necrosis (Fig.111a) (lesion score 3+ to 4+) were the predominant lesions which either persisted or increased in area and severity up to 48 hours. Necrosis became extensive and more marked. In the DCL, a similar type of reaction, though less intense, (Fig.112) was observed (lesion score 1+ to 4+), both at 24

Fig.108. PHA-induced DH response in fowl. Note severe reaction in the control (left). Swollen wattle and ear area. OA-treated bird (right) shows very depressed response.

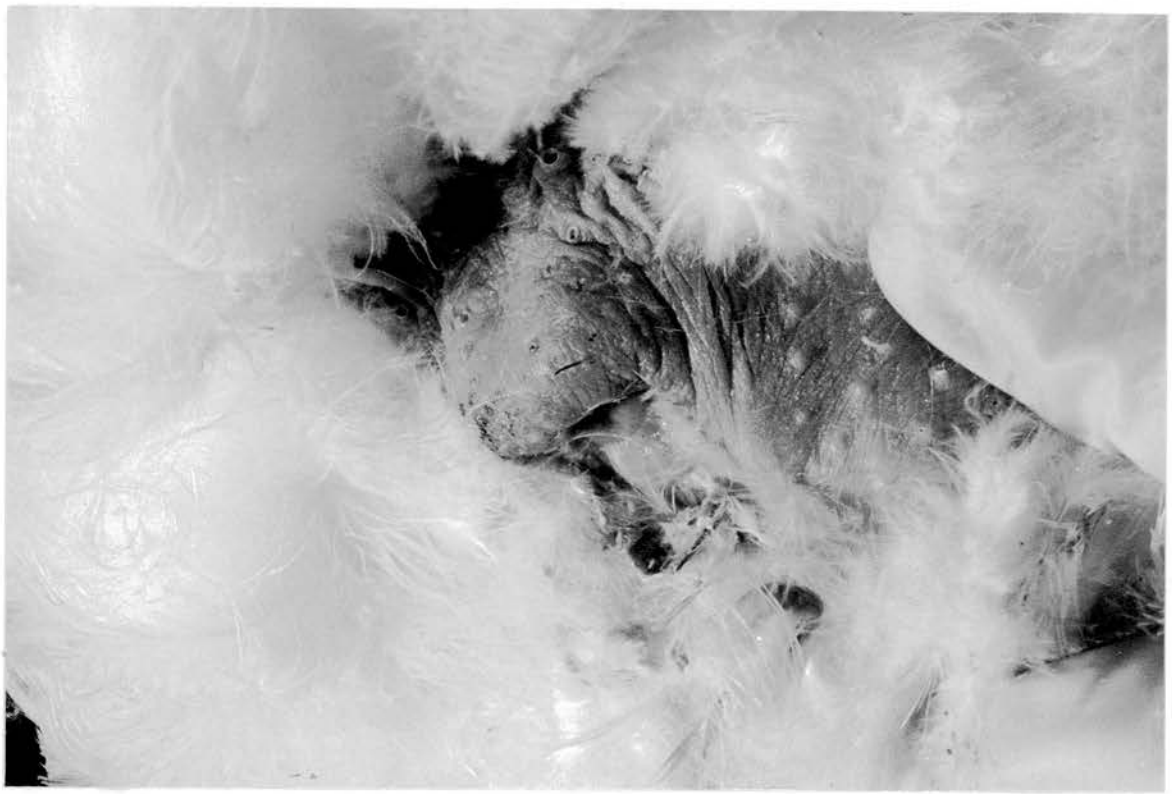


Fig.109a. PHA-induced DH response in dorsal cloacal lip of a control fowl. Note swelling.

Fig.109b. PHA-induced DH response in dorsal cloacal lip of an OA-fed fowl. Note minimal reaction.

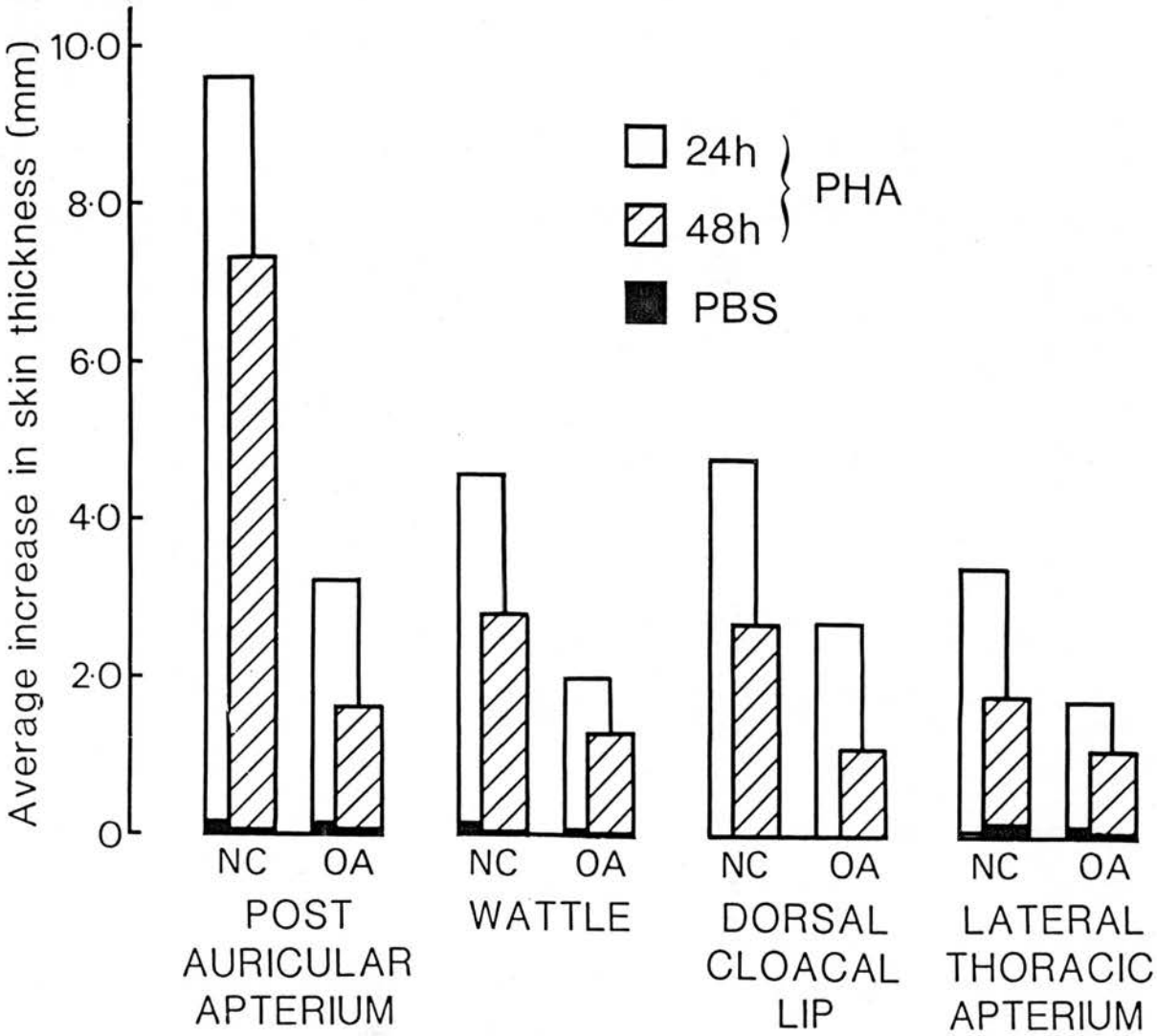


Fig.110. Average increases in skin thickness (mm) in sensitised broilers in response to phytohaemagglutin-P (PHA-P).

Table 15. Analysis of increases in skin thickness (mm) in broilers in response to PHA-P[a]

Test Sites	Treatment	OA - NC		PBS swelling	
		24hr	48hr	24hr	48hr
PAA	E.D.(S.E.)	-6.03 (2.41)	-3.48 (1.92)	9.73 (21.70)	-6.44 (32.40)
	95% C.I.	(-13.70, 1.60)	(-9.59, 2.63)	(-59.32, 78.78)	(-109.54, 96.66)
W	E.D.(S.E.)	-1.20 (1.77)	-1.51 (1.39)	26.02 (13.52)	22.51 (13.80)
	95% C.I.	(-6.83, 4.43)	(-5.84, 2.72)	(-17.0, 69.04)	(-21.40, 66.42)
LTS	E.D.(S.E.)	-1.29 (0.19)	-0.55 (0.27)	-1.76 (1.41)	-3.61 (3.71)
	95% C.I.	(-1.89, -0.69)	(-1.41, 0.31)	(-6.25, 2.73)	(-15.42, 8.20)
DCL	E.D.(S.E.)	-2.0 (0.41)	-1.63 (0.48)	-	-
	95% C.I.	(-3.14, -0.86)	(-2.96, -0.30)		

[a]Significant differences are underlined

and 48 hours. Injection of PBS did not elicit any lesions. In OA-treated birds, much less intense lesions were observed (Table 16). The severity and area of reaction were markedly reduced (lesion score 1+ to 2+) at both the skin sites tested. Necrotic lesions were not observed (Fig.111b) in these birds.

Histological examination revealed hyperkeratosis of the stratum corneum and necrosis of stratum germinativum layers of the epidermis. In the superficial dermis, the blood vessels were hyalinised and sometimes a heavy infiltration of thrombocytes and RBCs was evident. Many blood vessels were blocked with thrombus formation together with damage to the endothelial cells and vessel wall. Macrophage activity with phagocytosed material was also seen. Monocytes were frequently seen near vascular lesions. In the deep dermis, lymphoid aggregates, vascular proliferation and new capillary formation were noticed in many areas.

DH response to BSA. The DH response to the intradermal injection of BSA in PAA,wattle (Fig.113a) and DCL (Fig.112) was distinct with peak reaction at 24 hours (Fig.114a and Table 17). In OA-treated birds, the DH response was considerably reduced (Fig.113b), the estimated difference in lesion diameters ranging from 2.67 to 3.56 mm (Table 17) compared with the control birds. PBS injection resulted in a very slight and negligible increase (Fig.113c) in skin thickness.

Histologically, particularly at the post-auricular apterium, large numbers of mononuclear cells with small dark blue granules (phagocytic macrophages) were seen in the superficial and the deep dermis. The cells were negative for mast cell or basophil granules after toluidine blue or acridine orange (Jagatic and Weiskopf, 1966) staining. Lymphoid cells and heterophils were seen in the capillary layer at dilated venous sinuses. No peroxidase positive

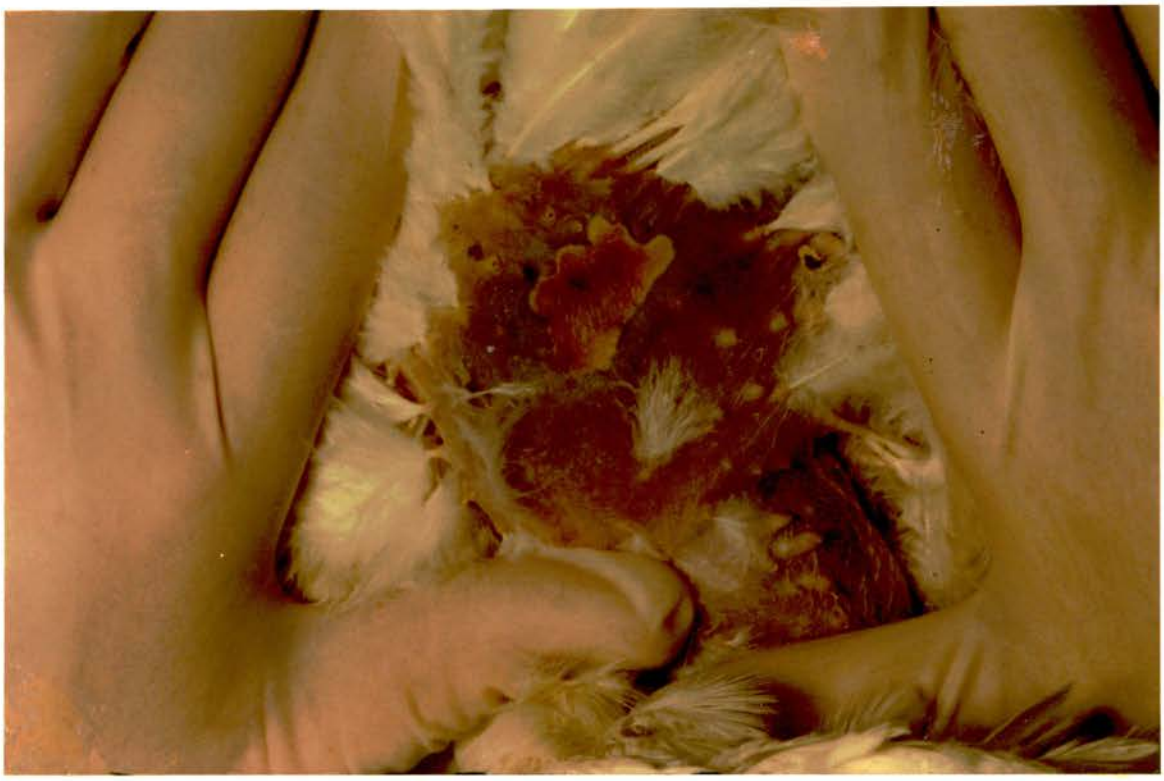


Fig.111a. Arthus reaction in a control fowl. Note widespread necrosis in the lateral thoracic apterium.

Fig.111b. Arthus reaction in an OA-treated fowl. Note minimal reaction.

Fig.112. BSA-induced response in dorsal cloacal lip in a control fowl. Note distinct haemorrhagic discolouration.

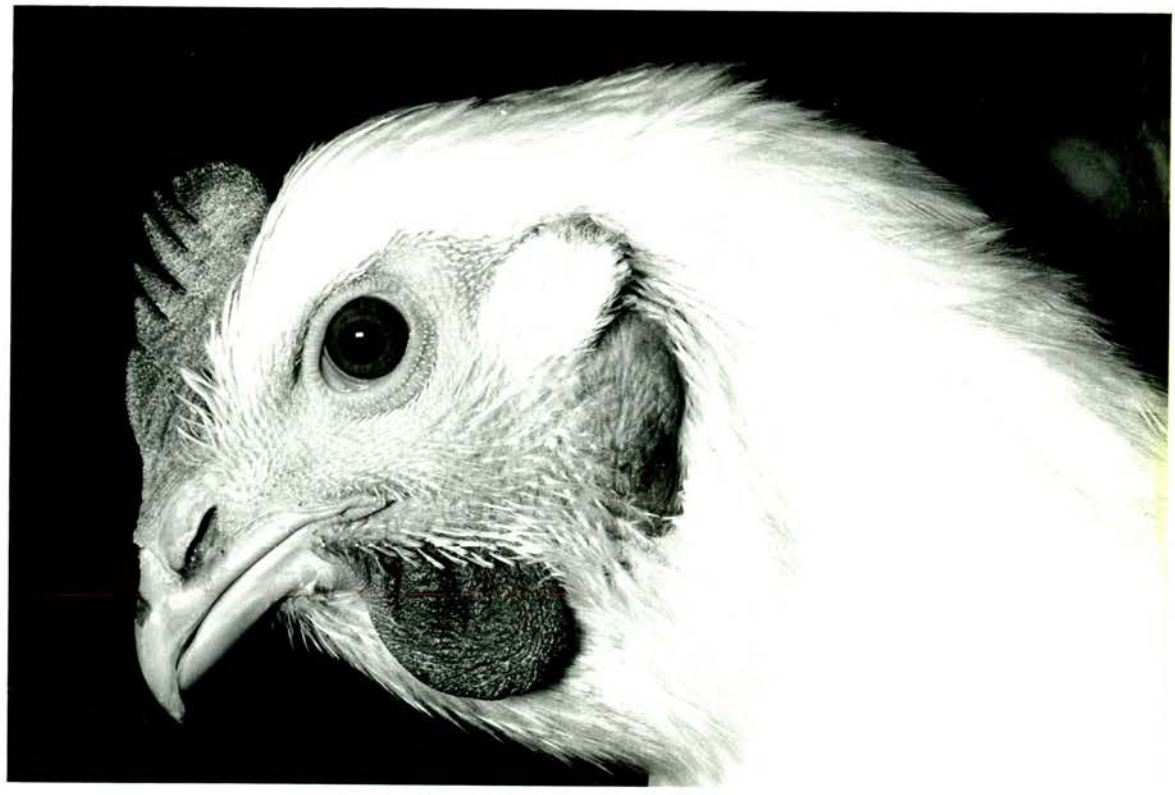


Fig.113a. BSA-induced DH response in a control fowl. Note swollen wattle and post-auricular apterium.

Fig.113b. BSA-induced DH response is depressed in the OA-treated fowl.

Fig.113c. Control (PBS-injected). Wattle and post-auricular apterium for Fig.113a.

Table 16. Arthus reaction in broilers

Lesions	0ppm OA		2ppm OA	
	24hr	48hr	24hr	48hr
I. LATERAL THORACIC APTERIUM[a]				
Macroscopic[a a]	Er,Hc,O, Ds,Ns	Hc,De,Ne	P,E,O, Ds	Hc,Ds or NL
Area (mm)	16x16 to 25x25x16	25x21x15 to 46x30x21	5 to 10	5 to 10
Score	3^+ to 4^+	4^+	1^+ to 2^+	0 to 2^+
II. DORSAL CLOACAL LIP				
Macroscopic	Hc,O,Ds	De,Ne	Hc,Ds or NL	Ds or NL
Area (mm)	15x15 to 40x22	20x17 to 35x13	3 to 14	0 to 12.5x14
Score	1^+ to 4^+	1^+ to 4^+	0 to 2^+	0 to 2^+

[a] PBS injected site did not elicit any reaction.

[a a] Er Erythema, Hs Slight haemorrhage, Hc confluent haemorrhage, O Oedema, Ns Slight necrosis, Ne Extensive necrosis, P Petechiae, E Ecchymosis, Ds Slight discolouration, De Extensive discolouration, NL No lesion

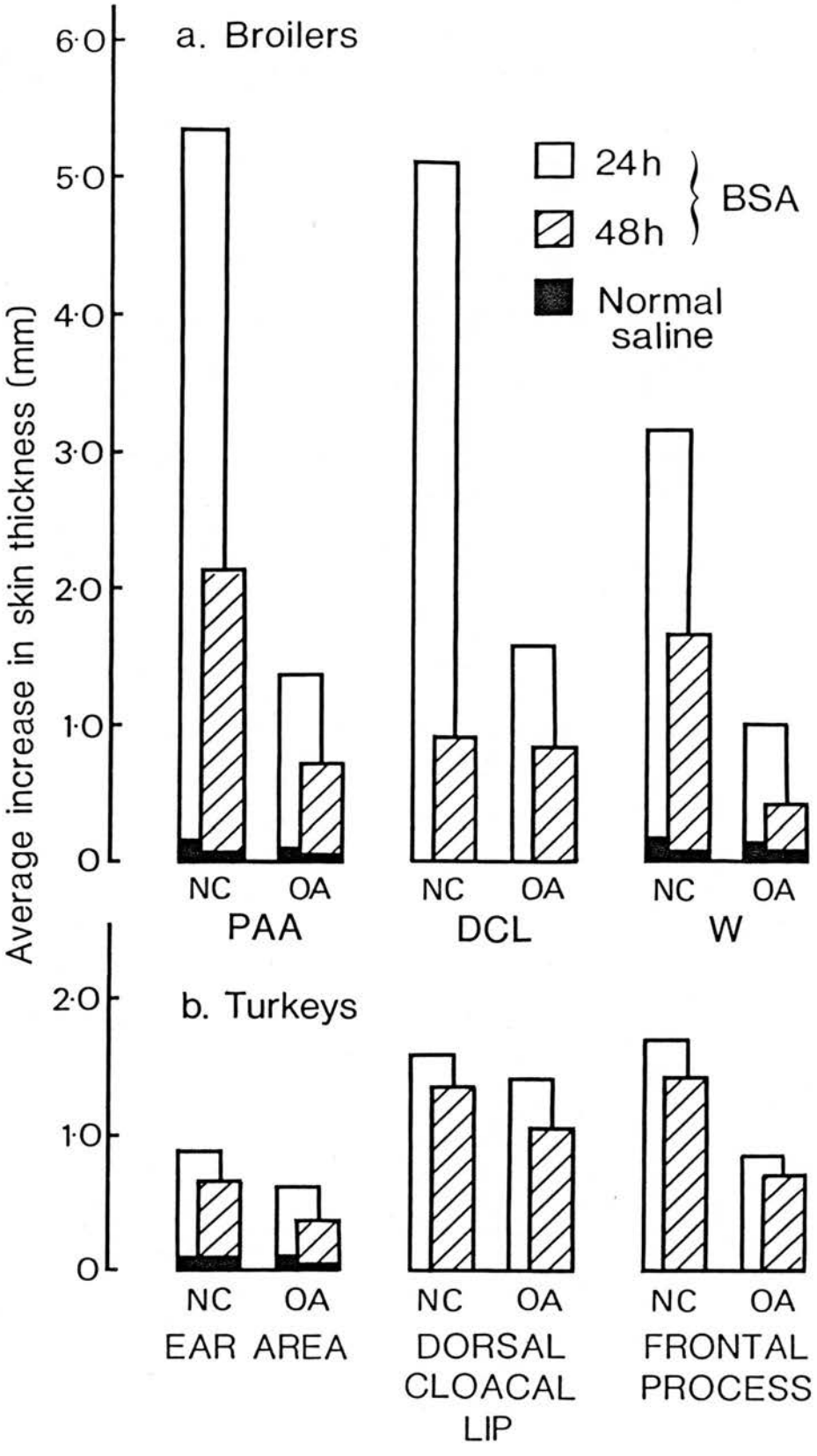


Fig.114. Average increases in skin thickness (mm) in (a) broilers and (b) turkeys in response to bovine serum albumin (BSA).

Table 17. Analysis of BSA-induced DH response in broilers

Skin site	Hours after test injection	OA - NC		PBS (Regression slope)	
		E.D.(S.E.)	95%C.I.	E.D.(S.E.)	95%C.I.
PAA	24	-2.76 (1.97)	(-8.23, 2.71)	14.31 (21.22)	(-44.60, 93.22)
	48	-1.29 (0.57)	(-2.87, 0.29)	2.79 (8.98)	(-22.14, 27.72)
Wattle	24	-2.67 (1.01)	(-5.47, 0.13)	-19.36 (12.91)	(-55.20, 16.48)
	48	-1.16 (0.74)	(-3.21, 0.89)	-1.22 (9.68)	(-28.09, 25.65)
DCL	24	-3.56 (1.48)	(-7.37, 0.25)		
	48	-0.08 (0.35)	(-0.98, 0.82)		

cells (eosinophils) were seen. The changes were less marked in OA-fed birds. In the dorsal cloacal lip, fewer granulated mononuclear cells, a large number of monocytes and heterophils were seen along with changes in the blood vessels, which were hypertrophied and had a swollen endothelium. Early necrotic and thrombotic changes similar to that in the Arthus reaction, were also seen.

4.10.2.1.4 Contact-hypersensitivity to DNCB

Challenge exposure with DNCB resulted in the appearance of macroscopic lesions after 24 hours (slight at 4-6 hours), particularly at the site of sensitisation, in the left lateral thoracic apterium (Figs.115a and 115b). In the control (0ppm OA) group, the birds had lesions ranging from 2+ to 4+, characterised by erythema, induration and small vesicle formation. In the OA-fed group, the lesion was scored between 1+ and 2+, the erythema and induration being slight and less marked. The intensity of the lesions decreased at 48 and 72 hours after challenge. At 72 hours, no macroscopic lesions could be seen in the OA-group. The application of vehicle alone did not elicit any visible macroscopic lesions.

Challenge with DNCB resulted in an increased thickness at all the skin sites in the sensitised birds; the peak response was at 24 hours (Fig.116). Further, the skin thickness was more marked at those sites which had been previously used for sensitisation, i.e., the left lateral thoracic apterium (LLTS) gave a better response (Fig.115a) than the right lateral thoracic apterium (RLTS) (Fig.115b) after challenge with the test antigen. Similarly the comb showed a better reaction than the wattle. At all the skin sites tested, the OA-treated birds had a depressed response as compared with the controls (Table 18). There was a significant difference, particularly at the PAA, in the increase in skin thickness between the control group (0ppm OA)

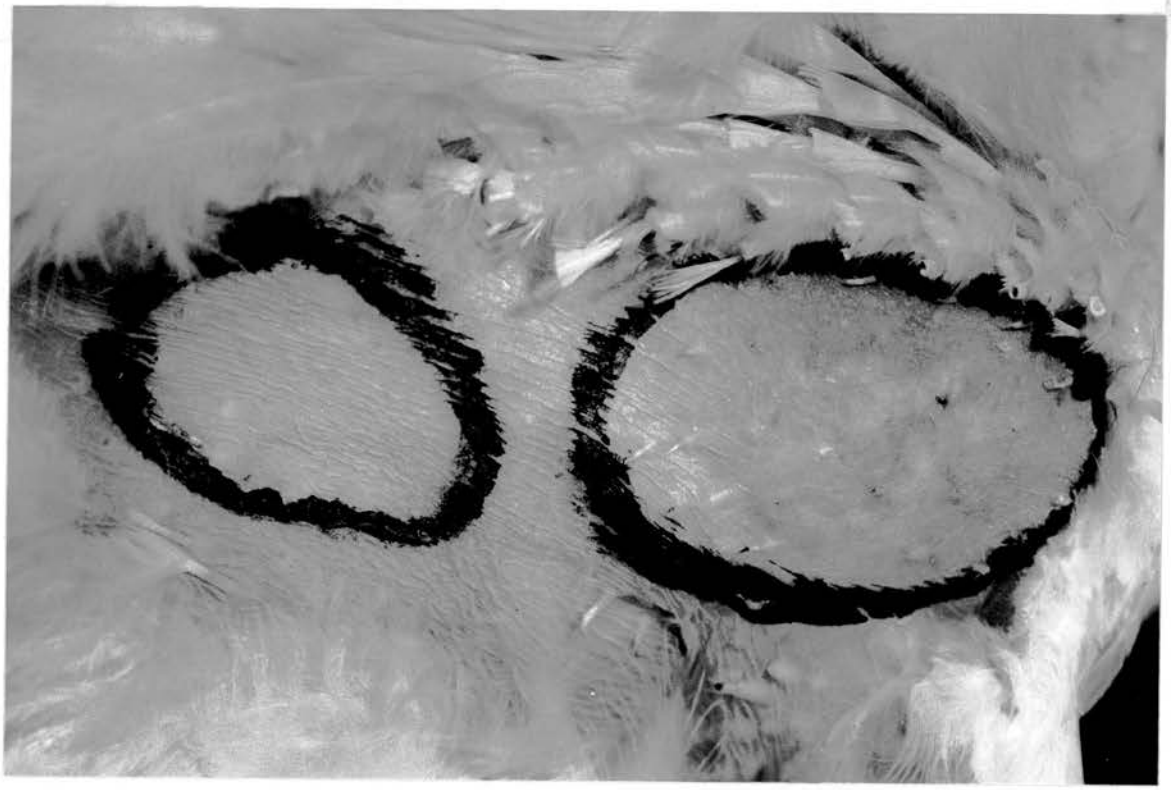


Fig.115a. DNCB-induced contact-hypersensitivity reaction in a control fowl. Left lateral thoracic apterium. Induration and vesicle formation. On left is the control (vehicle application) area.

Fig.115b. Right lateral thoracic apterium shows less response than in Fig.115a.

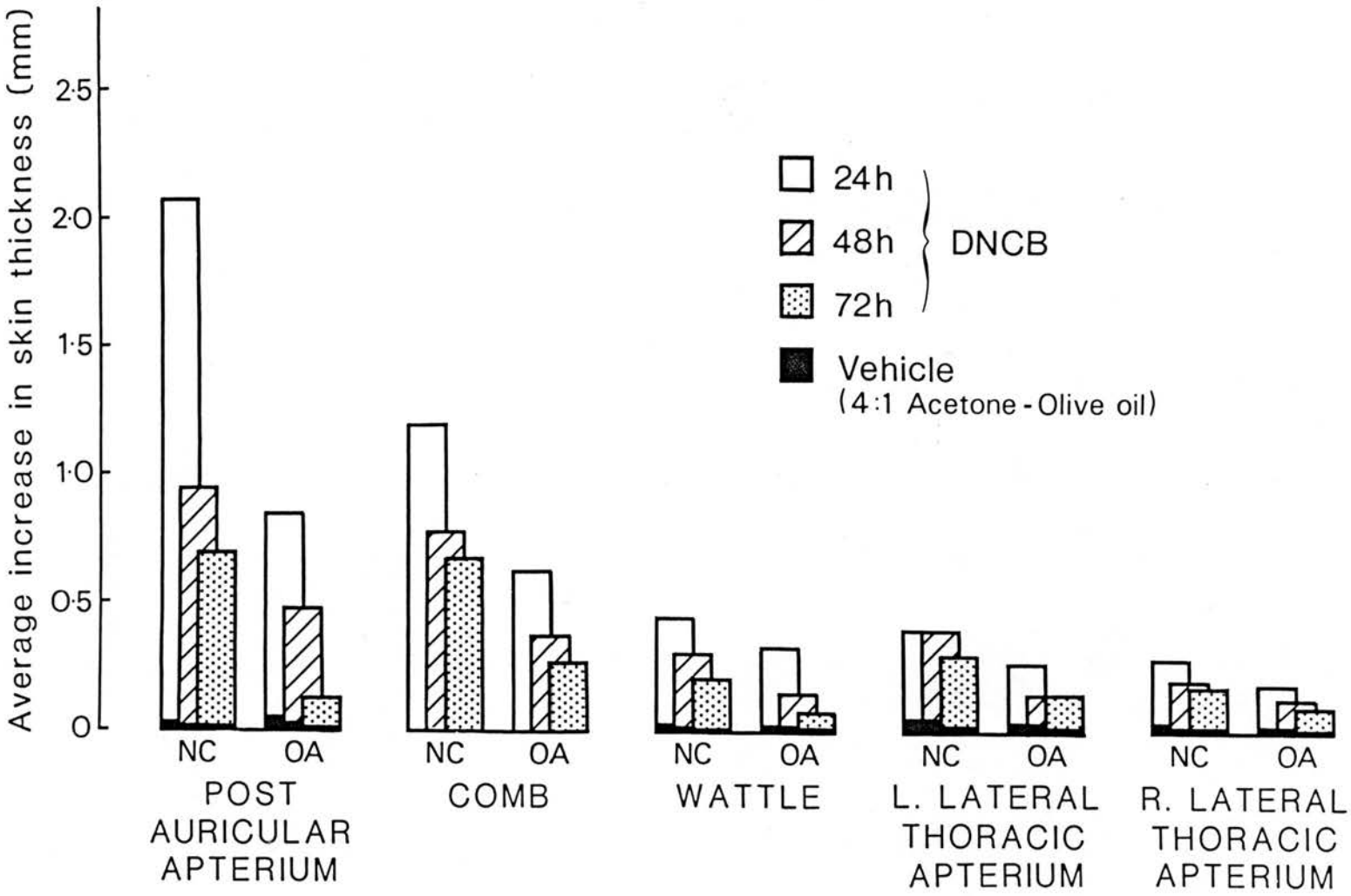


Fig.116. Contact-hypersensitivity reaction to dinitrochlorobenzene (DNCB) in broilers.

Fig.117a. DNCB-induced contact-hypersensitivity reaction in a control fowl. Note swelling in the post-auricular apterium.

Fig.117b. DNCB-induced contact hypersensitivity reaction in an OA-treated fowl. Less reaction than control (Fig.117a).

Fig.117c. Control (vehicle) for Fig.117b. No reaction.

Table 18. Analysis of DNCB-induced contact hyper sensitivity responses in broilers (increase in skin thickness, mm)*

Test sites	Hours after challenge	OA - NC		Vehicle (Regression slope)	
		E.D.(S.E.)	95%C.I.	E.D. (S.E.)	95%C.I.
PAA	24	-1.13(0.39)	(-2.13, -0.13)	-4.08 (6.86)	(-21.72,13.56)
	48	-0.54(0.28)	(-1.26, 0.18)	1.14 (6.30)	(-17.34,15.06)
	72	-0.50(0.06)	(-1.56, 0.56)	3.45 (2.16)	(-41.69,34.79)
Wattle	24	-0.12(0.11)	(-0.40, 0.16)	4.55 (3.22)	(-3.75,12.85)
	48	-0.15(0.09)	(-0.38, 0.08)	1.43 (2.39)	(-7.57,4.71)
	72	-0.10(0.13)	(-2.40, 2.20)	-5.40 (7.75)	(-142.62, 131.82)
LLTS	24	-0.11(0.09)	(-0.34, 0.12)	2.01 (1.58)	(-2.04,6.05)
	48	-0.16(0.09)	(-0.39, 0.07)	3.11 (1.93)	(-8.07,1.85)
	72	-0.14(0.08)	(-1.56, 1.28)	-12.75 (5.00)	(-101.28, 75.78)
RLTS	24	-0.07(0.06)	(-0.22, 0.08)	0.23 (2.36)	(-5.84,6.30)
	48	-0.07(0.03)	(-0.15, 0.01)	-1.03 (0.81)	(-3.11,1.05)
	72	-0.08(0.01)	(-0.26, 0.10)	1.15 (0.44)	(-6.64,8.94)
Comb	24	-0.55(0.26)	(-1.22, 0.12)		
	48	-0.37(0.15)	(-0.76, 0.02)		
	72	-0.53(0.04)	(-1.24, 0.18)		

* Significant differences are underlined.

(Fig.117a) and the 4ppm OA group (Fig.117b). The application of vehicle (acetone:olive oil) alone induced only a very slight increase in skin thickness at the test sites (Fig.117c).

Histopathological and histochemical examination. The cellular reaction was similar in nature at all the skin sites in all birds. A more severe reaction was seen at the skin sites originally sensitised and then challenged with DNCB (LLTS) compared with sites challenged but not sensitised (RLTS). The intensity of the reaction was very severe in the PAA. The skin from OA-treated birds had a much reduced cellular response. The reaction varied greatly in the superficial and deep layers of the skin sites.

Epidermis. The stratum corneum layer was thickened to 8-10 layers compared with 5-6 layers at control sites or at places where vehicle alone had been applied, and showed hyperkeratosis. Cells of the stratum germinativum were vacuolated and there were degenerative changes. A large number of cells were swollen and had cytoplasmic haloes around the nuclei both in the basal as well as in the intermediate layers. A few cells were seen undergoing mitosis. The dense connective tissue layer, just below the basement membrane of the epidermis, was infiltrated by mononuclear cells and a few heterophils.

Dermis. In the superficial dermis (stratum superficiale), the capillary layer was thickened and the capillary sinuses were dilated. There were circumscribed areas of cellular reaction around the capillary sinuses and blood vessels, composed of predominantly mononuclear cells, granulocytes and red blood cells (Fig.118). The mononuclear cells were mainly lymphocytes and macrophages. Many macrophages were very large and were widely distributed. They could be named as 'giant phagocytic macrophages' on the basis of their structure, size and phagocytic property. These giant macrophages had

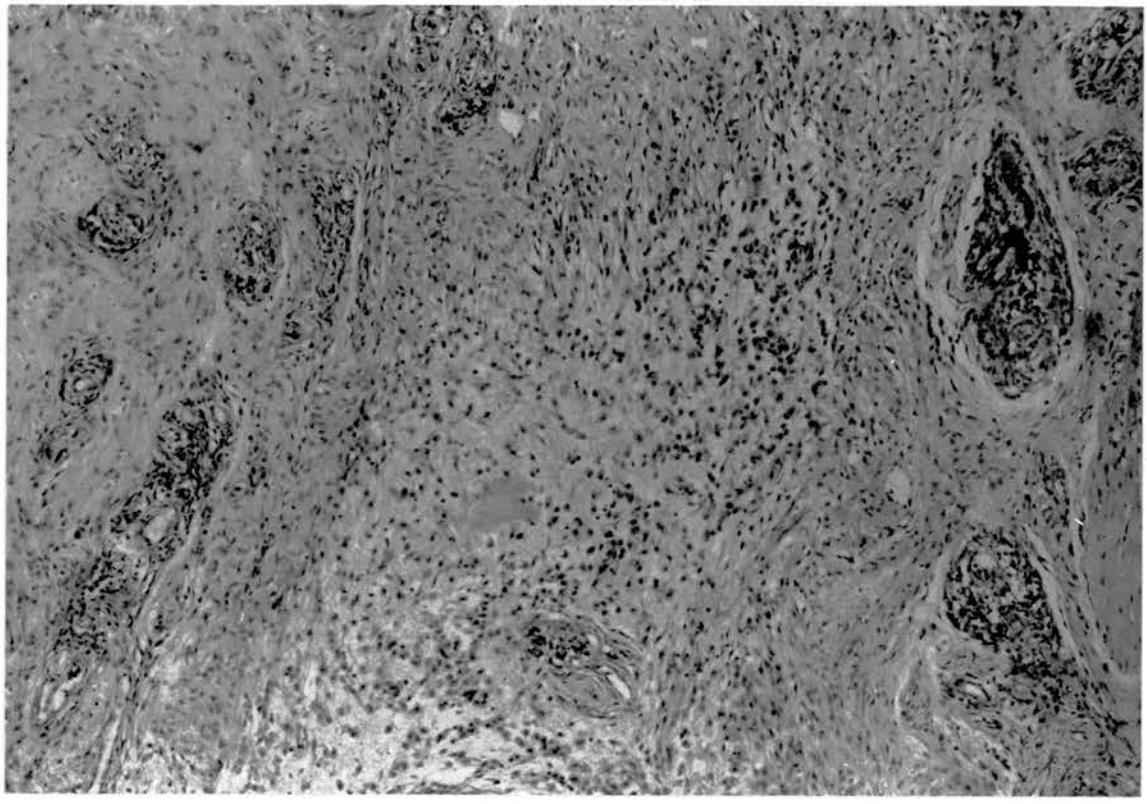


Fig.118. Fowl(Control). Lateral thoracic apterium (DNCB). 48 hr. Low power view of intense cellular reaction in the dermis. Note generalised and perivascular nodular reaction. Peroxidase - H &E x 75.

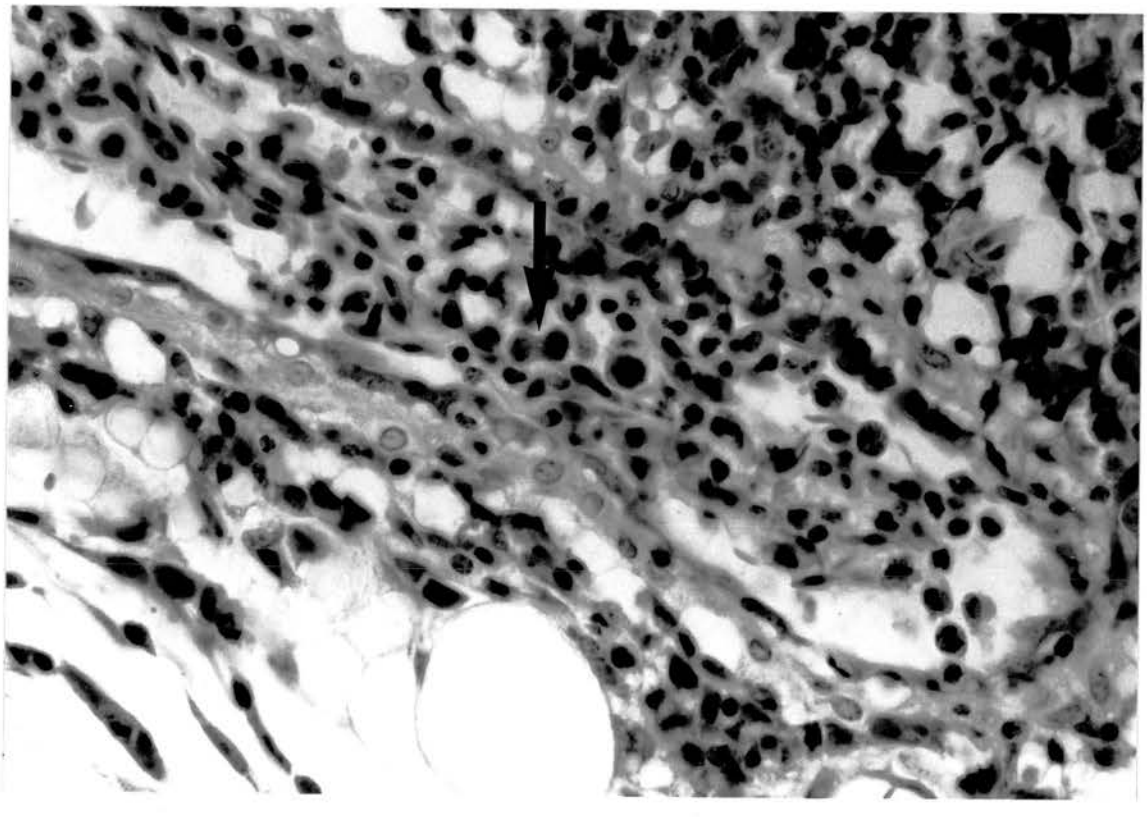


Fig.119. Fowl (Control). Post-auricular apterium (DNCB, 48hr). Hypertrophied blood vessel containing dividing lymphoblasts (arrow), granulocytes and red blood cells. Predominantly mono-nuclear cells are present in the perivascular area. Peroxidase - H & E x 400.

a foamy cytoplasm and always contained some phagocytosed material such as RBC's, dead or dying cells or tissue debris. The granulocytes were predominantly heterophils but frequently peroxidase-positive, brown staining eosinophils were seen at the periphery of the nodular areas of the cellular reaction. These nodular areas of cellular aggregates were sometimes limited by a fibroblastic proliferation but usually these perivascular aggregations, which varied in size, either coalesced with other similar areas to form a large zone of cellular reaction or extended further to the deep dermis. In many areas, dying cells were seen in various stages of pyknosis, karyorrhexis and karyolysis.

Blood vascular changes were common. The dilated blood vessels contained many lymphocytes, RBCs, heterophils, monocytes and a few eosinophils. Many of these showed margination and migration of leucocytes. Some cells were seen trapped in the wall of blood vessels. Dividing lymphoblasts could be seen inside the blood vessels (Fig.119) or even outside in a nodular zone of reaction. The wall of the blood vessels was usually thickened. The endothelial cells were hypertrophied and proliferating. In many areas the formation of new capillaries with the laying down of angioblasts was evident. Many newly formed small blood vessels were noticed (Fig.120). Vascular proliferation was a common feature.

Deep dermis (stratum profundum). The reaction was similar to that of the superficial dermis but was more severe in intensity and composition. The perivascular cellular reaction was generalised with an intense aggregation of mononuclear cells (Fig.118). Giant macrophages with engulfed cellular debris were more frequently seen in the semi-thin plastic sections. Occasionally, a few mast cells were seen in toluidine blue stained sections. Heterophils were sometimes

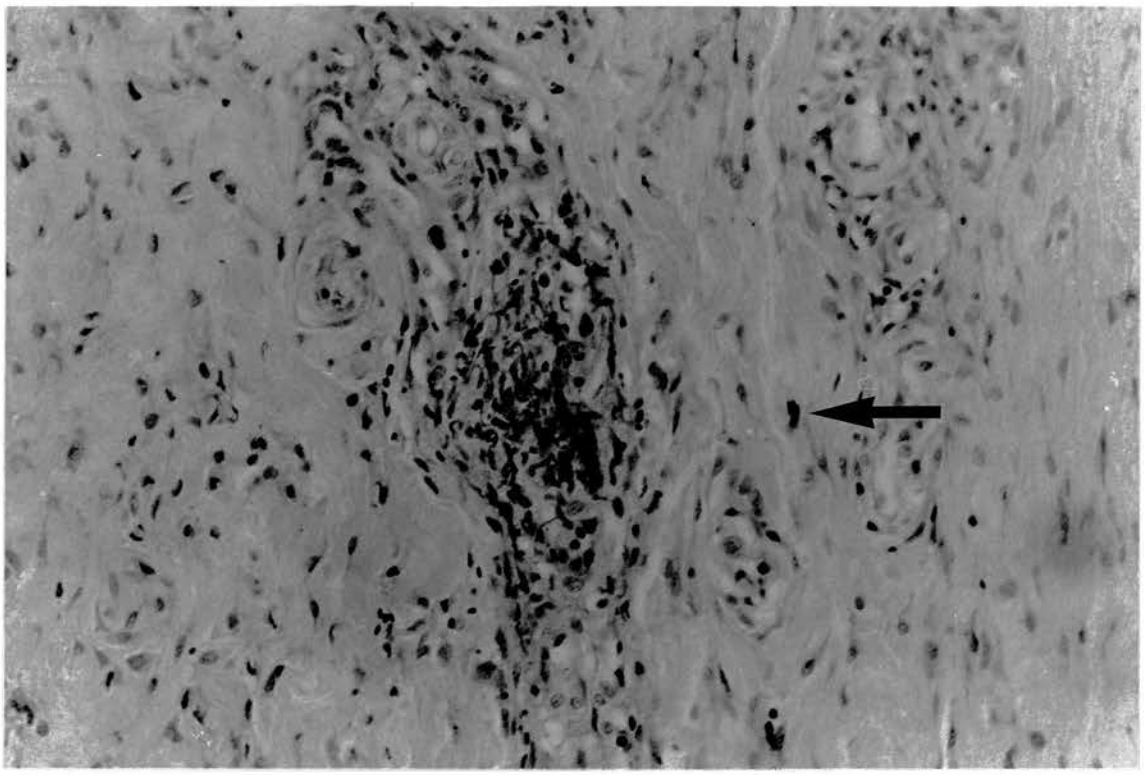


Fig.120. Fowl (Control). Lateral thoracic apterium (DNCB, 48hr). An area in the deep dermis showing vascular proliferation and new capillary formation. A peroxidase positive eosinophil (arrow) is also present. Peroxidase - H & E x 400.

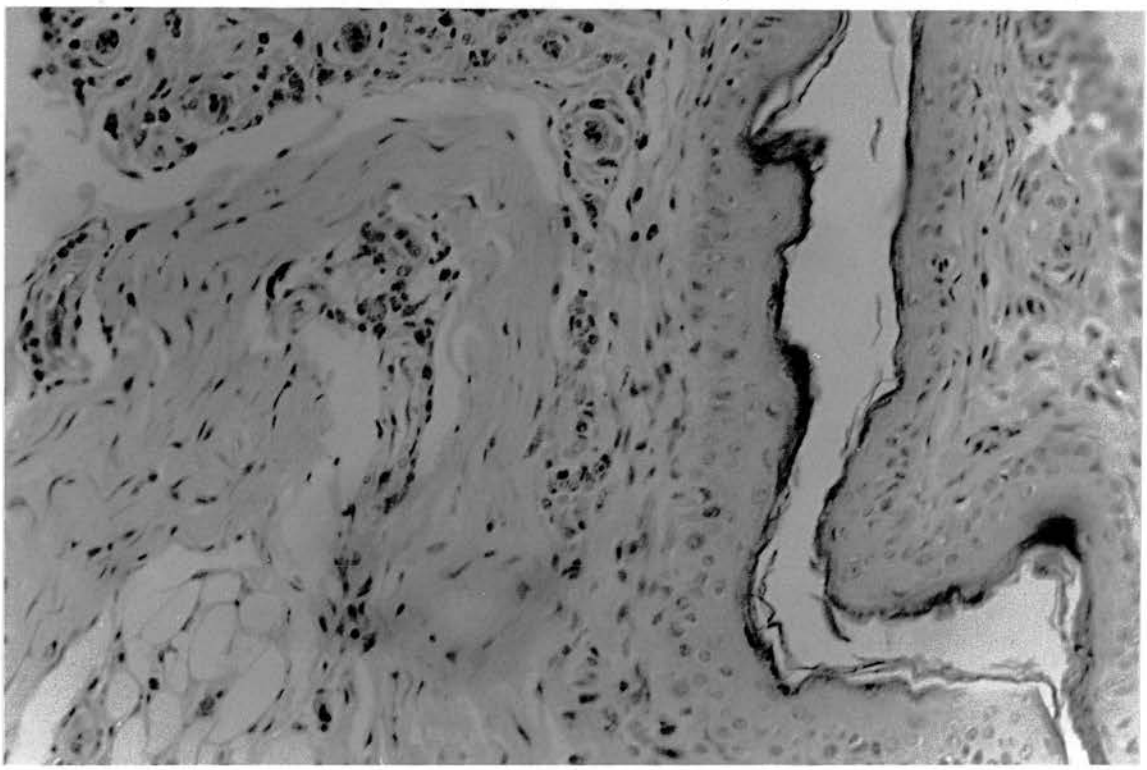


Fig.121. Fowl (4ppm OA). Lateral thoracic apterium (DNCB, 48hr) showing mild cellular reaction. H & E x 400.

degranulating. Dead and dying cells were frequently seen. Peroxidase-positive eosinophils were seen at the periphery of several reactive zones, though in smaller numbers than the heterophils. Fibroblastic proliferation, vascular changes and new capillary formation were also seen. The reaction sometimes extended to the subcutis or underlying muscular tissue. The microscopic picture of the sites challenged with vehicle only, did not differ greatly from that of the untreated sites. In the lateral thoracic apterium, occasionally a few leucocytes could be seen. In OA-treated birds the cellular reaction was similar though less marked in intensity and severity at all the skin sites. The reaction ranged from mild to moderate. Blood vessels were sometimes empty, but the vascular changes, such as endothelial cell hypertrophy and new capillary formation were frequently observed. Zones of cellular reaction were fewer in number and smaller in size (Fig.121). The number of lymphocytes, heterophils, eosinophils and macrophages appeared to be considerably less than in the control group, both in the superficial and deep dermis. Hyperkeratotic changes in the epidermis were less marked. Giant macrophages with engulfed debris were seen in many reaction zones.

Electron microscopic examination. In the epidermis, the epithelial cells of the stratum germinativum showed vacuolation with a signet ring appearance and pyknotic nuclei. Lymphocytes, lymphoblasts, heterophils, histiocytes and macrophages were seen. The dilated blood vessels contained heterophils, lymphocytes, thrombocytes and RBC's (Fig.122). A few cells were seen migrating through the wall of a blood vessel, which was thickened and vacuolated. Pericytes were swollen. The endothelial cells were also thickened and sometimes protruded into the lumen. In the deeper part of the dermis, fibroblastic proliferation with active fibroblasts synthesising collagen

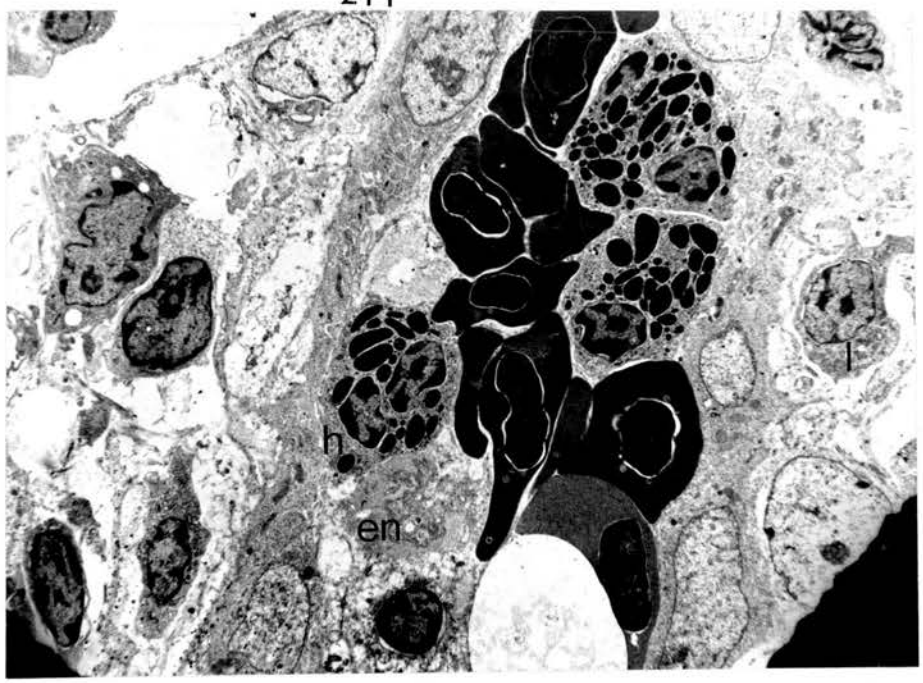


Fig.122. Fowl (Control). Lateral thoracic apterium (DNCB, 48 hr). An electron micrograph showing a small blood vessel with heterophils (h) and red blood cells, some of which are seen passing between the endothelial cells (en). Lymphocytes are present in perivascular region. x 4374.

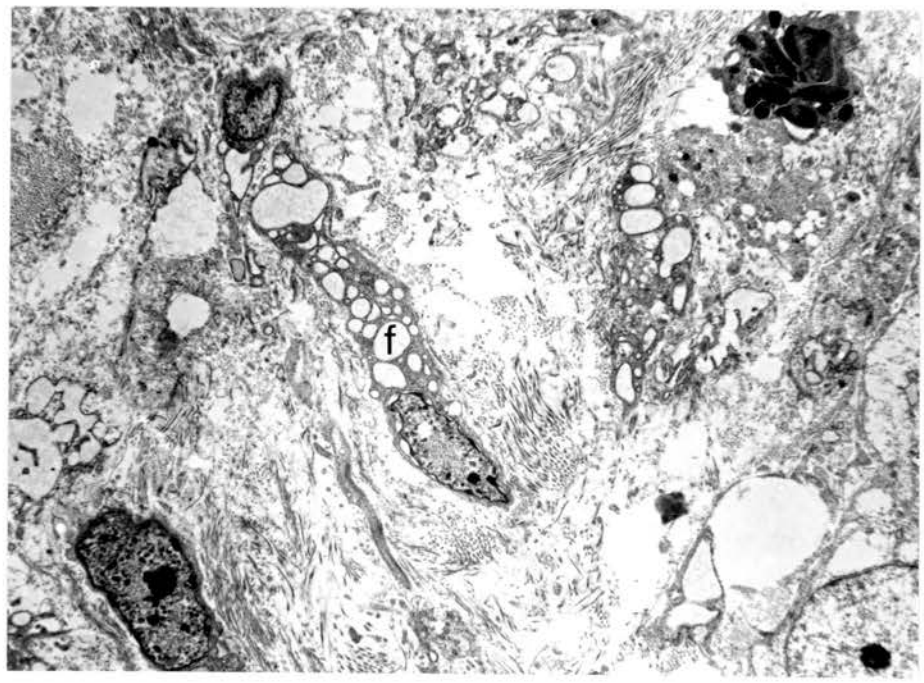


Fig.123. Fowl (Control). Lateral thoracic apterium. An area of fibroblastic proliferation in the deep dermis. Fibroblasts (f) have dilated endoplasmic reticulum. A heterophil is also present. x 4374.

fibrils in their cytoplasm was commonly seen (Fig.123). Macrophages with phagocytic material and dark granular debris (RBCs) were seen; they were pleiomorphic in shape and size. A similar reaction was seen in the skin of OA-treated birds.

4.10.2.1.5 Graft-versus-host reaction

GVH reaction as assessed by splenomegaly and hepatomegaly in the chick embryos (Table 19; Figs. 124a and 124b) was found to cause a four and half times increase in the relative size of the spleens of embryos inoculated with blood from the control group (0ppm OA). Hepatic enlargement was only one and a half times (Fig.125). When data were compared by taking absolute weights of spleens and livers, the increase in size and weight was about 2.77 and 1.33 times respectively (Table 19). Chick embryos inoculated with blood from the OA group of birds showed very low GVH response (splenomegaly, 1.64 to 1.83 times) in comparison with the control group (0ppm OA). Increase in the size of livers was slight. The GVH response was highly significant ($P < 0.01$) in embryos injected with cells from control (0ppm OA) donors compared with the uninoculated control embryos. Further, there was a highly significant depression ($P < 0.01$) in the GVH reaction in the OA-treated group compared with the control (0ppm OA) birds, but the GVH response in the OA-treated group did not differ significantly from the uninoculated group of embryos (Table 19).

4.10.2.2 Turkeys

Hypersensitivity responses were elicited in turkeys with all the test antigens, although at most of the skin sites tested, they were of a lesser intensity than in broilers. The responses were markedly depressed in OA-treated turkeys. The earskin area was found to be best and most convenient for

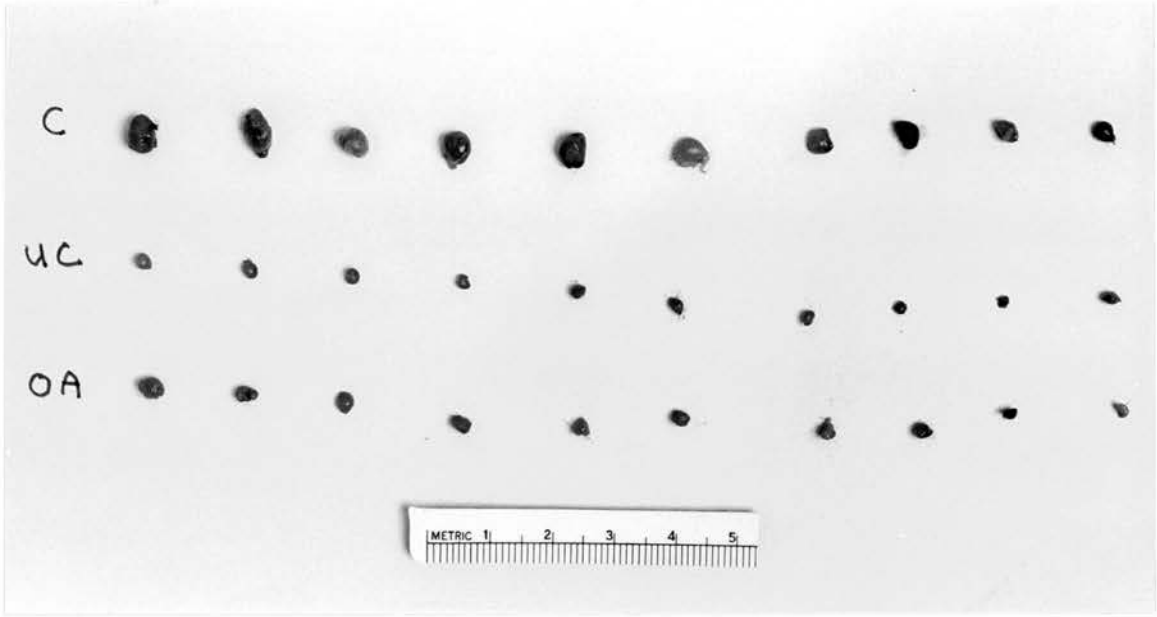


Fig.124a. Graft-versus-host (GVH) response in chick-embryos. Splenomegaly. Note enormous enlargement of spleens from inoculated control group (C) as compared with untreated control (UC). Response is very much depressed in OA-group.

Fig.124b. GVH response in chick embryos. Hepatomegaly. As in Fig.124a control group (C) livers gave better reaction than OA-group.

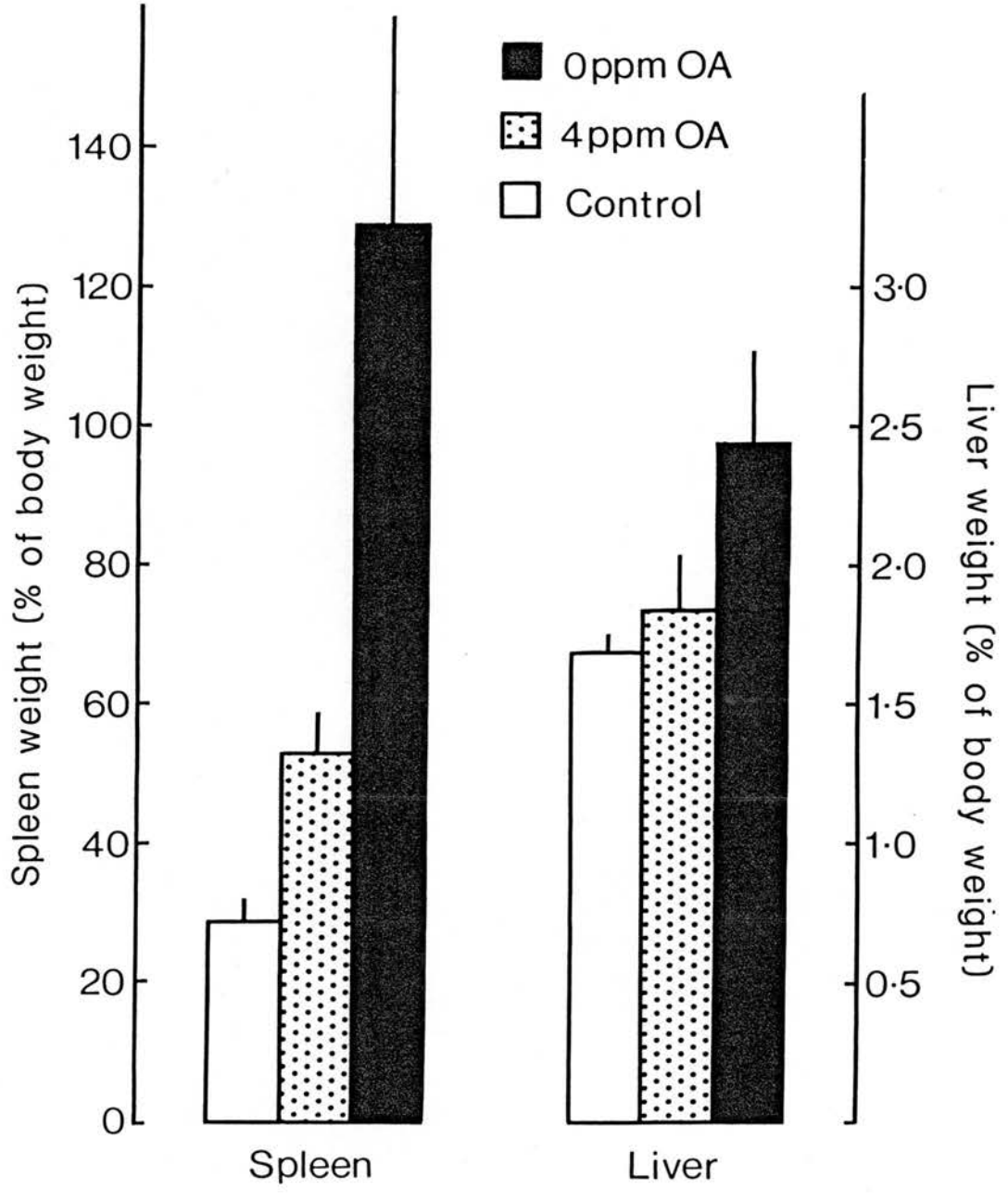


Fig.125. Graft-versus-host (GVH) response in embryos (splenomegaly and hepatomegaly).

Table 19. Graft-versus-host reaction induced by inoculation of blood cells of broilersinto embryos

Donor group	Spleen		Liver	
	wt. (mean±SEM) mg	Mean ratio	wt. (mean±SEM) mg	Mean ratio
A Absolute weight				
0ppm OA	33.80±3.37[a]	2.77	0.74±0.06[a]	1.33
4ppm OA	20.00±4.49[a]	1.64	0.66±0.10[b]	1.18
uninoculated controls	12.20±2.35[b]	1.00	0.56±0.04[b]	1.00
B Relative weight (per 100g body weight)				
0ppm OA	129.17±29.12[a]	4.44	2.44±0.28[a]	1.44
4ppm OA	53.15± 9.41[b]	1.83	1.84±0.19[b]	1.09
uninoculated controls	29.06± 2.55[b]	1.00	1.69±0.06[b]	1.00

a,b values with different superscript in a column differ significantly ($P < 0.01$).

intradermal injection, measurement and study of macroscopic and microscopic changes. The frontal process and dorsal cloacal lip offered considerable resistance to the injection. The lateral thoracic apterium (skin) was thin but accurate measurement of thickness was relatively difficult. The wing web was convenient for injection and measurement, but the DH response was found to be variable and inconsistent with the various antigens tested and the cellular response (microscopic changes) was not as marked as in the earskin area or even lateral thoracic apterium. Although a DH response was elicited in the frontal process and the dorsal cloacal lip, a suitable control injection of saline or application of vehicle was not possible in the same bird.

No differences between sexes were noticed in the development of hypersensitivity reactions in turkeys.

4.10.2.2.1 Delayed hypersensitivity to avian tuberculin, PPD

The mean increases in skin thickness at 0, 24 and 48 hours at the sites tested are presented in Fig.126. There was a significantly greater response in turkeys sensitised with killed Mycobacterium avium than in those sensitised with avian tuberculin, PPD, particularly in the earskin and frontal process sites both at 24 and 48 hours after the test injection. The reaction was seen best in the earskin area which was also easy to inject and convenient for measurement. Liquid paraffin and FCA injections did not cause any significant cutaneous reactions, though FCA resulted in a slight increase in skin thickness. Section 1 of Table 20 shows that the DH response was significantly depressed in OA-fed birds in both the M. avium - and avian tuberculin PPD-sensitised groups. Control saline injections caused a negligible

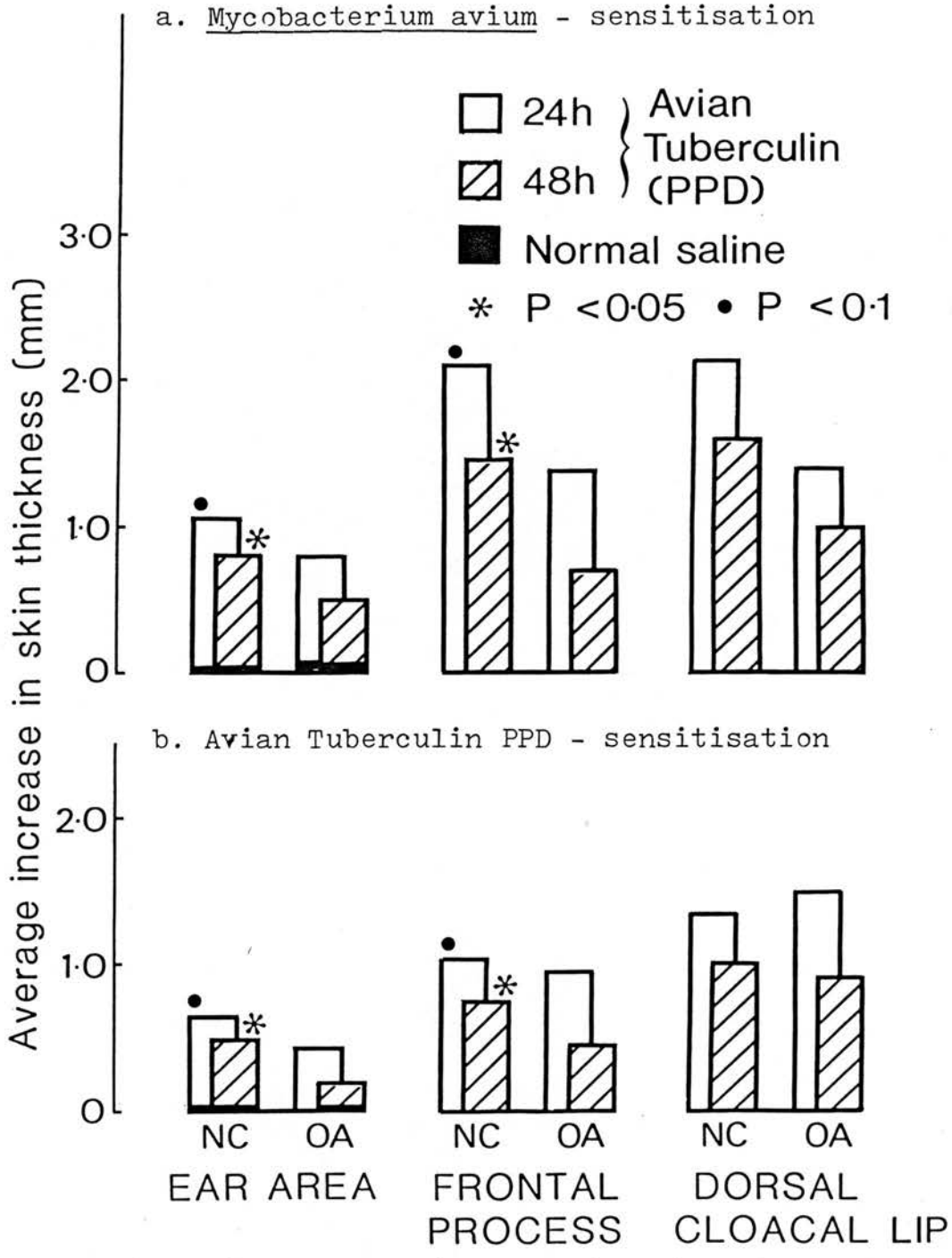


Fig.126. Average increase in skin thickness (mm) in sensitised turkeys in response to avian tuberculin, PPD.

Table 2.0 Analyses of delayed hypersensitivity responses in normal and ochratoxin A treated turkeys.

S.No	Treatment		Ear skin area 24h	Ear skin area 48h	Frontal process 24h	Frontal process 48h	Dorsal cloacal lip 24h	Dorsal cloacal lip 48h
	I. Tuberculin tested birds							
A.	OA - NC	E.D. (S.E.)	-0.20(0.10)	-0.26(0.09)	-0.41(0.20)	-0.50(0.18)	-0.23(0.43)	-0.34(0.46)
		95% C.I.	(-0.42, 0.02)	(-0.46, -0.06)	(-0.84, 0.02)	(-0.89, -0.11)	(-2.15, 1.69)	(-1.33, 0.65)
B.	MA - PPD	E.D. (S.E.)	0.36(0.11)	0.31(0.09)	0.69(0.21)	0.42(0.18)	0.27(0.45)	0.32(0.48)
		95% C.I.	(0.11, 0.51)	(0.12, 0.60)	(0.24, 1.14)	(0.03, 0.81)	(-0.70, 1.24)	(-8.72, 1.36)
C.	FCA - PPD	E.D. (S.E.)	-0.27(0.23)	-0.16(0.20)	-0.55(0.46)	-0.29(0.40)	-0.85(0.99)	-0.81(1.05)
		95% C.I.	(-0.77, 0.23)	(-0.60, 0.28)	(-1.54, 0.44)	(-1.15, 0.57)	(-2.99, 1.29)	(-3.08, 3.73)
D.	LP - MA	E.D. (S.E.)	-0.75(0.18)	-0.56(0.15)	-1.71(0.34)	-0.99(0.29)	-1.49(0.72)	-1.30(0.76)
		95% C.I.	(-1.14, -0.36)	(-0.89, -0.23)	(-2.44, -0.98)	(-1.62, -0.36)	(-3.05, 0.07)	(-2.94, 0.34)
E.	Saline swelling	Regression slope (S.E.)	-1.16(1.54)	-1.59(1.48)	-	-	-	-
		95% C.I.	(-4.52, 2.20)	(-4.81, 1.63)	-	-	-	-
F.	—	d.f.	12	12	13	13	13	13
	II. BSA tested birds							
A.	OA - NC	E.D. (S.E.)	-0.25(0.18)	-0.26(0.17)	-0.86(0.22)	-0.72(0.17)	-0.21(0.37)	-0.29(0.31)
		95% C.I.	(-0.63, 0.13)	(-0.62, 0.10)	(-1.32, -0.40)	(-1.08, -0.36)	(-0.99, 0.57)	(-0.94, 0.36)
B.	PBS - BSA	E.D. (S.E.)	-0.73(0.32)	-0.50(0.28)	-1.57(0.37)	-1.27(0.29)	-1.33(0.61)	-1.19(0.52)
		95% C.I.	(-1.41, -0.05)	(-1.09, 0.09)	(-2.35, -0.79)	(-1.34, -0.66)	(-2.62, -0.02)	(-2.29, -0.09)
C.	PBS swelling	Regression slope (S.E.)	-1.25(1.40)	0.12(1.37)	-	-	-	-
		95% C.I.	(-4.22, 1.72)	(-2.78, 3.02)	-	-	-	-
D.	—	d.f.	16	16	17	17	17	17

E.D. (S.E.) - Estimated differences of the mean increase in skin thickness (and their standard errors).

NC (Normal control) - 0 ppm OA; OA (Ochratoxin A) - 4 ppm OA.

MA - Mycobacterium avium; PPD - Avian tuberculin, Purified protein derivative; LP - Liquid paraffin; FCA - Freund's complete adjuvant.

BSA - Bovine serum albumin; PBS - Phosphate buffered saline.

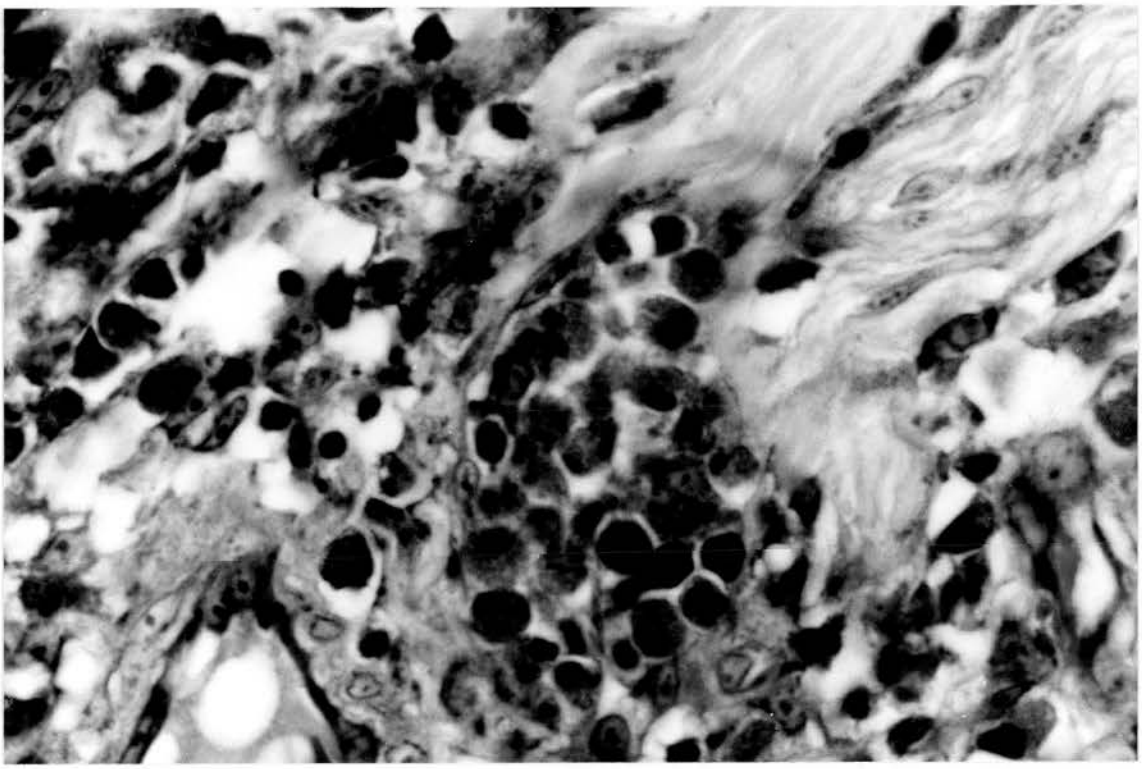


Fig.127. Turkey (Control). Frontal process (avian PPD). An area of dermis showing predominantly heterophilic reaction. May-Grunwald and Giemsa x 750.

reaction.

Histological examination of the skin sites tested revealed typical granulomatous reaction in both M. avium - and avian tuberculin PPD- sensitised birds. However the numbers of infiltrating cells (mononuclear cells and heterophils) were relatively small (Fig.127) compared with broilers.

4.10.2.2.2 DH to PHA-P

Early macroscopic lesions, such as erythema, oedema and slight swelling were detected at 6 hours after PHA-injection at the skin sites. The lesions became more pronounced and more severe by 24 hours when the injected sites were hard, indurated and oedematous although the erythema was less marked. The earskin area became so swollen that it had a puffy, pendulous appearance (Fig.128a). At 48 hours the reaction was still present but less marked. The average increases in skin thickness, 24 and 48 hours after injection of PHA in 0 and 4 ppm OA-treated turkeys, are presented in Fig.129. A maximum cutaneous response was observed at 24 hours, though the reaction was still marked at 48 hours. The most pronounced reactions were noticed in the earskin area followed by the lateral thoracic apterium. The ochratoxin group showed an appreciable depression in PHA response at all the skin sites except the wing web (Fig.129). The response was significantly depressed ($P < 0.05$) in the earskin area (Fig.128b) and lateral thoracic apterium (Table 21). Injections of PBS resulted in negligible responses (Fig.129).

Differential leucocyte counts. PHA-injection resulted in an increase in circulating heterophil counts from 42 (normal) to 60 per cent (range, 45-76 per cent) in 0 ppm OA group with corresponding decreases in lymphocytes (Table 22). In birds of the OA group treated with PHA, the

Fig.128a. PHA-induced DH response in a control turkey. Note good reaction (swelling) in the earskin area and frontal process.

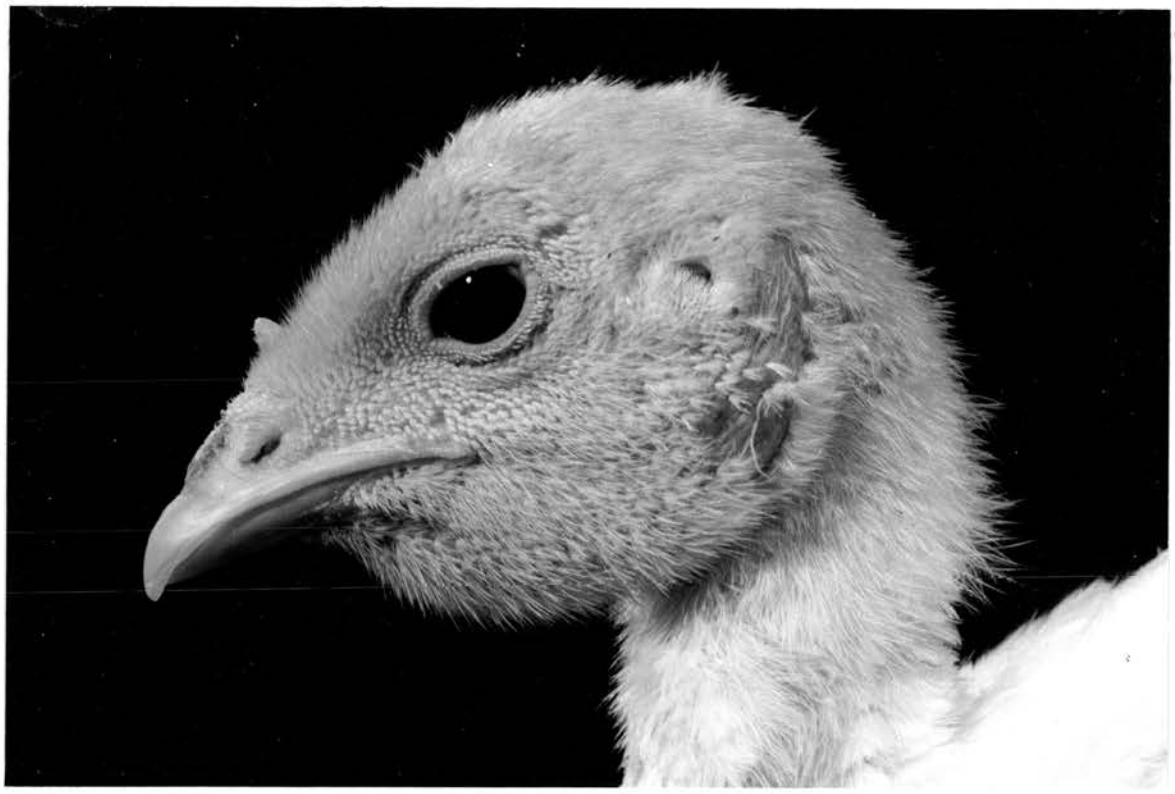


Fig.128b. PHA-induced DH response in an OA-treated turkey. Note depressed reaction in the earskin area and frontal process.

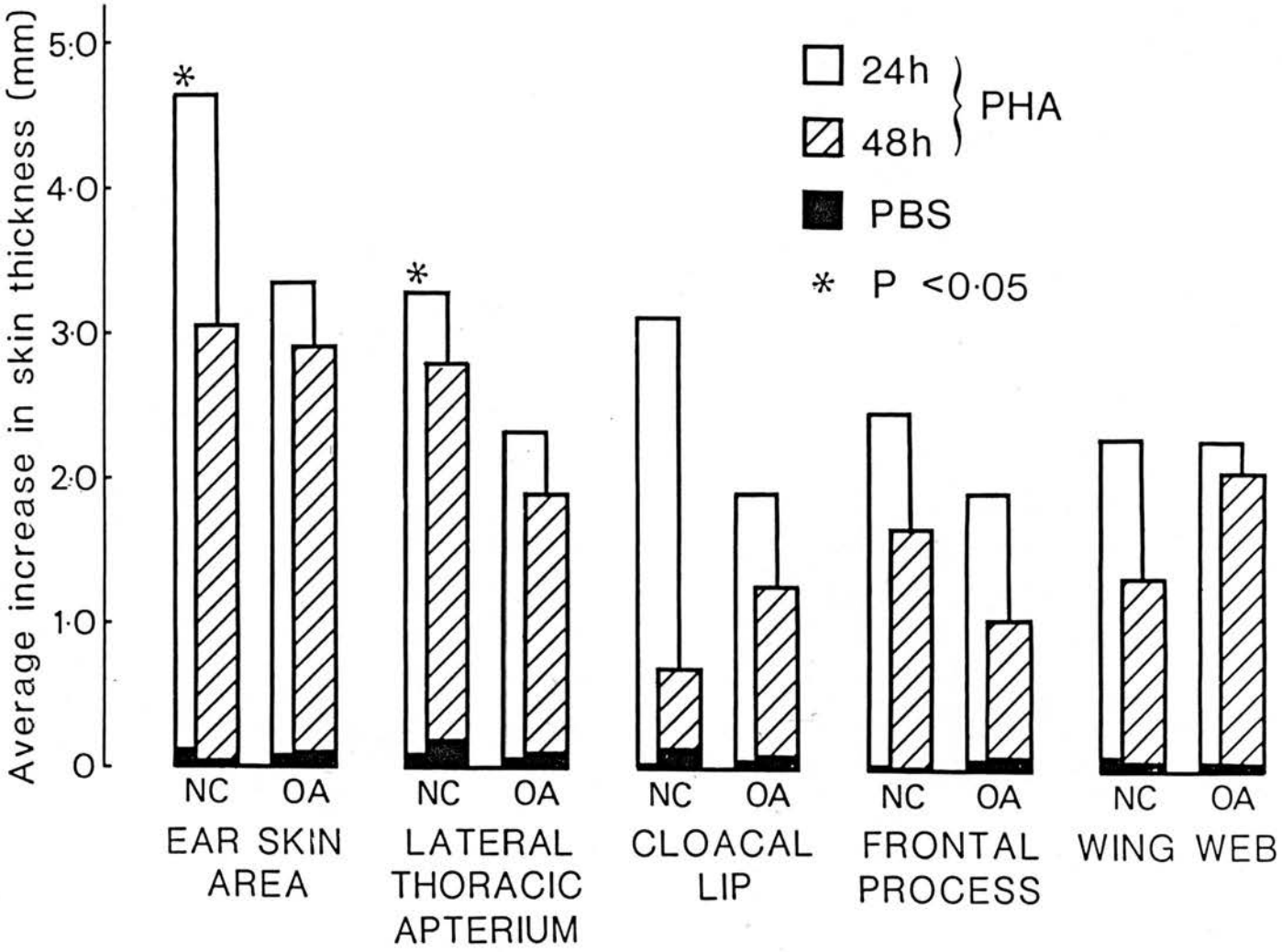


Fig.129. Average increase in skin thickness (mm) in turkeys in response to phytohaemagglutin-P (PHA-P).

Table 21. Analysis of PHA-induced DH response in turkeys*

Test sites	Hours after challenge	OA - NC	
		E.D. (S.E.)	95% C.I.
ES	24	-1.26 (0.37)	(-2.04, -0.47)
	48	-0.50 (0.47)	(-1.65, 0.65)
FP	24	-0.54 (0.30)	(-1.18, 0.10)
	48	-0.58 (0.56)	(-2.27, 0.79)
LTS	24	-1.01 (0.43)	(-1.94, -0.09)
	48	-1.00 (0.26)	(-1.64, -0.36)
WW	24	0.08 (0.26)	(-0.48, 0.65)
	48	0.76 (0.32)	(-1.54, 0.02)
DCL	24	-1.16 (0.58)	(-2.40, 0.08)
	48	0.58 (0.49)	(-1.78, 0.62)

*Significant differences are underlined.

heterophil levels were significantly lower than in the PHA-treated Oppm OA group and resembled control birds (Oppm OA) which had not been treated with PHA (Table 22).

Histological and histochemical examination. PHA induced a severe cellular reaction in the injected skin sites. Intradermal injections of PHA also elicited a systemic reaction; the most reactive organs being the lymphoid tissues, especially the thymus and spleen. The differentiation of heterophils and eosinophils both in the skin and in lymphoid tissues, was difficult in H and E stained sections because of the eosinophilic staining reaction of the granules in both cases. Some cells could be identified by morphological features, but confirmed identification was not possible because of the confusion of immature heterophilic myelocytes, appearing similar to eosinophils. The granules of eosinophils in turkeys, in contrast to those in fowls, were peroxidase and Sudan Black B negative (as also were heterophils and basophils). Occasionally in toluidine blue stained sections, metachromatic cells could be seen. In PAS-stained sections, some PAS-positive granules (heterophils) were seen in the skin and lymphoid tissues.

Skin. Histologically, the cellular reaction was essentially similar at all the skin sites tested, though most pronounced in the earskin area (Figs.130 and 131) and lateral thoracic apterium. A less intense reaction was noticed in the dorsal cloacal lip and frontal process. The cellular response in the wing web was variable. PBS-injected skin sites were either normal or had very few heterophils and mononuclear cells.

Epidermis. There was some hyperkeratosis and disruption of the stratum corneum layer in most of the skin sites. The prickle cell layer (stratum germinativum) had changes ranging from degeneration to necrosis. The cells were

Table 22. Differential leucocyte counts (mean ± S.E.M.) in PHA-treated turkeys

Group	No. of birds examined	Hetero-phils	Eosino-phils	Baso-phils	Lympho-cytes	Mono-cytes
0ppmOA (PHA)	5	59.8 ±6.3[a]	0.6 ±0.24[a]	3.8 ±1.11[a]	28.6 ±5.02[a]	7.2 ±1.02[a]
4ppmOA (PHA)	5	43.2 ±4.19[b]	2.0 ±1.30[a]	6.2 ± 0.56[a]	35.2 ±3.70[a]	13.4 ±1.75[a]
0ppmOA (nonPHA)	10	42.1 ±3.67[b]	0.6 ±0.28[a]	4.9 ±0.99[a]	46.6 ±3.38[b]	5.8 ±1.27[a]

a,b,c values with different superscripts in a column differ significantly from each other ($P < 0.02$)

usually vacuolated or swollen or had a foamy appearance. A halo formation around the nucleus was also observed and nuclei were pushed to one side. Many nuclei were pyknotic or karyorrhectic and even karyolytic. The prickle cell layer was also heavily infiltrated with granulocytes and lymphoid cells and, in places, the normal structure was completely replaced by these invading cells.

Dermis. In the superficial dermis (stratum superficiale), the normal capillary layer of venous sinuses was replaced by an accumulation of granulocytes and lymphoid cells. At 24 hours, there was severe oedema with infiltrating cells heavily dispersed throughout. Many lymphoid cells had lymphoblastic characteristics and some of the nuclei were pleiomorphic and budding (Fig.132). Many mononuclear cells, especially macrophages, were also seen, some containing phagosomes and engulfed material. The granulocytes had a monolobed or bilobed nucleus and large, usually round prominent granules. These cells were frequently seen degranulating. The presence of these granulocytes was a conspicuous and consistent feature of the lesions. By 48 hours, the oedema had subsided in most cases but the granulocytic infiltration appeared to increase relative to lymphoid and mononuclear cells (Fig.131).

In blood vessels, particularly venules and capillaries, marked changes were noticed. The endothelial cells were thickened and had protrusions into the lumen and in some severe cases, the walls of the larger vessels were thickened with prominent nuclei (pericytes) or even hyalinisation. Entrapped in the wall of affected blood vessels were small groups of granulocytes and lymphoid cells. A perivascular accumulation of these cells, or at some places of lymphoid cells, was also seen (Fig.130).

In the deep dermis (stratum profundum), these large

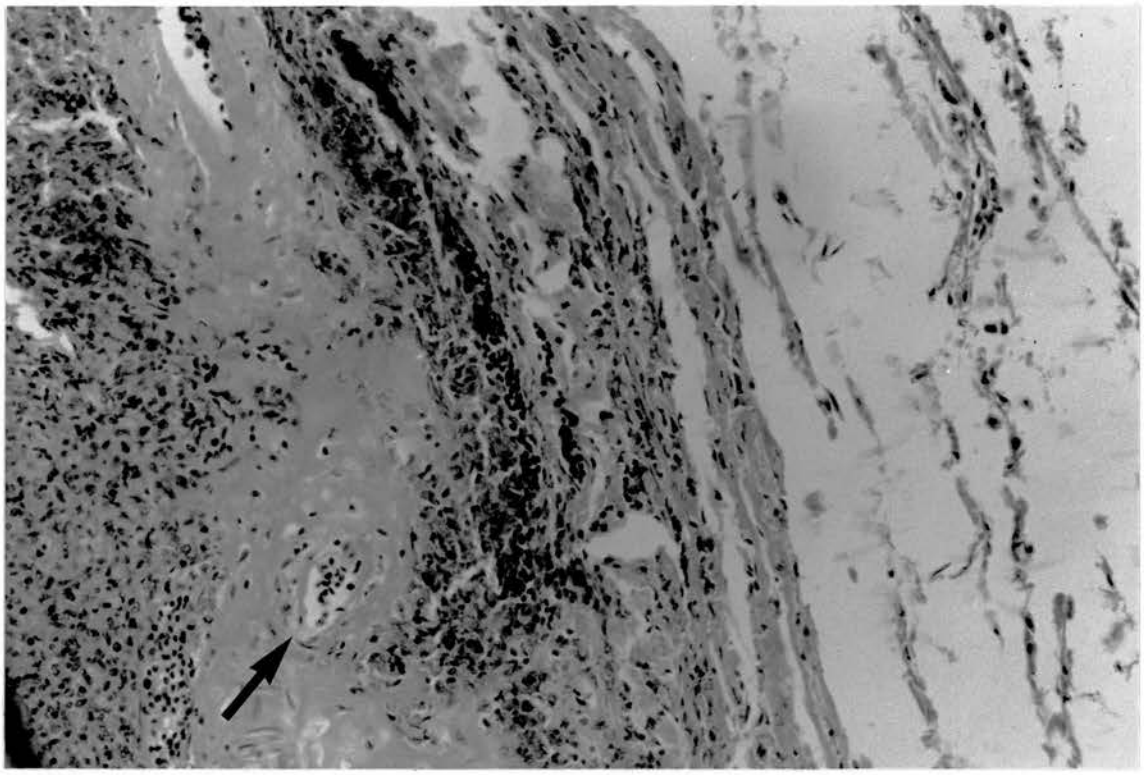


Fig.130. Turkey (Control). Earskin area (PHA). Intense cellular reaction in the dermis. Note the presence of inflammatory cells in the lumen, wall and outside the blood vessel (arrow).Tissue damage is also evident. H & E x 154.

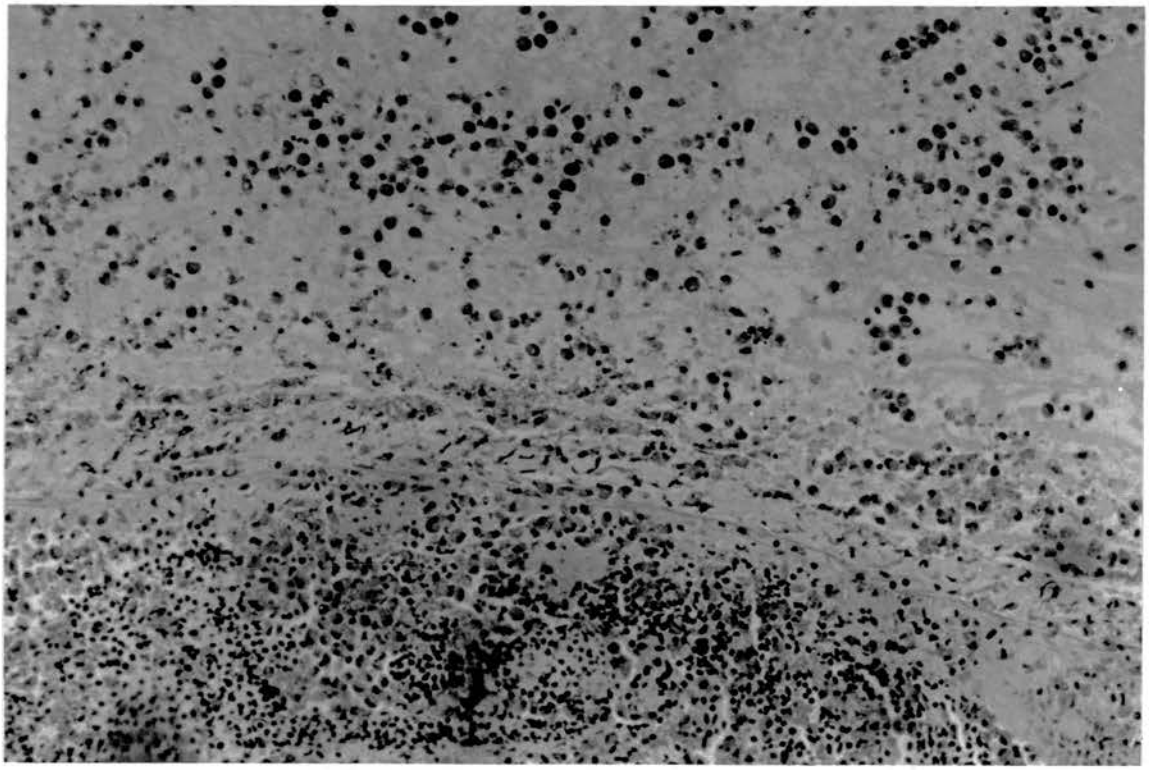


Fig.131. Turkey (Control). Earskin area (PHA) showing predominantly heterophilic reaction together with mononuclear cells in the dermis. H & E x 400.

granulocytes predominated. Changes were similar to those seen in the superficial dermis, but a more marked degranulation of granulocytes was evident, at times leaving empty membrane-bound vesicles in the cytoplasm of the site of degranulation. Free granules in the connective tissue stroma were a common and consistent feature. Fibroblastic proliferation was also noticed at places. In addition to showing thickening of endothelium and vessel walls many dilated blood vessels contained granulocytes and mononuclear cells, many of which were showing margination and even migration. The severe reaction progressed more deeply into the subcutis and muscular layers, resulting in disruption and damage to these tissues (Fig.130). An infiltration of considerable numbers of granulocytes appeared to be a constant feature in all the skin sites tested. Perivascular lymphoid aggregations were also occasionally seen. No basophils could be seen in the toluidine blue-stained sections, though a few metachromatic mast cells were noticed occasionally.

In OA-treated birds, the number of infiltrating granulocytic and lymphoid cells was considerably reduced in both the superficial (Fig.133) and deep dermis. The number of cells showing degranulation was also smaller. Oedema was mild in these birds. Blast forms were less common. The perivascular lymphoid accumulations seen in 0ppm OA birds were not seen very often. The cellular infiltration was rarely seen in the epidermis (Fig.133). Mononuclear cells, particularly macrophages and histiocytes with vacuolated cytoplasm and engulfed debris, were seen on the superficial and deep dermis as well as in the deeper muscular layer. A mild fibrous tissue proliferation was noticed in OA-treated turkeys. A few mast cells were occasionally seen in the dermis. Changes in the blood vessels were of moderate intensity and did not progress to hyalinisation.

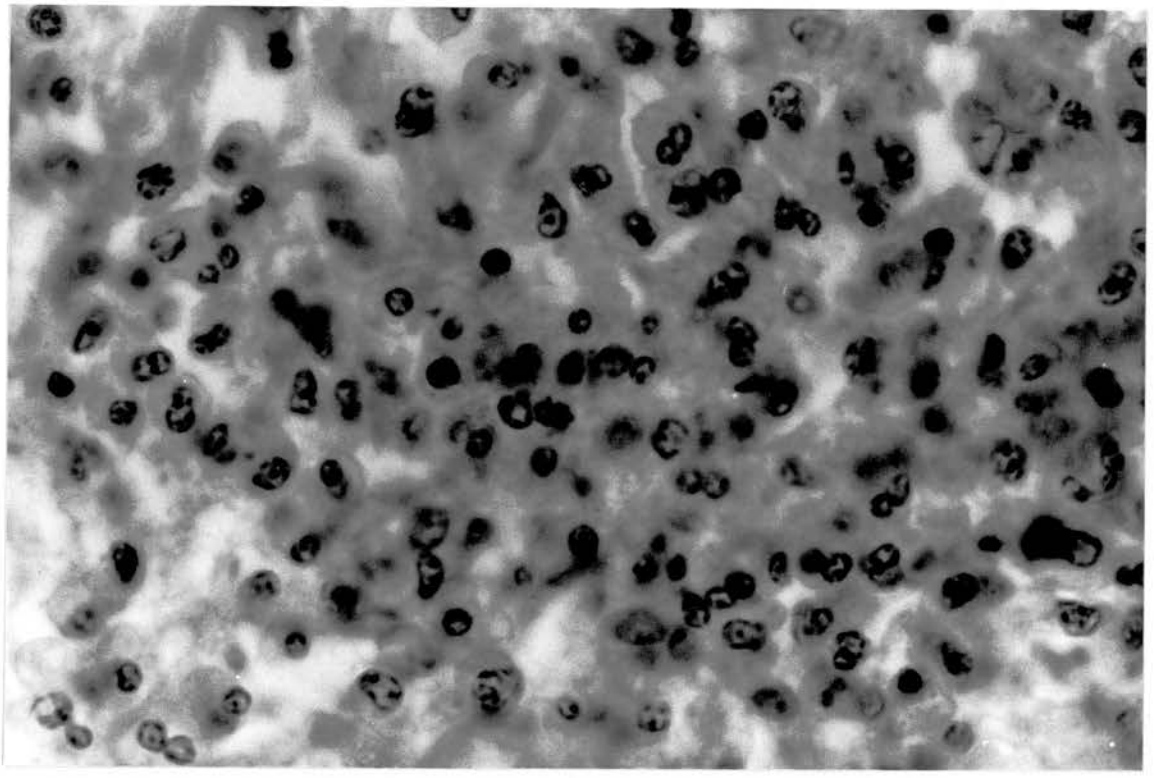


Fig.132. Turkey (Control). Earskin area (PHA) showing lymphoblastic reaction in the superficial dermis. Note the presence of many budding cells. H & E x 750.

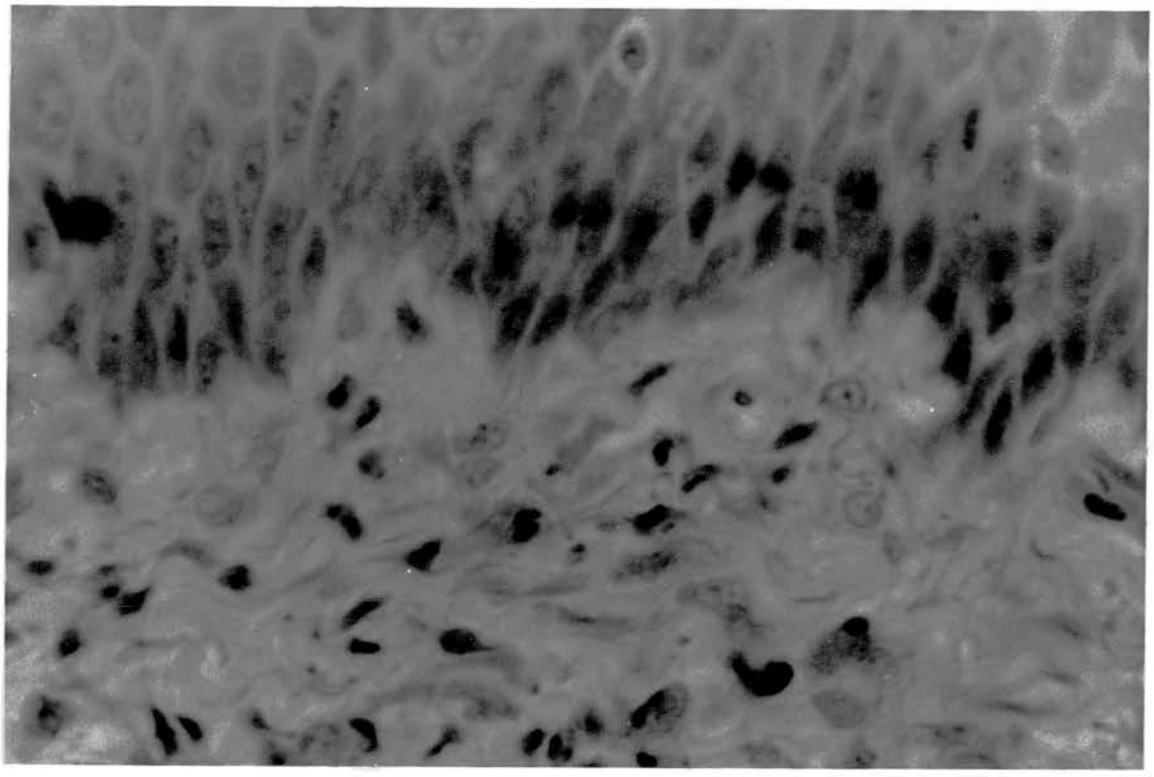


Fig.133. Earskin area (PHA) from an OA-treated (4ppm) turkey showing mild changes in the epidermis and slight cellular reaction in the superficial dermis. H & E x 750.

Spleen. Large numbers of granulocytes, similar to those observed in the skin, were seen in the red pulp and around the white pulp throughout the parenchyma. Usually the germinal centres and periarteriolar adenoid sheaths were not infiltrated by these cells. Degranulation was also observed in places. The white pulp, particularly the peripheral area, appeared prominent and distinct. Heterophilic granules and the walls of the blood vessels were PAS-positive. Large mononuclear cells and granulocytes were seen in and around the dilated blood vessels. A similar reaction, though of lesser intensity, was noticed in OA-treated turkeys. In addition, some lymphoid depletion was evident.

Thymus. The thymus appeared to be more reactive than the spleen and other internal organs. Though there was a good distinction between the cortex and medulla, a heavy infiltration of granulocytes was seen mostly in the medulla. Sometimes many granulocytes were observed around Hassal's corpuscles. Some cells were degranulated. At some places in the medulla nodules, made up of lymphoid cells and a few macrophages, were noted. Mitotic figures were noticed in the lymphocytes. In some areas the cortex was also infiltrated with granulocytes. In OA-treated birds, in addition to a depletion of lymphoid elements from both the cortex and medulla, a similar reaction but of lesser intensity was noticed. Free-lying granules, some of which were PAS-positive, were seen in some areas.

Bursa of Fabricius. The bursal follicles were not affected, though mononuclear cells together with some granulocytes were observed in the subepithelial areas and also in the interfollicular stroma. In OA-treated birds a similar reaction was seen and there were degenerating lymphocytes in many areas.

Small intestine, Peyer's patch (PP) and caecal tonsil (CT). A granulocytic infiltration was seen in the lamina propria and around the glands. In PP and CT, cells were usually seen in the diffuse lymphoid tissue (DLT) and around the germinal centres (GC). Many mononuclear cells, especially phagocytic macrophages, were frequently seen. Goblet cells and mucus cells were PAS-positive. A few heterophils were seen between the epithelial cells. In OA-treated birds, a similar reaction together with depleted GC and DLT was seen.

Liver. Congestion, dilatation of blood vessels and occasional granulocytic (heterophilic) infiltration were seen in the hepatic parenchyma in both 0ppm and 4ppm OA groups. No glycogen was seen in the hepatic cells after PAS and Best's Carmine staining. There was no accumulation of glycogen in the liver from OA-treated birds. In non-PHA-treated control birds, a small amount of glycogen was evident.

Kidney. Occasional infiltrations of granulocytes and a few mononuclear cells were observed in the subcapsular and interstitial tissues in both the groups.

Harderian gland. The gland appeared to be unaffected.

Immunofluorescence examination. Lymphocytes, ranging from a few to many, with positive staining for IgG and IgM (and a few IgA) were seen mostly in the dermis. In some skin sections many mulberry-like granulated cells fluoresced a bright apple green. Immunoglobulin-containing lymphocytes in varying numbers were also seen fluorescing in the spleen, thymus, bursa of Fabricius, Peyer's patches, caecal tonsils, and Harderian gland. In OA-treated birds a similar reaction was noted, but usually with fewer cells showing a weaker fluorescence.

Electron microscopic examination. Ultrastructural examination of PHA-treated skin sites (and internal organs) confirmed most of the light microscopic findings and facilitated a more detailed study of the nature of the cellular reaction. It was also possible to identify and differentiate heterophils and eosinophils in tissues.

The characteristic eosinophil granule in normal turkeys was larger, measuring up to 1.5μm in diameter and slightly irregular in shape. It appeared as one type, being homogeneous and membrane-bound. There were three types of membrane-bound granules in normal turkey heterophils. The most common granule was spherical to oval in shape, electron-dense, measuring up to 1.0μm in diameter. Sometimes as many as three central bodies could occur in these granules. Secondly, and less numerous, were less-densely-stained granules, oval in appearance, which measured up to 0.5μm in diameter. The third type of granule was small, round, electron-dense containing an intensely staining internum and measured up to 150nm in diameter.

Skin. Similar reactions were seen in a series of sections examined from all the skin sites. The most marked reactions were noticed in the sections from the ear skin area and the lateral thoracic apterium. Reactions were least marked in the frontal process. The reactions in the wing web were variable, sometimes showing very heavy cellular reaction or only slight infiltration of cells. Tissue damage was most marked in the dorsal cloacal lip. In OA-treated birds, these reactions were reduced both in intensity and severity.

A conspicuous and consistent feature in PHA-treated skin was the infiltration of a large number of heterophils and some eosinophils. A few normal heterophils were seen (Fig.134) but the majority of infiltrating heterophils had abnormalities in the nucleus and cytoplasm. The cytoplasmic

alterations were more pronounced than the nuclear changes. A characteristic alteration was seen in the common dense granules which became enlarged, pleiomorphic and often lost their central bodies (Fig.135). The number of granules was reduced. The other two types of granules, less dense and dense round granules were also reduced or sometimes even absent. The electron density of these granules varied considerably. The mitochondria were usually damaged and could be absent from many cells. These cells contained a large number of phagosomes and sometimes much glycogen. The majority of the cells were either undergoing degeneration or were in various stages of degranulation. Nuclear abnormality, though not frequent, was seen affecting particularly the shape, size and arrangement of heterochromatin, which was either vacuolated (Fig.136) or showed dense margination at the periphery.

In eosinophils, changes were seen in the cytoplasm, which showed vacuolations and enlarged dense round granules (Fig.137). Mitochondrial damage with disorganisation and loss of cristae was also noticed. Occasionally small, electron-dense round granules measuring about 95nm and the presence of lipid droplets (Fig.138) were also evident.

Many of the eosinophils were seen in various stages of degranulation.

A large number of heterophils and eosinophils were seen in PHA-treated skin sections, both in the superficial and deep dermis, together with lymphoid cells and macrophages (Fig.139). The heterophils varied in size from 5.0 - 8.0µm in diameter (normal heterophil, 6.5µm), with a mono - or bilobed nucleus. The cytoplasm usually contained large pleiomorphic granules with altered morphological characteristics. The common dense granules were wider than normal and their shape varied considerably from the usual common round or oval shape to bizarre irregular forms with a homogeneous electron dense appearance. Many of these

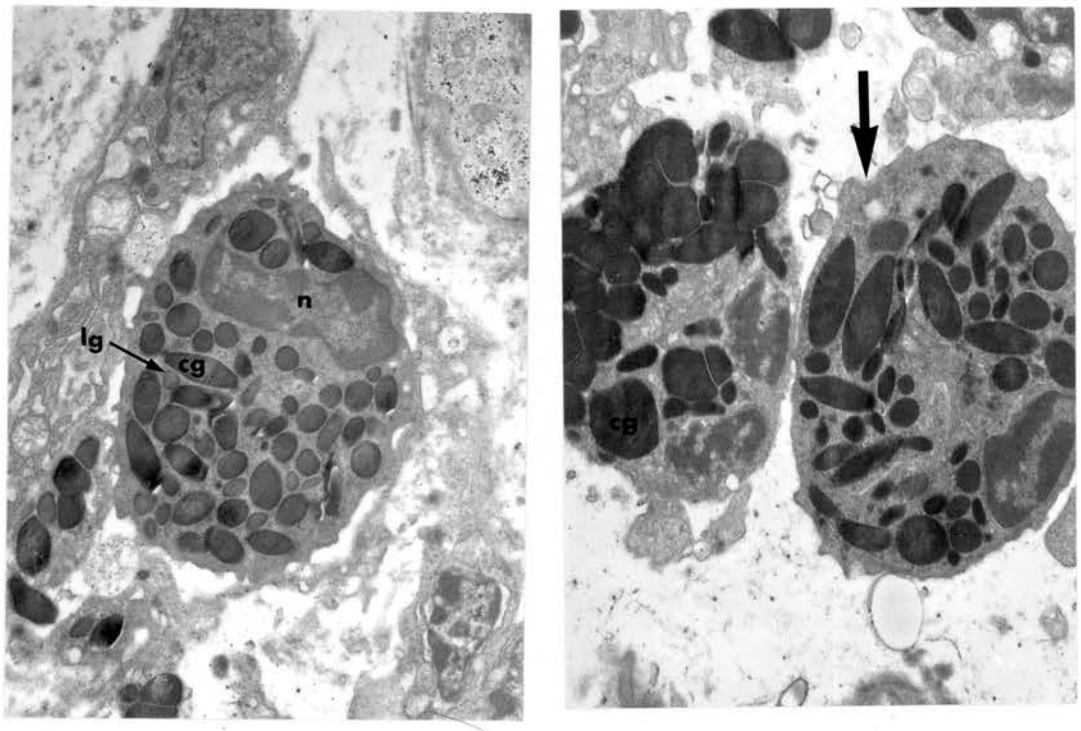


Fig.134. Turkey (Control) Skin. DCL(PHA). Normal heterophil in the epidermis. Common dense granules (cg) with central bodies and less dense granules (lg), nucleus (n). x 11664.

Fig.135. Turkey (Control). DCL(PHA). A relatively normal (arrow) and an abnormal heterophil showing pleiomorphic common dense granules (cg) some of which are degranulating. x 7776.

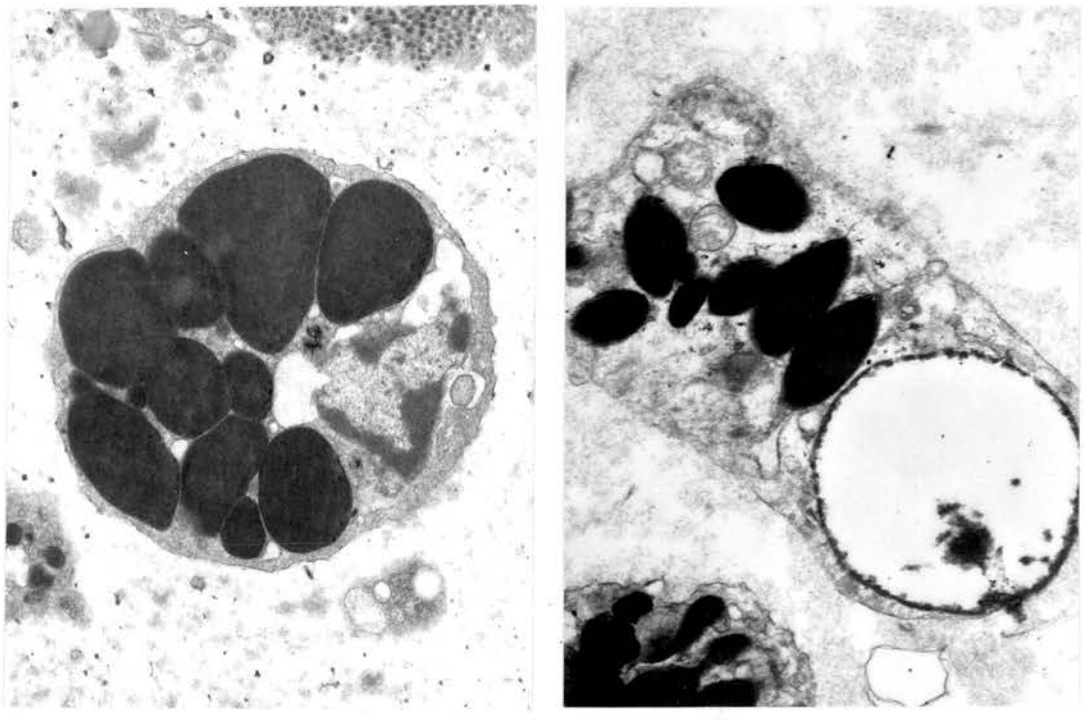


Fig.136. Turkey (Control). Earskin (PHA). An abnormal heterophil with damaged vacuolated round nucleus and a few common dense granules. x 12528.

Fig.137. Turkey (Control). DCL(PHA). An eosinophil showing large dense round granules and cytoplasmic vacuolation. x 10800.

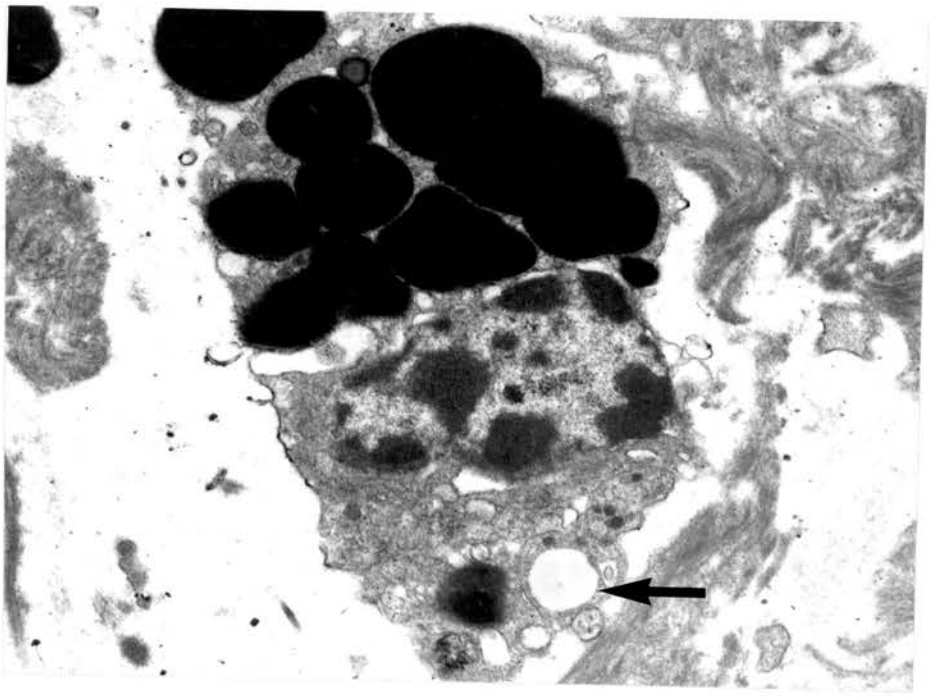


Fig.138. Turkey (Control). Earskin (PHA). An abnormal eosinophil showing the presence of some small, electron-dense round granules and lipid droplets (arrow). x 16200.

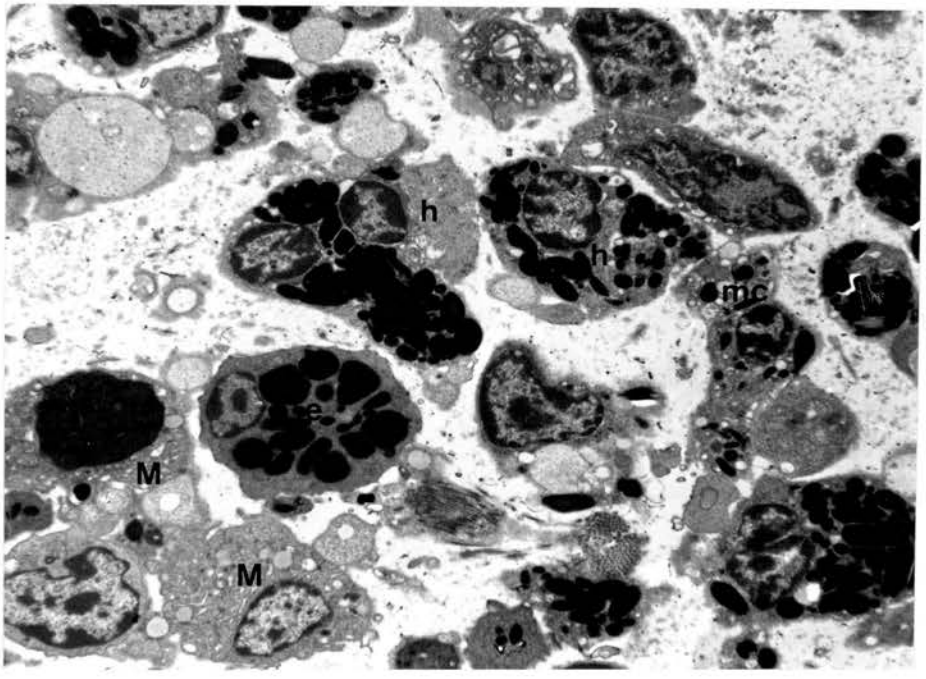


Fig.139. Turkey (Control). Lateral thoracic apterium (PHA). Note the presence of heterophils (h), eosinophil (e), mast cells (mc) and macrophages (M). x 4374.

granules either lacked central bodies or the bodies were very weakly stained (Fig.140). The size of these altered granules varied from 0.3 - 1.8um in length to 0.2 - 1.2µm in width (normal: length 0.09 - 1.6µm; width 0.09 - 0.56µm). Sometimes, the granules became very elongated or revealed a ring formation or even a 'spectacled' appearance (Fig.141), enclosing cytoplasmic structures. Such heterophils had much glycogen dispersed throughout the cytoplasm. A centriole and Golgi apparatus were present in only a few cells. Mitochondria were few in number and many were swollen or degenerated with a loss of cristae. In many cases vacuoles were observed containing remnants of mitochondrial cristae.

In the epidermis and superficial dermis, infiltrating heterophils and a few eosinophils were seen. Although many degranulating and degranulated cells were seen in these areas, heavier degranulation was noticed in the deep dermis, with granules frequently seen in the connective tissue stroma (Fig.142). In the cytoplasm of these degranulated cells, membrane-bound empty vesicles or vacuoles were observed. The number of granules in such cells was drastically reduced (on an average 12 in contrast to 48 in normal cells). Sometimes heterophils contained thin elongated processes on their surfaces. They were seen forming a vesicle, during the process of degranulation. Small vacuoles and glycogen granules were also present (Fig.143). Many of the cells were covered by an indistinct cell-membrane. Both heterophilic and eosinophilic granules were seen lying free in the cytoplasm, and often in association with mast cells (Fig.142). The mast cells were usually mature, rich in glycogen and in various stages of degranulation (Fig.144). Many lymphocytes with irregular nuclei and lymphoblasts were also seen.

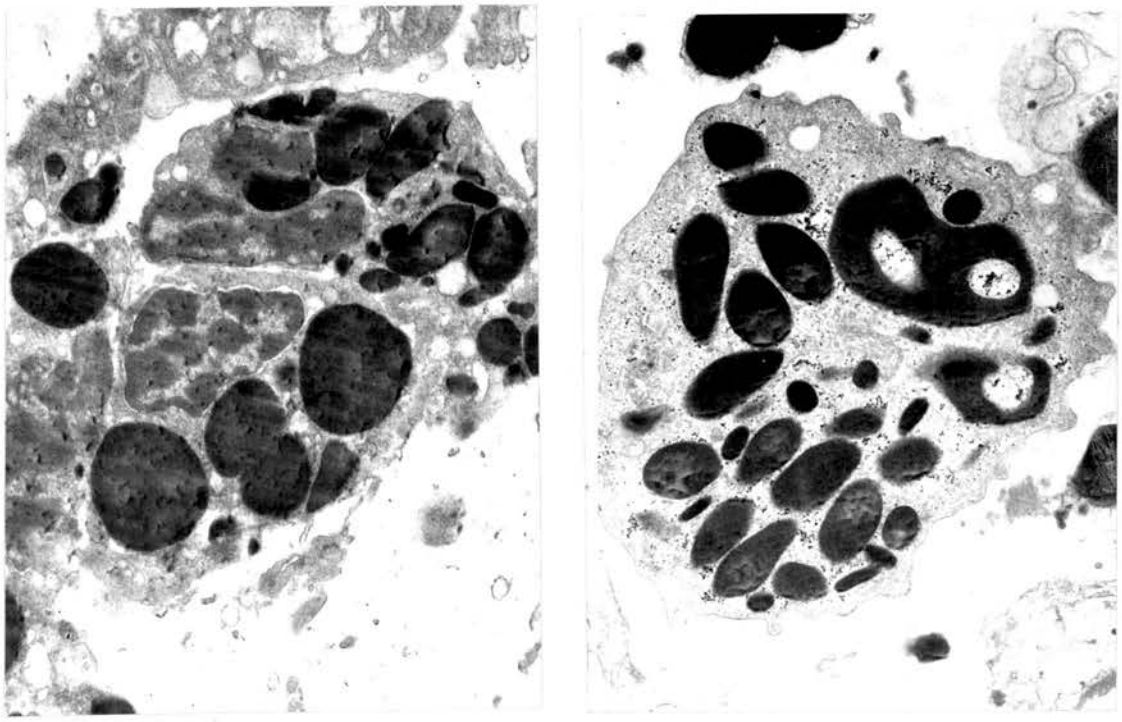


Fig.140. Turkey(Control). DCL (PHA). Abnormal heterophils. Very few large altered common dense granules with indistinct central body within the large granules. x 10800.

Fig.141. Turkey (Control) Earskin (PHA). An abnormal heterophil showing the presence of glycogen and abnormal granules giving "spectacled" appearance. x 12528.

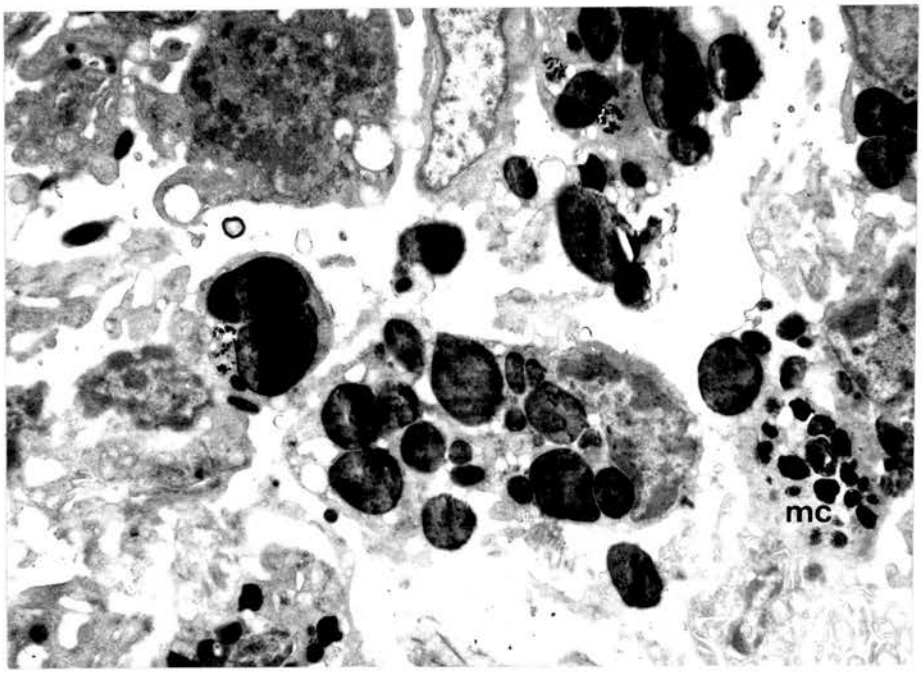


Fig.142. Turkey (Control). Lateral thoracic apterium (PHA). Heterophils showing degranulation. Free granules are seen in the connective tissue stroma and also near a mast cell (mc). x 7614.

Another feature was the presence of macrophages and histiocytes which were actively phagocytic. These large macrophages frequently contained several phagosomes, which varied in size. Within these phagosomes, intact heterophils, eosinophils, or their granules, dying or dead cells or tissue debris were seen (Fig.145). In addition to phagocytosed granules many macrophages had a considerable amount of glycogen in their cytoplasm as well as lipid containing inclusions and the endoplasmic reticulum was dilated (Fig.146). Rarely, multinucleated giant cells containing mast cells, heterophilic and eosinophilic granules were also seen. The degranulating cells and free granules were seen in the underlying subcutis and muscular layer causing damage and fragmentation to the tissues of the Z-lines. The damaged muscle fibres were disorganised and fragmented (Fig.147).

Changes were seen in the blood vessels. The endothelial cells contained an excess of glycogen, the vessel walls were thickened and thrombocyte plugs were seen. RBCs in the process of diapedesis and many heterophils were seen between the endothelium and pericytes, as well as in the perivascular areas. Fibroblastic proliferation with collagen formation was also seen in some areas of the deep dermis. Despite a thorough search, no basophils were seen in any of the tissues examined.

The mean values of counts of tissue heterophils, eosinophils and mast cells in 0ppm and 4ppm OA-treated turkeys are presented in Table 23. In OA-treated birds the numbers of heterophils and eosinophils were considerably reduced. The degranulation of these two types of cell was reduced. The mast cells were also fewer in number in OA-fed birds. Vascular reactions, such as haemorrhages, thrombocyte plugs in the vessels or sometimes empty venous sinuses, were a common feature.

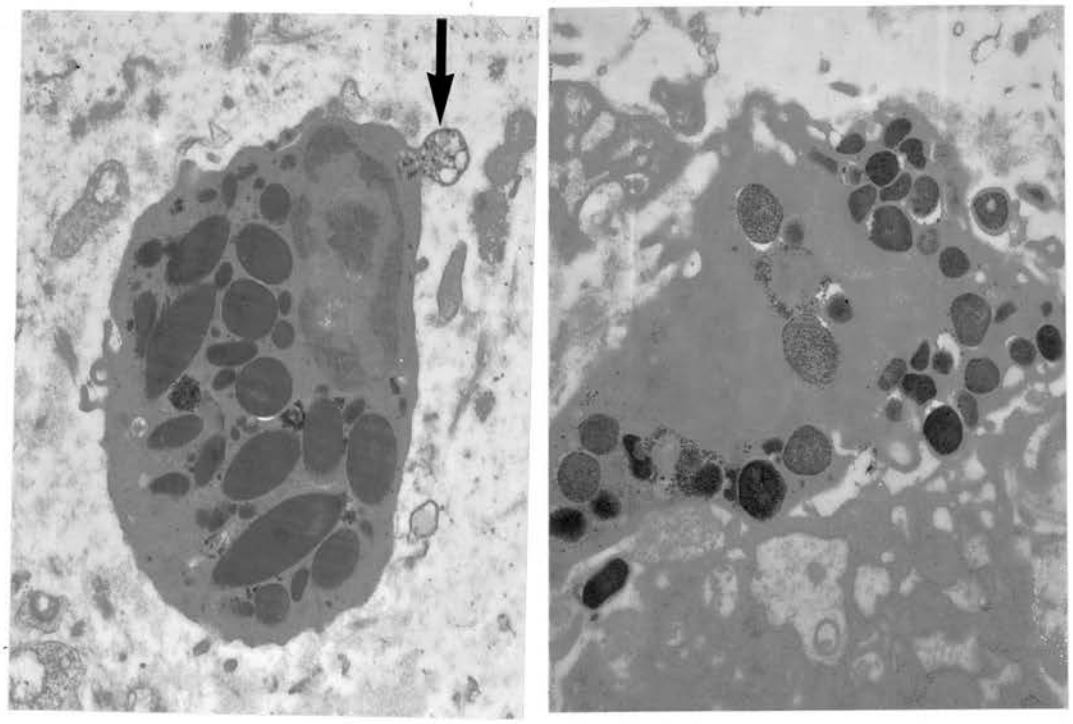


Fig.143. Turkey (Control). LTS (PHA). A heterophil containing many glycogen granules. A cytoplasmic vesicle formation (arrow) containing small vacuoles and glycogen is on the surface. x 10800.

Fig.144. Turkey (4ppm OA). FP (PHA). A mast cell containing glycogen is degranulating. x 12528.

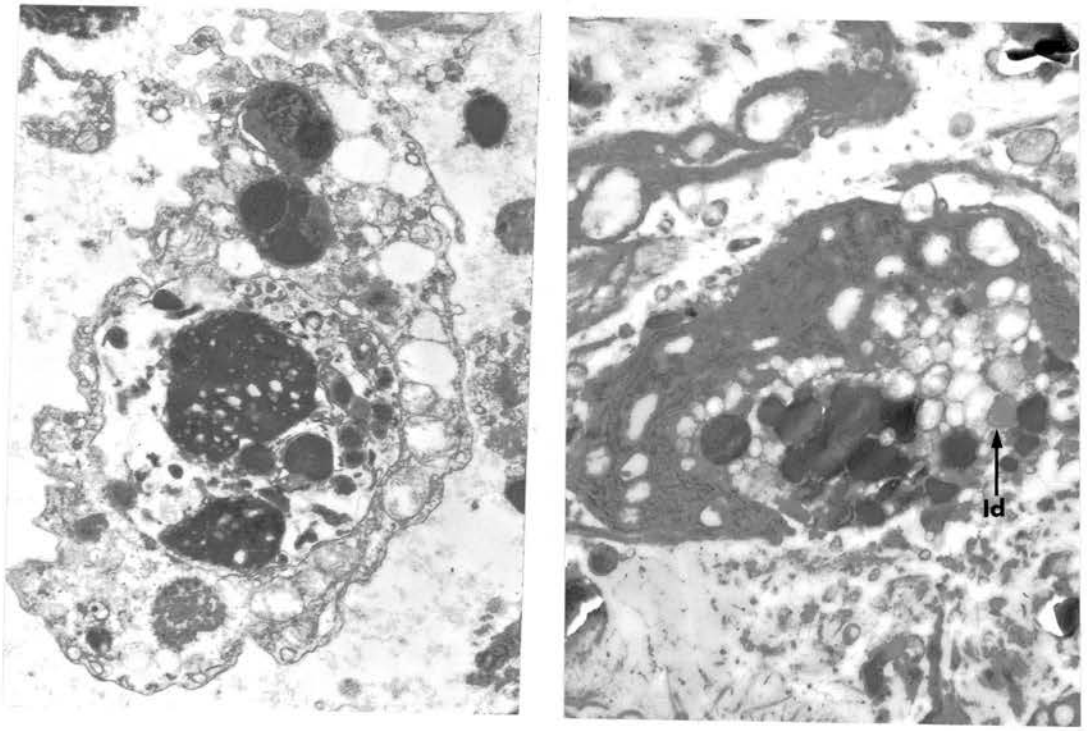


Fig.145. Turkey (4ppm OA). FP (PHA). A large macrophage with several phagosomes. Disintegrating granulocytes and cellular debris are within a large phagosome. x 7776.

Fig.146. Turkey (Control). DCL (PHA). A macrophage with phagocytosed granules of the degranulated cells and a lipid droplet (ld). x 7776.

Table 23. Means of counts of tissue heterophils, eosinophils and mast cells present in PHA-treated skin sites in turkeys

	Group (ppm OA)	
	0	4
Total grid squares examined	60	50
No.cells per grid square	126.16	10.20
Total No. Heterophils Degranulated	120.96(95.88)[a] 112.32(92.85)	9.72(95.29) 3.84(39.51)
Total No. Eosinophils Degranulated	4.82 (3.82) 3.32(68.81)	0.38 (3.73) 0.16(42.10)
Mast cells	0.38 (0.30)	0.10 (0.98)
Heterophil/ Eosinophil ratio	25.10:1.0	25.58:1.0

[a]Figures in brackets are percentages

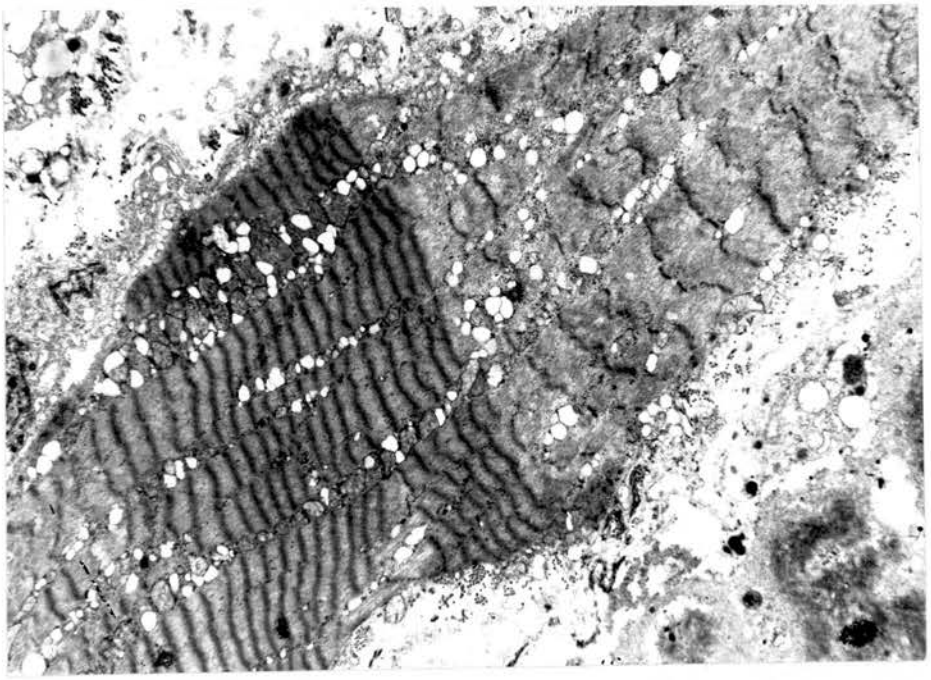
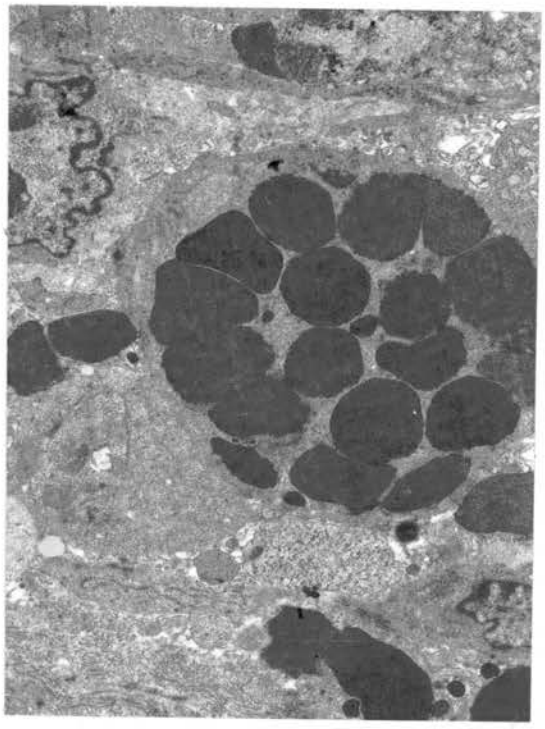


Fig.147. Turkey (4ppm OA). DCL (PHA). A muscle fibre showing zig-zag appearance and fragmentation of z line. On the left the muscle fibre is relatively normal. x 4374.



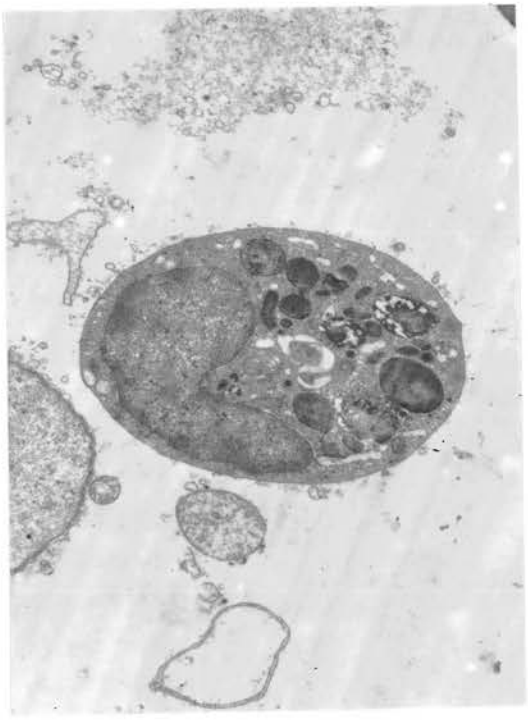


Fig.148. Turkey (Control). Caecal tonsil (PHA). An intact eosinophil with slightly enlarged and pleiomorphic granules. x 7776.

Fig.149. Turkey (4ppm OA). Kidney (PHA). A heterophil showing degeneration and vacuolation in the cytoplasmic granules. Endoplasmic reticulum is dilated. x 7776.

Thymus. Heterophils with altered granules and a few eosinophils were seen mostly in the medulla but also in the cortex. A few blood vessels contained these cell types. Both cell types were usually intact but sometimes degranulation was found. Lymphoblasts, with a large nucleus and nucleolus, were present in the cortex and medulla. A constant feature was the presence of very large macrophages with phagocytosed granules of heterophils or eosinophils in various stages of digestion and disintegration contained within unusually large phagosomes. The nuclei were of variable size, with irregularly distributed chromatin and a large nucleolus. These cells also had cytoplasmic lipid droplets. The reaction was less marked in OA-treated birds, though lymphoid cell damage was evident.

Spleen. The spleen also showed a strong reaction similar to that of the skin and thymus. The red pulp area had a large number of usually intact normal and altered heterophils and eosinophils. When the cells were counted, 14 per cent of the heterophils were normal whereas 52 per cent had altered granules. The eosinophils were usually normal (32 per cent) and only 2 per cent of cells showed cytoplasmic alterations and degranulation. In non-PHA turkey spleen, some normal heterophils and few eosinophils were present. Lymphoblastic forms and actively phagocytic macrophages were seen throughout the spleen. Erythrophagocytosis was rare and the white pulp was usually not affected. In OA-treated birds, granulocytes were markedly reduced. Vacuolated pyknotic lymphocytes were seen occasionally.

Bursa of Fabricius. The bursal stroma, particularly in the subepithelial regions, contained some heterophils and a few eosinophils. Macrophages with long cytoplasmic processes, were seen phagocytosing lymphoid cells, heterophils and cellular debris. Lymphoid follicles appeared to be unaffected. In OA-treated birds, a similar reaction

with many pyknotic and degenerating lymphoid cells was seen.

Peyer's patch and caecal tonsils. Changes were similar in both tissues. Many heterophils, a few eosinophils (Fig.148), actively phagocytic macrophages, containing granulocytes, and lymphoblasts were seen in the lamina propria, diffuse lymphoid tissue and around the glands. The germinal centres were largely unaffected. In OA-treated birds, granulocytes were reduced but vacuolation of lymphoid cells and mitochondrial damage or even pyknotic forms together with degenerating plasma cells were frequently seen. Globule leucocytes, mast cells, secretory cells and lymphocytes with granules were seen in both groups.

Kidney. A few heterophils, showing degeneration of cytoplasmic granules (Fig.149) were seen in the interstitial tissues. In OA-fed birds characteristic ring shaped transformation of mitochondria was also seen.

Liver. In sections from non-PHA treated control turkey poults, some glycogen particles were seen scattered in the cytoplasm of the hepatocytes but in the liver of non-PHA treated OA turkey poults, glycogen was considerably increased in these cells. In the liver of PHA-treated birds of both groups, however, no glycogen was found. A few intact heterophils and the occasional eosinophil and the Kupffer cell were identified.

Harderian Gland. No changes were seen in the Harderian gland.

4.10.2.2.3 Arthus reaction and DH to BSA

Arthus reaction. An Arthus reaction characterised by typical macroscopic lesions was not seen in any of the turkeys neither at the lateral thoracic apterium nor at the

dorsal cloacal lip skin sites. Only slight oedema and erythema was seen at the LTS both in 0 and 4ppm OA-treated birds.

DH to BSA. In the BSA-sensitised turkeys, a DH response was seen at all the skin sites tested (Fig.114b) with a peak response at 24 hours after test-injection. DH response was markedly depressed in 4ppm OA-treated turkeys (Fig.114b; Table 20, Section II). A difference, which was found to be significant, was noticed especially in the reaction in the frontal process. No reaction was elicited in turkeys after injections of PBS but the DH response was highly significant in turkeys sensitised by BSA (Table 20, Section II). No significant reactions were noticed at the PBS-injected sites.

4.10.2.2.4 Contact hypersensitivity to DNCB

Marked macroscopic lesions of induration and vesicle formation were not noticed in the DNCB-sensitised and challenged lateral thoracic apterium sites. Only slight erythema was seen in some birds.

However, DNCB resulted in the thickening of skin sites tested with the peak response at 24 hours (Fig.150). The earskin area had a more favourable response than the lateral thoracic apteria. Further the presensitised left lateral thoracic apterium gave a better reaction than the unsensitised right lateral thoracic apterium after a challenge application of DNCB (Fig.150). Contact-hypersensitivity responses appeared to be reduced in OA-treated turkeys (Fig.150). There was a significant difference, particularly at the earskin site both at 24 and 48 hours after challenge with DNCB in OA-treated turkeys (Table 24). The application of vehicle (acetone: olive oil) alone did not induce a significant reaction at the test sites (Fig.150).

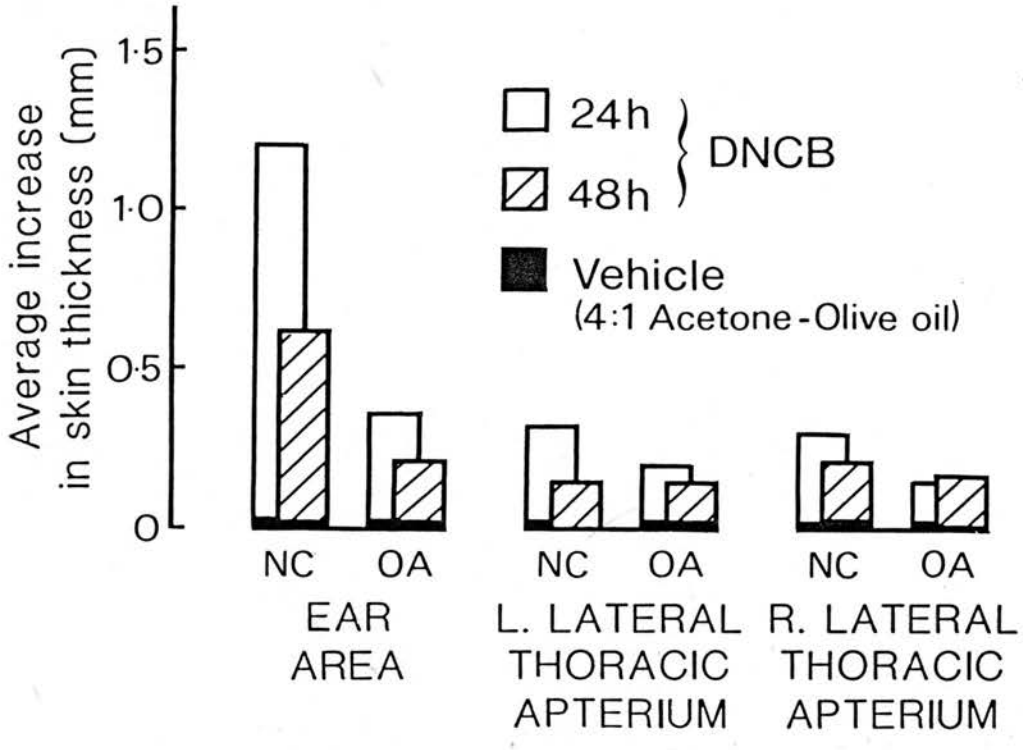


Fig.150. Contact hypersensitivity reaction to dinitrochlorobenzene (DNCB) in turkeys.

Table 24. Analysis of DNCB-induced contact hyper sensitivity response in turkeys*

Test sites	Hours after challenge	OA - NC		vehicle (regression slope)	
		E.D. (S.E.)	95% C.I.	E.D. (S.E.)	95% C.I.
Earskin area	24	-0.92 (0.13)	(-1.33, -0.51)	-2.76 (4.28)	(-16.38, 10.86)
	48	-0.46 (0.11)	(-0.81, -0.11)	-1.26 (5.34)	(-18.25, 15.73)
LLTS	24	-0.13 (0.10)	(-0.45, 0.19)	-3.24 (3.27)	(-13.65, 7.17)
	48	0.03 (0.10)	(-0.35, 0.29)	-1.06 (3.18)	(-11.18, 9.06)
RLTS	24	-0.13 (0.09)	(-0.42, 0.16)	3.75 (1.90)	(-9.80, 2.30)
	48	-0.12 (0.17)	(-0.66, 0.42)	-1.97 (5.03)	(-17.98, 14.04)

*Significant differences are underlined

4.11 OA Residues in Tissues

4.11.1 Quantitative analysis of OA

Table 25 shows the levels of OA residues detected in kidney, liver and muscles at 2,4 and 6 weeks after feeding 4ppm dietary OA to broilers and turkeys.

The highest concentration of OA was detected in the kidney and the lowest in the muscles. The highest concentration in all tissues was noticed after 4 weeks. Tissues from turkeys had higher levels of OA than those from broilers. The ranges of OA concentration in broilers and turkeys respectively were 6.7-22.3 ppb and 10.4-46.0ppb in the kidneys, 0-6.3ppb and 6-18.6ppb in the livers and 0.2-2.1ppb and 0.4 -6.6ppb in the breast muscles. In tissues from control birds, particularly the kidney and liver, some OA residues could be detected. In muscles, OA was either not detected or was present only as trace amounts.

4.11.2 Localisation of OA in tissues

4.11.2.1 Broilers

Cryostat sections of tissues (kidney, liver, muscles and small intestine) from broilers fed 0 and 4ppm OA for up to 10 weeks were treated with 1:50 and 1:10 dilutions of fluorescein iso-thiocyanate labelled anti-ochratoxin-antiserum to demonstrate the presence of OA, by the immunofluorescent antibody technique. With dilutions of 1:50, OA could not be detected; tissues from both the 0 and 4ppm group fluoresced nonspecifically. When 1:10 dilutions were used, there was weak specific fluorescence in the kidney, liver and muscles from OA-fed-birds. Cytoplasmic granular fluorescence was seen in the epithelial cells of the proximal convoluted tubules (PCT) and in hepatocytes.

Table 25. Residues of OA (ng/g,ppb) in tissues of control and OA-fed broilers and turkeys[a]

Tissues	Group (ppmOA)	Age (weeks)			
		2	4	6	Overall
I. BROILERS					
Kidney	0	3.9 (3.5-4.3)	1.68 (0.96-2.4)	0.34 (0-0.68)	1.97 (0-4.3)
	4	12.45 (6.7-18.2)	18.8 (15.7-21.9)	14.55 (6.8-22.3)	15.27 (6.7-22.3)
Liver	0	0.38 (0-0.75)	0	0.3 (0.3)	0.23 (0-0.75)
	4	3.15 (0-6.3)	3.55 (2.0-5.1)	3.1 (2.5-3.7)	3.27 (0-6.3)
Muscle	0	0.1 (0.1)	traces	0.25 (0.1-0.4)	traces (0-0.4)
	4	0.65 (0.6-0.7)	1.4 (0.7-2.1)	0.55 (0.2-0.9)	0.87 (0.2-2.1)
II. TURKEYS					
Kidney	0	4.8 (1.6-8.0)	7.2 (2.4-12)	0.14 (0-0.27)	4.05 (0-12.0)
	4	21.3 (15.6-27.0)	31.45 (61.9-46.0)	28.4 (10.4-18)	22.32 (10.4-46.0)
Liver	0	1.25 (0.7-1.8)	2.9 (2.3-3.5)	0	1.38 (0-3.5)
	4	11.45 (7.7-15.2)	16.3 (14-18.6)	6.35 (6-6.7)	11.37 (6-18.6)
Muscle	0	traces	traces	traces	traces
	4	3.9 (1.2-6.6)	1.6 (1.6)	0.9 (0.4-1.4)	2.13 (0.4-6.6)

[a] Mean values (range)

There was some non-specific staining in tissues from birds of both groups.

4.11.2.2 Quail

Immunofluorescence was used to show the localisation of OA tissues from quail kept on an 8ppm OA diet for 11 weeks. Non-specific staining was considerably reduced by precipitating the anti-ochratoxin-antiserum with BSA. When cryostat sections were incubated with cyanamide and then treated with a 1:5 dilution of the anti-OA-antiserum, OA-deposits in tissues fluoresced a bright apple-green colour. The OA-residues appeared as granules of varying size in the cytoplasm; sometimes a bright diffuse fluorescence was seen in the cytoplasm of the cells, leaving the nuclei unstained. In the kidney, the most intense specific fluorescence was seen in the epithelial cells of the PCT (Fig.152), but cells of the collecting tubules and some of the glomeruli also fluoresced strongly. Occasionally extracellular OA deposits were seen as though they had been washed out of the cells. Hepatocytes and muscle cells (from breast muscle) also fluoresced, though to a lesser degree than cells in the kidneys. In control sections, a non-specific weak fluorescence (Fig.151) could sometimes be detected. There was non-specific staining in connective tissues and in some of the blood vessels, necrotic and desquamated cells in sections from both control and OA treated birds.

4.12 Production Characteristics and Teratogenic Effects

4.12.1 Production characteristics

Egg Production. Quail from all the three experimental groups came into lay at 6 weeks of age. OA-fed birds laid fewer eggs than the controls in a dose-related fashion, as summarised in Table 26. Between the 6th and 8th weeks,

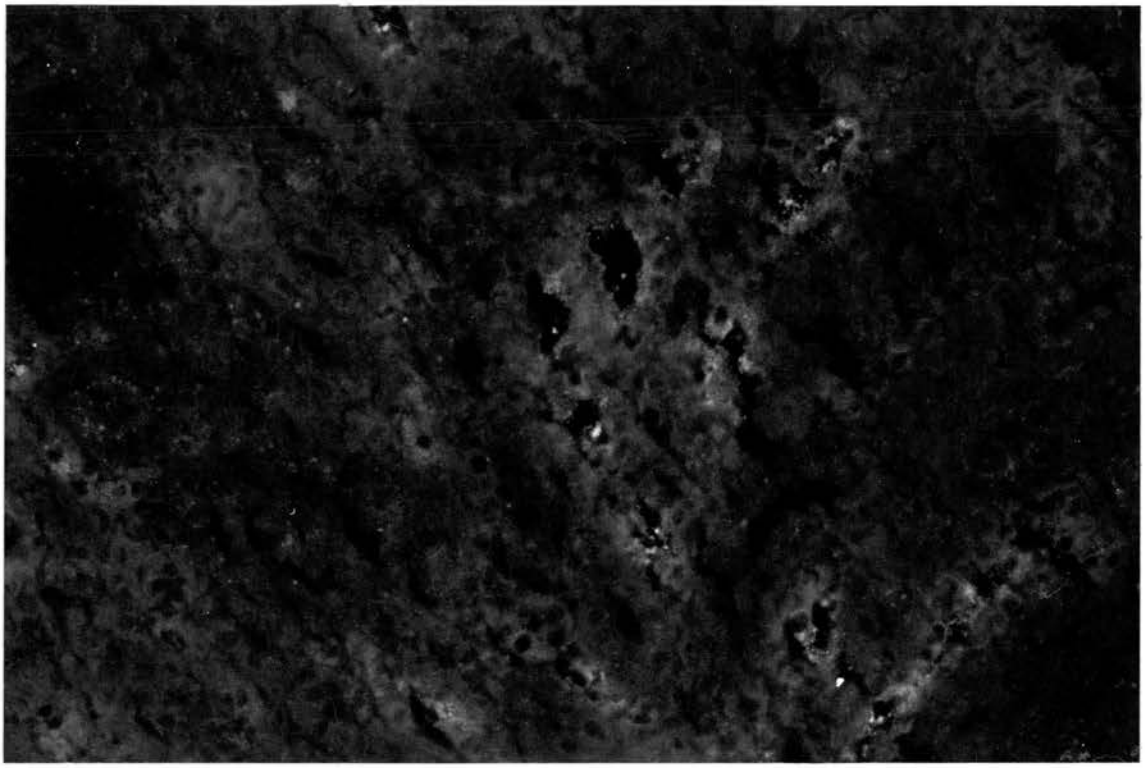


Fig.151. Quail (Control). 11 w.o. Kidney. Note the absence of specific cytoplasmic fluorescence in the tubules. Some non-specific granular fluorescence is present. Anti-ochratoxin A x 400.

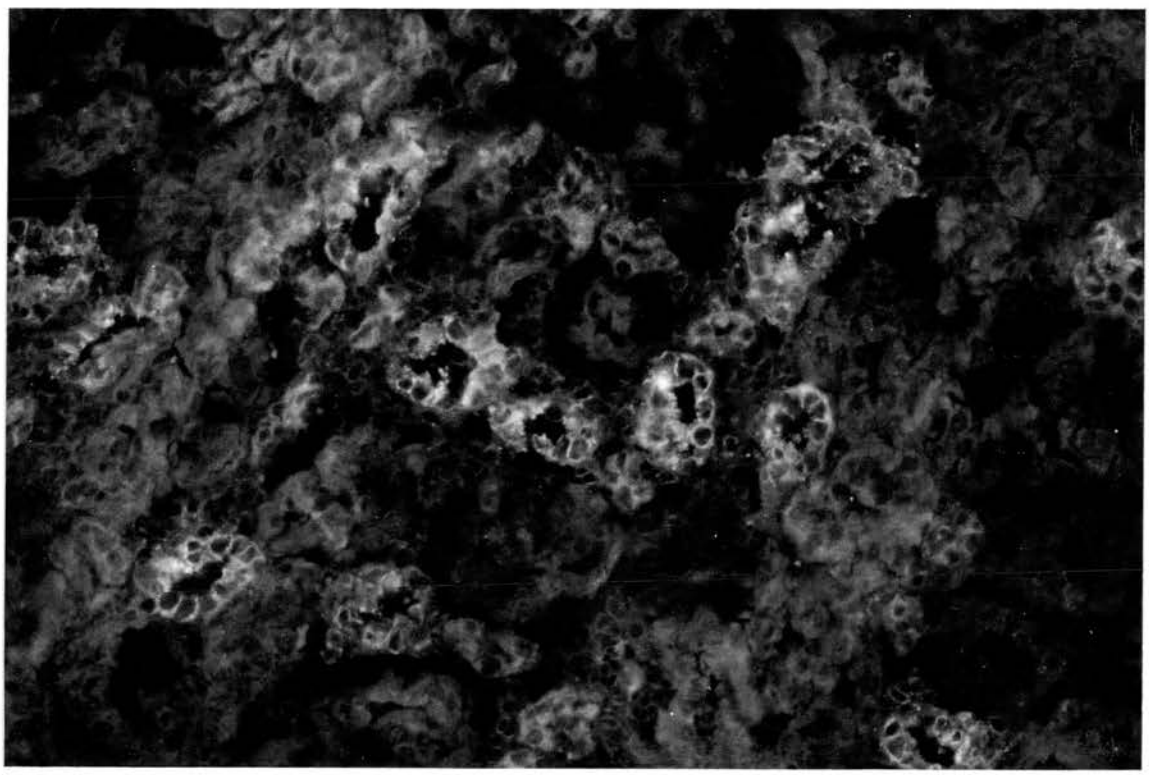


Fig.152. Quail 11 w.o. (8ppm OA). Kidney. Note the presence of bright specific cytoplasmic fluorescence particularly in the proximal convoluted tubules. Anti-ochratoxin A x 400.

laying birds from 0,4 and 8ppm OA groups produced 2.40, 2.19 and 1.38 eggs/bird/week respectively. Egg production was at its peak between weeks 8-11, when it reached 5.59 (0ppm OA), 5.27 (4ppm OA) and 4.36 (8ppm OA) eggs/bird/week. Though there was little adverse effect on egg production at 4ppm OA level, the 8ppm group consistently produced fewer eggs.

Eggs laid by birds in the 4 and 8ppm OA groups were smaller in size than those from controls and sometimes soft-shelled eggs were produced, particularly at 8 to 10 weeks of age. The natural pigmentation of the quail egg made the assessment of egg-shell stains difficult.

Fertility and hatchability. As can be seen from Table 27, fertility was not affected by feeding 4ppm OA, though it was reduced to some extent at 8ppm OA level (68.9 per cent compared with 74.6 per cent in the control group).

There was a marked difference in hatchability between the control and OA-treated groups (Table 27). In all the three batches of eggs set for hatching, there was a consistent dose-related reduction in hatchability with the most drastic effect being seen in eggs from the 8ppm OA group, where out of 73 fertile eggs, 9 (12.3 per cent) chicks hatched out.

4.12.2 Embryotoxic and teratogenic effects

Data on embryotoxic and teratogenic effects on developing embryos of Japanese quail fed dietary OA are presented in Table 28.

In general, the percentages of embryonic deaths were higher in eggs from birds fed OA and this occurred in a dose-related fashion, being 14 per cent in the 4ppm OA group and 26 per cent in the 8ppm OA group compared with 8.7

Table 26 Egg production in Japanese quail fed OA

Age (weeks)		Group (ppm OA)		
		0	4	8
6-7	No. females	15	13	13
	Total eggs	72	57	36
	Eggs[a]	2.40	2.19	1.38
8-11	No. females	13	11	11
	Total eggs	218	174	144
	Eggs[a]	5.59	5.27	4.36
6-11 (overall total)	Total eggs	290	231	180
	Eggs[a]	4.31	4.04	3.17

[a] No. of eggs per bird per week

Table 27. Fertility and hatchability of eggs from Japanese quail fed OA

Hatch No.	Group	No. eggs set	Fertility		Hatchability		No. pipped
			No. Fertile	%	No. hatched	%	
1	0ppmOA	56	45	80.35	30	66.67	3
	4ppmOA	46	38	82.61	15	39.47	2
	8ppmOA	29	22	75.86	2	9.09	1
2	0ppmOA	46	33	71.74	22	66.67	3
	4ppmOA	56	42	75.0	23	54.76	5
	8ppmOA	35	25	71.43	2	8.0	3
3	0ppmOA	36	25	69.44	21	84.0	0
	4ppmOA	37	27	72.70	13	48.15	3
	8ppmOA	42	26	61.90	5	19.23	4
overall total	0ppmOA	138	103	74.64	73	70.87	6
	4ppmOA	139	107	76.98	51	47.66	10
	8ppmOA	106	73	68.86	9	12.33	8

per cent in the control group.

Developmental malformation were observed during the period of incubation between day 8 to 12 in embryos from both the 4 and 8ppm OA groups. More frequent and more severe abnormalities were noticed in embryos from the 8ppm OA groups (48 per cent of fertile eggs). Developmental defects were seen both in dead-in-shell embryos as well as in those which had pipped but had failed to hatch.

No developmental defects were noticed in the control (0ppm OA) group but in comparison changes which were more severe in the 8ppm OA were often observed in the OA-treated groups and are described below.

External appearance. Embryos were smaller in size, weak, thin and emaciated. There was either poor feathering or the feathers were ruffled. Poor feather pigmentation was a common feature, the feathers being greyer than in the controls. The neck, head and abdomen were usually scantily feathered. Urate deposits were frequently noticed near the cloaca.

Head. Embryos from the OA groups always had enlarged heads and were either hydrocephalic or exencephalic (Figs.153 and 155). The brain, when not exposed, had a thin pliable covering and in exencephalic (exposed) embryos it was sometimes protruding. Haemorrhages were often present on the surface of the cerebral cortex. The cranial region was either poorly feathered or had no feathers (Fig.154). The eyes were often small with microphthalmia being more prevalent in embryos from the 8ppm OA group.

The beak was often misshapen and could be small, soft and blunt, or bifurcated in either the upper or lower part (cleft beak)(Fig.154), or completely curved.

Table 28. Embryotoxicity and teratogenicity in the fertile eggs from Japanese quail fed OA

Hatch No.	Group	No. fertile eggs	Early embryonic deaths (%)	Dead in shell	Embryonic malformations (%)
1	0ppmOA	45	6(13.3)	1	0(0.0)
	4ppmOA	38	10(26.3)	2	9(23.7)
	8ppmOA	22	10(45.5)	2	9(40.9)
2	0ppmOA	33	2(8.7)	1	0(0.0)
	4ppmOA	42	3(7.1)	2	10(23.8)
	8ppmOA	25	5(20.0)	2	13(52.0)
3	0ppmOA	25	1(4.0)	1	0(0.0)
	4ppmOA	27	3(11.1)	2	8(29.6)
	8ppmOA	26	4(15.4)	2	13(50.0)
over-all total	0ppmOA	103	9(8.7)	3	0(0.0)
	4ppmOA	107	16(15.0)	6	27(25.2)
	8ppmOA	73	19(26.0)	6	35(47.9)

Fig.153. Quail embryo (from 8 ppm OA-fed quail). 8 d.o. Note the exposed brain, short lower beak, poor feathering and exposed abdominal organs.

Fig.154. Quail embryo (from 8ppm OA-fed quail). 10 d.o. showing poor feathering, cleft beak and complete exposure of abdominal organs.

Neck. The neck was thick and stumpy, sometimes with poor feathering.

Abdomen. Gastroschisis was the most noticeable defect characterised by incomplete closure of the abdominal wall (Figs. 153 and 154). The viscera were most exposed in embryos from the 8ppm OA group (Fig.154). When present, the abdominal covering was thin, soft and pliable and could be either pink or dark in colour. Abdominal feathering was poor (Fig.154).

Congestion and enlargement was seen in the alimentary tract, heart, jugular vein, venacava and gall bladder. Neither ventricular septal defects nor any other cardiac anomaly were seen.

Limbs. The limbs, particularly the legs, were always small and were twisted or curved and weak. The digits were also either curved, twisted or fused (syndactyly).

Newly-hatched chicks. Chicks which hatched from 4ppm OA group, were small, emaciated, weak and poorly feathered. They had difficulty in standing and showed signs of nervous incoordination, such as trembling, falling and an inability to walk. The legs were usually splayed. The few chicks which hatched from the 8ppm OA group were more severely affected. They were small, emaciated, moribund and had splayed legs with twisted digits. A circular head movement accompanied by a sideways twitch was often seen. They could not stand or walk. Living chicks from pipped eggs showed similar symptoms. Feathering was generally poor and was not well pigmented, being a dull greyish colour.

No septal or other ventricular heart defects were seen in hatched chicks from either OA group.

No abnormalities were seen in chicks from eggs of the control group which hatched or were only pipped.

Fig.155. Quail embryo (from 4ppm OA-fed quail). 10 d.o. showing complete exposure of brain (exencephaly).

CHAPTER 5

DISCUSSION

DISCUSSION

The main objective of the work presented in this thesis was to study in detail the clinical, pathological, ultrastructural and immunological changes in broilers and turkeys and the production characteristics and teratogenic effects in quail induced by ochratoxin A (OA). Compared with aflatoxins, far less is known about the immunopathological effects of OA in poultry, although the incidence of the occurrence of OA in feedstuffs (Tables 1 and 2) and its association with natural disease outbreaks are increasingly being reported (Hamilton et al., 1982; Visconti and Bottalico, 1983; Bodnarchuk and Kaspruk, 1984). Immunosuppressive effects of mycotoxins have been a matter of great concern owing to its anticipated adverse affects on vaccination programmes and resistance against secondary infections. OA-induced immunosuppression in poultry has been strongly suspected but not authenticated, though increased incidence of secondary infections during natural disease conditions has been reported (Hamilton et al., 1982). Better understanding of the disease syndrome caused by OA, its pathogenesis and its effect on the immune system will be helpful in looking for a better diagnostic tool so that efforts can be directed towards further prevention and control of the economically important and emerging OA-induced disease conditions in man, domestic animals and poultry.

The dose levels selected for the present experiments are those frequently observed in the contaminated feed under natural disease conditions in broilers and turkeys (Hamilton et al., 1982).

5.1 Clinical Signs and Growth Response

OA was found to be extremely toxic to young growing broiler chicks and to a lesser extent to turkeys. Clinical

signs and growth depression were not noticeable in quail up to 8ppm OA feeding for 11 weeks. No specific clinical signs of diagnostic significance were noticed in either of the species. In broilers, the most noticeable clinical effect was a loss of carotenoid pigmentation from the shank, but this effect was not obvious in turkeys. Loss of carotenoid pigmentation maybe correlated with the hypocarotenoidaemia observed in experimental ochratoxicosis in broiler chicks (Huff and Hamilton, 1975a; Osborne et al., 1982) but not in turkeys (Chang et al., 1981).

Increased susceptibility to bruising and petechiae as observed in young broiler chicks (while handling them) is consistent with the earlier reports of bruising and impairment of clotting factors of blood (Doerr et al., 1974; 1981; Huff et al., 1983) during experimental ochratoxicosis. Decreased pigmentation and petechiae would downgrade the carcass and even lead to its condemnation.

Growth depression in both broilers and turkeys became more marked with age, being detectable at 2 weeks of age in broilers and 3 weeks in turkeys. This may be due partly to decrease in feed consumption, as also noticed by Bitay et al.(1979), Prior et al.(1980) and Kubena, Phillips, Witzel and Heidelbaugh (1984) in broilers and by Chang et al.(1981) in turkeys. Other reason for growth retardation may be decrease in protein synthesis, an effect commonly observed in OA-toxicosis in in vivo (Svendsen and Skadhauge, 1976; Manning and Wyatt, 1984) and in vitro conditions (Konrad and Röschenthaler, 1977; Bunge, Dirheimer and Röschenthaler, 1978; Heller and Röschenthaler, 1978; Creppy et al., 1979a, 1979b). No increased susceptibility related to the sex was noticed in either species of poultry except in the long-term feeding of OA in broilers (Experiment II, Fig.6) where males appeared to be more susceptible than females. Prior et al.(1980) also reported males to be more susceptible to OA than females.

The clinical signs, behavioural changes and growth response as observed in present studies are in general agreement with earlier reports in fowls (Huff et al., 1974; Prior et al., 1976) and turkeys (Chang et al., 1981). These effects were dose-dependent in broilers, being more severe at the 4ppm OA level than at 2ppm OA.

There is variation between species in susceptibility to OA. In the present study, broiler fowls were found to be most susceptible followed by turkeys. Japanese quail were relatively most resistant. Prior et al.(1976) also noticed a similar type of susceptibility using the LD_{50} as the criterion. Similarly, susceptibility varies greatly in laboratory animals. Guinea pigs appear to be most susceptible (Thacker, Carlton and Sansing, 1975), followed by rat (Purchase and Theron, 1968) and mice are least susceptible (Galtier, Moré and Bodin, 1974).

5.2 Organ Weight

Huff et al.(1974) detected an increase in weight of the liver, kidney, crop, proventriculus and gizzard and a regression of the bursa of Fabricius (only at 8ppm OA) in broilers. Chang et al.(1981) observed an enlargement of proventriculus and gizzard and a regression of the thymus at 4 and 8ppm OA in turkeys. The present study has shown that OA, even at the minimum growth inhibitory level of 2ppm (Wyatt, 1981), could adversely affect the vital organs of young broiler chicks. Enlargement of the kidney and liver was noticed with greater frequency and consistency in broilers than in turkeys. The most consistent effect seen both in broilers and turkeys was the suppressed development and regression of the lymphoid organs, viz. thymus, bursa of Fabricius and even spleen. The comparative analyses of the effects of OA on the lymphoid organs (Table 6, section C) indicated that the bursa of Fabricius and spleen were more severely affected in

broilers than in turkeys and the effects were most severe in the case of the thymus in both species. The depression of growth and development of lymphoid organs became more severe as age advanced (Table 6). Chang et al.(1981) did not notice any effect on the bursa of Fabricius on the basis of relative organ weight analysis (expressed as a percentage of body weight). In the present experiments (III, IV, V), the data were treated both by absolute organ weight and by corrected organ weight analyses, both of which have been demonstrated to be superior to relative organ weight analysis (Stevens, 1976; Shirley, 1977). Corrected organ weight analysis was able to pick up those differences which could not be detected by absolute organ weight analysis.

The inconsistent results obtained in the weights of heart, proventriculus, crop and gizzard suggest that these organs cannot be used as sensitive indicators of OA-toxicity. Huff et al. (1974) and Chang et al.(1981), however, noted an enlargement of upper digestive tract organs (on the basis of relative organ weight) and attributed it to the affinity of OA to the gastrointestinal tract.

5.3 Haematological Changes

In the present studies, no consistent change was seen in the blood-profile in both broilers and turkeys. At 4 weeks of age in broilers, there was no difference on any haematological parameters studied (Table 9). This observation is inconsistent with the lymphocytopaenia reported in broilers (Chang et al., 1979) and turkeys (Chang et al., 1981). However, there was an indication of lymphocytosis with corresponding heteropaenia in quail in the present study. This discrepancy might be due to differences in strain susceptibility and experimental conditions.

5.4 Biotin Status

Blood pyruvate carboxylase (PC) activity (located in mitochondria and associated with glucose metabolism) has been reported to be a promising criterion for assessing the status of biotin, a member of the vitamin B complex group, in growing broilers and turkeys (Whitehead and Bannister, 1978). In the present study, blood PC activity and biotin levels tended to increase in broilers (Experiment II) and turkeys (Experiment V) after 3 weeks of OA treatment in birds fed a biotin deficient diet. This unexpected increase in the enzymic activity as well as plasma biotin levels is difficult to explain. Nevertheless, there is an indication of a disturbance of biotin metabolism in OA-fed birds particularly when the diet is low in biotin. The exact mechanism remains to be further investigated. The more drastic effect seen in turkeys may be due to a higher requirement of biotin in the fast growing turkeys raised on litter (Fig.9) during OA-toxicosis, as suggested by Whitehead and Bannister (1978).

Lack of correlation between PC activity and plasma biotin levels in turkeys (Experiment IV) or even broilers (Experiment III) may possibly be due to the fact that the plasma samples examined for biotin levels were pooled samples rather than from individual birds.

The effects of OA on vitamin status have not been studied, though carotenoid levels were found to be reduced in broilers (Huff et al., 1975a; Osborne et al., 1982) but not in turkeys (Chang et al., 1981).

5.5 Plasma Steroid Hormone Levels

Aflatoxin B_1 has been reported to depress the growth of the testes and the levels of androgens. Ottinger and Doerr (1982) fed this toxin at 10ppm level for 1 week to 2 and 3 week-old male Japanese quail and observed these

depressive effects in the period when the birds were 4-6 weeks of age. In the present study, however, testosterone and progesterone levels were not affected in 6 week-old broilers of either sex by feeding a 2ppm OA from hatching. The difference between sexes in the depressive effect of OA on growth does not seem to depend on differences in levels of steroid hormones. The testes and ovaries of experimental birds fed OA for 16 weeks (Experiment II) were smaller than those of control birds which could be associated with the general stunting of growth found in the OA-fed birds.

However, a direct effect of OA on the testes has been reported by More and Camguilhem (1979) in rats when they observed severe pathological changes in the testes after injection of OA intratesticularly. Intraperitoneal injection of OA into female rats resulted in an arrest of the oestrus cycle in the dioestrus phase and a fall in the activity of the enzymes involved in ovarian steroidogenesis (Gupta, Bandopadhyay, Mazumdar and Paul, 1980).

5.6 Gross Lesions

Gross lesions such as emaciation, enlargement and change in the colour of the kidney (and liver), catarrhal enteritis, dry and firm gizzard mucosal lining and diminished size of lymphoid organs as observed particularly in broilers in the present studies are in general agreement with earlier reports (Doupnik and Peckham, 1970; Peckham et al., 1971; Huff et al., 1974; Chang et al., 1981). Generalised visceral gout which was reported by Peckham et al.(1971) and Prior et al.(1976) in chicks or decreased breaking strength and rupture of the large intestine noted by Warren and Hamilton (1980b) in broilers were not observed in either species in the present experiments. Contrary to the observation of Huff et al.(1974) proventricular lesions were not apparent in the present study.

5.7 Histopathological Changes

Despite a variation in susceptibility between species the kidney appears to be the target organ for OA in rats (Munro et al., 1973), mice (Galtier, Moré and Bodin, 1974), guinea pigs (Thacker, Carlton and Sansing, 1975), rainbow trout (Doster and Sinnhuber, 1972), Beagle dogs (Szczech et al., 1973c), swine (Krogh et al., 1974a; Elling, 1981) and poultry (Peckham et al., 1971; Huff et al., 1975; Prior et al., 1976). In the present studies, in addition to the kidney, where predominantly the proximal convoluted tubules and glomeruli were affected, consistent changes were also noticed in the liver and lymphoid organs. As with the clinical signs, the depression of growth and the gross lesions, the histopathological changes were most severe in broilers as compared with turkeys and quail.

Contrary to the report of Peckham et al.(1971) and Huff et al,(1975) neither visceral gout nor oedema were seen in any of the OA-treated birds. This further contradicts Huff et al.(1975) who suggested that oedema might be responsible for the increase in kidney size. The present study suggests that this increase is more likely to have been due to the enormous enlargement and hypertrophy of the PCT's and to an infiltration of lymphoid cells. The toxin seemed to have an affinity for the PCT's, as evidenced by the demonstration of OA in the PCT (Section 4.11.2) and by the mitochondrial abnormalities observed with the electron microscope (Sections 4.8.1.1 and 4.8.2.1). Other lesions noted in the kidney such as localised necrosis, mitotic figures, congestion and haemorrhage are in accord with previous studies (Peckham et al., 1971; Galtier et al., 1976; Krogh et al., 1976a; Gylstorff and Rolf, 1978.). The homogeneous hyaline globules noted in the present study do not correspond to the hyaline casts described by Elling et al.(1975) and Galtier et al. (1976) nor are they the eosinophilic granular casts of

Szczech et al.(1973b). Huff et al. (1975) observed proteinaceous material in the lumina of the tubules. The effects of these tubular changes together with the thickened GBM might be reflected in impaired renal functions shown as decreased tubular excretion and urine concentrating capacity and phenol red clearance in OA-treated chicks (Huff et al., 1975 ; Svendsen and Skadhauge, 1976). The thickened GBM has been studied at the ultra-structural level and will be discussed elsewhere (Section 5.8). The finding of the glomerular lesions in the present work differs from the observations of Krogh et al. (1976a) who failed to detect any such lesions.

Warren and Hamilton (1980a)reported an enzymic inhibition of glycogenolysis in OA-toxicosis and this could account for the excess glycogen observed in the liver in the present study. Both decreased (Munro et al., 1973; Suzuki and Satoh, 1973; Chu, 1974) and increased (Purchase and Theron, 1968) amounts of liver glycogen have been reported in rats treated with OA. Recently Huff et al.(1979b) noted an increase in glycogen in the livers of OA-fed broilers (at 4 and 8ppm OA but not at lower levels). The observation of vacuolation of the hepatic cells agrees with the findings of Peckham et al. (1971) and Prior et al. (1976)although no hepatic necrosis was observed in the present study. Bile duct proliferation and diffuse vacuolation of hepatocytes were noticed in quail chicks during OA-toxicosis (Doster et al., 1973). Similar changes as well as the increased accumulation of glycogen in a dose-related fashion were observed in quail during the present study.

The pathological changes reported here indicate and confirm that OA is more of a nephrotoxin than a hepatotoxin in broilers and turkeys in contrast to aflatoxin which is considered to be more hepatotoxic.

The decrease in the size of the lymphoid organs seen in the present study may have been due to the depletion of lymphoid cells and the more drastic changes seen in the thymus from OA-treated birds suggest a stronger effect on cell-mediated immune responses. Lymphoid depletion has also been observed in the thymus, spleen, tonsils and Peyer's patches in OA-fed dogs and swine (Szczech et al., 1973b; 1973c) and in the spleen and bursa of Fabricius from OA-treated fowls (Peckham et al., 1971; Gylstorff and Rolf, 1978).

The heterophilic reactions noted in the alimentary tract and other organs from OA-fed birds in the present study is in accord with the observations of Choudhury et al. (1971) and Peckham et al. (1971). Chang et al. (1979) found an increase in heterophils in broilers in ochratoxicosis, though their phagocytic ability was impaired (Chang and Hamilton, 1980). These changes together with the lymphoid depletion of immunological organs as observed in the present study lend support to the suggestion of Prior and Sisodia (1982) and of Hamilton et al. (1982) that OA may reduce the capability of an animal to respond to immunological challenge.

The prsent study suggests that OA is a potent nephrotoxin in broilers and turkeys, principally causing distension, enlargement and hypertrophy of the PCT and thickening of the GBM. In the liver, it caused lymphoid cell infiltration and glycogen accumulation in a dose-related fashion. Furthermore, OA resulted in a regression of the lymphoid organs and a drastic reduction of the lymphoid cell population.

5.8 Ultrastructural Changes

Ultrastructural examination of the kidney, liver and lymphoid organs from broilers and turkeys fed OA confirmed much of the light microscopical findings and demonstrated a possible site of injurey.

On the basis of the ultrastructural studies, it is postulated that the following sequential cellular changes and pathogenetic mechanism occured during OA-toxicosis in broilers and turkeys. The principal target site in the kidney was the PCT where the first organelles to be affected were the mitochondria which became swollen and enlarged, lost their cristae and became misshapen, undergoing progressive ring-transformation with increasing age of the lesions. Correspondingly, lysosomal activity started to increase and became more pronounced after 4 weeks of age. Later degeneration of the mitochondria and reduction in their population became obvious, with lysosomes possibly playing a role in their elimination as evidenced by the presence of degenerating mitochondria in the lysosomal bodies. GBM thickening also progressed and was sometimes so severe as to completely occlude the capillary lumen. Changes in the collecting tubules primarily affecting the mitochondria were noticed in the later stages.

In the liver, the accumulation of glycogen granules was the principal alteration and it increased with advancing age. In the later stages lysosomal and Kupffer cell activity became marked as in the kidney. These cells were also associated with phagocytic activity and sometimes contained large amounts of glycogen.

In the lymphoid organs, the most consistent effect was a degeneration and necrosis of lymphocytes and a corresponding increased phagocytic activity of macrophages.

Lymphoid follicles in the bursa of Fabricius, spleen, Peyer's patch, caecal tonsils and thymus had a vacuolated appearance and were empty due to drastic lymphoid degeneration suggesting a direct effect of OA on lymphocytes. Plasma cell degeneration also became marked and sometimes these cells were completely disintegrated. Mitochondrial damage in the lymphoid cells and plasma cells was a consistent feature. Lysosomal activity also appeared to increase in the later stages though not as markedly as in the kidney or in the liver.

The intestinal mucosal surface was usually not altered except in birds which had been fed OA for 8 weeks.

The changes were similar in both broilers and turkeys suggesting a common mechanism of toxicity in both the species.

In the kidney, abnormal mitochondria were common in the PCT of OA-fed fowls. Less severe changes have been reported in the kidney of pigs (Elling, 1977a) and mice (Shadmi and Nobel, 1981) during ochratoxicosis and of the fowl in phenoxy-acid toxicity (Björklund and Erne, 1971). Ring and 'U'-shaped mitochondria have been observed in the liver of fowls with fatty liver and kidney syndrome (FLKS) but not in the kidney (Siller and Wight, 1976). Ring-shaped mitochondria have been reported in the liver from rats treated with carbon tetrachloride (Reynolds, 1960), ammonium carbonate (David and Kettler, 1961) and alcohol (Koch, Rotta de Conti, Bolanos and Stoppani, 1978) and in clear cell carcinoma of the kidney (Seljelid and Ericsson, 1965). However, the significance of such transformation of mitochondrial morphology is not known (Ghadially, 1982) but this could be the early stages of a degenerative phenomenon as swollen mitochondria later showed degeneration. Ring-form mitochondria, a more or less constant feature in PCT cells in the present study, appeared to be formed by fusion of the membranes at the

tips of curved elongated mitochondria. The spaces or vacuoles, which could be of varying size and shape, were lined by a double membrane similar to the mitochondrial membrane, further supporting the notion of their formation by this process.

Since OA is known to inhibit enzyme activity (Elling, 1979) and mitochondrial respiration (Moore and Truelove, 1970; Meisner and Chan, 1974; Meisner, 1976), the mitochondrial changes seen in the present study might account for the impairment in renal function in ochratoxicosis of the fowl, reported by Huff et al. (1975) and by Svendsen and Skadhauge (1976). There is increasing evidence that the mitochondrial inner membrane plays an important role in the regulation of renal metabolism (Simpson, 1983). Renal phosphoenolpyruvate carboxykinase enzyme (present in the mitochondria) has been found to be specifically inhibited by OA in rats and pigs (Meisner and Meisner, 1981; Meisner and Krogh, 1982).

Mitochondrial dense granules are related to mineral metabolism, representing sites of divalent cation accumulation (Ghadially, 1982). Since Huff et al. (1975) found plasma electrolyte changes, particularly a decrease in potassium levels, at higher levels of dietary OA in broiler fowls, it seems to be of considerable relevance that these granules were greatly increased, both in number and in size, in the present study.

Normally there is very little lipid in the PCT of the fowl, but in OA-fed birds there was a moderate deposition of cytoplasmic lipid, similar to that reported in OA-treated Beagle dogs (Szczech et al., 1974). In the present study, lipid droplets were also found in the nuclei, which occurs under certain pathological conditions and is usually accompanied by lipid accumulation in the cytoplasm (Ghadially, 1982). The nature of round electron-dense bodies seen in the SER is unknown but their density is

similar to some of the lipid droplets and this could be evidence of lipid synthesis in the ER (Stein, Bar-On and Stein, 1972). Similar bodies were demonstrated in the ER of fowls with FLKS (Siller and Wight, 1976).

The increase in number and size of peroxisomes is interesting, since Ghadially (1982) relates peroxisomes with lipid metabolism and Björklund and Erne (1971) found an increase in the number of peroxisomes (microbodies) in the PCT from phenoxy acid-treated broilers.

Thickening of the glomerular basement membrane (GBM) has not often been reported in fowls. Focal thickening was seen in endotheliosis (Simpson, Pritchard and Harms, 1959) and diffuse thickening and degeneration with extensive collagen deposition was observed in salt toxicity (Siller, 1981; Sokkar, Hussein and Mohammed, 1983) in fowls. In the present study, thickened GBM was often found to be accompanied by swelling of the glomerular capillary endothelium and these changes sometimes led to the occlusion of the lumen. This could be associated with the decreased glomerular filtration found in ochratoxicosis of fowls by Huff et al. (1975) and Svendsen and Skadhauge (1976). In the present study increase in the number of lysosomes, usually containing lipids (known as residual bodies) or degenerating mitochondria, was a consistent feature noticed in the PCT of the kidney and also in other organs particularly in the later stages of the disease process when mitochondrial and cellular damage became eminent. This increased lysosomal activity may be related to their intracellular phagocytic property (autophagocytosis) for removal of the damaged organelles, as the lysosomes contain acid hydrolases and are considered to be the main part of an intracellular digestive system capable of digesting or degrading a variety of endogenous and exogenous substances (de Duve and Wattiaux, 1966). This could possibly also account for the decreased number of mitochondria observed in the PCT. An increased number of

lysosomes coupled with reduced numbers of mitochondria, many of which had lost their cristae, were noticed by Elling (1977a) in the PCT of OA-fed pigs. However, neither mitochondrial ring transformation nor thickened GBM were observed. Prior (1978) also recorded increased numbers of lysosomes, though no changes in the mitochondria in the cells exposed to OA in the tissue culture studies. The present findings of lysosomal activity confirm the speculation of Elling (1979) and Shadmi and Nobel (1981) who suggested that there would be an increase of lysosomes in the PCT.

However, Szczech et al.(1974) reported cytomorphological alterations in the endoplasmic reticulum in the PCT of Beagle dogs during experimental ochratoxicosis, whereas the mitochondria and lysosomes appeared normal. Interestingly, swollen mitochondria were noticed in the kidney of Beagle dogs given citrinin alone or in combination with OA (Kitchen et al., 1977c). Cytoplasmic vacuolation and myelin figures, features of ochratoxicosis in dogs (Szczech et al., 1974; Kitchen et al., 1977c), were not noticed in the present study. It is, therefore, likely that the mechanism of OA-induced nephrotoxicity in fowls and turkeys may, in some respects, differ from those in mammals.

Huff et al. (1979b) observed an increase in glycogen at the periphery of the hepatic lobules of fowls fed 4 and 8ppm OA levels (but not at lower levels), and it was suggested (Warren and Hamilton, 1980a) that the accumulation of glycogen in ochratoxicosis was due to an inhibition of protein-kinase enzyme and glycogenolysis. In the present study an accumulation of excessive amounts of monoparticulate and rosette forms of glycogen was observed in the cytoplasm of the hepatocytes of all the OA-treated birds, even at 2ppm, and throughout the liver. This increase in the amount of glycogen in the liver could perhaps be due to diminished usage and increased storage rather than as a result of altered metabolic activity. This is supported by the absence of appreciable changes in the mitochondria, ER and other cell

organelles in the present study. The only other report of ultrastructural studies in birds fed OA is that of Theron et al. (1966) in ducklings; they did not find any accumulation of glycogen in the liver. However, their findings are based on a single dose of OA (100 ug) over a short period.

Severe lymphoid depletion noticed in the lymphoid organs by light microscopy in the present study and by other workers in the fowl (Peckham et al., 1971; Gylstorff and Rolf, 1978), dogs (Szczech et al., 1973b; Kitchen et al., 1977b) and pigs (Szczech et al., 1973c) can be explained by the severe damage caused to the lymphoid population leading to the degeneration and disintegration of lymphocytes (and plasma cells) at the ultrastructural level. As in the kidney, mitochondria were found to be consistently damaged in lymphocytes in all the primary and secondary lymphoid organs studied.

The present study suggests that in fowls and turkeys OA is more nephrotoxic than hepatotoxic and that the mitochondria of the PCT are the organelles which are most sensitive to OA-injury. The mitochondrial ring forms in the kidney PCT and the accumulation of glycogen in the liver may be of diagnostic significance. In addition to hepatorenal changes, OA caused severe damage to lymphoid cells in the primary and secondary lymphoid organs, suggesting a depressive effect on the immune system.

5.9 Skeletal Development

Skeletons from OA-treated broilers and turkeys in the present study were stunted and the weights of tarsometatarsi and middle toes were consistently lower (Table 13a and 13b). Endochondral bone growth must obviously be involved. Rachitic changes have been reported during ochratoxicosis and aflatoxicosis (Huff et al., 1980) and during Fusarium

moniliformae toxicosis (Gedek, Huttner, Kahlau, Köhler and Vielitz, 1978; Köhler, Huttner, Vielitz, Kahlau and Gedek, 1978). However, there was no evidence of gross morphological osteomalacic changes in the present study. Stunted bones were of normal shape and size.

Tibial dyschondroplasia has been reported in broilers fed a Fusarium roseum-contaminated diet (Walser, Allen, Mirocha, Hanlon and Newman, 1982). Impairment of skeletal mineralisation has also been associated with diets containing aflatoxin B_1 (Halama, 1981). There are indications that intestinal absorption of calcium and phosphorus is decreased (Huff, Chang, Garlich and Hamilton, 1977) and vitamin D_3 metabolism is disturbed (Bird, 1978; Britton and Wyatt, 1978) in aflatoxin-treated broilers. In the present study, neither dyschondroplastic nor chondrodystrophic changes were noticed, which is in agreement with the absence of dyschondroplastic lesions in aflatoxin-fed broilers (Huff, 1980b). However, the possibility of OA affecting vitamin D_3 metabolism as in the case of aflatoxin B_1, can not be ruled out. The liver and particularly the kidneys were severely affected in the present study and electrolyte imbalance or altered vitamin D_3 metabolism may have resulted in skeletal changes.

Bone ash appeared to be slightly reduced (about 6-7 per cent), particularly in broilers. Toe ash, which is considered to be a measure of bone mineralisation (Fritz and Roberts, 1968; Yoshida and Hoshii, 1982), was only marginally reduced in broilers but not in turkeys. Huff (1980a), however, noticed a discrepancy between bone ash (tibiotarsi) and toe ash during aflatoxicosis.

Histological examination of bones from turkeys fed OA indicated developmental abnormalities. Appositional bone growth was affected in such a way that the increase in the diaphyseal width resulted in normal bone shape but the character of the bone was altered. This could be due to

a) a direct effect of the toxin on bone cells (osteoblasts and osteoclasts) and periosteal cells, b) a direct effect on mineralisation of osteoid (bone matrix) and c) abnormally increased osteoclastic resorption. Decreased bone ash could be accounted for by any of these three possibilities. Decreased breaking strength of the bones has been reported in broilers during experimental ochratoxicosis (Huff et al; 1980) and aflatoxicosis (Maurice, Bodine and Rehrer, 1983). If the mechanical tests are applied on bones with altered characteristics (widened vascular canals and thin seams of bone formation), as observed in the present study, the breaking strength would predictably be reduced due to altered appositional (intramembranous) bone growth. However, Huff et al. (1980) and Pepeljnjak et al. (1982b) failed to detect any histological changes in bones from OA-treated fowls.

Though the turkeys (Experiment V) were fed a diet deficient in biotin, symptoms and skeletal lesions characteristic of biotin deficiency (Jensen and Martinson, 1969; Arends, 1970) were not noticed either in controls or OA-treated birds. Yet the possibility of biotin deficiency enhancing the OA-induced bone changes cannot be ruled out.

Unfortunately, no authentic and detailed histopathological studies have been conducted on the skeleton in mycotoxic conditions in poultry. Most of the reports suggest an indirect effect of mycotoxin on the bone (Huff et al., 1977; Bird, 1978; Britton and Wyatt, 1978). In pathological studies conducted on the skeleton in birds fed diets contaminated with fungal cultures (Gedek et al., 1978; Köhler et al., 1978; Walser et al., 1982), the possibility of a variety of toxic components causing the changes cannot be ruled out.

The results of the present study suggest that OA interferes with deposition of bone and/or its maturation, causing a direct effect primarily on periosteal new bone

formation, i.e., on endochondral and intramembranous ossification. The study further suggests that the toxin does not cause a marked effect on bone already laid down, as the diaphyseal bone existing prior to OA treatment is of relatively normal structure. The exact mechanism of these changes remains to be investigated and further detailed studies are in progress. Depressed endochondral and intramembranous ossification in developing bones have been reported in animals in intoxications due to aldrin (Singh and Jha, 1982) and uranyl acetate (Guglielmotti, Ubios, de Rey and Cabrini, 1984).

5.10 Humoral and Cell-Mediated Immune Responses

The effects of OA on humoral and CMI responses, were assessed by investigating immunoglobulin levels in sera and tissues and DH responses to a variety of antigens and GVH reaction both in broilers and in turkeys.

Dohms and Saif (1984) have recently defined criteria for the evaluation of immunosuppression in poultry. The results from one assay (fulfilment of one of the criteria) are not sufficient to assign the term immunosuppression to a particular agent. The evidence should be provided from several approaches including morphometric changes in central (primary) and/or peripheral (secondary) lymphoid tissues, changes in concentrations or ratios of immunoglobulin classes within serum and changes in the functional activity of the immune responses (*in vitro* and *in vivo* tests of humoral and CMI responses). The present study has attempted to fulfill all the above criteria in assessing immunosuppressive effects of OA in broilers and turkeys.

5.10.1 Immunoglobulin levels

Antisera against chicken immunoglobulins were used for RID and immunofluorescent studies in turkeys, as a cross

reaction has been reported between avian species immunoglobulins (Leslie and Benedict, 1970). It is difficult to explain the failure in the present work to detect IgA levels by RID whilst IgA was shown to be present in tissues by means of immunofluorescence. This might be due to faulty technique.

While the levels of all the three immunoglobulins (IgA, IgG and IgM) were reduced in fowls, only IgM was depressed in turkeys. This could be explained by a decreased susceptibility to OA, or by selective inhibition of IgM, as has been reported in mice (Haubeck et al., 1981).

Regression of the immunological organs and of their lymphoid cell population and the severe damage to lymphocytes and plasma cells (at ultrastructural level) in the OA-treated birds, has been mirrored in the reduction of immunoglobulin-containing cells, as demonstrated by immunofluorescence, and in the decrease of serum immunoglobulins observed in the present study. The germinal centres and lymphoid follicles had very few fluorescing cells. The significance of the apparent migration of immunoglobulin-containing cells to subepithelial regions, especially in the bursa of Fabricius, is not known but it does suggest an altered immune mechanism. OA in sublethal quantities has been shown to be an immunosuppressant in mice (Haubeck et al., 1981; Creppy et al., 1982), although Prior and Sisodia (1982) did not find changes in antibody titres to Brucella abortus or sheep red blood cells in immunised mice fed 4ppm OA. However, intraperitoneal injections of OA (5mg/kg for 50 days) caused a depressed antibody response to Br. abortus but not to sheep erythrocytes. These workers suggested that OA, like cyclophosphamide, has a non-selective immunosuppressive effect in mice. In guinea pigs neither antibody titres to Br. abortus nor complement activity were affected by OA (Richard et al., 1975b).

Patterson et al. (1981) could not find any alteration

in immunoglobulin levels or titres in calves given subclinical doses of OA. An immunosuppressive effect has, however, been demonstrated in fowls in aflatoxicosis (Pier, 1973; Thaxton, Tung and Hamilton, 1974; Giambrone, Ewert, Wyatt and Eidson, 1978).

Bolton, Tucker and Sturgill (1980) have shown that there is a deposition of IgG along the glomerular basement membrane (GBM) in experimental autoimmune glomerulonephritis in the fowl. Siller (1981) is of the opinion that if all kidneys from routine autopsy material were to be examined histologically, lesions of proliferative glomerulonephritis would be seen with great frequency. Thus, staining of the GBM does not necessarily indicate autoimmune glomerulonephritis in the fowl though the findings of more glomeruli showing positive fluorescence for IgG in kidneys from OA-fed birds might be taken as an indication of renal involvement. This correlates well with the observation of thickened GBM in OA-treated broilers and turkeys both at light microscopic and at ultrastructural levels. The presence of immunoglobulin containing cells in kidneys from OA-fed birds further corroborates the frequent lymphoid cell infiltration observed in the renal parenchyma.

Immunoglobulin production in the fowl is dependent on the bursa of Fabricius and other associated lymphoid organs and an intact thymus is necessary for the switch mechanism of IgM to IgG and/or IgA to operate successfully (Bienenstock, Gauldie and Perey, 1973). Thus, the decreased production of immunoglobulin-containing cells and the lowered levels of serum immunoglobulins can be considered to be a sequel to the regression of the lymphoid organs due to severe damage of lymphocytes and immunoglobulin forming plasma cells during ochratoxicosis. Reduced IgM and IgG levels have also been observed in OA-treated mice (Creppy et al., 1982, 1983). Creppy et al. (1979a,1979b) related the immunosuppressive effect of OA in mice to its

inhibitory action on protein synthesis. The mechanism of immunosuppression in the present study appears to be a direct effect of OA on lymphocytes and plasma cells which were found to be very sensitive to OA. IgE levels were not affected, nevertheless the present study shows that some IgE is present in the sera of even 2-3 week old broiler chicks and young turkeys.

The immunosuppressive action of OA has practical implications because of the enhanced susceptiblity of birds to secondary infections as seen in the increased incidence of air sacculitis in field outbreaks of ochratoxicosis in broiler fowls (Hamilton et al., 1982). The possibility of adverse effects of OA on anticipated protection by vaccination programmes should not be discounted.

5.10.2 Cell-mediated immunity

CMI responses, as assessed by cutaneous delayed hypersensitivity reactions to a variety of antigens (avian tuberculin PPD, PHA, DNCB, BSA), were found to be significantly reduced in OA-treated broilers and turkeys (Section 4.10.2). The depression was generally more marked in broilers than in turkeys. GVH response, a T-cell mediated reaction, was also significantly reduced in OA-fed broilers. These results indicate a marked suppression of cell-mediated immune responses in ochratoxicosis A. This may be due to the regression of the thymus and other lymphoid organs together with a depletion of lymphoid cells from these organs seen in the present study.

T-lymphocytes, the principal mediators of cell-mediated immunity, develop and mature in the thymus and then migrate to colonise the secondary lymphoid tissues (Weber, 1975 ; Odend'hal and Breazile, 1980; Sharma and Tizard, 1984). They constitue between 60 and 70 per cent of the lymphocytes in the peripheral blood (Albini and Wick, 1974), 55 per cent in the spleen, where they are located in

the periarteriolar lymphoid sheaths, red pulp and sinuses (Boyd and Ward, 1978; Hoffman-Fezer, Rodt, Gotze and Thierfelder, 1977), and considerable numbers of the lymphoid cells in the bursa of Fabricius, Peyer's patches and caecal tonsils (Albini and Wick, 1974; R.B. Burns, personal communication).

The degenerative and necrotic changes seen in the thymus and other lymphoid organs of the OA-fed birds of the present study must be reflected in the reduced responses to tests of T-cell function. There are no reports that show the effects of OA on CMI in poultry, though a lymphocytopaenia (Chang et al., 1979; Campbell et al., 1983) and regression of the thymus (Chang et al., 1981), spleen and other lymphoid organs (Peckham et al., 1971; Gylstorff and Rolf, 1978) have been taken to suggest an effect on T-cell responses. Klinkert et al.(1981) are the only workers to study T-cell function directly in ochratoxicosis A. They reported 56 per cent inhibition of macrophages from guinea pigs in an in vitro study. CMI responses in fowls have been reported to be depressed due to aflatoxin B_1 (Pier, Fichtner and Cysewski, 1977; Giambrone et al., 1978).

Besides measuring skin thickness at the test sites the histology of the reactions to the test antigens were also studied; they were found to be consistently reduced in skins from OA-fed birds. When studying DH responses in turkeys McCorkle et al. (1983) concluded that there was no response to avian PPD challenge in their birds as assessed by skin thickness measurement and gross lesion. However, in the present study turkeys developed a visible macroscopic reaction and a good microscopic response with avian tuberculin PPD, though not as strongly as broilers. This, then, suggests the importance of histological examination particularly before deriving a negative conclusion.

During the present study some features of the immune response of both fowls and turkeys have emerged which warrant further discussion.

Because of reported regional variations in skin sites used for DH tests in the fowl, turkey and quail (Benedict, 1957; Karlson, Thoen and Harrington, 1970; Rose and Bradley, 1977; Lasley, 1980) different areas were investigated for DH responses. The most suitable sites were found to be the post-auricular apterium in the fowl and the earskin area in the turkey and these can be recommended for routine use in skin testing. Characteristic histological reactions could always be seen at these sties.

Difficulties to varying degrees were encountered when other skin sites were used for DH reactions. The wattle usually gave a good response but was difficult to inject and is absent in young chicks. The lateral thoracic apterium was suitable for following the macroscopic lesions induced by DNCB and for testing Arthus reactions but it was difficult to inject intradermally and to measure. The wing web, which has been used in turkeys (McCorckle et al., 1982; McCorckle et al., 1983), was found to give inconsistent results. The dorsal cloacal lip, frontal process and comb can be used if necessary but suffer from the absence of control sites and in being difficult to inject (Rose and Bradley, 1977).

Histological studies of cutaneous delayed hypersensitivity reactions in birds have been hampered by difficulties in identifying two of the principal cells, eosinophils and heterophils, involved in inflamatory lesions. The problem is more difficult in turkeys as their eosinophil granules were both peroxidase- and Sudan black B- negative, in contrast to those of the fowl (Maxwell, 1984). Thus, it was possible to distinguish

eosinophils from heterophils in tissue sections from DNCB-treated fowls using the indirect peroxidase staining method of Maxwell (1984), but not in turkey tissues. The only way available at present to differentiate between these two cells in turkey tissues is by electron microscopy (Maxwell and Trejo, 1970) and this method was used in the present study both in turkeys and fowls.

Dvorak, Galli and Dvorak (1980) have advocated the use of semi-thin plastic sections in conjunction with immunofluorescence and electron microscopy for the study of inflammatory reactions characteristic of CMI. These methods were used in the present study.

DH responses in both broilers and turkeys were characterised by the participation of large numbers of granulocytes, mostly heterophils with some eosinophils, and by structural alterations in the microvasculature. The role of these granulocytes in CMI responses has recently been emphasised by Colvin and Dvorak (1979) and by Dvorak et al. (1980). They appeared to be a regular, though minor, component of DH reactions in guinea pigs and mice and have been thought to be dependent on the presence of sensitised lymphocytes (Colvin and Dvorak, 1979).

Recently, an epidermal cell-derived thymocyte-activating factor (ETAF), a cytokine indistinguishable from macrophage-derived lymphokine (interleukin 1), has been isolated from murine and human keratinocytes, the principal cell in the epidermis (Lugar, Charon, Colot, Micksche and Oppenheim, 1983a; Luger, Sztein, Schmidt, Murphy, Grabner and Oppenheim, 1983b). ETAF production can be induced by mitogenic agents and cell damage or by injurious agents, and has been found to be responsible for interleukin 2 production by T-lymphocytes and to be chemotactic for neutrophils. If a similar mechanism operates in birds then the inflammatory and immunological

cellular reactions observed in the present study can be more readily explained. A lymphocyte-derived chemotactic factor or eosinophil migration factor has also been described (Colvin and Dvorak, 1979; Moriya and Ichikawa, 1981, 1982).

Though a cutaneous basophil hypersensitivity (CBH) has been described by Dvorak, Colvin and Churchill (1975) and reported in DH responses of fowls (Stadecker, Lukic, Dvorak and Leskowitz, 1977; McCorckle et al., 1980), basophils were not seen in either control or OA-fed turkeys or fowls in the present study; mast cells were sometimes encountered. The heterophil was the principal granulocyte seen in DNCB-treated fowls and PHA-stimulated turkeys. Chand, Carlson and Eyre (1976) have described heterophils, mononuclear cells and basophils in passive cutaneous anaphylaxis induced in fowls by BSA. Specialised staining techniques failed to identify with certainty the large numbers of granulated cells seen in the present study and they were considered to be phagocytic macrophages.

In the present study, the structural alterations noticed in the blood vessels especially in the skin from control birds, probably indicated the activation and participation of endothelial cells in DH reactions in poultry. Endothelial proliferation has been seen in guinea pig skin after challenge with DNCB or with HPD (Polverini, Cotran and Sholley, 1977). Vascular endothelium is considered to play a part in T-lymphocyte immunity (Burger and Vetto, 1982), particularly in antigen presentation to virgin lymphocytes (Groenewegen and Buurman, 1984).

The present observation of the participation of considerable numbers of eosinophils in DNCB-induced contact hypersensitivity reactions is consistent with the

reports of Awadhiya et al. (1982) and Maxwell (1984). Giant macrophages, many of which were actively phagocytic, could be seen throughout the reaction zone suggesting that they play a role in DH reactions. The production of macrophages is controlled by colony stimulating factors produced by fibroblasts, lymphocytes and mononuclear phagocytes (Weir, 1984). Macrophages play an important part in immune reactions (Sharma and Tizard, 1984) so that their presence in the skin and lymphoid tissues of stimulated birds in the present study should not be surprising.

GVH reactions are often used for the evaluation of CMI in poultry (Giambrone et al., 1978; Chauhan and Verma, 1983). They were significantly depressed in OA-treated broilers. Technically it was found best to use blood collected in Alsever's solution and washed three times in either saline or PBS to remove the anti-coagulant. Strict sterile precautions were observed. The cells injected into embryos attacked the embryonic tissues (Biggs and Payne, 1961), particularly the haematopoietic cells which were stimulated to divide so that the proliferative events involved both donor and recipient cells, resulting in splenomegaly and hepatomegaly. Thus, the GVH reaction was a useful experimental model (Sharma and Tizard, 1984).

It was not possible to elicit a typical Arthus reaction in turkeys, with only a slight erythema being noticed at the lateral thoracic skin site (recommended by Luoma and Benedict, 1977, and by Palladino et al., 1978). A distinctive Arthus reaction which even led to necrosis, was seen in fowls. The DH response to BSA in turkeys was much less intense than that seen in the fowl. Palladino et al.(1978) have shown that in the fowl an Arthus reaction does not interfere with the measurement of DH

responses. Sensitisation with killed M. avium gave a better response than tuberculin PPD in both fowls and turkeys. Giambrone et al. (1978) though obtained good reactions with mammalian tuberculin.

M. avium- and avian tuberculin, PPD-sensitisation did not affect organ weights of OA-treated turkeys, though BSA immunisation appeared to cause a significant enlargement of the spleen and liver (Table 8). This might be attributed to a possible systemic effect.

A severe cutaneous DH reaction was obtained by PHA in both fowls and turkey. This is in agreement with earlier reports (Stadecker et al., 1977; McCorckle et al., 1983).

In sections it could be seen in the turkey that PHA had a marked effect on the heterophils. There was a large infiltration of heterophils into the reaction zone. At the level of the light microscope it was not possible to differentiate between eosinophils and heterophils but at the ultrastructural level it could be seen that the granulocytes were indeed heterophils with altered granule morphology. A heavy infiltration of heterophils into the skin and internal organs of turkeys treated with PHA was associated with an increase in the numbers of circulating heterophils (Table 22). This might suggest that turkeys react differently from other animals when stimulated with PHA as in guinea pigs, for example, the response was mainly a basophilic one (Dvorak et al., 1975) rather than the heterophilic picture of the turkey. A basophilic reaction has also been seen in mice (Hurtado and Urbina, 1983) and in the domestic fowl (Stadecker et al., 1977; McCorckle et al., 1980). This finding contradicts reports of PHA-induced CBH in neonatal (McCorckle, Luginbuhl and Simmons, 1981) and young turkeys (McCorckle, et al., 1983), probably because these reports were based on a presumption of identification taking into consideration earlier reports of CBH in fowls rather than a detailed ultrastructural study (McCorckle

et al., 1982).

PHA induced a generalised reaction in turkeys. There was a heavy infiltration of heterophils, eosinophils, lymphocytic cells, lymphoblastic cells and macrophages at the site of injection and in the lymphoid organs. Gamble (1966) has reported an increase in splenic weight in mice stimulated by PHA and this was also the case in the turkeys of the present study.

Subba Rao and Glick (1975) have shown that PHA when given i.v. enhanced the activity of T-lymphocytes and peritoneal macrophages as measured by their increased oxygen consumption, whereas intraperitoneal injection resulted in decreased activity. The present study suggests that intradermal injection of PHA in turkeys parallels i.v. administration. The bursal lymphoid follicles and germinal centres in the spleen, Peyer's patches and caecal tonsils were not affected by PHA suggesting little effect on B-lymphocytes. This is consistent with other work (Subba Rao and Glick, 1975).

PHA induced a transformation of lymphocytes at the sites of injection similar to that recorded in in vitro studies (Stewart and Ingram, 1967). Lymphocytes as perivascular lymphoid aggregates have been considered to be a characteristic feature of DH responses (Dvorak et al., 1980). The presence of cells fluorescing for IgG, IgA and IgM at the dermal site of injection indicates a possible location of B-T lymphocyte interaction (Sharma and Tizard, 1984). Subba Rao and Glick (1975) have suggested that PHA-stimulated lymphocytes might interact with antibody-forming precursor B-cells. The mulberry-like fluorescing cells might be eosinophils as their granules are known to fluoresce when treated with immunoglobulin-fluorescein conjugates (Floyd, Suter and Lutz, 1983).

The function of the avian heterophil granule is not known (Maxwell and Trejo, 1970), but the excessive accumulation and heavy degranulation of these cells in PHA-stimulated fowls and turkeys indicates a close relationship with mitogens (McCorckle et al., 1980). Heterophils have been reported in fowls after PHA treatment, especially in the early stages of the reaction (McCorckle et al., 1980; Goto, Kodama, Okada and Fujimoto, 1978).

There was an increase in eosinophils in PHA treated turkeys (Table 23) and since turkey eosinophils were found to differ in their histochemical reactions from those of the fowl, it suggests a possible different involvement for the same cell in the inflammatory response.

Moriya and Ichikawa (1979) reported an accumulation of eosinophils in the bursa of Fabricius and spleen in PHA-treated chick embryos but in the present study and according to Maxwell (M.H. Maxwell, personal communication) the infiltration was mainly by heterophils.

The majority of eosinophils and heterophils in tissues of PHA-stimulated birds were seen to be degranulated or to be degranulating. Free granules were seen in the tissue parenchyma. Empty vesicles were seen in many granulocytes which is similar to the picture of CBH given by Dvorak et al. (1980). It can be seen, then, that PHA and the activity of the granulocytes and their granules appeared to stimulate intense phagocytic activity in the macrophages of the skin. This might be considered to be similar to PHA-induced cytotoxicity described by Dallegri, Frumento, Maggi and Patrone (1983). The presence of many mast cells at the reaction sites might

suppose that they released a factor chemotactic for granulocytes.

The present study suggests that OA causes an immunosuppression in both turkeys and fowls that affects both CMI and humoral immune responses confirming the suggestion of Chang et al. (1981), Prior and Sisodia (1982) and Hamilton et al. (1982) that OA causes reduced resistance in animals. The effects that OA might have on expected levels of protection that might be given by vaccination must be recognised especially when the mycotoxin is present at sub-clinical levels.

5.11 OA residues in Tissues

It was possible to demonstrate the localisation of OA in kidney, liver, muscles and small intestine by indirect fluorescence, both in broilers and quail. The weak fluorescence found in the tissues from broilers could have been due to use of higher dilutions of anti-OA-antiserum and also to the fact that these tissues were not preincubated with cyanamide. Preincubation of quail tissues with cyanamide prior to specific staining seems to have contributed significantly to the preservation of microscopic structure and in the intensity of fluorescence, a fact that was observed by Elling (1977b) in rat kidney though not in porcine tissues. Cyanamide is known to couple compounds containing many types of functional groups such as carboxylic acid (Goodfriend, Levine and Fasman, 1964). In the present study, preincubation with cyanamide should couple OA via its carboxylic acid group to lysine of the proteins in the tissue sections thus binding the OA to tissues and preventing its disappearance during the washing procedure. Some of OA could also be demonstrated in the process of being washed away from the cells, particularly in the kidney, thereby suggesting the value of pretreatment of tissue sections with cyanamide in such studies.

In broiler tissues (and to some extent in quail), the non-specific staining noticed frequently both in controls and OA-fed birds could have been due to anti-BSA-antibodies both because these birds had been immunised with BSA and also because the anti-OA-antiserum was prepared in rabbits using OA coupled with BSA. In order to eliminate anti-BSA-antibodies (and false positive fluorescence), the antiserum was absorbed with BSA, and this proved to be effective when used on quail tissues.

In the kidney OA localised mostly in the PCT, though its presence could be demonstrated in some of the glomeruli, suggesting that PCT is the target part of the nephron. This

is consistent with observations in pigs and rats (Elling, 1977b) and further substantiates and explains the severe lesions noticed in the present study, particularly in the kidney, in the light and electron microscopy studies. In addition to the kidney, OA was demonstrated in other tissues, many of which showed pathological changes. OA is known to bind to plasma albumin and is very rapidly distributed widely in various tissues of chickens (Prior, 1978; Galtier et al., 1981). It is possible that OA-albumin complex (Prior, 1978) passes the damaged glomerular capillaries and the OA then exerts a further toxic effect upon the PCT during reabsorption.

Quantitatively, the highest amount of OA was found to be in the kidney, followed by the liver and the breast muscle in both broilers and turkeys, which is consistent with earlier reports in fowls (Krogh et al.,1976a; Prior et al.,1980). No studies seem to have been made in turkeys in this respect. OA residues have been found in various tissues of fowls and quail including ovarian yolk in a dose-related fashion (Piskorska-Pliszczyńska and Juszkiewicz, 1979; Juszkiewicz et al., 1982). Prior and Sisodia (1978) have reported unusually high values for OA in the liver, muscle and kidney (up to 106.9ppm), although in a subsequent report (Prior et al.,1980) they quoted markedly lower values (up to 41 ppb). In the present study and reports by other workers, concentrations at ppb levels (up to 114 ppb) have been reported. OA residues at similar levels and distributions have been demonstrated in pigs (Krogh et al., 1974a, Shreeve et al., 1977; Madsen et al., 1982b; Mortensen et al., 1983). In the present work, interestingly enough, higher OA residues were observed in the tissues of turkeys as compared with broilers. The quantitative findings in the various tissues correlate well with the microscopic results showing the localisation of OA in the tissues by means of immunofluorescence.

The highest and most consistent residues of OA were encountered in the kidney and liver, the two organs which receive about 50 per cent of the cardiac output. The residues in muscles were of a lower magnitude. The fact that renal cells were exposed to the highest levels of OA for a longer period coupled with subsequent localisation in the kidney PCT (and glomeruli) and hepatocytes may explain the severe changes encountered in these organs during light and electron microscopy.

It is not possible to assess the public health significance of the OA residues because of the very limited information available at present. However, in view of the teratogenic (Section 4.12; Arora, 1982) and carcinogenic (Kanisawa and Suzuki, 1978; P. Krogh, personal communication) potentials of OA, the present study does point to the public health problem posed by the possibility of OA-contaminated poultry meat, a problem on which comment has already been made in connection with pig meat (Krogh et al., 1974a; Krogh, 1976) and eggs (Piskorska-Pliszczyńska and Juszkiewicz, 1979; Juszkiewicz et al., 1982).

5.12 Production Characteristics and Teratogenic Effects

Japanese quail are a useful experimental model because of their small size, requiring less space and feed, and because of their relatively short generation time. Quail attain sexual maturity by six weeks of age, reach 50 per cent production by 8 weeks and peak production by 10 weeks of age (Anon., 1983), as confirmed in the present study (Table 26). Because of the high cost of OA, it was felt that Japanese quail would offer an opportunity to study production characteristics and teratogenic effects of ochratoxin A, without requiring the large quantities of toxin needed for fowls and turkeys.

Despite the lack of adverse effects on growth and the lack of apparent clinical signs, egg production appeared to be reduced in OA-fed birds in a dose-related manner (Table 26). Prior et al. (1978) reported no adverse effects on body weight, feed conversion or egg production in quail fed up to 16ppm dietary OA. Egg production has also been found to be depressed in fowls due to OA by other workers (Choudhury et al., 1971; Prior and Sisodia, 1978; Page et al., 1980). The smaller eggs from OA-fed birds observed in the present study are consistent with the reports of Prior and Sisodia (1978), Prior et al. (1981) and Tohala (1983). It was not possible to assess whether there were increased egg shell stains (spots) in the present work as reported in fowl eggs (Page et al., 1980; Tohala, 1983) because of the pigmented nature of the quail egg.

Fertility was depressed only at 8ppm OA level whereas hatchability was severely depressed in both groups (4 and 8ppm) in a dose-related fashion (Table 27), as was also observed by Prior et al. (1978) in quail at the 16ppm OA levels. The decreased egg production (subsequently resulting in decreased fertility and hatchability) in OA-treated birds may be due to the effect of OA on the ovary, as suggested by the presence of higher amounts of OA in the ovarian follicles of quail (Piskorska-Pliszczyńska and Juszkiewicz, 1979) and fowls (Juszkiewicz et al., 1982) after oral feeding of OA either as a single dose or for up to 10 days. The amount of OA detected in the ovarian follicle was ten fold higher than that in whole eggs (Piskorska-Pliszczyńska and Juszkiewicz, 1979). Since OA is transmitted to the eggs via the ovary in both quail and fowls, as reported by these workers, its effect in causing, in addition to decreased fertility and hatchability, malformations in

the developing embryo and increased embryonic mortality could be related to a direct effect of the toxin. Embryotoxic and teratogenic effects (Table 28) appear to be exerted through the presence of OA in the environment of the embryo and not through an alteration of gametes as suggested by Prior et al.(1979). Increased embryonic mortality (Veselý et al, 1982), embryocidal effects (Brown, 1970, Gedek, 1972, Gilani et al., 1978) and teratogenic effects (Gilani et al., 1978; Veselá et al., 1983) observed in earlier work were induced by the administration of OA directly into the egg. However, the route of administration of OA in the present work appears to be more realistic because natural exposure to mycotoxins occurs chiefly via the ingestion of contaminated feed. The embryonic malformations and developmental defects observed in the present experiment are consistent with those reported by Gilani et al. (1978) and Veselá et al. (1983) in fowl embryos, indicating a similar mechanism operating in both species of bird. However, in the present work the cardiac anomalies observed by Gilani et al. (1978) in 6 out of 30 embryos, were not noticed despite a thorough search. Prior et al. (1978, 1979), however, failed to detect any developmental defect in quail embryos. The embryonic malformations were usually noticed at 8 to 10 days of incubation, which is in agreement with earlier work in the fowl (Veselá et al., 1983) and in mice (Arora, 1982). Malformations occurred mainly in the cephalic region, particularly an impaired development of the cranial bone resulting in exposure of the brain (exencephaly) in a dose-related manner. This correlates with the impaired intramembranous ossification noticed in young growing turkeys (section 5.9). A similar deformity has been reported in mammals (Still et al.,1971; Moré and Galtier, 1974, 1975, 1978; Brown et al.; 1976, Hayes et al., 1974; Hood et al., 1976; Arora, 1982). The mouse embryo appeared to be the most susceptible amongst the mammals investigated. Moreover, OA has been considered to have a much more pronounced teratogenic potential than

either aflatoxin B_1 or Zearalenone (Arora, Frölén and Nilsson, 1981; Arora and Frölén, 1981). The peak sensitivity of the mouse embryo to OA was on gestation day 9 and it was related to the effect of toxin primarily on neurulation (Arora, 1982). It is not possible to correlate the effect noticed in the mouse directly with those found in the quail, but the similar mechanism which appears to be operating in the avian species requires further investigation.

The present work suggests that OA, even in sub-clinical doses where no apparent clinical signs or growth depression are evident, can cause drastic effects on egg production, fertility and particularly hatchability. This may pose a great problem to the poultry producers and even diagnosticians in determining the cause. In addition, it possibly passes to the egg through the ovary and exerts severe embryocidal effects and causes developmental abnormalities in the embryo. The practical implications of these findings must be evaluated from the point of view of human safety because of the presence of OA residues in the edible tissues and meat of poultry (Section 5.11) and eggs. When more than two toxins are present in the diet the possibility of their causing a synergistic effect on these parameters, as reported for OA and citrinin in fowl embryos (Veselá et al., 1983), should not be ruled out.

5.13 Concluding Remarks

1. OA was found to be toxic to broilers, turkeys and quail in decreasing order of susceptibility. Loss of carotenoid pigmentation from the shank (leg) was a characteristic sign of the disease in broilers. Growth was markedly depressed in broilers and turkeys.

2. OA was nephrotoxic and resulted in distension and

enlargement of the kidney PCT with individual cell necrosis and thickening of the GBM.

3. It also caused an accumulation of glycogen in the liver in the three avian species and regression of the primary and secondary lymphoid organs coupled with depletion of the lymphoid cells.

4. OA appeared to affect skeletal development by interfering in endochondral and intramembranous ossificiation.

5. Mitochondria were principally affected in the PCT. Thickening of the GBM coupled with extensive degeneration usually caused obliteration of the capillary lumen. In the later stages, increased lysosomal activity was noticed in all the organs. In lymphoid organs, degeneration and necrosis of lymphoid cells including plasma cells was a conspicuous feature. Ring-form mitochondria in the kidney PCT and accumulation of glycogen in the liver were considered to be of diagnostic significance.

6. OA caused a drop in egg production, fertility and hatchability in quail usually in a dose-related fashion. It was also found to cause increased embryonic mortality and developmental deformities including exencephaly, crooked or cleft beak, gastroschisis, syndactyly, microphthalmia and poor feathering.

7. The highest concentration of OA residues were found in the kidney followed by liver and muscles, with levels being higher in turkeys than in broilers. OA was found to localise in the PCT and in the glomerulus in kidney and also in liver, muscles and small intestine.

8. OA was found to cause a significant depression of humoral and cell-mediated immune responses both in broilers and turkeys. Total serum immunoglobulin levels (IgG, IgM and IgA in broilers and IgM in turkeys) and tissue immunoglobulin levels as well as anti-BSA-antibodies were markedly reduced due to the severe damage to the precursor cells (lymphocytes and plasma cells) in the bursa of Fabricius, thymus, spleen, Peyer's patches, caecal tonsils and even Harderian gland.

9. Correspondingly, cutaneous DH responses to avian tuberculin PPD, PHA, DNCB and BSA and Arthus reaction were also significantly reduced in OA-fed birds. GVH reaction, as assessed by a splenomegaly response in chick embryos, was found to be markedly depressed in OA-treated broilers.

10. A number of immunological features, which indicate different mechanisms playing a role in fowls and particularly in turkeys, emerged from the present work. The post-auricular apterium in fowls and the earskin area in the turkey were found to be most suitable and convenient sites for testing cutaneous DH responses and could be recommended for routine use in birds. Sensitisation with killed M. avium was found to be better than sensitisation by avian tuberculin, PPD for the evaluation of classical DH responses.

11. In turkeys, despite a less intense reaction, a characteristic histological reaction could be seen even after avian tuberculin PPD-sensitisation indicating that an absence of gross thickening should not be interpreted as a negative response. PHA-induced responses were severe in both species. In turkeys, PHA caused a cutaneous heterophil

hypersensitivity , rather than a cutaneous basophil hypersensitivity (CBH), characterised by a marked heterophilic reaction in which were seen heterophils with altered granules and frequently degranulating together with eosinophils and mononuclear cells (lymphocytes and macrophages) causing intense tissue damage. A generalised reaction involving lymphoid organs was also induced by PHA.

12. Turkeys did not exhibit a distinct Arthus reaction, as did fowls, though BSA could be used to study DH response in sensitised birds. DNCB induced a typical contact-hypersensitivity response,particularly in broilers, characterised by an infiltration of mononuclear cells, heterophils and eosinophils and by alterations of the microvasculature.

13. In general the granulocytes (particularly heterophils and eosinophils), together with vascular endothelial proliferation and new capillary formation appear to be characteristic features in cutaneous DH responses in fowls and turkeys. This suggests that granulocytes and endothelial cells play a role in immunologically-mediated reactions

14. Since OA caused both humoral and cellular immunosuppression, it is suggested that fowl may serve as a useful experimental model for study of the effect of mycotoxins on the immune system.

5.14 Future Perspectives

This study has laid the ground work for possible avenues of future research on OA. Some of these are:-

1. The immunopathological and immunosuppressive effects of OA combined with other common mycotoxins, known to occur simultaneously in contaminated feedstuffs

under natural conditions, should be investigated.

2. It should be established whether immunosuppression caused by OA (alone or in combination with other mycotoxins) is strong enough to cause a break in immunity to infectious agents and thus whether the toxin might interfere with the vaccination programmes.

3. The teratogenic effects of OA in fowl, turkeys and ducks require to be studied by feeding OA, a natural mode of toxicity. The mechanism of teratogenesis remains to be investigated in birds.

4. The interaction of OA, alone or in combination, with common avian pathogens (such as Escherichia coli, Mycoplasma spp; Eimeria species, and the viruses of Marek's disease, infections bursal disease and Newcastle disease) is another area for further investigation.

5. The effects of OA on vitamin status and nutrients need to be studied.

6. The effect of OA (alone or in combination with other mycotoxins) in causing skeletal developmental defects, particularly the mechanism involved in the process seems to be an important area for future studies.

7. Since OA and other mycotoxins have been found to be carcinogenic, it is desirable to study the role of OA in carcinogenesis in poultry.

8. Efforts should be directed to find a suitable diagnostic method, particularly a quick, reliable and cheap method which may be applicable under field conditions.

9. Another area, which needs further attention, is to find substances or methods which could effectively control, prevent and detoxify the mycotoxin(s) under field conditions.

CHAPTER 6

Appendix

APPENDIX

Reagents

Phosphate Buffered Saline pH 7.34

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	1 litre

Alsever's Solution pH 6.1

Dextrose	2.05 g
Sodium citrate	0.8 g
Sodium chloride	0.42 g
Distilled water	100 ml

Millonig's Buffer (0.1M) pH 7.2

$NaH_2PO_4 \cdot H_2O$	2.26 g
NaOH	2.52 g
Glucose	5.4 g
Distilled water	100 ml

Acridine Orange (Fluorescent Method) stain

a)	Haematoxylin	1.0 g
	Alcohol (95%)	100 ml
b)	Ferric Chloride (29% aqueous)	4 ml
	Distilled water	95 ml
	Hydrochloric acid	1 ml
c)	Acridine orange (aqueous solution)	1 : 1000

Substrate for Peroxidase Staining (Incubating Medium)

p - phenylenediamine dihydrochloride	7.5 - 15 mg (1 part)
Pyrocatechol	15 - 30 mg (2 parts)
Tris buffer (0.1M) pH 7.6	10 ml
Hydrogen peroxide (1%)	0.1 ml

Lead Citrate Stain

Sodium citrate	2.08 g
CO_2 - free distilled water	84 ml
N NaOH (carbonate free)	16 ml
Lead citrate	1.35 g

Uranyl Acetate Stain

2% Uranyl acetate in 50% ethyl alcohol (Saturated solution).

CHAPTER 7

REFERENCES

REFERENCES

Abramson,D., Mills,J.T. and Boycott,B.R. (1983). Mycotoxins and mycoflora in animal feedstuffs in Western Canada. Canadian Journal of Comparative Medicine, 47:23-26.

Albini,B. and Wick,G. (1974). Delineation of B- and T-lymphoid cells in the chicken. Journal of Immunology, 112:444-450.

Alderman,G. (1981). Effects of mycotoxins on food utilization and animal production. Proceedings of a Seminar, Mycotoxins in Animal Feedstuffs held at Metropole Hotel, Llandrindadwells. Ministry of Agriculture, Fisheries and Food, pp.36-39.

Allcroft,R. and Carnaghan,R.B.A. (1962). Groundnut toxicity - Aspergillus flavus toxin (aflatoxin) in animal products: Preliminary communication. Veterinary Record, 74:863-864.

Anon. (1978). Animal health. Report of the Chief Veterinary Officer, London. U.K., HMSO, 1980. p.53.

Anon. (1980). Survey of mycotoxins in the United Kingdom. Food Surveillance Paper No.4, Ministry of Agriculture, Fisheries and Food, London. HMSO, pp.13-21.

Anon. (1983). Annual Report of the Central Avian Research Institue, Izatnagar, India. Publication No.7/1983.

Applegate,K.L. and Chipley,J.R. (1973). Ochratoxins. Advances in Applied Microbiology, 16:97-109.

Arends,L.G. (1970). Gross and microscopic lesions induced by biotin deficiency in the poult. Poultry Science,49: 1364 (Abstract).

Arora,R.G. (1982). Mycotoxin induced effects on the prenatal development in mice : a teratopathologic and autoradiographic study of aflatoxin B_1, ochratoxin A and zearalenone. Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Arora,R.G. and Frölén,H. (1981). Interference of mycotoxins with prenatal development of the mouse. II. Ochratoxin A-induced teratogenic effects in relation to the dose and stage of gestation. Acta Veterinaria Scandinavica, 22:535-552.

Arora,R.G., Frölén,H. and Nilsson,A. (1981). Interference of mycotoxins with prenatal development of the mouse. I. Influence of aflatoxin B_1, ochratoxin A and zearalenone. Acta Veterinaria Scandinavica, 22:524-534.

Ashton,P.S., Meselson,M., Robinson,J.P.P. and Seeley,T.D. (1983). Origin of yellow rain. Science, 222:366,368.

Austwick,P.K.C. (1977). Aflatoxicosis in poultry. In: Wyllie,T.D. and Morehouse,L.G. (Eds.), Mycotoxic Fungi, Mycotoxins and Mycotoxicoses. An Encyclopedic Handbook. Volume 2. Marcel Dekker, Inc., New York and Basel, pp.279-301.

Awadhiya,R.P., Vegad,J.L. and Kolte,G.N.(1982). Eosinophil leucocytic response in dinitrochlorobenzene-skin hypersensitivity reaction in the chicken. Avian Pathology, 11:187-194.

Bacon,C.W., Sweeney,J.G., Robbins,J.D. and Burdick,D. (1973). Production of penicillic acid and ochratoxin A in poultry feed by Aspergillus Ochraceus : temperature and moisture requirements. Applied Microbiology, 26:155-160.

Balžer,I., Bogdanić,C. and Mužic,S. (1977). Natural contamination of corn (Zea mais) with mycotoxins in Yugoslavia. Anna de la Nutrion et de l'alimentation, 31:425-430.

Bannister,D.W. and Whitehead,C.C. (1976). Presence of pyruvate carboxylase in the blood of the domestic fowl (Gallus domesticus) and an assay technique. International Journal of Biochemistry,7:619-627.

Barnes,J.M., Austwick,P.K.C., Carter,R.L., Flynns,F.V., Peristianis,G.C. and Aldridge,W.N. (1977). Balkan (endemic) nephropathy and a toxin-producing strain of Penicillium verrucosum var cyclopium: an experimental model in rats. Lancet,i:671-675.

Benedict,A.A. (1957). The intradermal test for epidemiological studies of turkey ornithosis. American Journal of Hygiene, 66:245-252.

Benham,C.L. (1982). Moulds and mycotoxins in animal feed stuffs. Proceedings of 4th meeting on mycotoxins in Animal Disease, Weybridge, April 1981, pp.38-40.

Bienenstock,J., Gauldie,J. and Perey, D.Y.E. (1973). Synthesis of IgG, IgA, IgM by chicken tissues: immunofluorescent and ^{14}C amino acid incorporation studies. Journal of Immunology, 111:1112-1118.

Biggs,P.M. and Payne,L.N. (1961). Pathological changes following the inoculation of chick embryos with adult cells. II. Blood cells. Immunology, 4:38-48.

Bird,F.H. (1978). The effect of aflatoxin B_1 on the utilization of cholecalciferol by chicks. Poultry Science, 57: 1293-1296.

Bitaý,Z., Glavits,R. and Sellyey,G. (1979). Feeding experiments on broiler chickens with ochratoxin A, patulin, T-2 and butenolide. Magyar Allatorvosok Lapja (1979) 34:417-422.

Björklund,N.-E. and Erne,K. (1971). Phenoxy-acid-induced renal changes in the chicken. I. Ultrastructure. Acta Veterinaria Scandinavica, 12:243-256.

Blount,W.P. (1961). Turkey "X" disease. Turkeys, (Journal of British Turkey Federation), 9:52,55-58,61,77.

Blythman,H.E. and White,R.G. (1977). Effect of early bursectomy on germinal centre and immunoglobulin production in chickens. Immunology, 33:671-677.

Bodnarchuk,A.I. and Kaspruk,I.M. (1984). (Ochratoxicoses in ducks). Veterinariya, Moscow, 1984, 2:67-68. Abstracted in Poultry Abstracts (1984), 10:135. Abstract No.1092.

Bolton,W.K., Tucker,F.L. and Sturgill,B.C. (1980). Experimental autoimmune glomerulonephritis in chickens. Journal of Clinical and Laboratory Immunology, 3:179-184.

Boyd,R.L. and Ward,H.A. (1978). Lymphoid antigenic determinants of the chicken. Cellular representation and tissue localization. Immunology, 34:9-17.

Brandtzaeg,P. (1981). Prolonged incubation time in histochemistry: Effect on fluorescence staining of immunoglobulins and epithelial components in ethanol - and formaldehyde - fixed paraffin-embedded tissues. Journal of Histochemistry and Cytochemistry, 29:1302-1315.

Britton,W.M. and Wyatt,R.D. (1978). Effect of dietary aflatoxin on vitamin D_3 metabolism in chicks. Poultry Science, 57:163-165.

Brown,M.H., Szczech,G.M. and Purmalis,B.P. (1976). Teratogenic and toxic effects of ochratoxin A in rats. Toxicology and Applied Pharmacology, 37:331-338.

Brown,R.F. (1970). Some bioassay methods for mycotoxins. In : Herzburg,M. (Ed.), Toxic Micro-organisms - UJNR - USDI, Washington, D.C. p.12.

Buckle,A.E. (1982). The occurrence of mycotoxins in barley stored on farms in England and Wales. Proceedings of 4th Meeting on Mycotoxins in Animal Disease. Weybridge, April, 1981, pp.41-43.

Buckle,A.E. (1983). The occurrence of mycotoxins in cereals and animal feedstuffs. Veterinary Research Communications, 7:171-186.

Buckley,H.G. (1971). Fungal nephrotoxicity in Swine. Irish Veterinary Journal, 25:194-196.

Bunge,I., Dirheimer,G. and Röschenthaler,R. (1978). In vivo and in vitro inhibition of protein synthesis in Bacillus stearothermophilus by ochratoxin A. Biochemical and Biophysical Research Communications, 83:398-405.

Burger,D.R. and Vetto,R.M. (1982). Vascular endothelium as a major participant in T-lymphocyte immunity. Cellular Immunology, 70:357-361.

Burns,R.B. and Maxwell,M.H. (1981). Probable occurrence of IgE in the adult domestic fowl (Gallus domesticus) after horse serum inoculation. Veterinary Research Communications, 5:67-72.

Campbell,M.L., Jr., May,J.D., Huff,W.E. and Doerr,J.A. (1983). Evaluation of immunity of young broiler chickens during simultaneous aflatoxicosis and ochratoxicosis. Poultry Science, 62: 2138-2144.

Carlton,W.W. and Krogh,P. (1979). Ochratoxins, a review. In: Shimoda,W. (Ed.), Proceedings of Conference on Mycotoxins in Animal Feeds and Grains Related to Animal Health. BVM, FDA, Rockville, pp.165-287.

Carlton,W.W. and Tuite,J. (1970). Nephropathy and oedema syndrome induced in miniature swine by corn cultures of Penicillium viridicatum. Pathologia Veterinaria, 7:68-70.

Carlton,W.W., Tuite,J. and Mislivec,P. (1968). Investigations of the toxic effects in mice of certain species of Penicillium. Toxicology and Applied Pharmacology. 13:372.

CAST (1979). Aflatoxin and other mycotoxins : an agricultural prospective. Council for Agricultural Science and Technology. Report No. 80, pp.56.

Caxton-Martins,A.E. and Daimon,T. (1976). Histochemical observations on chicken blood and bone marrow cells. Journal of Anatomy, 122:553-558.

Chand,N., Carlson,H.C. and Eyre,P. (1976). Passive cutaneous anaphylaxis in the domestic fowl. International Archives of Allergy and Applied Immunology, 57: 508-517.

Chang,C.-F. (1980). Survey for Ochratoxin A in mixed feeds (Abstract). Scientific Research Abstracts in Republic of China, 1:657-658. Abstracted in Nutrition Abstracts and Reviews (1982), 52:253, Abstract No.2128.

Chang,C.-F., Doerr,J.A. and Hamilton,P.B. (1981). Experimental ochratoxicosis in turkey poults. Poultry Science, 60:114-119.

Chang,C.-F. and Hamilton,PB. (1980). Impairment of phagocytosis by heterophils from chickens during ochratoxicosis. Applied and Environmental Microbiology, 39:572-575.

Chang,C.-F., Huff,W.E. and Hamilton, P.B. (1979). A leucocytopenia induced in chickens by dietary ochratoxin A. Poultry Science, 58:555-558.

Chauhan,H.V.S. and Verma,K.C. (1983). Evaluation of cell-mediated immunity to Marek's disease. British Veterinary Journal, 139:6-14.

Chelkowski,J. and Golinski,P. (1982). Mycotoxins and toxinogenic fungi in mixed feeds and their cereal components. Proceedings of V International IUPAC Symposium on Mycotoxins and Phycotoxins, Vienna, 1982, pp.68-71.

Choudhury,H. and Carlson,C.W. (1973). The lethal dose of ochratoxin for chick embryos. Poultry Science, 52:1202-1203.

Choudhury,H., Carlson,C.W. and Semeniuk,G. (1971). A study of ochratoxin toxicity in hens. Poultry Science, 50:1855-1859.

Christensen,C.M., Fanse,F.A., Nelson,G.H., Bates,F. and Mirocha,C.J. (1967). Microflora of black and red peppers. Applied Microbiology, 15:622-626.

Christensen,C.M. and Kaufmann,H.H. (1969). Grain storage: the role of fungi in quality loss. University of Minnesota Press, Minneapolis. p.82.

Christensen,C.M., Nelson,G.H., Mirocha,C.J. and Bates,F. (1968). Toxicity of experimental animals of 943 isolates of fungi. Cancer Research, 28:2293-2295.

Chu,F.S. (1971). Interaction of ochratoxin A with bovine serum albumin. Archives of Biochemistry and Biophysics, 147:359-366.

Chu,F.S. (1974). Studies on ochratoxins. CRC Critical Reviews on Toxicology, 2:499-524.

Chu,F.S. and Butz,M.E. (1970). Spectrophotofluoro-densitometric measurement of ochratoxin A in cereal products. Journal of the Association of Official Analytical Chemists, 53:1253-1257.

Chu,F.S. and Chang,C.C. (1971). Sensitivity of chicks to ochratoxins. Journal of the Association of the Official Agricultural Chemistry, 54:1032-1034.

Chu,F.S., Chang,F.C.C. and Hinsdill,R.D. (1976). Production of antibody against ochratoxin A. Applied and Environmental Microbiology, 31:831-835.

Ciegler,A., (1972). Bioproduction of ochratoxin A and penicillic acid by members of the Aspergillus ochraceus group. Canadian Journal of Microbiology , 18:631-636.

Ciegler,A. and Bennett,J.W.(1980). Mycotoxins and mycotoxicoses. Bio Science, 30:512-515.

Ciegler,A., Burmeister,H.R. and Vesonder,R.F. (1983). Poisonous fungi : mycotoxins and mycotoxicosis. In: Howard, D.H. (Ed.), Fungi pathogenic for human and animals. Part B. Pathogenicity and detection. Volume I., Marcel Dekker, New York, pp.413-469.

Ciegler,A., Fennell,D.I., Mintzlaff,H.J. and Leistner,L. (1972). Ochratoxin synthesis by Penicillium species. Die Naturwissenschaften, 59:365-366.

Ciegler,A., Kadis,S. and Ajl,S.J. (1971). Microbial Toxins, VI. Fungal Toxins. Academic Press, New York and London.pp.179-205.

Ciegler,A. and Lillehoj,E.B. (1968). Mycotoxins. Applied Microbiology, 10:155-219.

Clarke,J.H.C. and Niles, E.V. (1978). Fungi mycotoxins detected in samples from the infestation control service, 1976-77 and recent laboratory experiments on Mycotoxins at Pest Infestation Control Laboratory, Slough (U.K.), pp. 8-12.

Colin,R.B. and Dvorak,H.F. (1979). Role of granulocytes in cell-mediated immunity. In: Ward,P., McClusky, T. and Cohen,S. (Eds.), Mechanisms of Immunopathology. Wiley, New York, pp.69-89.

Connole,M.D., Blaney,B.J. and McEwan,T. (1981). Mycotoxins in animal feeds and toxic fungi in Queensland 1971-80. Australian Veterinary Journal, 57:314-318.

Coons,A.H., Leduc,E.H. and Connoly,J.M. (1955). Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. Journal of Experimental Medicine, 102:49-60.

Cooper,P. (1979). The kidney and ochratoxin A. Food and Cosmetics Toxicology, 17:406-408.

Cooper, S.J., Wood, G.M., Chapman, W.B. and Williams, A.P. (1982). Mycotoxins occurring in mould damaged foods. Proceedings of V International IUPAC Symposium on Mycotoxins and Phycotoxins, Vienna, pp.64-67.

Creppy,E.-E., Lorkowski,G., Röschenthaler,R. and Dirheimer, G. (1982). Kinetics of the immunosuppressive action of ochratoxin A on mice. Proceedings of V International Symposium on Mycotoxins and Phycotoxins, Vienna, pp.289-292.

Creppy,E.-E., Lugnier,A.A.J., Beck,G., Röschenthaler, R. and Dirheimer,G. (1979a). Action of ochratoxin A on cultured hepatoma cells - reversion of inhibition by phenylalanine. FEBS Letters, 104:287-290.

Creppy,E.-E., Lugnier,A.A.J., Fasiolo,F., Heller,K., Röschenthaler,R. and Dirheimer,G. (1979b). In vitro inhibition of yeast phenylalanyl - t RNA synthetase by ochratoxin A. Chemico - Biological Interactions, 24: 257-261.

Creppy,E.-E., Størmer,F.C., Röschenthaler,R. and Dirheimer, G. (1983). Effects of two metabolites of ochratoxin A, (4R)-4-hydroxyochratoxin A and ochratoxin α on immune response in mice. Infection and Immunity, 39:1015-1018.

Culling,C.F.A. (1966). Handbook of histopathological techniques. 2nd Ed., Butterworth, London. pp.173-272.

Dallegri,F., Frumento,G., Maggi,A. and Patrone,F. (1983) PHA-induced neutrophil-mediated cytotoxicity. Journal of Laboratory and Clinical Immunology, 11:203-206.

David,H. and Kettler,L.H. (1961). Degeneration von lebermitochondrien nach ammonium-intoxikation. Zeitschrift für Mikroscopische Anatomie, 53:857-866.

de Duve,C. and Wattiaux,R. (1966). Functions of lysosomes. Annual Review of Physiology, 28:435-492.

Dighe,K.K. and Hunter,W.M. (1974). A solid phase radioimmunoassay for plasma progesterone. Biochemical Journal, 143:219-231.

Doerr,J.A. and Campbell,M.L., Jr. (1982). Interaction between dietary citrinin and ochratoxin A in broiler chickens. Poultry Science, 61:1453 (Abstract).

Doerr,J.A., Huff,W.E., Hamilton, P.B. and Lillehoj,E.B. (1981). Severe coagulopathy in young chickens produced by ochratoxin A. Toxicology and Applied Pharmacology, 59:157-163.

Doerr,J.A., Huff,W.E.,Tung,R.D., Wyatt,R.D. and Hamilton, P.B. (1974). A survey of T-2 toxin, ochratoxin and aflatoxin for their effects on the coagulation of blood in young broiler chickens. Poultry Science, 53:1728-1734.

Dohms,J.E. and Saif, Y.M. (1984). Criteria for evaluating immunosuppression. Avian Diseases, 28: 305-310.

Doster,R.C., Arscott,G.H. and Sinnhuber,R.O. (1973). Comparative toxicity of ochratoxin A and crude Aspergillus ochraceus culture extract in Japanese quail (Coturnix coturnix japonica). Poultry Science, 52:2351-2353.

Doster,R.C. and Sinnhuber,R.O. (1972). Comparative rates of hydrolysis of ochratoxin A and B in vitro. Food and Cosmetics Toxicology, 10:389-394.

Doupnik,B. and Bell,D.K. (1971). Toxicity to chicks of Aspergillus and Penicillium species isolated from mouldy pecans. Applied Microbiology, 21:1104-1106.

Doupnik,B.,Jr. and Peckham,J.C. (1970). Mycotoxicity of Aspergillus ochraceus to chicks. Applied Microbiology, 19:594-597.

Drury,R.A.B.,Wallington,E.A. and Cameron,S.R. (1976) Carleton's histological technique. 4th Ed., Oxford University Press, London, NewYork and Toronto. pp.138-147.

Duff,S.R.I. (1979). Induced abnormal bone growth with particular reference to the growth plate. Ph.D. Thesis, University of Glasgow.

Dvorak,H.F., Colvin,R.B. and Churchill,W.H. (1975). Specificity of basophils and lymphocytes in cutaneous basophil hypersensitivity. Journal of Immunology, 114:507-511.

Dvorak,H.F., Galli,S.J. and Dvorak,A.M. (1980). Expression of cell-mediated hypersensitivity *in vivo* - recent advances. International Review of Experimental Pathology, 21:119-194.

Eaker,D. and Wadström,T. (1979). Natural Toxins. Proceedings of 6th International Symposium on Animal, Plant and Microbial Toxins, Uppsala, Sweden. Pergamon Press Ltd. Oxford, pp.691-699.

Elling,F. (1977a). Morphological aspects of mycotoxic nephropathy. In: Rodricks, J.V., Hesseltine,C.W. and Mehlman,M.A. (Eds.), Mycotoxins in human and animal health. Panthotox Publishers Inc., Illinois, pp.499-506.

Elling,F. (1977b). Demonstration of ochratoxin A in kidneys of pigs and rats by immunofluorescence microscopy. Acta pathologica et Microbiologica Scandinavica, Section A, 85:151-156.

Elling,F. (1979). Ochratoxin A - induced mycotoxic porcine nephropathy - alterations in enzymic activity in tubular cells. Acta Pathologica et Microbiologica Scandinavica, Section A,87:237-243.

Elling,F. (1981). Ochratoxin A - induced porcine nephropathy : structural changes and pathogenesis. DSR Forlag, Copenhagen, pp.1-32.

Elling,F., Hald,B., Jacobsen,C. and Krogh,P. (1975). Spontaneous toxic nephropathy in poultry associated with ochratoxin A. Acta Pathologica et Microbiologica Scandinavica, Section A, 83:739-741.

Elling,F. and Møller,T. (1973). Mycotoxic nephrophathy in pigs. Bulletin of the World Health Organisation, 49:411-418.

Escher,F.E., Köehler,P.E. and Ayres,J.C. (1973). Production of Ochratoxins A and B on country cured ham. Applied microbiology, 26:27-30.

Fischback,H. and Rodricks,J.H. (1973). Current efforts of the Food and Drug Administration to control mycotoxins in food. Journal of the Association of Official Analytical Chemists, 56:767-770.

Floyd,K., Suter,P.F. and Lutz,H, (1983). Granules of blood eosinophils are stained directly by antiglobulin fluorescein isothiocyanate conjugates. American Journal of Veterinary Research, 44:2060-2063.

Frangioni,G. and Borgioli,G. (1979). Polystyrene embedding : a new method for light and electron microscopy. Stain Technology, 54:167-172.

Fritz,J.C. and Roberts,T. (1968). Use of toe ash as a measure of calcification in the chick. Journal of the Association of official Analytical Chemists, 51: 591-594.

Galtier,P. (1975). Metabolisme et mode d'action d'ochratoxine A. In: Les Mycotoxines. Edition INSERM, PARIS, pp.69-78.

Galtier,P., Alvinerie,M. and Charpenteau,J.L. (1981). The pharmacokinetic profiles of ochratoxin A in pigs, rabbits and chickens. Food and Cosmetics Toxicology, 19:735-738.

Galtier,P., Moré,J. and Alvinerie,M. (1976). Acute and short-term toxicity of ochratoxin A in 10-day-old chicks. Food and Cosmetics Toxicology, 14:129-131.

Galtier,P., Moré,J. and Bodin,G. (1974). Toxines D'Aspergillus ochraceus Wilhelm. III. Toxicite aigue de l'ochratoxine A chez le rat et la souris adultes. Annales de Recherches Vétérinaires, 5:233-247.

Gamble,C.N. (1966). The effect of phytohaemagglutinin on mouse spleen cells in vivo. Blood, 28:175.

Gedek,B. (1972). Biologischer Nachweis von Mykotoxinen. Zentralblatt für Veterinarmedizin, B,19:15-32.

Gedek,B.,Huttner,B., Kahlau,D., Köhler,H. and Vielitz,E. (1978). (Rickets in broilers after contamination of the feed with Fusarium moniliformae Sheldon. 1. Field observations, reproduction of disease picture and treatment studies). Zentralblatt für Veterinar Medizin, B,25:29-33.

Ghadially,F.N. (1982). Ultrastructural Pathology of the Cell and Matrix, 2nd Ed. Butterworth, London, pp. 80-83,196,222-225,581-590.

Giambrone,J.J., Ewert,D.L., Wyatt,R.D. and Eidson,C.S. (1978). Effect of aflatoxin on the humoral and cell-mediated immune systems of the chicken. American Journal of Veterinary Research, 39:305-308.

Gilani,S.H., Bancroft,J. and Reily,M. (1978). Teratogenicity of OA in chick embryos. Toxicology and Applied Pharmacology, 46:543-546.

Goldblatt,L.A. (1969). Aflatoxin - Scientific background, control, and implications. Academic Press, New York and London, pp.1-11.

Goodfriend,T.L., Levine,L. and Fasman,G.D. (1964). Antibodies to bradykinin and angiotensin: A use of carbodiimides in immunology. Science, 144:1344-1346.

Goto,N., Kodama,H., Okada,K. and Fujimoto,Y. (1978). Suppression of phytohaemagglutinin skin response in thymectomised chickens. Poultry Science, 57:246-250.

Groenewegen,G. and Buurman,W.A. (1984). Vascular endothelial cells present alloantigens to unprimed lymphocytes. Scandinavian Journal of Immunology, 19:269-273.

Guglielmotti,M.B., Ubios,A.M., de Rey,B.M. and Cabrini, R.L. (1984). Effects of acute intoxication with uranyl acetate on bone formation. Experientia, 40: 474-476,

Gupta,M., Bandopadhyay,S., Mazumdar,S.K. and Paul,B. (1980). Ovarian steroidogenesis in rats following ochratoxin A treatment. Toxicology and Applied Pharmacology, 53:515-520.

Gylstorff, I. and Rolf,J. (1978). Zur diagnose von Mycotoxikosen bei Junghühnern. Proceedings and Abstracts of XVI World's Poultry Congress, Brazil, 1978, vol.III-D.3,pp.333-335.

Halama,A.K. (1981). (Mycotoxin-induced impairments of skeletal mineralization in young broiler chickens.) Wiener Tierarztliche Monatsschrift, 68:196-200.

Hald,B., and Krogh,P., (1972). Residues of ochratoxin A in bacon pigs. Proceedings of IUPAC Symposium on Control of Mycotoxins, Kungälv, Sweden, p.18.

Hamilton,P.B., Huff,W.E., Harris,J.R. and Wyatt,R.D. (1982). Natural occurrences of ochratoxicosis in poultry. Poultry Science, 61:1832-1841.

Hanker,J.S., Yates,P.E., Metz,C.B. and Rostioni,A. (1977). A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochemical Journal, 9:789-792.

Harwig,J. (1974). Ochratoxin A and related metabolites. In: Purchase,I.F.H. (Ed.), Mycotoxins. Alsevier Scientific Publishing Company, Oxford, pp.345-367.

Harwig,J., Chen,Y.-K. and Collins-Thompson,D.L. (1974). Stability of ochratoxin A in beans during canning. Canadian Institute of Food Science and Technology Journal, 7:288-289.

Haubeck,H.-D., Lorkowski,G., Kölsch,E. and Röschenthaler, R. (1981). Immunosuppression by ochratoxin A and its prevention by phenylalanine. Applied and Environmental Microbiology, 41:1040-1042.

Hayes,A.W. (1981). Mycotoxin teratogenicity and mutagenicity. CRC Press, Boca Raton, Florida. pp.47-49.

Hayes,A.W., Hood,R.D. and Lee,H.L. (1974). Teratogenic effects of ochratoxin A in mice. Teratology, 9:93-97.

Heller,K. and Röschenthaler,R. (1978). Inhibition of protein synthesis in Streptococcus faecalis by ochratoxin A. Canadian Journal of Microbiology, 24:466-472.

Hesseltine,C.W., Vandegraft,E.I., Fennell,D.L., Smith,M.C. and Shotwell,O.L. (1972). Aspergilli as ochratoxin producers. Mycologia, 64:539-550.

Heyndrickx,A. (1984). Scientists in turmoil over yellow rain. (cited by Wilkie,T.). New Scientist, 95:3,8.

Hoffman-Fezer,G., Rodt,H., Gotze,D. and Thierfelder,S. (1977). Anatomical distribution of T- and B-lymphocytes identified by immunochemistry in the chicken spleen. International Archives of Allergy and Applied Immunology, 55:86-95.

Hökby,E., Hult,K., Gatenbeck,F. and Rutqvist,L. (1979). Ochratoxin A and citrinin in 1976 crop of barley stored on farms in Sweden. Acta Agriculturae Scandinavica, 29:174-178.

Hood,R.D., Naughton,M.J. and Hayes,A.W. (1976). Prenatal effects of ochratoxin A in hamsters. Teratology, 13:11-14.

Howell,M.V. (1982). Mycotoxin monitoring in the feed industry. In: Pepin,G.A., Patterson,D.S.P. and Gray, D.E., (Eds.), Proceedings of a fourth meeting on Mycotoxins in Animal Disease, April, 1981, Weybridge, Ministry of Agriculture, Fisheries and Food, pp.44-45.

Huff,W.E. (1980a). Discrepancies between bone ash and toe ash during aflatoxicosis. Poultry Science, 59: 2213-2215.

Huff,W.E. (1980b). Evaluation of tibial dyschondroplasia during aflatoxicosis and feed restriction in young broiler chickens. Poultry Science, 59:991-995.

Huff,W.E., Chang, C.-F., Garlich,J.D. and Hamilton,P.B. (1977). Aflatoxicosis in chickens fed diets varying in calcium and phosphorus. Poultry Science, 56: 1724 (Abstract).

Huff,W.E., Chang,C.-F., Warren,M.F. and Hamilton,P.B. (1979a). Ochratoxin A-induced iron deficiency anaemia. Applied and Environmental Microbiology, 37:601-604.

Huff,W.E. and Doerr,J.A. (1981). Synergism between Aflatoxin and ochratoxin in broiler chickens. Poultry Science, 60:550-555.

Huff,W.E., Doerr,J.A. and Hamilton, P.B. (1979b). Decreased glycogen mobilization during ochratoxicosis in broiler chickens. Applied and Environmental Microbiology, 37:122-126.

Huff,W.E., Doerr,J.A., Hamilton,P.B., Hamann,D.D., Peterson,R.E. and Ciegler,A. (1980). Evaluation of bone strength during aflatoxicosis and ochratoxicosis. Applied and Environmental Microbiology, 40:102-107.

Huff,W.E., Doerr,J.A., Wabeck,C.J., Chaloupka,G.W., May, J.D. and Merk ley,J.W. (1983). Individual and combined effects of aflatoxin and ochratoxin A and bruising in broiler chicken. Poultry Science, 62:1764-1771.

Huff,W.E. and Hamilton, P.B., (1974). Nephrotoxicity of ochratoxin A in broiler chicks. Poultry Science, 53: 2140-2155.

Huff,W.E. and Hamilton,P.B. (1975a). Decreased plasma carotenoids during ochratoxicosis. Poultry Science, 54:1308-1310.

Huff,W.E. and Hamilton,P.B. (1975b). The interaction of ochratoxin A with some environmental extremes. Poultry Science, 54:1659-1662.

Huff,W.E. and Ruff,M.D. (1982). Eimeria acervulina and Eimeria tenella infections in ochratoxin A- compromised broiler chickens. Poultry Science, 61:685-692.

Huff,W.E., Wyatt, R.D. and Hamilton,P.B. (1975). Nephrotoxicity of dietary ochratoxin A in broiler chickens. Applied Microbiology, 30:48-51.

Huff,W.E., Wyatt,R.D., Tucker,T.L. and Hamilton,P.B. (1974). Ochratoxicosis in the broiler chicken. Poultry Science, 53:1585-1591.

Hult,K., Teiling,A. and Gatenbeck,S. (1976). Degradation of ochratoxin A by ruminants. Applied and Environmental Microbiology, 32:443-444.

Hurtado,I. and Urbina,C. (1983). Presence of basophils in PHA-reacted skin sites of mice: an electron microscopic study. International Archives of Allergy and Applied Immunology, 72:116-118.

Hutchinson,R.D., Steyn,P.S. and Thompson,D.C. (1971) The isolation and structure of 4-hydroxyochratoxin A and 7-carboxy-3,4-dihydro-8-hydroxy-3-methyl isocoumarin from Penicillium viridicatum. Tetrahydron Letters, 43:4033-4036.

IARC (1976). The evaluation of the carcinogenic risk of chemicals to man: some naturally occurring substances. International Agency for Research on Cancer. Monograph, Lyon.,p.191-197.

Ichinoe,M., Udagawa,S.-S., Tazawa,M. and Kurata,H. (1970). Some considerations in a biological method for the detection of mycotoxins in Japanese foods. In: Herzburg, M.(Ed.), Toxic Micro-organisms,UJNR-USDI, Washington, D.C. p.191.

Jagatic,J. and Weiskopf,R. (1966). A fluorescent method for staining mast cells. Archives of Pathology, 82:430-431.

Jemmali,M. (1978). Mycotoxins. III. International Symposium on Mycotoxins in Food stuffs. Editions du CNRS, Paris.

Jensen,L.S. and Martinson,R. (1969). Requirements of turkey poults for biotin and effect of deficiency of incidence of leg weakness in developing turkeys. Poultry Science, 48:222-230.

Joffe,A.Z. (1977). Fusarium toxicosis in poultry. In: Wyllie,T.D. and Morehouse,L.G. (Eds.), Mycotoxic fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbood. Volume 2. Marcel Dekker, Inc., New York and Basel, pp.309-321.

Juszkiewicz,T. and Piskorska-Pliszczyńska,J. (1976). (Occurrence of aflatoxin B_1,B_2,G_1 and G_2, ochratoxin A and B; sterigmatocystin and zearalenone in cereals). Medycyna Weterynaryjna, 32:617-619.

Juszkiewicz,R. and Piskorska-Pliszczyńska,J. (1977). (Occurrence of mycotoxins in mixed feed and concentrates). Medycyna Weterynaryjna, 33:193-196.

Juszkiewicz,T., Piskorska-Pliszczyńska,J. and Wisniewska,H. (1982). Ochratoxin A in laying hens: tissue deposition and passage into eggs. Proceedings of the V International IUPAC symposium on Mycotoxins and Phycotoxins, Vienna, pp.122-125.

Kanisawa,M. and Suzuki,S. (1978). Induction of renal and hepatic tumours in mice by ochratoxin A. Gann (Tokyo), 69:599.

Karlson,A.G., Thoen,C.O. and Harrington,R. (1970). Japanese quail: susceptibility to avian tuberculosis. Avian Diseases, 14:39-44.

Kitchen,D.N., Carlton,W.W. and Hinsman,E.J. (1977a). Ochratoxin A and citrinin-induced nephrosis in Beagle dogs. III. Terminal renal ultrastructural alterations. Veterinary Pathology, 14:392-406.

Kitchen, D.N., Carlton,W.W. and Sansing,G.N. (1974). Ochratoxin A and citrinin-induced nephropathy in Beagle dogs. Toxicology and Applied Pharmacology, 33:156.

Kitchen,D.N., Carlton,W.W. and Tuite,J. (1977b). Ochratoxin A and citrinin-induced nephrosis in Beagle dogs. I. Clinical and clinicopathological features. Veterinary Pathology, 14:154-172.

Kitchen,D.N., Carlton,W.W. and Tuite,J. (1977c). Ochratoxin A and citrinin-induced nephrosis in Beagle dogs. II. Pathology. Veterinary Pathology,14:261-272.

K linkert,W., Lorkowski,G., Creppy, E.-E., Dirheimer,G. and Röschenthaler,R. (1981). Inhibition of macrophage migration by ochratoxin A and citrinin, and prevention by phenylalanine of the ochratoxin A-induced inhibition. Toxicological European Research, 3:187-189.

Koch,O.R., Roatta, deConti,L.L. Bolaños,L.P. and Stoppani,A.O.M. (1978). Ultrastructural and biochemical aspects of liver mitochondria during recovery from ethanol-induced alterations: experimental evidence of mitochondrial division. American Journal of Pathology, 90:35-44.

Köhler,H., Huttner,B., Vielitz,E., Kahlau,D. and Gedek, B. (1978). (Rickets in broilers after contamination of the feed with Fusarium moniliforme.2.Histological and mycotoxicological study). Zentralblatt für Veterinar Medizin, B,25:89-109.

Konrad,I. and Röschenthaler,R. (1977). Inhibition of phenylalanine TRNA synthetase from Bacillus subtilis by ochratoxin A. FEBS Letters, 83:341-347.

Krogh,P. (1976). Mycotoxic nephropathy. Advances in Veterinary Science and Comparative Medicine, 20:147-170.

Krogh,P. (1977a). Ochratoxin A residues in tissues of slaughter pigs with nephropathy. Nordisk Veterinaer-Medicin., 29:402-405.

Krogh,P. (1977b). Mycotoxic nephropathy. In: Wyllie,T.D. and Morehouse,L.G. (Eds.), Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. - An Encyclopedic Handbood. Volume, 2. Marcel. Dekker, Inc., New York and Basel, pp.236-256.

Krogh,P. (1978). Causal associations of mycotoxic nephropathy. Acta Pathologica et Microbiologica Scandinavica, Section A, Supplement No.269, pp.1-28.

Krogh,P. (1980). Ochratoxins: Occurrence, biological effects and causal role in diseases. In: Eaker,D. and Wadström,T. (Eds.), Natural Toxins. Pergamon Press, Oxford, pp.673-680.

Krogh,P. (1982). Surveillance and occurrence of mycotoxins in animal feed. In: Pepin,G.A., Patterson,D.S.P. and Gray, D.E. (Eds.), Proceedings of a fourth meeting on Mycotoxins in Animal Disease. Ministry of Agriculture, Fisheries and Food, p.53.

Krogh,P., Axelsen,N.H., Elling,F., Gyrd Hansen,N., Hald, B., Hyldgaard-Jensen,J., Larsen,A.E., Madsen,A., Mortensen,H.P., Møller,T., Petersen,O.K., Ravnskov,U., Rostgaard,M. and Aalund,O. (1974a). Experimental porcine nephropathy: changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Pathologica et Microbiologica Scandinavica, Section A, Supplement No.246,pp.1-21.

Krogh,P., Elling,F., Hald,B., Jylling,B., Petersen,V.E., Skadhauge,E. and Svendsen,C.K. (1976a). Experimental avian nephropathy. Changes of renal function and structure induced by ochratoxin A-contaminated feed. Acta Pathologica et Microbiologica Scandinavica, Section A, 84:215-221.

Krogh,P., Elling,F., Hald,B., Larsen,A.E., Lillehoj, E.B., Madsen,A. and Mortensen,H.P. (1976b). Time-dependent disappearance of ochratoxin A residues in tissues of bacon pigs. Toxicology,6:235-242.

Krogh,P., Hald,B., England,L., Rutqvist,L. and Swahn O. (1974b). Contamination of Swedish cereals with ochratoxin A. Acta Pathologica et Microbiologica Scandinavica, Section B, 82:301-302.

Krogh,P., Hald,B. and Pedersen,H.E. (1973). Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Pathologica et Microbiologica Scandinavica, Section B, 81:689-695.

Krogh,P., Hald,B., Plestina,R. and Ceovic,S. (1977). Balkan (endemic)nephropathy and foodborne ochratoxin A: Preliminary results of a survey of foodstuffs. Acta Pathologica et Microbiologica Scandinavica, Section B, 85:238-240.

Krogh,P. and Hasselager,E. (1968). Studies on nephrotoxicity. Royal Veterinary Agricultural College, Yearbook, Copenhagen,pp.198-214.

Kubena,L.F., Phillips,F.D., Creger,C.R., Witzel,D.A. and Heidelbaugh,N.D. (1983). Toxicity of ochratoxin A and tannic acid to growing chicks. Poultry Science, 62: 1786-1792.

Kubena,L.F., Phillips,T.D., Witzel,D.A. and Heidelbaugh, N.D. (1984). Toxicity of ochratoxin A and penicillic acid to chicks. Bulletin of Environmental Contamination and Toxicology, 32:711-716.

Lai,M., Semeniuk,G. and Hesseltine,C.W. (1968). Nutrients affecting ochratoxin A production by Aspergillus Spp. Phytopathology, 58:1056 (Abstract).

Lasley,J.A. (1980). An assessment of cell-mediated immune responses in Japanese quail. M.S. Thesis. North Carolina State University, U.S.A.

Leslie,G.A. and Benedict,A.A. (1970). Structural and antigenic relationhsip between avian immunoglobulins. III. Antigenic relationship of the immunoglobulins of the chicken, pheasant and Japanese quail. Journal of Immunology, 105:1215-1222.

Levi,C.P., Trenk,H.L. and Mohr,K.H. (1974). Study of occurrence of ochratoxin A in green coffee beans. Journal of the Association of Official Analytical Chemists, 57:866-870.

Lillie,R.D. (1965). Histopathologic Technic and Practical Histochemistry, 3rd Ed., McGraw-Hill Book Company, New York, Toronto, Sydney, London. pp.176-177,626-637.

Lindsay,D.G. (1976). Food surveillance in the United Kingdom and abroad. Reports of ADAS Microbiologists. Ministry of Agriculture, Fisheries and Food, U.K. p.13.

Lindsay,D.G. (1978). Food surveillance in the U.K. and abroad. Reports of ADAS Microbiologists. Ministry of Agriculture, Fisheries and Food, U.K. pp.13-16.

Lindsay,D.G. (1981). Hazards from mycotoxins in the food chain. Proceedings of a seminar 'Mycotoxins in Animal Feedstuffs' held at the Metropole Hotel, LLandrindadwells, March 25-26, 1981. Ministry of Agriculture, Fisheries and Food (ADAS). Agricultural Science and Veterinary Services, Wales and Midlands and Western Region, pp.10-20.

Lucas,A.M. and Jamroz,C. (1961). Atlas of Avian haematology. United States Department of Agriculture, Washington, D.C. p.230.

Luger,T.A., Charon,J.A., Colot,M., Micksche,M. and Oppenheim,J.J. (1983a). Chemotactic properties of partially purified human epidermal cell-derived thymocyte-activating factor (ETAF) for polymorphonuclear and mononuclear cells. Journal of Immunology, 131:816-820.

Luger,T.A., Sztein,M.B., Schmidt,J.A., Murphy,P., Grabner,G. and Oppenheim,J.J. (1983b). Properties of murine and human epidermal cell-derived thymocyte-activating factor. Federation Proceedings, 10:2772-2776.

Luoma,B. and Benedict,A.A. (1977). Arthus reaction in chickens. Developmental and Camparative Immunology, 1:33-40.

Madsen,A., Hald,B., Lillehoj,E. and Mortensen,H.P. (1982a). Feeding experiments with ochratoxin A-contaminated barley for bacon pigs. 2. naturally contaminated barley given for 6 weeks from 20kg compared with normal barley supplemented with crystalline ochratoxin A and/or citrinin. Acta Agriculturae Scandinavica, 32:369-372.

Madsen,A., Mortensen,H.P. and Hald,B. (1982b). Feeding experiments with ochratoxin A-contaminated barley for bacon pigs. 1. Influence on pig performance and residues. Acta Agriculturae Scandinavica, 32:225-239.

Mancini,G., Carbonara,A.O. and Heremans, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, 2:235-254.

Manning,R.O. and Wyatt,R.D. (1984). Toxicity of Aspergillus ochraceus contaminated wheat and different chemical forms of ochratoxin A in broiler chicks. Poultry Science, 63:458-465.

Maurice,D.V., Bodine,A.B. and Rehrer,N.J. (1983). Metabolic effects of low aflatoxin B_1 levels on broiler chicks. Applied and Environmental Microbiology, 45:980-984.

Maxwell, M.H. (1978). Safer substitutes for xylene and propylene oxide in histology, haematology and electron microscopy. Medical Laboratory Sciences, 35: 401-403.

Maxwell,M.H. (1981). The production of a Heinz body anaemia in the domestic fowl after the ingestion of dimethyl disulphide: a haematological and ultrastructural study. Research in Veterinary Science, 31: 113-115.

Maxwell,M.H. (1984). The histochemical identification of tissue eosinophils in the inflammatory response of the fowl (Gallus domesticus). Research in Veterinary Science, 37:7-11.

Maxwell,M.H. and Trejo,F. (1970). The ultrastructure of white blood cells and thrombocytes of the domestic fowl. British Veterinary Journal, 126:583-592.

McCorkle,F.M., Luginbuhl,G.H. and Simmons,D.G. (1981). Effect of Alcaligenes faecalis on cutaneous basophil hypersensitivity in neonatal turkeys. Proceedings of Conference on Research Work on Animal Diseases, 62:36 (Abstract).

McCorkle,F.M., Luginbuhl,G.H., Simmons,D.G., Morgan,G.W. and Thaxton, J.P. (1983). Ontogeny of delayed hypersensitivity in the turkey. Developmental and Comparative Immunology, 7:517-524.

McCorkle,F., Jr., Olah,I. and Glick,B. (1980). The morphology of the phytohaemagglutinin-induced cell response in the chicken's wattle. Poultry Science, 59:616-623.

McCorkle,F.M., Simmons,D.G. and Luginbuhl,G.H. (1982). Delayed hypersensitivity response in Alcaligenes faecalis - infected turkey poults. Avian Diseases, 26:782-786.

Meisner,H. (1976). Energy-dependent uptake of ochratoxin A by mitochondria. Archives of Biochemistry and Biophysics, 173:132-140.

Meisner,H. and Chan,S. (1974). Ochratoxin A, an inhibitor of mitochondrial transport systems. Biochemistry, 13:2795-2800.

Meisner,H. and Meisner,P. (1981). Ochratoxin A, an in vivo inhibitor of renal phosphoenolpyruvate carboxykinase. Archives of Biochemistry and Biophysics, 208:146-153.

Mehdi,N.A.Q., Carlton,W.W. and Tuite,J. (1981). Citrinin mycotoxicosis in broiler chickens. Food and Cosmetics Toxicology, 19:723-733.

Mehdi,N.A.Q., Carlton,W.W. and Tuite,J. (1983). Acute toxicity of citrinin in turkeys and ducklings. Avian Pathology, 12:221-233.

Mehdi,N.A.Q., Carlton,W.W. and Tuite,J. (1984). Mycotoxicoses produced in ducklings and turkeys by dietary and multiple doses of citrinin. Avian Pathology, 13:37-50.

Meisner,H. and Krogh,P. (1982). Inhibition of renal phosphoenolpyruvate carboxykinase in pigs by alimentary exposure to ochratoxin A. Proceedings of V International Symposium on Mycotoxins and Phycotoxins, Vienna, pp.342-345.

Mintzlaff,H.J., Ciegler,A. and Leistner L. (1972) Potential mycotoxin problems in mould-fermented sausage. Zeitschrift für Labensmittel-untersuchung und-Forschung, 150:133.

Mirocha,C.J. (1982). Hazards of scientific investigation. Analysis of samples implicated in biological warfare. Journal of Toxicology - Toxin Reviews, 1:199-203.

Mirocha,C.J., Pathre,S.V. and Christensen,C.M. (1979). Mycotoxins. In: Pomeranz,Y. (Ed.), Advances in Cereal Science and Technology, III; American Association of Cereal Chemists Inc., St. Paul, Minnesota. PP.159-225.

Moore,J.H. and Truelove,B. (1970). Ochratoxin A: inhibition of mitochondrial respiration. Science, 168: 1102-1103.

Moré,F. and Camguilhem,R. (1979). Effect of low doses of ochratoxin A after intratesticular injection in the rat. Experientia, 35:890-892.

Moré,J. and Galtier,P. (1974) Toxicité de l'ochratoxine A. I. Effect embryotoxique et tératogène chez le rat. Annales de Recherches Vétérinaires, 5:167-178.

Moré,J. and Galtier,P. (1975) Toxicité de l'ochratoxine A. II. Effects du traitment sur la descendance (F_1 et F_2) de rattes intoxiqués. Annales de Recherches Vétérinaires, 6:379-389.

Moré,J. and Galtier,P. (1978). Embryotoxic and teratogenic effects of ochratoxin A in rats. In: Klika, E. (Ed.) XIXth, Morphological Congress Symposia, University Karlova, Prague, pp.321-326.

Moreau,C. (1984). Vingt ans de mycotoxines en France. Les Dossiers de L'Élevage, 5:5-20.

Morgan,M.R.A., Matthew,J.A., McNerney,R. and Chan, H.W.-S. (1982). The immunoassay of ochratoxin A. Proceedings of V International IUPAC Symposium on Mycotoxins and Phycotoxins. Vienna, PP.32-35.

Moriya,O. and Ichikawa,Y. (1979). Accumulation of eosinophils and monocytes in lymphoid organs of chick embryos. II. Effect of mitogenic stimulation. Microbiology and Immunology, 23:523-531.

Moriya,O. and Ichikawa,Y. (1981). Production of an eosinophil migration factor in lymphocytes of mitogen-treated chick embryos. Microbiology and Immunology, 25:417-421.

Moriya,O. and Ichikawa,Y. (1982). Isolation of chicken eosinophils and their migration response in chick embryos. Developmental and Comparative Immunology, 6:717-726.

Mortensen,H.P., Hald,B.,Larsen,A.E. and Madsen,A. (1983). Ochratoxin A--contaminated barley for sows and piglets - Pig performance and residues in milk and pigs. Acta Agriculturae Scandinavica, 33:349-352.

Mortensen,H.P., Madsen,A. and Hald,B. (1982). Ochratoxin A in pig blood. Proceedings of 4th Meeing on Mycotoxins in Animal Disease, Weybridge, April,1981, Ministry of Agriculture, Fisheries and Food. pp.30-32.

Munk,M. and Topolko,S. (1975). Ispitivanje frekvencije mikotoksina u Krmivima. Krmiva,5:92-94. (Cited by Pepeljnjak et al., 1982).

Munro,I.B. (1982). Field observations on possible mycotoxicosis. Proceedings of 4th Meeting on Mycotoxins in Animal Disease, Weybridge, April, 1981. p.51.

Munro,I.E., Scott,P.M., Moodie,C.A. and Willes,R.F. (1973). Ochratoxin A - occurrence and toxicity. Journal of the American Veterinary Medical Association, 163:1269-1273.

National Academy of Sciences (1979). Interactions of Mycotoxins in Animal Production. National Academy of Sciences, Washington,D.C., pp.87-95.

Natori,S.S., Sakaki,S., Kurata,H.,Udagawa,S.-T., Ichinoe,M., Saito,M. and Umeda,M. (1970). Chemical and cytotoxicity survey on the production of ochratoxins and penicillic acid by Aspergillus ochraceus Wilhelm. Chemical and Pharmaceutical Bulletin, 18: 2259-2268.

Natt,M.P. and Herrick,C.A. (1952). A new blood diluent for counting the erythrocytes and leucocytes of the chicken. Poultry Science, 31:735-738.

Neely,W.C. and West,A.D. (1972). Spectroanalytical parameters of fungal metabolites. III. Ochratoxin A. Journal of the Association of Official Analytical Chemists, 55:1305-1309.

Nelson,T.S., Johnson,Z.B., Kirby,L.K. and Beasley, J.N. (1982). Digestion of dry matter and amino acids and energy utilization by chicks fed moulded corn containing mycotoxins. Poultry Science, 61:584-585.

Nesheim,S., (1971). Ochratoxins: occurrence, production, analysis and toxicity. 85th Annual Meeting of the Association of official Analytical Chemists, Wahsington D.C., p.23 (Abstract).

Newberne,P.M. (1974). The new world of mycotoxins - Animal and human health. Clinical Toxicology, 7: 161-177.

Nip,W.K. and Chu,F.S. (1979). Fate of ochratoxin A in goats. Journal of Environmental Science and Health, B,14:319-333.

Norton,D.M., Toule,G.M., Cooper,S.J., Partington,S.R. and Chapman,W.B. (1982). The surveillance of mycotoxins in human food. In: Pepin, G.A., Patterson, D.S.P. and Gray,D.E. (Eds.), Proceedings of 4th Meeing on Mycotoxins in Animal Disease, Ministry of Agriculture, Fisheries and Food, pp.77-81.

Nowicke,J.W. and Meselson,M. (1984). Yellow rain - a palynological analysis. Nature, 309:205-206.

Odend'hal,S. and Breazile, J.E. (1980). An area of T-cell localization in the cloacal bursa of White Leghorn chickens. American Journal of Veterinary Research, 41:255-258.

Osborne, B.G. (1980). The occurrence of Ochratoxin A in mouldy flour and bread. Food and Cosmetics Toxicology, 18:615-617.

Osborne,D.J., Huff, W.E., Hamilton, P.B. and Burmeister, H.R. (1982). Comparison of ochratoxin, aflatoxin and T-2 toxin for their effects on selected parameters related to digestion and evidence for specific metabolism of carotenoids in chickens. Poultry Science, 61:1646-1652.

Ottinger,M.A. and Doerr,J.A. (1982). Effect of mycotoxins on avian reproduction. In: Scanes et al., 1982 (Eds.), Aspects of Avian Endocrinology: Practical and Theoretical Implications. Graduate Studies Texas Technical University, No.26,pp.217-229.

Page,R.K., Stewart,G., Wyatt,R., Buch,P., Fletcher,O.J. and Brown,J. (1980). Influence of low levels of ochratoxin A on egg production, egg shell stains, and serum uric-acid levels in leghorn type hens. Avian Diseases, 24:777-780.

Palladino,M.A., Grebenau,M.D. and Thorbecke,G.J. (1978). Requirements for induction of delayed hypersensitivity in the chicken. Developmental and comparative immunology, 2:121-132.

Patterson,D.S.P., Shreeve,B.J., Roberts,B.A., Berrett,S., Brush,P.J., Glancy,E.M. and Krogh,P. (1981). Effect on calves of barley naturally contaminated with ochratoxin A and groundnut meal contaminated with low concentration of aflatoxin B_1. Research in Veterinary Science, 31:213-218.

Peckham,J.C. (1977). Ochratoxicosis in poultry. In: Wyllie,T.D. and Morehouse,L.(Eds.), Mycotoxic Fungi, Mycotoxins and Mycotoxicoses - An encyclopedic handbood. Volume 2. Marcel Dekker, Inc., New York and Basel, pp.301-307.

Peckham,J.C., Doupnik,B. and Jones,O.H. (1971) Acute toxicity of ochratoxins A and B in chicks. Applied Microbiology, 21:492-494.

Pegram,R.A. and Wyatt,R.D. (1981). Avian gout caused by oosporein, a mycotoxin produced by Chaetomium trilaterale. Poultry Science, 60:2429-2440.

Pegram,R.A., Wyatt,R.D. and Smith,T.L. (1982). Oosporein-toxicosis in the turkey-poult. Avian Diseases,26:47-59.

Pepeljnjak,S. and Blaževic,N. (1982). Contamination with moulds and occurrence of ochratoxin A in smoked meat products from endemic nephropathy region of Yugoslavia. Proceedings of V Internation IUPAC Symposium on Mycotoxins and Phycotoxins, Vienna, pp.102-105.

Pepeljnjak,S., Blaževic,N. and Čuljak,K. (1982a). Histopathological changes and findings of ochra-toxin A in organs of pigs, in the area of endemic nephropathy in Yugoslavia. Proceedings of V International Symposium on Mycotoxins and Phyco-toxins, Vienna, pp.346-348.

Pepeljnjak,S., Mužic,S. and Herceg,M. (1982b). Patho-genic influence of ochratoxin A and B on chicken growth. Veterinarski Arhiv, 52:235-240.

Pettersson,H. and Kiessling,K.-H.(1976). Effect of alfatoxin, ochratoxin and sterigmatocystin on micro-organisms from sheep rumen. Swedish Journal of Agricultural Research, 6:161-162.

Pier,A.C. (1973). Effects of aflatoxin on immunity. Journal of American Veterinary Medical Association, 163:1268-1269.

Pier,A.C. (1981). Mycotoxins and animal health. Advances in Veterinary Science and Comparative Medicine, 25: 185-243.

Pier,A.C., Cysewski,S.J., Richard,J.C., Baetz,A.L. and Mitchell,L. (1976). In: Proceedings of 80th Annual Meeting, U.S. Animal Health Association,pp.130-148. (Cited by Pier,A.C. 1981).

Pier,A.C., Fichtner,R.E. and Cysewski,S.J. (1977). Effects of aflatoxin on the cellular immune response. Annales de la Nutrition et de l'Alimentation, 31: 781-788.

Pier,A.C., Richard,J.L. and Cysewski,S.J. (1980a). Implications of mycotoxins in animal disease. Journal of the American Veterinary Medical Association, 176:719-724.

Pier,A.C., Richard,J.L. and Thurston,J.R. (1979). The influence of mycotoxins on resistance and immunity. In: National Academy of Sciences, Interaction of Mycotoxins in Animal Production, National Academy of Sciences, Washington,D.C.,pp.56-66.

Pier,A.C., Richard,J.L. and Thurston,J.R. (1980b). Effects of mycotoxins on immunity, and resistance of animals. In: Eaker,D. and Wadström,T. (Eds.), 'Natural Toxins.' Pergamon Press, Oxford and New York, pp.691-699.

Piskorska-Pliszczyńska,J. and Juszkiewicz,T. (1979). Tissue deposition and passage into eggs of ochratoxin A in Japanese quail. Proceedings of IUPAC Symposium on Mycotoxins and Phycotoxins, Lausanne, 1979.

Polverini,P.J., Cotran,R.S. and Sholley,M.M. (1977). Endothelial proliferation in the delayed hypersensitivity reaction: an autoradiographic study. Journal of Immunology, 118:529-532.

Prescott,C.A., Wilkie,B.N., Hunter,B. and Julian,R.J. (1982). Influence of a purified grade of pentachlorophenol on the immune response of chickens. American Journal of Veterinary Research, 43:481-487.

Prior,M.G. (1976). Mycotoxin determinations on animal feedstuffs and tissues in Western Canada. Canadian Journal of Comparative Medicine, 40:73-79.

Prior,M.G. (1978). Toxicological studies on ochratoxin A. PhD. Thesis, University of Saskatchewan, Canada.

Prior,M.G. (1981). Mycotoxins in animal feedstuffs and tissues in Western Canada, 1975-1979. Canadian Journal of Comparative Medicine, 45:116-119.

Prior,M.G., O'Neil,J.B. and Sisodia,C.S. (1978). Effects of ochratoxin A on production characteristics and hatchability of Japanese quail.Canadian Journal of Animal Science, 58:29-33.

Prior,M.G., O'Neil,J.B., Sisodia,C.S. (1980). Effect of ochratoxin A on growth response and residues in broilers. Poultry Science, 59:1254-1257.

Prior,M.G. and Sisodia,C.S. (1978). Ochratoxicosis in White Leghorn hens. Poultry Science, 57:619-623.

Prior,M.G. and Sisodia,C.S. (1982). The effects of ochratoxin A on the immune response of swiss mice. Canadian Journal of Comparative Medicine, 46: 91-96.

Prior,M.G., Sisodia,C.S. and O'Neil,J.B. (1976). Acute oral ochratoxicosis in day-old White Leghorns, turkeys and Japanese quail. Poultry Science, 55: 786-790.

Prior,M.G., Sisodia,C.S. and O'Neil,J.B. (1981). Effects of ochratoxin A on egg production, body weight and feed intake in White Leghorn hens. Poultry Science, 60: 1145-1148.

Prior,M.G., Sisodia,C.S., O'Neil,J.B. and Hrudka,F. (1979), Effect of ochratoxin A on fertility and embryo viability of Japanese quail (Coturnix coturnix japonica). Canadian Journal of Animal Science, 59: 605-609.

Purchase,H.G., Chubb,R.C. and Biggs,P.M. (1968). Effect of lymphoid leukosis and Marek's disease on the immunological responsiveness of the chicken. Journal of National Cancer Institute, 40:583-592.

Purchase,I.F.H. (1974). Mycotoxins. Alsevier Scientific Publishing Company, Oxford. pp.345-367.

Purchase,I.F.H. and Nel,W. (1967). Recent advances in research on ochratoxin. PartI. Toxicological Aspects. In: Mateles,R.I. and Wogan,G.N. (Eds.), Biochemistry of some food borne microbial toxins. M.I.T. Press, Cambridge, Massachusetts, pp.153-156.

Purchase,I.F.H. and Theron,J.J. (1968). The acute toxicity of ochratoxin A to rats. Food and Cosmetics Toxicology, 6:479-483.

Raper,K.B. and Fennell,D.I. (1965). The genus Aspergillus. Williams and Wikens, Baltimore,p.37.

Reichmann,K.G., Blaney,B.J., Connor,J.K. and Runge,B.M. (1982). The significance of aflatoxin and ochratoxin in the diet of Australian chickens. Australian Veterinary Journal, 58:211-212.

Reynolds,E.S.(1960). Cellular localisation of calcium deposition in liver of rat poisoned with carbon tetrachloride. Journal of Histochemistry and Cyto-chemistry, 8:331-332.

Reynolds,E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, 17:208-212.

Ribelin,W.E., Fukushima,K. and Still,P.(1978). Toxicity of ochratoxin to ruminants. Canadian Journal of Comparative Medicine, 42:172-176.

Ribelin,W.E., Smalley,E.B. and Strong,F.M. (1973). Effect of ochratoxin and other mycotoxins on cattle. 2nd International congress in Plant Pathology, Minneapolis, Minnesota, Abstract No.0865.

Richard,J.L., Thurston,J.R., Deyoe,B.L. and Booth,G.D. (1975a). Effect of ochratoxin and aflatoxin on serum proteins, complement activity and antibody production to Brucella abortus in guinea pigs. Applied Microbiology, 29:27-29.

Richard,J.L., Tiffany,L.H. and Pier,A.C. (1969). Toxigenic Fungi associated with stored corn. Mycopathologia et Mycologia Applicata, 38: 313.

Richard,J.L., Thurston,J.R. and Pier,A.C. (1975b). Mycotoxin-induced alterations of immunity. In: Microbiology. ASM Publications, Washington D.C., pp.388-396.

Richard,J.L., Thurston,J.R. and Pier,A.C. (1978). Effects of mycotoxins on immunity. In: Rosenberg,P. (Ed.), Proceedings of V International symposium on 'Toxins: Animals, Plant and Microbial.' Pergamon Press, Oxford, New York, pp.801-817.

Richardi,J.C. and Huff,W.E. (1983). Effects of acute ochratoxicosis on blood pressure and heart rate of broiler chickens. Poultry Science, 62:2164-2168.

Richardson,E.A.,Flude,P.J., Patterson,D.S.P., Mackenzie, D.W.R. and Wakefield,E.M. (1978). Ochratoxin A in retail flour, Lancet, II:366.

Robb,J., Kirkpatrick,K.S., and Norval,M. (1982). Association of toxin-producing fungi with disease in broilers. Veterinary Record, 111:389-390.

Roberts,J.C. and Woollven,P. (1970). Studies in mycological chemistry. Part XXIV. Synthesis of ochratoxin A, a metabolite of Aspergillus ochraceus Wilh. Journal of chemical society, Section C:278-281.

Rodricks,J.V., Hesseltine,C.W. and Mehlman,M.A. (1977) Mycotoxins in Human and Animal Health. Pathotox Publ. Inc., Park Forest South, Illinois. pp.438-498.

Rose,M.E. and Bradley,J.W.A. (1977). Delayed hypersensitivity in the fowl, turkey and quail. Avian Pathology, 6: 313-326.

Rowell,J.G. and Walters,D.E. (1976). Analysing data with repeated observations on each experimental unit. Journal of Agricultural Science, Cambridge, 87:423-432.

Rutqvist,L., Björklund,N.-E., Hult,K. and Gatenbeck,S. (1977). Spontaneous occurrence of ochratoxin residues in kidneys of fattening pigs. Zentralblatt für Veterinarmedizin, A, 24:402-408.

Rutqvist,L., Björklund,N.-E., Hult,K., Hökby,E. and Carlsson, B.(1978).Ochratoxin as a cause of spontaneous nephropathy in fattening pigs. Applied and Environmental Microbiology, 36:920-925.

Sainte-Marie,G. (1962). A paraffin embedding technique for studies employing immunofluorescence. Journal of Histochemistry and Cytochemistry, 10:250-256.

Sandor,G., Glavits,R. Vajda,L, Vanýi,A. and Krogh,P. (1982). Epidemiologic study of ochratoxin A - associated porcine nephropathy in Hungary. Proceedings of V International Symposium on Mycotoxins and Phycotoxins, Vienna, pp.349-352.

Sanwall,P.C., Sundby,A. and Edqvist,L.E. (1974). Diurnal variations of peripheral plasma levels of testosterone in bulls measured by a rapid radioimmunoassay procedure. Acta veterinaria Scandinavica, 15:90-99.

Scanes,C.G., Ottinger,M.A., Kenny,A.D. Balthazart,J., Cronshaw,J. and Jones, I.C. (1982). Aspects of Avian Endocrinology: Practical and Theoretic Implications. Graduate Studies Texas Technical University, No.26, pp.217-229.

Scott,De B. (1965a). Toxigenic fungi isolated from cereal and legume crops. Mycopathologia et Mycologia Applicata, 25:213-222.

Scott,De B. (1965b). Investigation of toxigenicity of moulds from cereal and legume crops. South African Medical Journal, 39:767.

Scott,P.M. (1977). Penicillium mycotoxins. In: Wyllie,T.D. and Morehouse,L.G. (Eds.), Mycotoxic Fungi, Mycotoxins, Mycotoxicoses - An Encyclopedic Handbook,Vol.I. Marcel Dekker, Inc., New York. pp.283-336.

Scott,P.M., Van Walbeek,W., Harwig,J. and Fenell,D.I. (1970). Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin producing strains of Penicillium viridicatum. Canadian Journal of Plant Science, 50:583-585.

Scott,P.M., Van Walbeek,W., Kennedy,W. and Anyeti,D. (1972). Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. Journal of Agricultural and Food Chemistry, 20:1103-1108.

Seagrave,S. (1981). Yellow rain - a journey through the terror of chemical warfare. M. Evans and Company, Inc., New York.

Seljelid,R. and Ericsson,J.L.E. (1965). An electron microscopic study of mitochondria in renal cell carcinoma. Journal de microscopie, 4:759.

Shadmi,A. and Nobel,T.N. (1981). Kidney lesions from mycotoxins as observed by electron microscope. Annales de Recherches Vétérinaires, 12:75-83.

Sharma,J.M. and Tizard,I. (1984). Avian cellular immune effector mechanisms - a review. Avian Pathology, 13:357-376.

Shirley,E. (1977). The analysis of organ weight data. Toxicology,8:13-22.

Shotwell,O.L., Goulden,M.L. and Hesseltine,C.W. (1976) Survey of U.S. wheat for ochratoxin and aflatoxin. Journal of the Association of Official Analytical Chemists, 59:122-124.

Shotwell,O.L., Hesseltine,C.W. and Goulden,M.L. (1969). Ochratoxin A : occurrence as a natural contaminant of a corn sample. Applied Microbiology, 17:765-766.

Shotwell,O.L., Hesseltine,C.W., Vandegraft,E.E. and Goulden,M.L. (1971). Survey of corn from different regions for aflatoxin, ochratoxin and Zearalenone. Cereal Science Today, 16:266-273.

Shreeve,B.J., Patterson,D.S.P. and Roberts,B.A. (1975) Investigation of suspected cases of mycotoxicosis in farm animals in Britain. Veterinary Record, 97: 275-278.

Shreeve,B.J., Patterson,D.S.P., Pepin ,G.A., Roberts, B.A. and Wrathall,A.E. (1977). Effect of feeding ochratoxin to pigs during early pregnancy. British Veterinary Journal, 133:412-417.

Siller,W.G. (1981). Renal pathology of the fowl - a review. Avian Pathology, 10:187-262.

Siller,W.G. and Ostler,D.C. (1961). The histopathology of an entero-hepatic syndrome of turkey poults. Veterinary Record, 73:134-138.

Siller,W.G. and Wight,P.A.L. (1976). An ultrastructural study of the liver, kidney and myocardium in the fatty liver and kidney syndrome in the fowl. Research in Veterinary Science, 21:79-89.

Simpson,C.F., Pritchard,W.R. and Harms,R.H. (1959). An endotheliosis in chickens and turkeys caused by an unidentified dietary factor. Journal of American Veterinary Medical Association, 134:410-416.

Simpson,D.P. (1983). Mitochondrial transport functions and renal metabolism. Kidney International 23:785-793.

Singh,K.K. and Jha,G.J. (1982). Bone growth during chronic aldrin intoxication in goats. Research in Veterinary Science, 32:283-288.

Smith,J.E. (1982). Mycotoxins and poultry management. World's Poultry Science Journal, 38:201-212.

Snedecor,G.W. and Cochran,W.G. (1973). Statistical Methods 6th Edition (A reprint of 1967). The Iowa State University Press, Ames, Iowa.

Sokkar,S.M., Hussein,B.M. and Mohammed,M.A. (1983). Renal lesions in baby chicks due to sodium chloride poisoning. Avian Pathology, 12:277-285.

Stack,M.E., Mislivec,P.B., Gibson,R. and Pohland,A.E. (1982). Mycotoxins produced by isolates of Aspergillus ochraceus found in coffee. Proceedings of V Internation Symposium on Mycotoxins and Phycotoxins, Vienna, pp.196-199.

Stadecker,M.J., Lukic,M., Dvorak,A. and Leskowitz,S. (1977). The cutaneous basophil response to phyto-haemagglutinin in chickens. Journal of Immunology, 118:1564-1568.

Stein,O., Bar-On,H. and Stein,Y. (1972). Progress in liver diseases. In : Popper,H. and Schaffner,F. (Eds.) Grune and Stratton, New York and London, 4:45.

Stevens, M.T. (1976). The value of relative organ weights. Toxicology, 5:311-318.

Stewart,C.C. and Ingram,M. (1967). A method for counting phytohaemagglutinin-stimulated lymphocytes. Blood, 29:628.

Steyn,P.S. (1971). Ochratoxin and other dihydroisocoumarins. In: Ciegler,A., Kadis,S. and Ajl,S.J. (Eds.), Microbial Toxins, VI, Academic Press, New York and London, pp.179-205.

Steyn,P.S. and Halzapfel,C.W. (1967a). The isolation of the methyl and ethyl esters of ochratoxins A and B, metabolites of Aspergillus ochraceus. Wilh. Journal of South African Chemical Institute, 20:186-189.

Steyn,P.S. and Halzapfel,C.W. (1967b). The synthesis of ochratoxins A and B, metabolites of Aspergillus ochraceus Wilh. Tetrahydron,23:4449-4461.

Still,P.E. (1973). Mycotoxins as possible causes of abortion in dairy cattle. Ph.D. Thesis, University of Wisconsin, Madisen,U.S.A.

Still,P.E., Macklin,A.W., Ribelin,W.E. and Smalley,E.B. (1971). Relationship of ochratoxin A to foetal death in laboratory and domestic animals. Nature, 234 (5331): 563-564.

Stuart,B.P. and Bedell,D.M. (1982). Mycotoxicosis in Swine. Veterinary Clinics of North America, Large Animal Practice, 4:377-388.

Subba Rao,D.S.V. and Glick,B. (1975). Antibody and cell-mediated immunity in phytohaemagglutin - treated chickens. International Archives of Allergy and Applied Immunology, 48:30-48.

Sugimoto,T., Minamisawa,M., Takano,K., Sasamura,Y. and Tsuruta,O. (1977). Detection of ochratoxin A, citrinin and sterigmatocystin from stored rice by natural occurrence of Penicillium viridicatum and Aspergillus ochraceus. Shokuhio Eisegaku Zasshi, 18:176-181.

Sundby,A., Tollman,R. and Velle,W. (1975). Long-term effect of HCG on plasma testosterone in bulls. Journal of Reproduction and Fertility, 45:249-254.

Suzuki,S. and Satoh,T. (1973). Effects of ochratoxin A on tissue glycogen level in rats. Japanese Journal of Pharmacology, 23:415-419.

Svendsen,C. and Skadhauge,E. (1976). Renal functions in hens fed graded dietary levels of ochratoxin A. Acta Pharmacologica et Toxicologica, 38:186-194.

Syrett,R. (1978). Moulds and Mycotoxins in Animal foodstuffs - Reports of ADAS microbiologists. Ministry of Agriculture, Fisheries and Food, U.K.

Szczech, G.M., Carlton,W.W. and Hinsman,E.J. (1974). Ochratoxicosis in Beagle dogs. III. Terminal renal ultrastuctural alterations. Veterinary Pathology,11: 389-409.

Szczech,G.M., Carlton,W.W. and Tuite,J. (1972). Ochratoxicosis in Beagle dogs. Laboratory Investigation, 20:492-493.

Szczech,G.M., Carlton,W.W. and Tuite,J. (1973a) Ochratoxicosis in Beagle dogs. I. Clinical and clinicopathological features. Veterinary Pathology, 10:135-154.

Szczech,G.M., Carlton,W.W. and Tuite,J. (1973b) Ochratoxicosis A in Beagle dogs. II. Pathology. Veterinary Pathology, 10:219-231.

Szczech,G.M., Carlton,W.W., Tuite,J. and Caldwell,R. (1973c). Ochratoxin A toxicosis in Swine. Veterinary Pathology, 10:347-364.

Szemerédi,G. and Agüero,A. (1983). Nephropathy in swine associated with feeding ochratoxin containing faecal meal prepared from bovine faeces. Magyar Allatorvosok Lapja, 38:45-49.

Thacker,H.L., Carlton,W.W. and Sansing,G.N. (1975). Toxicity of citrinin and ochratoxin A in guinea pigs. Toxicology and Applied Pharmacology, 33:155 (Abstract).

Thaxton,J.P., Tung,H.T. and Hamilton,P.B. (1974). Immunosuppression in chickens by aflatoxin. Poultry Science, 53:721-725.

Theron,J.J., Van der Merwe,K.J., Liebenberg,H., Joubert, H.J.B. and Nel,W. (1966). Acute liver injury in ducklings and rats as a result of ochratoxin poisoning. Journal of Pathology and Bacteriology, 91:521-529.

Tohala,S.H. (1983). A study of ochratoxin toxicity in laying hens. Dissertation Abstracts International,B, 44:655.

Trenk,H.L., Butz,M.E. and Chu,F.S. (1971). Production of ochratoxins in different cereal products by Aspergillus ochraceus. Applied Microbiology, 21:1032-1035.

Tucker,T.L. and Hamilton,P.B. (1971). The effect of ochratoxin in broilers. Poultry Science, 50:1637 (Abstract).

Uchiyama,M., Ishohata,E. and Takeda,Y. (1976). A case report on the detection of ochratoxin A from rice. Journal of the Food Hygienic Society of Japan, 17:100-101.

Udagawa,S. I., Ichinoe,M. and Kurata,H. (1970). Occurrence and distribution of mycotoxin producers in Japanese foods. In: Herzberg,M. (Ed.), Toxic Micro-organisms. UJNR-USDI, Washington,D.C. p.174.

Van der Merwe,K.J., Steyn,P.S., Fourie,L., Scott,D.B. and Theron,J.J. (1965a). Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. Nature, 205: 1112-1113.

Van der Merwe,K.J., Steyn,P.S. and Fourie,L. (1965b). Mycotoxins, Part III. The constitution of Ochratoxins A, B and C, metabolites of Aspergillus ochraceus. Wilh. Journal of Chemical Society, Part V:7083-7088.

Van Walbeek,W., Scott,P.M. and Thatcher,F.S. (1968). Mycotoxins from food-borne fungi. Canadian Journal of Microbiology, 14:131-137.

Van Walbeek,W., Scott,P.M., Harwig,J. and Lawrence,J.W. (1969). Penicillium viridicatum: a new source of ochratoxin A. Canadian Journal of Microbiology, 15: 1281-1285.

Vanyi,A., Bata,Á. and Lasztity,R. (1980). The effect of mycotoxins (T-2,ochratoxin A) and sodium chloride deficiency on the egg production in chickens. MTA-MEM Kutatasi Beszámolók, 7:17.

Veselý,D., Veselá,D. and Jelinek,R. (1982). Nineteen mycotoxins tested on chicken embryos. Toxicology Letters, 13:239-243.

Veselá,D., Veselý,O. and Jelinek,R. (1983). Toxic effects of ochratoxin A and citrinin, alone and in combination, on chicken embryos. Applied and Environmental Microbiology, 45:91-93.

Visconti,A. and Bottalico,A. (1983). High levels of ochratoxins A and B in mouldy bread responsible for mycotoxicosis in farm animals. Journal of Agricultural and Food Chemistry, 31:1122-1123.

Walser,M.M., Allen,N.K., Mirocha,C.J., Hanlon,G.F. and Newman,J.A. (1982). Fusarium-induced osteochondrosis (tibial dyschondroplasia) in chickens. Veterinary Pathology, 19:544-550.

Warren,M.F. and Hamilton,P.B. (1980a). Inhibition of the glycogen phosphorylase system during ochratoxicosis in chickens. Applied and Environmental Microbiology, 40:522-525.

Warren,M.F. and Hamilton,P.B. (1980b). Intestinal fragility during ochratoxicosis and aflatoxicosis in broiler chickens. Applied and Environmental Microbiology, 40:641-645.

Warren,M.F. and Hamilton,P.B. (1981). Glycogen storage disease type -X caused by ochratoxicosis A in broiler chickens. Poultry Science,60:120-123.

Watson,M.L. (1958). Staining of tissue sections for electron microscopy with heavy metals. Journal of Biophysical and Biochemical cytology, 4:475-478.

Weber,W.T. (1975). Avian B-lymphocyte subpopulations : origins and functional capacities. Transplantation Reviews, 24:113-158.

Weir,D.M. (1984). Macrophage signal recognition. Agents and Actions, 15:2-11.

Whitehead,C.C., Armstrong,J.A. and Waddington,D. (1982). The determination of the availability to chicks of biotin in feed ingredients by a bioassay based on the response of blood pyruvate carboxylase (EC 6.4.1.1) activity. British Journal of Nutrition, 48:81-88.

Whitehead,C.C. and Bannister,D.W. (1978). Blood pyruvate carboxylase activity as a criterion of biotin status in chickens and turkeys. British Journal of Nutrition, 39:547-556.

Whittaker,P.G., Fuller,B.P. and Morgan, M.R.A. (1981) Rabbit anti-steroid antisera: a study of titers and specificities over a 22-week period. Experientia, 37:1203-1204.

WHO (1979). Environmental Health Criteria. II. Mycotoxins. World Health Organisation, Geneva, pp.1-127.

Williams, J.B. and Sharp,P.J. (1978). Age-dependent changes in the hypothalamo-pituitary-ovarian axis of the laying hen. Journal of Reproduction and Fertility, 53:141-146.

Willoughby,D.A., Walters,M.N.I. and Spector,W.G. (1965). Lymphnode permeability factor in the dinitrochlorobenzene skin hypersensitivity reaction in guinea-pigs. Immunology, 8:578-584.

Wogan,G. (1975). Mycotoxins. Annual Review of Pharmacology, 15:437-451.

Wyatt,R.D. (1981). Mycotoxins can cause severe losses in animal production. Proceedings of 3rd European Symposium on Poultry Nutrition, World's Poultry Science Association, Peeble. pp.84-89.

Wyllie,T.D. and Morehouse,L.G. (1977). Mycotoxic Fungi, Mycotoxins, Mycotoxicoses: An Encyclopedic Handbook. Vol.2. Mycotoxicoses of Domestic and laboratory Animal, Poultry and Aquatic Invertebrates and Vertebrates. Marcel Dekker, Inc. New York and Basel. pp.301-307.

Yamazaki,M., Maebayashi,Y. and Miyaki,K. (1970). Production of Ochratoxin A by Aspergillus ochraceus isolated in Japan from mouldy rice. Applied Microbiology, 20: 452-454.

Yamazaki,M., Suzuki,S., Sakakibara,Y. and Miyaki,K. (1971). The toxicity of 5-chloro-8-hydroxy-3,4-dihydro-3methyl-isocoumarin-7-carboxylic acid, a hydrolysate of Ochratoxin A. Japanese Journal of Medical Science and Biology, 24:245-250.

Yoshida,M. and Hoshii,H. (1982). Relationship between ash content of the toe and hardness of the tibia bone of meat type chicks. Japanese Poultry Science, 19:126-128.

Zimmermann,J.L., Carlton,W.W. and Tuite,J. (1979a). Mycotoxicosis produced in swine by cultural products of an isolate of Aspergillus ochraceus.I. Clinical observations and pathology. Veterinary Pathology, 16:583-592.

Zimmermann,J.L., Carlton,W.W. and Tuite,J. (1979b). Mycotoxicosis produced in swine by cultural products of Aspergillus ochraceus.II. Veterinary Pathology, 16: 702-709.

PUBLISHED PAPERS

Material from this thesis has been used in the following publications.

Dwivedi, P. and Burns, R.B. (1984). Pathology of ochratoxicosis A in young broiler chicks. Research in Veterinary Science, 36: 92-103.

Dwivedi, P. and Burns, R.B. (1984). Effect of ochratoxin A on immunoglobulins in broiler chicks. Research in Veterinary Science, 36: 117-121.

Dwivedi, P. and Burns, R.B. (1984). Ochratoxicosis A in broilers and turkeys: a comparative immunopathological study. Proceedings and Abstracts of the XVII World's Poultry Congress and Exhibition, held in Helsinki. Finland (August 8-12, 1984). Section 4B10: 559-561.

Dwivedi, P., Burns, R.B. and Maxwell, M.H. (1984). Ultrastructural study of the liver and kidney in ochratoxicosis A in young broiler chicks. Research in Veterinary Science, 36: 104-116.

Dwivedi, P. and Burns, R.B. (1984). Immunosuppressive effects of ochratoxin A in young turkeys. Avian Pathology (in press).

ABBREVIATIONS USED IN TEXT

BSA	Bovine serum albumin
b.w.	Body weight
C	Control (0 ppm OA)
CBH	Cutaneous basophilic hypersensitivity
C.I.	Confidence interval
CMI	Cell-mediated immunity
CT	Collecting tubule
DCL	Dorsal cloacal lip
DCT	Distal convoluted tubule
DH	Delayed hypersensitivity
DLC	Differential leucocyte counts
DLT	Diffuse lymphoid tissue
DNCB	Dinitrochlorobenzene
E.D.	Estimated difference
ELISA	Enzyme linked immuno-sorbent assay
EPV	Estimated proportional value
ER	Endoplasmic reticulum
FCA	Freund's complete adjuvant
FP	Frontal process
G	Guage
g	gram(s)
GBM	Glomerular basement membrane
GC	Germinal centre
GVH	Graft-versus-host
H and E	Haematoxylin and eosin
hr	Hour
i.d.	Intradermal(ly)
IF	Immunofluorescence
Ig	Immunoglobulin(s)
i.m.	Intramuscular(ly)
i.p.	Intraperitoneal(ly)
i.u.	International unit(s)
i.v.	Intravenous(ly)
Kg	Kilogram
ld	Lipid droplets
LD_{50}	Median lethal dose
LP	Liquid paraffin
LTS	Lateral thoracic skin(apterium)
M	Molar
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mOsm	Milli Osmoles
MPN	Mycotoxic porcine nephropathy
NC	Normal control (0 ppm OA)
ng	Nanogram(s)
nm	Nanometre(s)
NSS	Normal Saline Solution
OA	Ochratoxin A
PAA	Post auricular apterium
PAS	Periodic acid Schiff's stain
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
PCT	Proximal convoluted tubule
pg	Picogram(s)
PHA or PHA-P	Phytohaemagglutinin(P)
ppb	Parts per billion
PPD	Purified protein derivative
ppm	Parts per million
PRC	Poultry Research Centre, Roslin
PRIST	Paper radio-immunosorbent
RBC	Red blood cell
RID/RIA	Radial immunodiffusion assay
RNA	Ribonucleic acid
s.c.	Subcutaneous(ly)
S.E./SEM	Standard error of mean
SER	Smooth endoplasmic reticulum
SOFG	Safranin orange fast green
TB	Toluidine blue
ug	Microgram(s)
uM	Micromolar
um	Micron(s) or micrometre(s)
wk	Week(s)
w.o.	Weeks old
WLH	White Leghorn

Research in Veterinary Science 1984, 36, 92-103

Pathology of ochratoxicosis A in young broiler chicks

P. DWIVEDI, R. B. BURNS, *Agricultural Research Council's Poultry Research Centre, Roslin, Midlothian EH25 9PS*

Ochratoxin A (OA), a potent nephrotoxin, was fed in the diet in sublethal concentrations (2 and 4 ppm) to broiler chicks for 20 days from hatch. Its effects on growth, relative organ size and histology of the liver, kidney and lymphoid organs were investigated. In young broiler chicks, OA caused a significant enlargement of the kidney, liver and proventriculus, whereas the thymus and bursa of Fabricius were reduced in size. It principally affected the proximal convoluted tubules of the kidney causing severe distension, enlargement and hypertrophy, and it also resulted in thickening of the glomerular basement membrane. In the liver, vacuolation and glycogen accumulation in the hepatocytes were seen. The toxin also caused regression of and a drastic reduction in the lymphoid cell population in the immunological organs. The toxicity of OA was found to be dose related.

OCHRATOXIN A (OA), a mycotoxin mainly produced by *Aspergillus ochraceus* and by *Penicillium viridicatum*, has been shown to be extremely toxic to dogs (Szczech et al 1973a), ducks (Theron et al 1966), turkeys (Chang et al 1981), rats (Purchase and Theron 1968, Suzuki et al 1975), swine (Szczech et al 1973b, Krogh 1976) and domestic fowl (Pekham et al 1971, Huff et al 1974). Natural field outbreaks of ochratoxicosis have been reported in poultry in the United States with OA levels in the feed ranging from 0·3 to 16 parts per million (ppm) (Hamilton et al 1982), as well as spontaneously occurring OA induced nephropathy (Elling et al 1975). OA caused impaired blood coagulation (Doerr et al 1974), bruising (Wabeck and Doerr 1974), anaemia (Huff et al 1979a), bone abnormalities (Huff et al 1980) and type X glycogen storage disease (Warren and Hamilton 1980). It is also presumed to be involved in Balkan (endemic) nephropathy, a fatal human kidney disease in parts of Bulgaria, Romania and Yugoslavia (Krogh et al 1977).

An attempt was made to study the clinicopathological effects of OA in young broiler chicks. The present communication deals with the effects of OA on the growth rate, clinical manifestations, relative organ size and detailed histopathological changes in the kidney, liver and lymphoid organs of young broiler chicks.

Materials and methods

Experimental birds

Diets containing 0, 2 and 4 ppm OA were fed to groups of 22, 20 and 10 broiler chicks respectively from hatch. Each group contained both male and female birds and was housed in a heated brooder with water and food available ad libitum.

Experimental feed

A broiler starter ration, which had been confirmed free of contaminating mycotoxins, was used. Crystalline OA (Calbiochem-Behring, CP Laboratories, or Sigma Chemical Company) was dissolved in chloroform and mixed with the diet to give the desired concentration per kg; this was confirmed analytically.

Experimental procedure

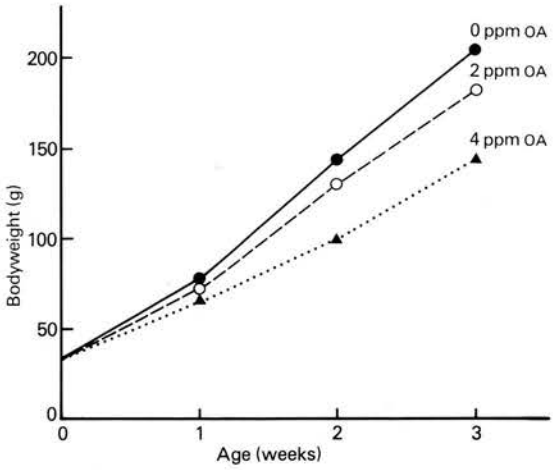
Birds, maintained on the diets until day 20, were weighed at weekly intervals and blood samples were taken for differential white cell counts. The birds were killed and various organs (liver, kidney, spleen, thymus, bursa of Fabricius, proventriculus, crop, gizzard, heart and Harderian gland) were removed, blotted dry and weighed.

Histology and histochemistry

Pieces of thymus, bursa of Fabricius, spleen, Peyer's patch, caecal tonsil, Harderian gland, liver and kidney were fixed in formol saline and wax-embedded. Sections, 5 μm thick, were stained with haematoxylin and eosin. Sections of liver were stained with Best's carmine for the demonstration of glycogen (Culling 1966) and kidney and liver sections were treated by the periodic acid-Schiff reaction (PAS).

Statistical analyses

The $\log_e$ of the organ weights as a percentage of body weight was subjected to analysis of variance.



OA level in diet	0 week	1 week	2 weeks	3 weeks	Average slope (log_e weight)
0 ppm	34·59 ± 2·94*	75·32 ± 13·08	143·16 ± 18·39	201·32 ± 21·71	0·100[a]
2 ppm	34·10 ± 2·07	72·90 ± 10·43	131·05 ± 20·50	181·10 ± 23·43	0·087[b]
4 ppm	32·62 ± 2·26	66·87 ± 10·57	99·50 ± 29·50	142·75 ± 45·52	0·073[c]

a,b,c Values in a column with a different superscript differ significantly ($P<0·05$) from each other
* Mean ± SD

FIG 1a: Effect of ochratoxin A on growth rate of broiler chicks

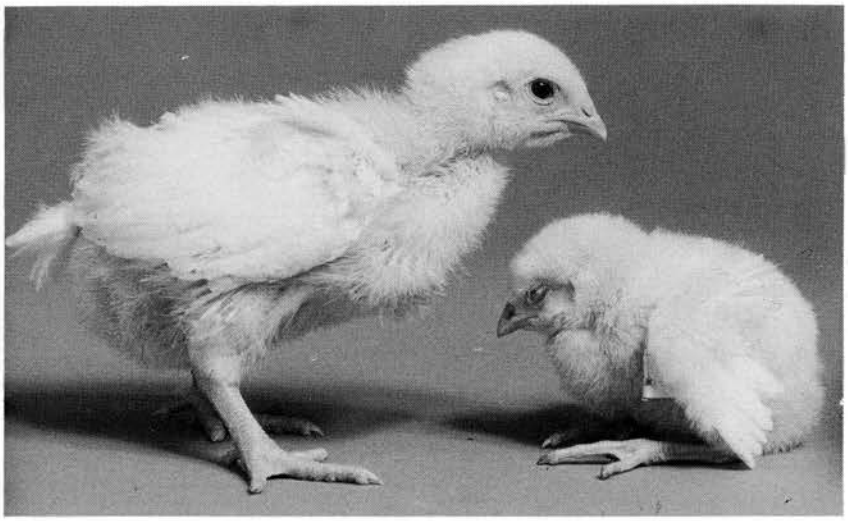


FIG 1b: Depressed growth (at 4 ppm OA) in a seven-day-old fowl. On the left is a normal control bird

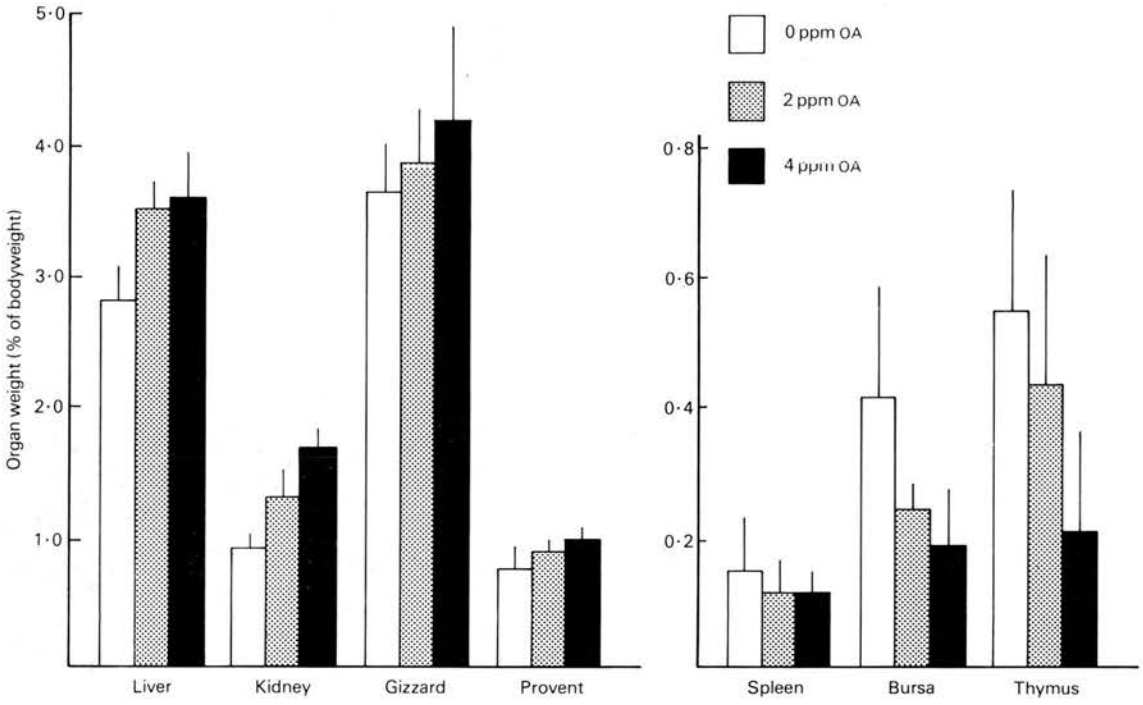
The growth of the chicks up to day 20 was approximately linear on the log scale. Thus, the slope of each bird's growth was calculated, and the differences in slope for different OA levels were examined using analysis of variance.

Results

The effects of OA on the fowls were more severe at 4 ppm than at 2 ppm. Analyses of feed samples confirmed the expected levels of OA and also showed an absence of other contaminating mycotoxins.

Clinical observations

Growth was depressed in the OA treated chicks (Fig 1a, b). Dullness, huddling, decreased appetite, reduced feed intake, weakness and diarrhoea were



OA level in diet	Liver		Kidney		Gizzard		Proventriculus		Spleen		Bursa		Thymus	
	Weight	EPV	Weight	EPV	Weight	EPV	Weight	EPV	Weight	EPV	Weight	EPV	Weight	EPV
0 ppm	2·81 ± 0·31*	1·00[a]	0·92 ± 0·10	1·00[a]	3·65 ± 0·39	1·00[a]	0·76 ± 0·19	1·00[a]	0·15 ± 0·09	1·00[a]	0·42 ± 0·17	1·00[a]	0·55 ± 0·19	1·00[a]
2 ppm	3·52 ± 0·22	1·25[b]	1·32 ± 0·23	1·40[b]	3·88 ± 0·43	1·06[a]	0·89 ± 0·11	1·17[b]	0·12 ± 0·05	0·82[a]	0·25 ± 0·04	0·61[b]	0·44 ± 0·20	0·75[a]
4 ppm	3·60 ± 0·36	1·31[b]	1·69 ± 0·16	1·86[c]	4·20 ± 0·72	1·18[a]	1·00 ± 0·07	1·35[c]	0·12 ± 0·03	0·92[a]	0·19 ± 0·09	0·47[b]	0·21 ± 0·15	0·33[b]

a,b,c Values in a column with different superscripts differ significantly ($P<0·05$)
EPV Estimated Proportional Value
* Mean ± SD

FIG 2: Effect of OA on the relative organ weight of broiler chicks

seen in the birds on the OA diets. No nervous symptoms were seen. One bird (4 ppm OA) died on day 15.

There were no signifiant differences in the differential white blood cell counts of OA birds compared to controls.

Bodyweight and relative organ weights

The effect of OA on the growth of broiler chicks is shown in Fig 1a and 1b. Regression analysis of the data showed that there was a significant depression of bodyweight at both 2 and 4 ppm OA. No sex differences were noted in the reduced growth rate of the OA fed birds.

The effect of the two levels of OA on the organ weights (as a percentage of bodyweight) and its estimated proportional value is shown in Fig 2. The liver, kidneys and proventriculus increased in size in the presence of OA. The effect was most marked in the kidneys, which were enlarged to about one and a half times at 2 ppm and to almost twice the size of those of the controls at 4 ppm OA. The thymus and bursa of Fabricius were significantly decreased in size, the effect being most marked in the thymus which was reduced by two thirds at 4 ppm OA. The spleen, crop and Harderian gland, however, did not show any significant differences in their relative sizes as compared to the controls. Increases in the relative weights of the gizzard at both OA levels were not significant and heart weights were inconsistent. No sex differences were noted in the relative organ weights.

Gross pathology

The OA treated fowls were emaciated and their bones were thin, soft and pliable. The kidneys were pale and enlarged; the liver was also enlarged. The bile was paler and less viscous than normal. There

was a slight catarrhal enteritis in some birds. The lining of the gizzard was often dry. Gross lesions were not detected in other organs.

Histopathology

Kidneys. In sections of kidney from OA fed birds, many of the proximal convoluted tubules (PCT) and some of the distal convoluted tubules were enlarged, distended and hypertrophied. The majority of the enlarged tubules, many of which were misshapen, contained translucent, homogeneous, round, hyaline globules (Fig 3a). These globules were periodic acid Schiff negative and varied from a few to many in the tubules. Nodular infiltrations of lymphoid cells were common. In some areas, the PCT were compressed and dense, with an increased number of cells per tubule. Very often at 4 ppm OA, there was necrosis of individual cells in the PCTs (Fib 3b), though other degenerative changes, except for ballooning of some cells, were not usually observed. Many mitotic figures (Fig 3c) were also seen in kidneys from OA treated birds. Neither oedema nor inflammatory reactions were detected. There was thickening of the glomerular basement membrane in either one or more capillary loops; this was most easily seen in PAS treated sections (Fig 3d). The glomerular basement membrane was thickened in 22, 29 and 2·5 per cent of glomeruli examined (491, 391 and 412) from birds given 2, 4 or 0 ppm OA, respectively.

Liver. Changes varied from a slight degeneration to vacuolation of the hepatic cells (Fig 4a). Sometimes a perivascular inflammatory (heterophilic) reaction and nodular lymphoid infiltration were seen. Some congestion and haemorrhage were also evident. Glycogen was increased throughout the liver tissue (Fig 4b), although this was more marked at the periphery of the lobule. The increase appeared to be dose related.

Bursa of Fabricius. There was severe lymphoid depletion from the follicles (Figs 5a, 5b), which appeared small and rounded and were often surrounded by fibrous connective tissue (Fig 5b). The increased cellularity of the subepithelial and interfollicular areas was due to heterophils and mononuclear cells, including plasma cells and lymphocytes. Haemorrhage had occurred in some cases. However, the epithelium appeared to be intact and unaffected by the OA (Fig 5a).

Thymus. There was depletion of lymphoid cells from the thymus. It was more apparent in the cortex than in the medulla (Fig 6a) and often occurred together with some congestion, haemorrhage and heterophilic infiltration (Fig 6b).

Spleen. There was a marked reduction in the numbers of lymphocytes in the white pulp (Fig 7a). In some areas the distinction between red and white pulps had become obscure. Germinal centres were reduced both in number and in size in sections from OA-treated birds (Fig 7b).

Harderian gland. In sections of Harder's gland from OA fed birds the plasma cell population was reduced and there was congestion, haemorrhage and sometimes an infiltration of heterophils. Frequently the epithelial lining of the secondary tubules was broken and plasma cells, epithelial cell debris and erythrocytes were present in the lumen.

Intestinal lymphoid organs. In the caecal tonsils and Peyer's patches, there was some depletion of lymphoid cells from both the diffuse lymphoid tissue and from the germinal centres, which were also reduced in number. Heterophilic infiltration was commonly seen in the lamina propria, muscularis mucosa, submucosa and even in the muscular layers (Fig 8). Heterophils were also seen in the intestinal glands and crypts. In some cases a degeneration of the villi and glands was apparent.

Discussion

Ochratoxin A was found to be extremely toxic to young growing broiler chicks and the severity of the clinical signs, behavioural changes, growth response (Fig 1a) and lesions were all dose dependent. This is in general agreement with Huff et al (1974) and Prior et al (1976). However, there was no consistent change in the blood profile as reported by Chang et al (1979).

Huff et al (1974) detected an increase in the weight of the liver and a regression of the bursa of Fabricius only at 8 ppm OA. Chang et al (1981) observed an enlargement of the proventriculus and gizzard and a regression of the thymus at 4 and 8 ppm OA in turkeys. The present study has shown that OA, even at the minimum growth inhibitory level of 2 ppm (Wyatt 1981), could adversely affect the vital organs of young broiler chicks. No significant sex linked differences in susceptibility in relation to body or organ weight were noted, though Prior et al (1980) reported males to be more susceptible to OA than females.

Contrary to the report of Pekham et al (1971) and Huff et al (1975), neither visceral gout nor oedema were seen in any of the OA treated birds. This further contradicts Huff et al (1975) who suggested that oedema might be responsible for the increase in kidney size. The results of this study suggest that this increase is more likely to have been due to the enormous enlargement and hypertrophy of the PCTs and to an infiltration of lymphoid cells. The toxin

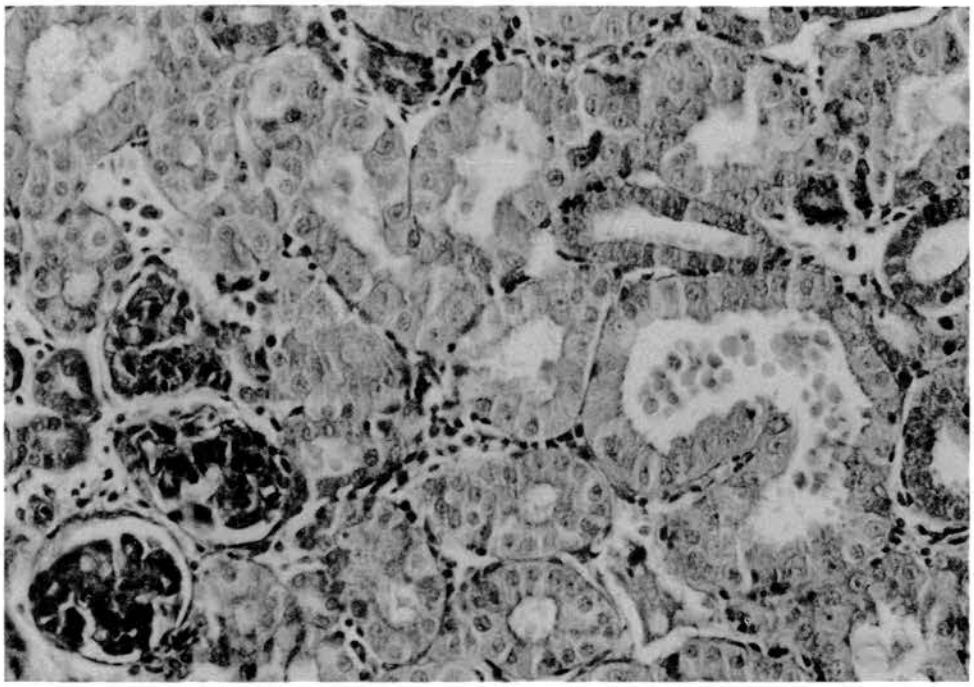


FIG 3a: Kidney PCT (4 ppm OA). Distension, enlargement and hypertrophy of PCT and the presence of hyaline globules in the lumen. H&E ×400

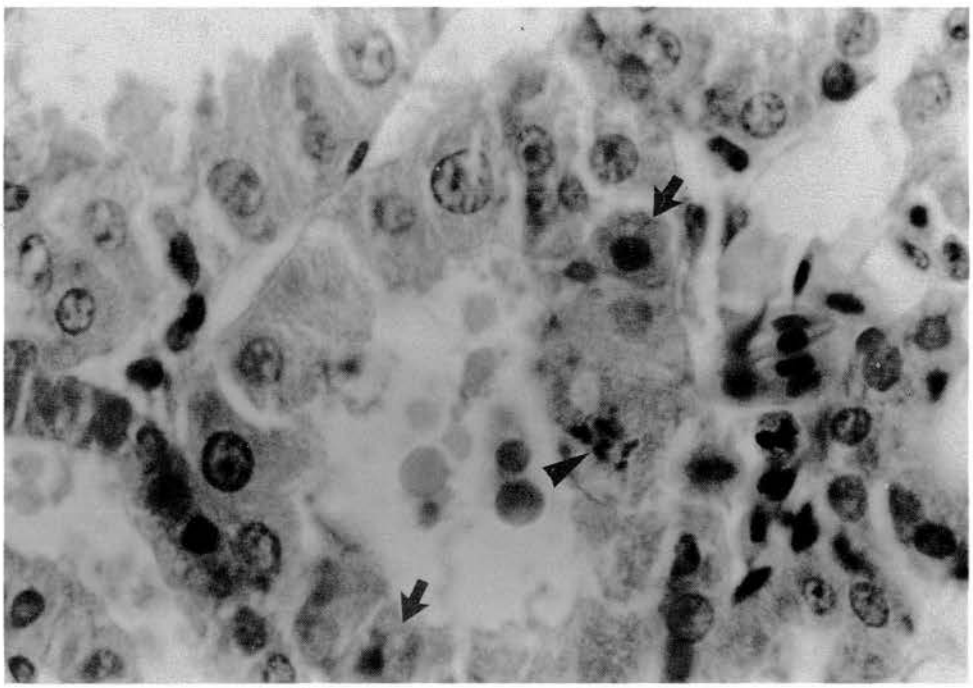


FIG 3b: Kidney PCT. Individual cell necrosis. Note the presence of pyknotic (arrow) and karyorrhectic (arrow head) nuclei in the tubule. Hyaline globules are also present in the lumen. A heterophil and a few red blood cells are adjacent to the affected tubule. H&E ×1185

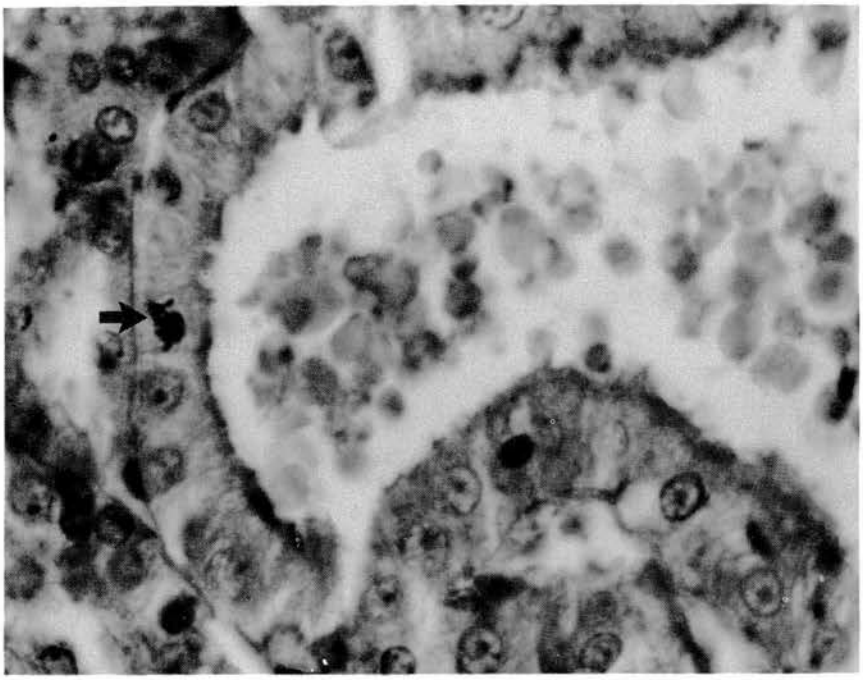


FIG 3c: Kidney PCT. An enlarged, hypertrophied and misshapen tubule with a mitotic figure (arrow) in a cell and many hyaline globules in the lumen. The brush border is intact. PAS × 1185

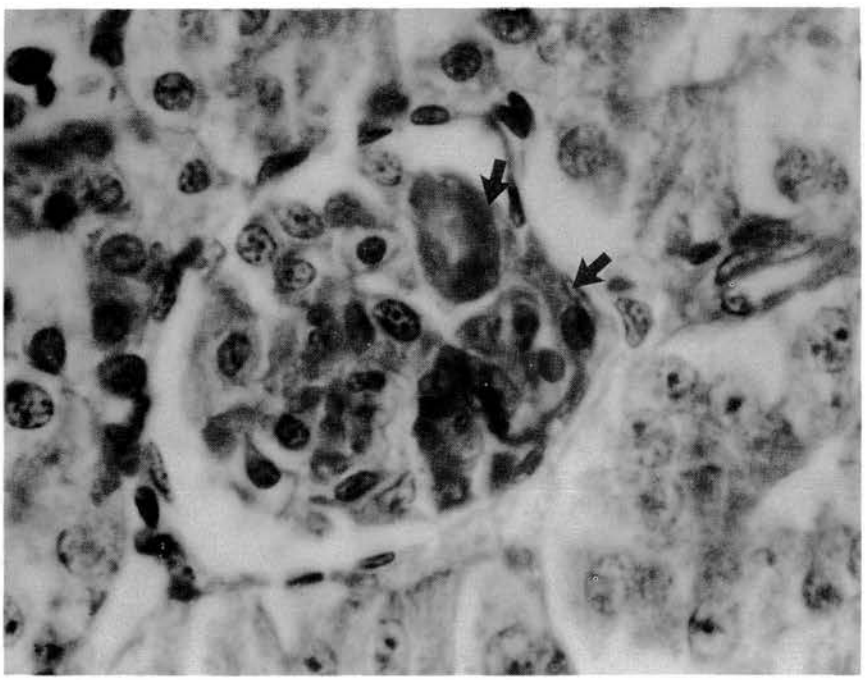


FIG 3d: Kidney glomerulus (4 ppm OA) showing thickening of the glomerular basement membrane in the capillary loop (arrow). PAS × 1185

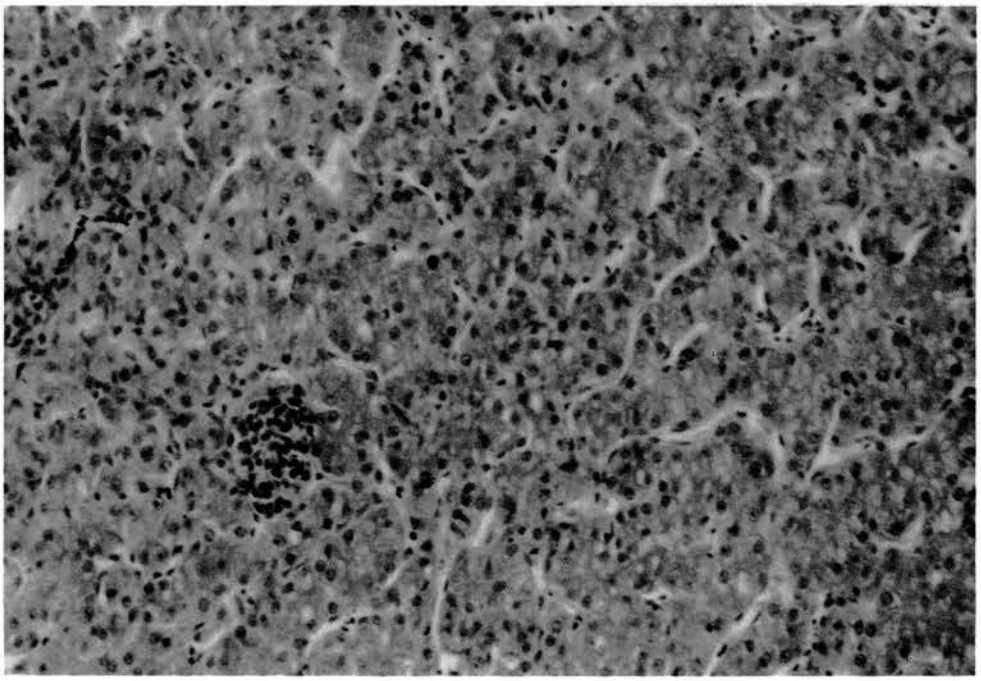


FIG 4a: Liver (2 ppm OA). Note the vacuolated hepatic cells and also the presence of a lymphoid nodule. H&E ×300

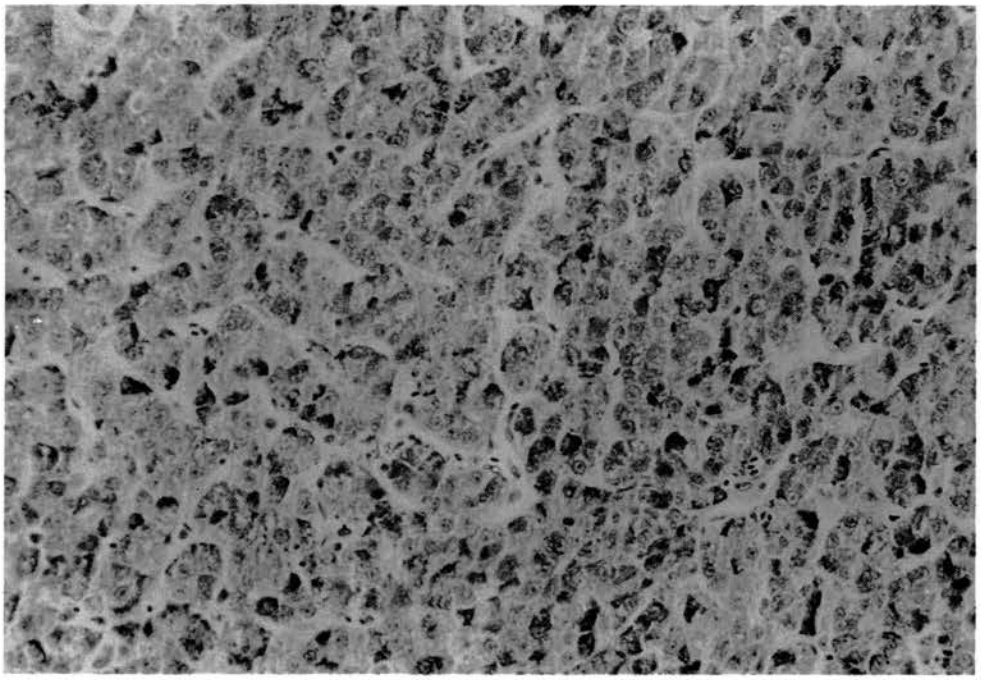


FIG 4b: Liver (4 ppm OA) showing the accumulation of glycogen in the hepatic cells. Best Carmine staining ×300

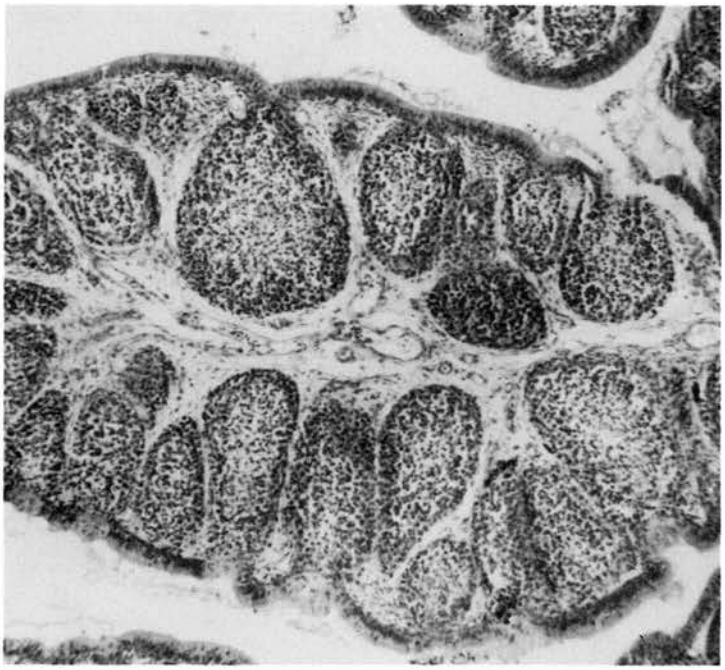


FIG 5a: Bursa of Fabricius (4 ppm OA). A low power view showing general lymphoid cell depletion and reduction in the size of the follicles. The epithelium seems to be unaffected. H&E × 115

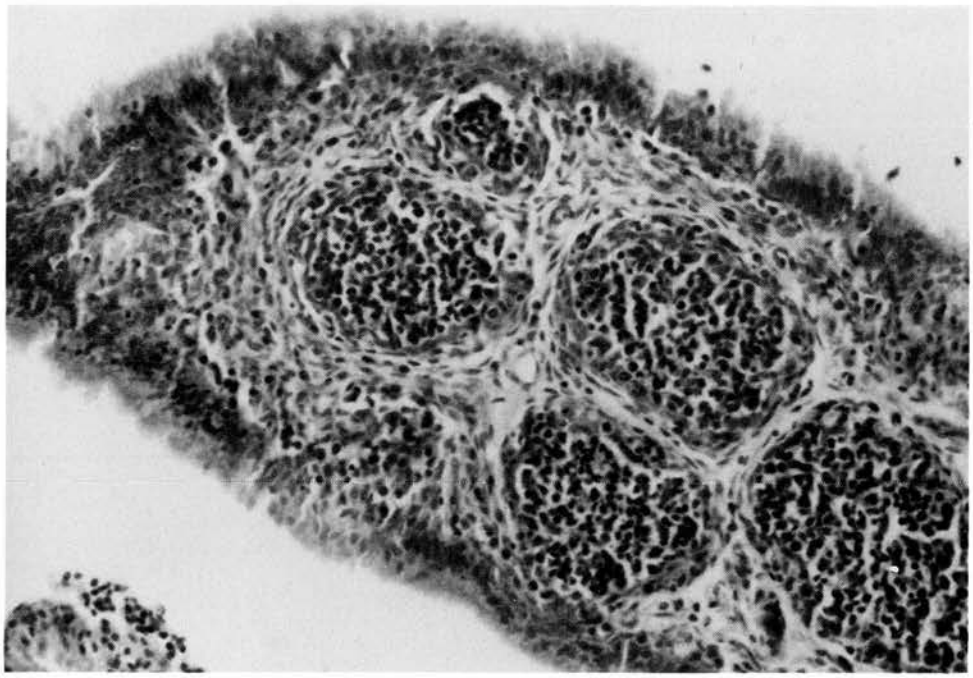


FIG 5b: Bursa (4 ppm OA) showing small, round, depleted lymphoid follicles surrounded by fibrous connective tissue. The cortex and medulla indistinguishable. Interfollicular cellularity has been increased. H&E × 300

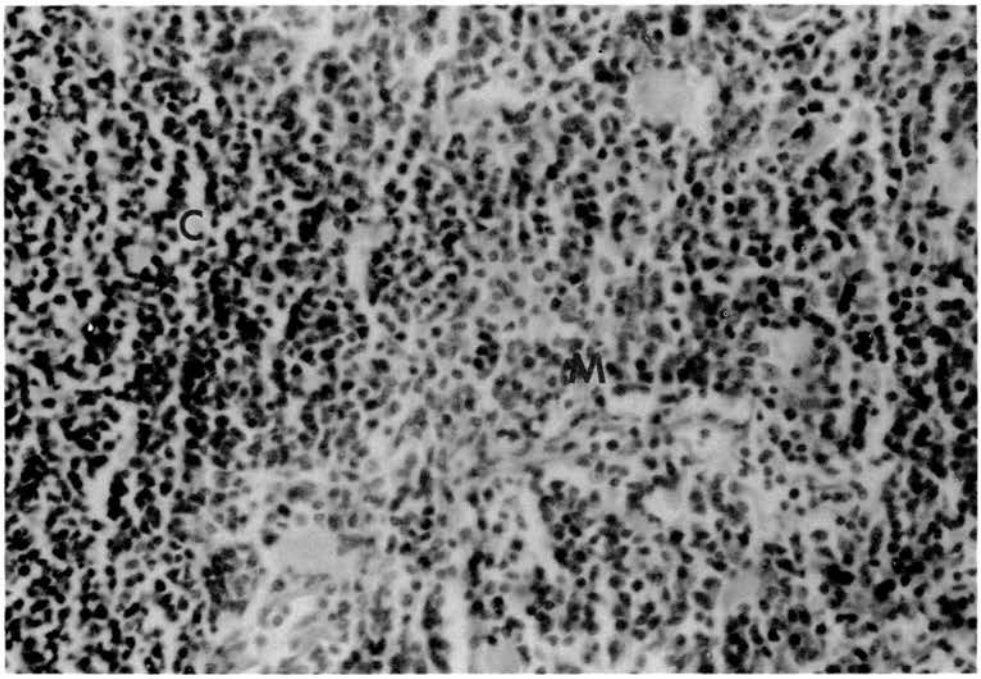


FIG 6a: Thymus (2 ppm OA) showing general depletion of lymphoid cells. Cortex (C) and medulla (M) are not well demarcated. H&E ×480

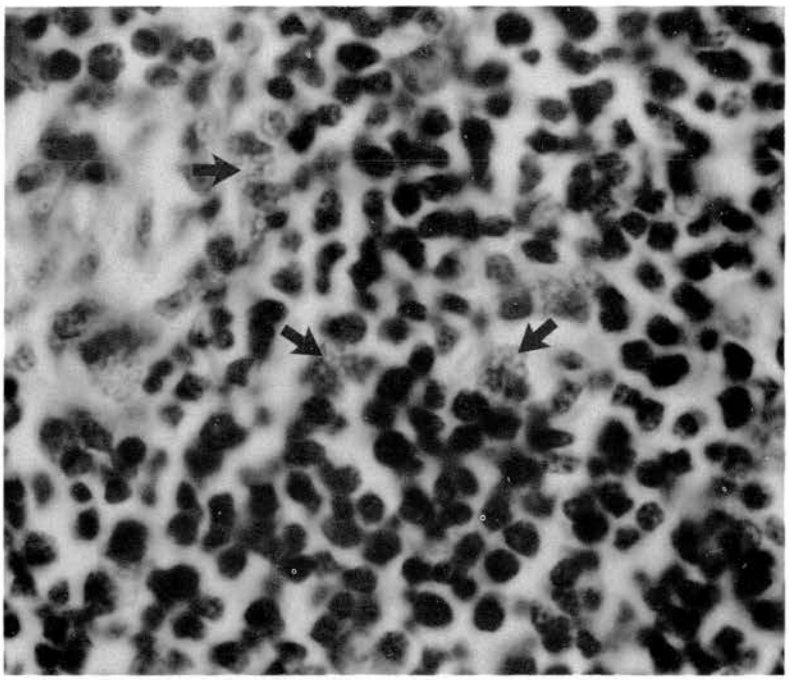


FIG 6b: Thymus (2 ppm OA). Note the presence of many heterophils (arrows) in the cortex. H&E ×1185

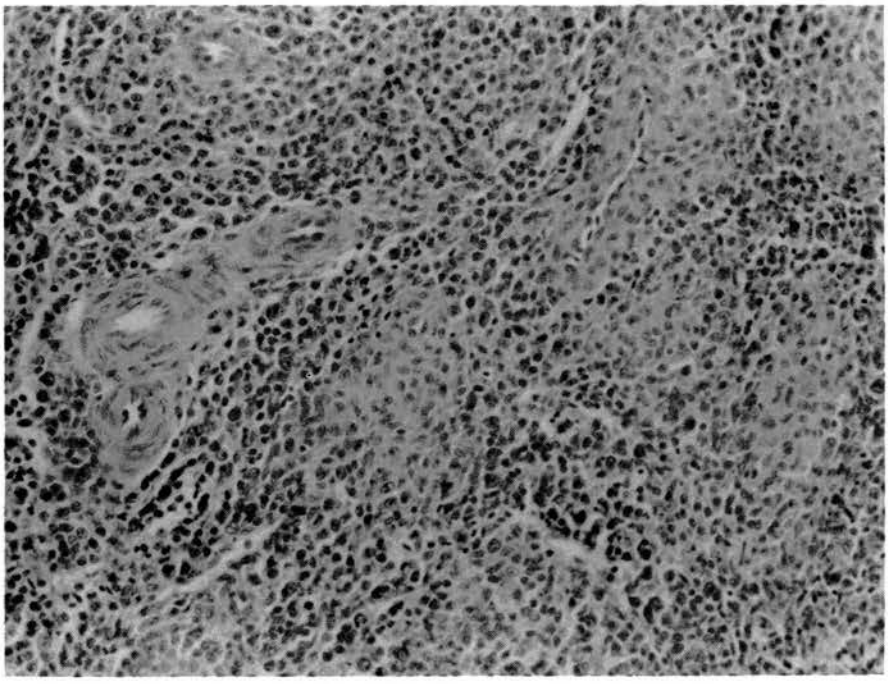


FIG 7a: Spleen (2 ppm OA). Note the depletion of lymphoid follicles from the white pulp and their disorganisation. H&E ×300

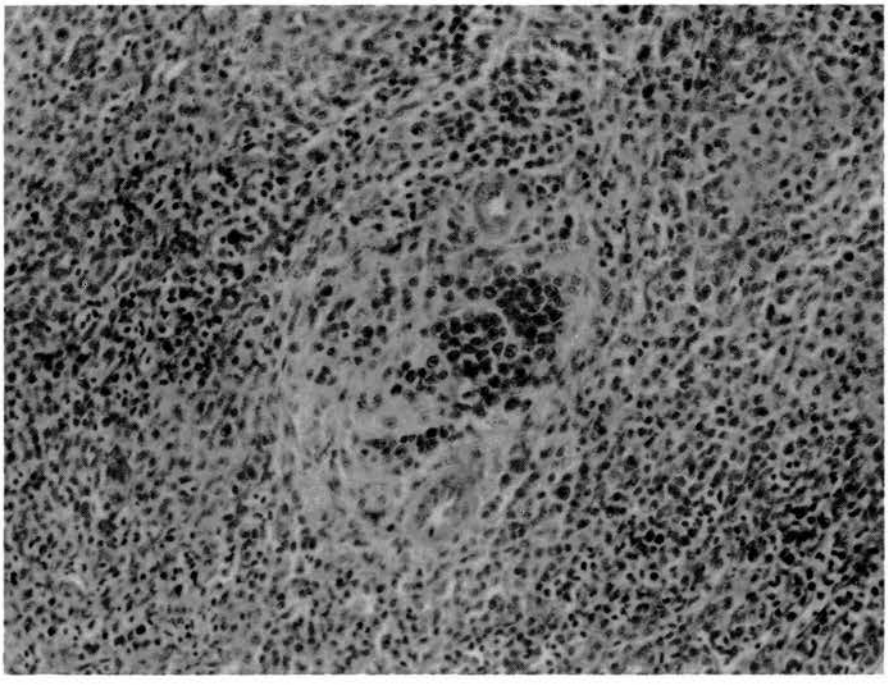


FIG 7b: Spleen (2 ppm OA) showing lymphoid depletion in a germinal centre near a blood vessel and indistinguishable white pulp. H&E ×300

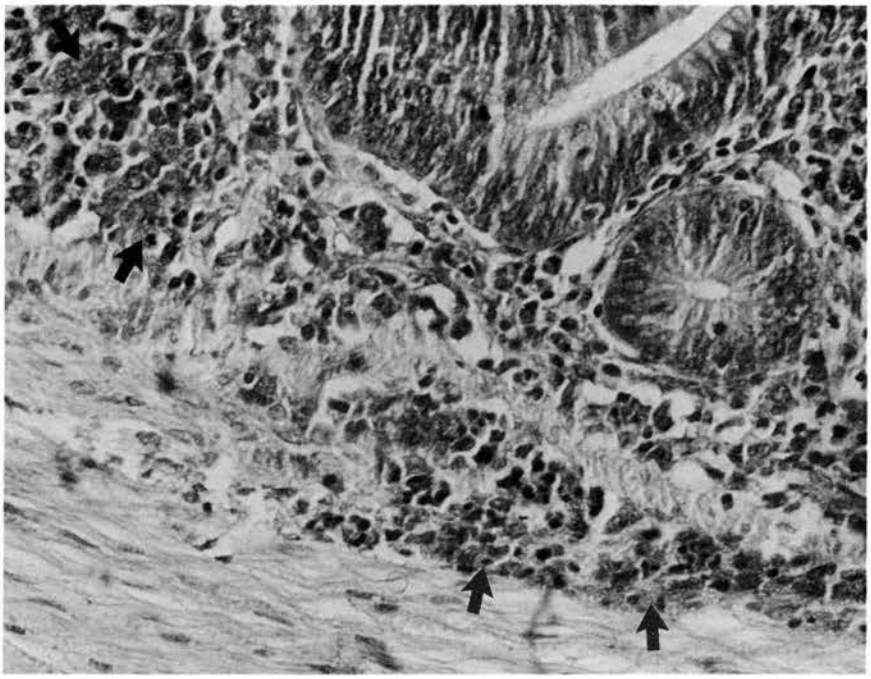


FIG 8: Caecal tonsil (4 ppm OA). Note the presence of heterophils (arrows) in the lamina propria, muscularis mucosa and submucosa. H&E × 480

seemed to have an affinity for the PCTs, as was shown by the mitochondrial abnormalities observed with the electron microscope (Dwivedi et al 1984). Other lesions noted in the kidney such as localised necrosis, mitotic figures, congestion and haemorrhage are in accord with previous studies (Pekham et al 1971, Galtier et al 1976, Krogh et al 1976).

The homogeneous hyaline globules noted in the present study do not correspond to hyaline casts described by Elling et al (1975) and Galtier et al (1976) nor are they eosinophilic granular casts (Szczech et al 1973a). Huff et al (1975) observed proteinaceous material in the lumina of the tubules. The effects of these tubular changes together with the thickened glomerular basement membrane might be reflected in impaired renal functions shown as decreased tubular excretion and urine concentrating capacity and phenol red clearance in OA treated chicks (Huff et al 1975, Svendsen and Skadhauge 1976). The thickened glomerular basement membrane has been studied at the ultrastructural level and discussed elsewhere (Dwivedi et al 1984).

Warren and Hamilton (1980) reported an enzymic inhibition of glycogenolysis in OA toxicosis and this could account for the excess glycogen observed in the liver in the present study. Both decreased (Munro et al 1973, Suzuki and Satoh 1973, Chu 1974) and increased (Purchase and Theron 1968) amounts of liver glycogen have been reported in rats treated with OA. Recently Huff et al (1979b) noted an increase in glycogen in the livers of OA fed broilers (at 4 and 8 ppm OA but not at lower levels). Observation of vacuolation of the hepatic cells agrees with the findings of Pekham et al (1971), though no hepatic necrosis was observed in the present study.

The pathological changes reported here indicate and confirm that OA is more of a nephrotoxin than a hepatotoxin in young broiler chicks in contrast to aflatoxin which is considered to be more hepatotoxic.

The decrease in the size of the lymphoid organs seen in the present study may have been due to the depletion of lymphoid cells and the more drastic changes seen in the thymus from OA treated birds suggest a stronger effect on cell mediated immune responses. Lymphoid depletion has also been observed in the thymus, spleen, tonsils and Peyer's patches from OA fed dogs and swine (Szczech et al 1973a, b) and in the spleen and bursa of Fabricius from OA treated fowls (Pekham et al 1971).

The heterophilic reaction noted in the alimentary tract and other organs from OA fed birds in the present study is in accord with the observations of Choudhury et al (1971) and Pekham et al (1971). Chang et al (1979) found an increase in heterophils in broilers in ochratoxicosis, though their phagocytic ability was impaired (Chang and Hamilton 1980).

These changes together with the lymphoid depletion of immunological organs as observed in the present study lend support to the suggestion of Prior and Sisodia (1982) and of Hamilton et al (1982) that OA may reduce the capability of an animal to respond to immunological challenge. This is further substantiated by the reduced immunoglobulin containing cells in the lymphoid organs and lowered immunoglobulin levels in sera from OA fed birds (Dwivedi and Burns 1984).

The present study suggests that OA is a potent nephrotoxin in broilers, principally causing distension, enlargement and hypertrophy of the PCT and thickening of the glomerular basement membrane. In the liver, it caused lymphoid cell infiltration and glycogen accumulation in a dose related fashion. Furthermore, OA resulted in a regression of the lymphoid organs and a drastic reduction of the lymphoid cell population.

Acknowledgements

The authors wish to thank Mr D. Waddington, Poultry Research Centre, for statistical analysis of the data and Miss J. Robb, East of Scotland College of Agriculture, University of Edinburgh, for anlaysis of mycotoxin(s) in the feed samples. Thanks are also due to the Association of Commonwealth Universities, London, for a Commonwealth Scholarship to P.D.

References

CHANG, C.-F., DOERR, J. A. & HAMILTON, P. B. (1981) *Poultry Science* **60,** 114–119

CHANG, C.-F. & HAMILTON, P. B. (1980) *Applied and Environmental Microbiology* **39,** 572–575

CHANG, C.-F., HUFF, W. E. & HAMILTON, P. B. (1979) *Poultry Science* **58,** 555–558

CHOUDHURY, H., CARLSON, C. W. & SEMENIUK, G. (1971) *Poultry Science* **50,** 1855–1859

CHU, F. S. (1974) *Critical Review of Toxicology* **3,** 499–524

CULLING, C. F. A. (1966) Handbook of Histopathological Techniques. 2nd edn. London, Butterworths. p 272

DOERR, J. A., HUFF, W. E., TUNG, H. T., WYATT, R. D. & HAMILTON, P. B. (1974) *Poultry Science* **53,** 1728–1734

DWIVEDI, P. & BURNS, R. B. (1984) *Research in Veterinary Science* **36,** 117–121

DWIVEDI, P., BURNS, R. B. & MAXWELL, M. H. (1984) *Research in Veterinary Science* **36,** 104–116

ELLING, F., HALD, B., JACOBSEN, C. & KROGH, P. (1975) *Acta Pathologica et Microbiologica Scandinavica* Section A **83,** 739–741

GALTIER, P., MORE, J. & ALVENERIE, M. (1976) *Food and Cosmetics Toxicology* **14,** 129–131

HAMILTON, P. B., HUFF, W. E., HARRIS, J. R. & WYATT, R. D. (1982) *Poultry Science* **61,** 1832–1841

HUFF, W. E., CHANG, C.-F., WARREN, M. F. & HAMILTON, P. B. (1979a) *Applied and Environmental Microbiology* **37,** 601–604

HUFF, W. E., DOERR, J. A. & HAMILTON, P. B. (1979b) *Applied and Environmental Microbiology* **37,** 122–126

HUFF, W. E., DOERR, J. A., HAMILTON, P. B., HAMANN, D. D., PETERSON, R. E. & CIEGLER, E. (1980) *Applied and Environmental Microbiology* **40,** 102–107

HUFF, W. E., WYATT, R. D. & HAMILTON, P. B. (1975) *Applied and Environmental Microbiology* **30,** 48–51

HUFF, W. E., WYATT, R. D., TUCKER, T. L. & HAMILTON, P. B. (1974) *Poultry Science* **53,** 1585–1591

KROGH, P. (1976) *Nordisk Veterinaer Medicin* **28,** 452–458

KROGH, P., ELLING, F., HALD, B., JYLLING, B., PETERSON, W. E., SKADHAUGE, E. & SVENDSEN, C. K. (1976) *Acta Pathologica et Microbiologica Scandinavica* Section A **84,** 215–221

KROGH, P., HALD, B., PLESTINA, R. & CEOVIĆ, S. (1977) *Acta Pathologica et Microbiologica Scandinavica* Section B **85,** 238–240

MUNRO, I. C., SCOTT, P. M., MOODIE, C. A. & WILLES, R. F. (1973) *Journal of American Veterinary Medical Association* **163,** 1269–1273

PEKHAM, J. C., DOUPNIK, B., Jr & JONES, O. H., Jr (1971) *Applied Microbiology* **21,** 492–494

PRIOR, M. G., O'NEIL, J. B. & SISODIA, C. S. (1980) *Poultry Science* **59,** 1254–1257

PRIOR, M. G. & SISODIA, C. S. (1982) *Canadian Journal of Comparative Medicine* **46,** 91–96

PRIOR, M. G., SISODIA, C. S. & O'NEIL, J. B. (1976) *Poultry Science* **55,** 786–790

PURCHASE, J. F. H. & THERON, J. J. (1968) *Food and Cosmetics Toxicology* **6,** 479–483

SUZUKI, S., KOZUKA, Y., SATOH, T. & YAMAZAKI, M. (1975) *Toxicology and Applied Pharmacology* **34,** 479–490

SUZUKI, S. & SATOH, T. (1973) *Japanese Journal of Pharmacology* **23,** 415–419

SVENDSEN, C. & SKADHAUGE, E. (1976) *Acta Pharmacologica et Toxicologica* **38,** 186–194

SZCZECH, G. M., CARLTON, W. W. & TUITE, J. (1973a) *Veterinary Pathology* **10,** 219–231

SZCZECH, G. M., CARLTON, W. W., TUITE, J. & CALDWELL, R. (1973b) *Veterinary Pathology* **10,** 347–364

THERON, J. J., VAN DER MERWE, K. J., LIEBENBERG, N., JOUBERT, H. J. B. & NEL, W. (1966) *Journal of Pathology and Bacteriology* **91,** 521–529

WABECK, C. J. & DOERR, J. A. (1982) *Poultry Science* **61,** 1563

WARREN, M. F. & HAMILTON, P. B. (1980) *Applied and Environmental Microbiology* **40,** 522–525

WYATT, R. D. (1981) Proceedings of 3rd European Symposium on Poultry Nutrition, World Poultry Science Association, Peebles. pp 84–89

Received for publication July 6, 1983

Research in Veterinary Science 1984, 36, 117-121

Effect of ochratoxin A on immunoglobulins in broiler chicks

P. DWIVEDI, R. B. BURNS, *Agricultural Research Council's Poultry Research Centre, Roslin, Midlothian EH25 9PS*

The effect of ochratoxin A (OA) on the immune system was investigated in broiler chicks fed graded dietary levels of OA up to 4 ppm for 20 days from hatch. Immunofluorescent demonstration of IgG, IgA and IgM in sections of liver, kidney and lymphoid tissues was used to assess immune competence. Serum immunoglobulin levels were measured by radial immunodiffusion. Ochratoxin caused a significant depression in immunoglobulin-containing cells in all the lymphoid organs studied. Correspondingly, total immunoglobulin levels were also reduced in the sera from OA fed birds, immunosuppression being similar at both the 2 and 4 ppm OA levels. Deposition of immunoglobulins, especially IgG, in the glomerular basement membrane was seen with greater frequency in kidneys from OA fed fowls. Immunoglobulin-containing lymphoid cells occurred more frequently in the kidney parenchyma of these birds.

OCHRATOXIN A (OA), a nephrotoxic mycotoxin, has long been recognised as a natural contaminant of feedstuffs and cereals (Shotwell et al 1969, Krogh et al 1977, Prior 1981), thereby posing a possible health hazard to both humans and animals, including poultry. Hamilton et al (1982) observed an increased incidence of air-sacculitis in several field outbreaks of ochratoxicosis in broiler fowls and related it to the possible immunosuppressive effects of the mycotoxin.

No systematic study has been conducted on the effects of OA on the avian immune system. There are a few reports, some of which are contradictory, on the effects of ochratoxicosis A on the immunology of mice (Prior and Sisodia 1982, Creppy et al 1982, 1983), guinea pigs (Van der Merwe et al 1965, Richard et al 1975) and calves (Patterson et al 1981). In the bird, lymphoid cell depletion in the bursa of Fabricius (Pekham et al 1971, Huff et al 1974), thymus regression (Chang et al 1981), impaired phagocytic ability of heterophils (Chang and Hamilton 1980) and leucopenia (Chang et al 1979) after OA treatment suggest an immunosuppressive effect. In a related study (Dwivedi and Burns 1984), regression and depletion of lymphoid cells from immunogenic and lymphoid organs were also noted.

The present paper forms part of a study on the effects of OA on the immune system of poultry and reports on immunoglobulins in sera and tissues of young broiler chicks after feeding OA.

Materials and methods

Birds, feed and experimental procedure have been described elsewhere (Dwivedi and Burns 1984). Briefly, broiler chicks were fed diets containing 0, 2 or 4 ppm OA (in groups of 22, 20 and 10) for 20 days from hatch. At the end of the experiment blood samples were taken for the collection of serum and the birds were killed with an overdose of pentobarbitone sodium. Pieces of liver, kidney, spleen, bursa of Fabricius, thymus, Peyer's patch, caecal tonsil and Harderian gland were fixed in ethanol.

Immunofluorescence

Ethanol-fixed tissues were wax-embedded according to the method of Sainte-Marie (1962), sectioned at 5 μm and treated for the immunofluorescent demonstration of IgA, IgG and IgM, using specific antisera (Miles Laboratories) by a modification (Brandtzaeg 1981) of the sandwich method of Coons et al (1955).

Serology

Relative concentrations of IgG, IgA and IgM were determined in sera by Mancini's radial immunodiffusion assay (Blythman and White 1977) using 1·5 per cent agarose in 8 per cent saline with 10 per cent rabbit anti-fowl IgG, IgA or IgM. The wells were filled with 5 μl test serum. Immunoglobulin levels at 36 hours were expressed as a percentage of that determined for the control birds. Serum samples were also screened for IgE by the Phadebas IgE paper radioimmunosorbent (PRIST) method (Pharmacia) (Burns and Maxwell 1981).

Results

Area and intensity of fluorescence

Differences in area and intensity of fluorescence

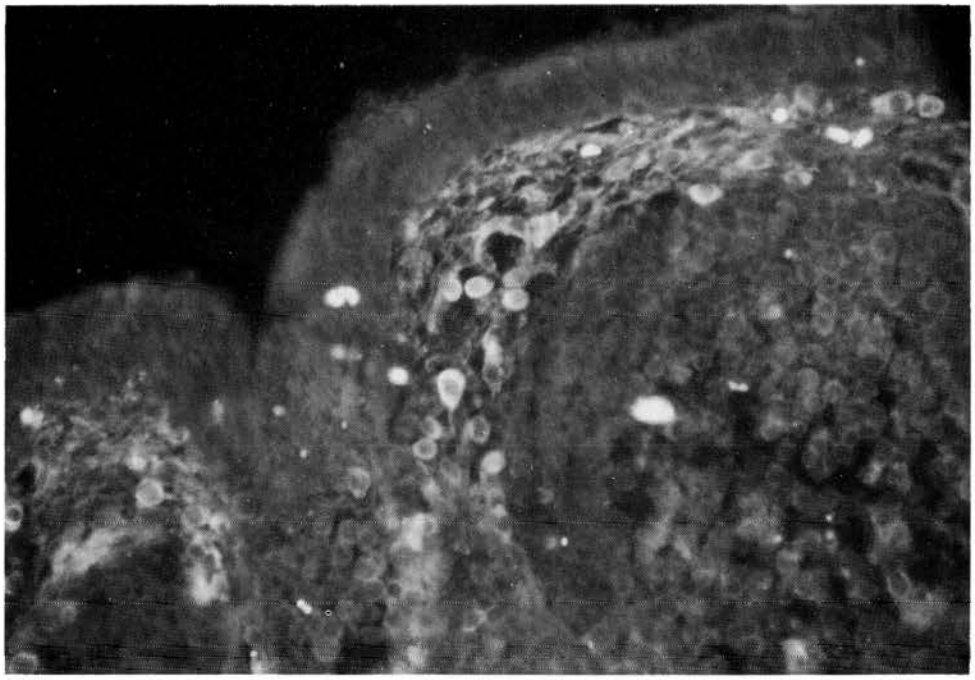


FIG 1: Bursa of Fabricius (4ppm OA) showing fluorescing lymphocytes in the subepithelial region. Lymphoid follicles appear to be negative. Anti-IgG × 480

were noted in tissues from OA-fed birds treated for the immunofluorescent demonstration of IgA, IgG and IgM when compared to those from controls.

Bursa of Fabricius. In sections of bursa of Fabricius from control birds, fluorescence was mainly confined to the medulla of the follicles for all three immunoglobulins, whereas in sections from OA treated birds cells with cytoplasmic fluorescence were found in the subepithelial regions (Fig 1) and interfollicular areas; these cells tended to be large and few in number. The reaction was similar for all three immunoglobulins although in both control and OA treated bursae fewer cells were positive for IgM than for IgG or IgA. The differences in fluorescence between control and OA bursae were most marked in those from birds fed 4 ppm OA.

Spleen. Immunofluorescent staining for immunoglobulins was very variable in sections of spleen from OA fed birds and fluorescing cells appeared to be fewer in number than in sections from control birds. Germinal centres were also reduced both in number and size in the OA spleens.

Intestinal lymphoid organs. In caecal tonsils and Peyer's patches from OA treated fowls, cells with cytoplasmic fluorescence for IgG and IgM were larger and fewer in number than in sections from control birds (Fig 2). A similar reaction for IgA was also seen but the mucous lining and often the goblet cells fluoresced a bright apple green. Very few positive cells were found in the germinal centres (Fig 2).

Thymus. Cells with cytoplasmic fluorescence for all three immunoglobulins were relatively reduced in numbers in sections from OA treated birds. Hassall's corpuscles fluoresced non-specifically more frequently in sections from control birds.

Harderian gland. In sections of Harderian gland from OA fed birds, plasma cells fluorescing for all three immunoglobulins were markedly reduced in numbers. Fluorescent plasma cells were seen in the lumina of the secondary tubules, which often had a break in the integrity of their epithelium.

Liver. In the liver immunoglobulin-containing cells were seen only occasionally and there appeared to be no difference between sections from control and OA treated birds.

Kidney. In sections of kidney from OA and control fowls, no differences were seen in immunoglobulin localisation in renal tubules though there was often

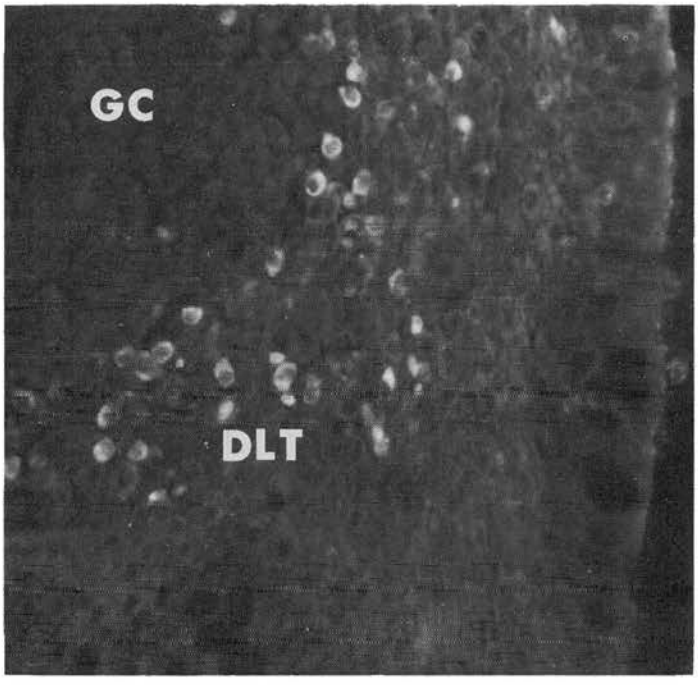


FIG 2: Caecal tonsil (2 ppm OA) showing a few isolated positive lymphocytes in the diffuse lymphoid tissue (DLT). The germinal centre (GC) has no positive cells. Anti-IgM × 115

an infiltration of immunoglobulin-positive, especially IgG, cells (Fig 3) into kidneys from OA fed birds. However, IgG staining of the glomeruli was seen more frequently and more strongly in sections from OA fed birds.

Serum immunoglobulins

Immunoglobulin concentrations were lower in sera from OA fed birds than in control sera (Fig 4). IgA levels were 66·35 per cent (± 2·77) and 62·04 per cent (± 5·80) of normal concentration at 2 and 4 ppm OA respectively. IgG levels were reduced to 63·77 per cent (± 4·88) and 62·29 per cent (± 7·75) of normal at 2 and 4 ppm OA and IgM values were 64·88 per cent (± 3·98) and 56·91 per cent (± 3·69) of normal values.

The PRIST IgE technique using antisera to human IgE showed no differences in IgE levels in sera from the two OA fed groups and from the controls. Very low levels of IgE, ranging from 400 to 695 counts per minute of ^{125}I labelled activity, were measured in all sera tested; it was not possible to calculate the exact concentration of IgE per litre of serum because of the very low counts.

Discussion

Regression of the immunological organs and of their lymphoid cell population in the OA treated birds (Dwivedi and Burns 1984) has been mirrored in the reduction of immunoglobulin-containing cells, as visualised by immunofluorescence, and in the decrease of serum immunoglobulins observed in the present study. The germinal centres and lymphoid follicles had very few fluorescing cells. The significance of the apparent migration of immunoglobulin-containing cells to subepithelial regions, especially in the bursa of Fabricius, is not known but it does suggest an altered immune mechanism.

Ochratoxin A has been shown to be an immunosuppressant in sublethal quantities in mice (Haubeck et al 1981, Creppy et al 1982), though Prior and Sisodia (1982) did not find changes in antibody titres to *Brucella abortus* or sheep red blood cells in mice fed 4 ppm OA. However, intraperitoneal injections of OA (5 mg kg^{-1} for 50 days) caused a depressed antibody response to *B abortus* but not to sheep erythrocytes. They suggested that OA, like cyclophosphamide, has a non-selective immunosuppressive effect in mice. In guinea pigs neither antibody titres to *B*

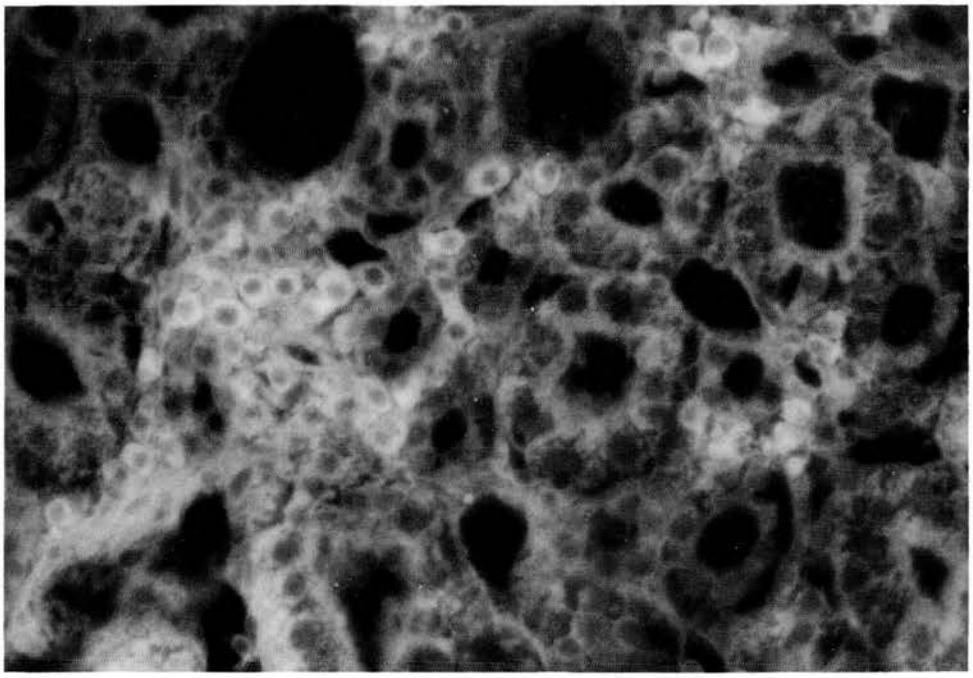


FIG 3: Kidney (2 ppm OA). Note the presence of fluorescing lymphocytes in the parenchyma. Anti-IgG ×570

abortus nor complement activity were affected by OA (Van der Merwe et al 1965, Richard et al 1975). Patterson et al (1981) could not find any alteration in immunoglobulin levels or titres in calves given sub-clinical doses of OA. An immunosuppressive effect has, however, been demonstrated in fowls in aflatoxicosis (Pier 1973, Thaxton et al 1974, Giambrone et al 1978).

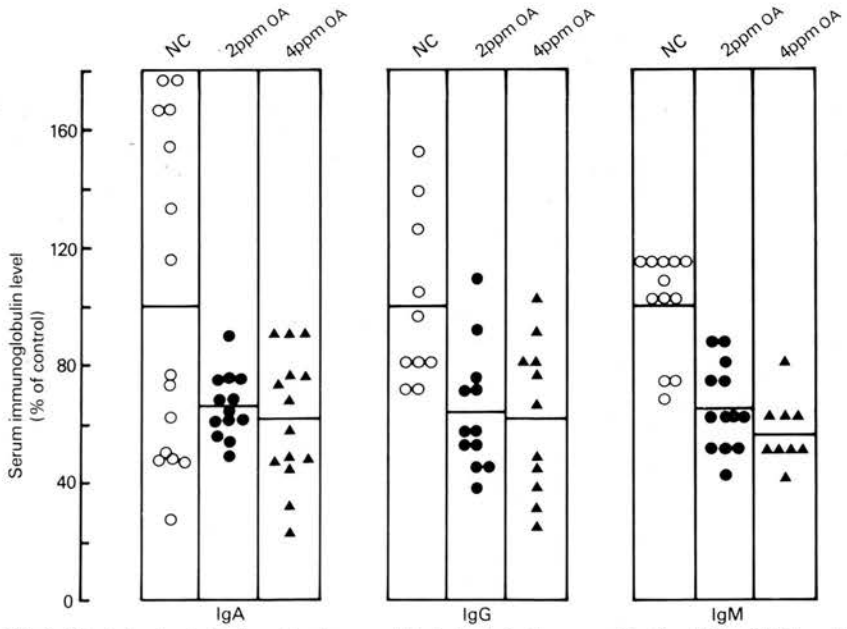


FIG 4: Effect of ochratoxin A on the immunoglobulin levels in the sera of broiler chicks. NC Normal concentration

Bolton et al (1980) have shown that there is a deposition of IgG along the glomerular basement membrane in experimental autoimmune glomerulonephritis in the fowl. Siller (1981) is of the opinion that, if all kidneys from routine autopsy material were to be examined histologically, lesions of proliferative glomerulonephritis would be seen with great frequency. Thus, staining of the glomerular basement membrane does not necessarily indicate autoimmune glomerulonephritis in the fowl though our findings of more glomeruli showing positive fluorescence for IgG in kidneys from OA fed birds might be taken as an indication of renal involvement. This correlates well with the reports of thickened glomerular basement membrane in ochratoxicosis (Dwivedi and Burns 1984, Dwivedi et al 1984). The presence of immunoglobulin-containing cells in kidneys from OA fed birds further corroborates the frequent lymphoid cell infiltration in the renal parenchyma (Dwivedi and Burns 1984).

Immunoglobulin production in the fowl is dependent on the bursa of Fabricius and other associated lymphoid organs and an intact thymus is necessary for the switch mechanism of IgM to IgG and, or, IgA to operate successfully (Bienenstock et al 1973). Thus, the decreased production of immunoglobulin-containing cells and the lowered levels of serum immunoglobulins can be considered to be a sequel to the regression of the lymphoid organs found in fowls with ochratoxicosis (Dwivedi and Burns 1984). Reduced IgM and IgG levels have also been observed in OA treated mice (Creppy et al 1982, 1983). Creppy et al (1979) related the immunosuppressive effect of OA in mice to its inhibitory action on protein synthesis.

IgE levels were not affected, nevertheless the present study shows that some IgE is present in the sera of even two- to three-week-old broiler chicks.

The immunosuppressive action of OA has practical implications because of the enhanced susceptibility of birds to secondary infections as seen in the increased incidence of air sacculitis in field outbreaks of ochratoxicosis in broiler fowls (Hamilton et al 1982). The possibility of adverse effects of OA on anticipated protection from vaccination programmes should not be discounted.

References

BIENENSTOCK, J., GAULDIE, J. & PEREY, D. (1973) *Journal of Immunology* **111,** 1112–1118

BLYTHMAN, H. E. & WHITE, R. G. (1977) *Immunology* **33,** 671–677

BOLTON, W. K., TUCKER, F. L. & STURGILL, B. C. (1980) *Journal of Clinical Laboratory Immunology* **3,** 179–184

BRANDTZAEG, P. (1981) *Journal of Histochemistry and Cytochemistry* **29,** 1302–1315

BURNS, R. B. & MAXWELL, M. H. (1981) *Veterinary Research Communications* **5,** 67–72

CHANG, C.-F., DOERR, J. A. & HAMILTON, P. B. (1981) *Poultry Science* **60,** 114–119

CHANG, C.-F. & HAMILTON, P. B. (1980) *Applied and Environmental Microbiology* **39,** 572–575

CHANG, C.-F., HUFF, W. E. & HAMILTON, P. B. (1979) *Poultry Science* **58,** 555–558

COONS, A. H., LEDUC, E. H. & CONNALY, J. M. (1955) *Journal of Experimental Medicine* **102,** 49–60

CREPPY, E.-E., LORKOWSKI, G., RÖSCHENTHALER, R. & DIRHEIMER, G. (1982) Proceedings of 5th International IUPAC Symposium on Mycotoxins and Phytotoxins. Wien (Austria), September 1–3, 1982, pp 289–292

CREPPY, E.-E., LUGNIER, A. A. J., FASIOLO, F., HELLER, K., RÖSCHENTHALER, R. & DIRHEIMER, G. (1979) *Chemical and Biological Interactions* **24,** 257–261

CREPPY, E.-E., STØRMER, F. C., RÖSCHENTHALER, R. & DIRHEIMER, G. (1983) *Infection and Immunity* **39,** 1015–1018

DWIVEDI, P. & BURNS, R. B. (1984) *Research in Veterinary Science* **36,** 92–103

DWIVEDI, P., BURNS, R. B. & MAXWELL, M. H. (1984) *Research in Veterinary Science* **36,** 104–116

GIAMBRONE, J. J., EWERT, D. L., WYATT, R. D. & EIDSON, C. S. (1978) *American Journal of Veterinary Research* **39,** 305–307

HAMILTON, P. B., HUFF, W. E., HARRIS, J. R. & WYATT, R. D. (1982) *Poultry Science* **61,** 1832–1841

HAUBECK, H. D., LORKOWSKI, G., KOLSCH, E. & RÖSCHENTHALER, R. (1981) *Applied and Environmental Microbiology* **41,** 1040–1042

HUFF, W. E., WYATT, R. D., TUCKER, T. L. & HAMILTON, P. B. (1974) *Poultry Science* **53,** 1585–1591

KROGH, P., HALD, B., PLESTINA, R. & CEOVIĆ, S. (1977) *Acta Pathologica et Microbiologica Scandinavica,* Section B **85,** 238–240

PATTERSON, D. S. P., SHREEVE, B. J., ROBERTS, B. A., BERRETT, S., BRUSH, P. J. & GLANCY, E. M. (1981) *Research in Veterinary Science* **31,** 213–218

PEKHAM, J. C., DOUPNIK, B., Jr & JONES, O. H., Jr (1971) *Applied Microbiology* **21,** 492–494

PIER, A. C. (1973) *Journal of the American Veterinary Medical Association* **163,** 1268–1269

PRIOR, M. G. (1981) *Canadian Journal of Comparative Medicine* **45,** 116–119

PRIOR, M. G. & SISODIA, C. S. (1982) *Canadian Journal of Comparative Medicine* **46,** 91–96

RICHARD, J. L., THURSTON, J. R., DEYOE, B. L. & BOOTH, G. D. (1975) *Applied Microbiology* **29,** 27–29

SAINTE-MARIE, G. (1962) *Journal of Histochemistry and Cytochemistry* **10,** 250–256

SHOTWELL, O. L., HESSELTINE, C. W. & GOULDEN, M. L. (1969) *Applied Microbiology* **17,** 765–766

SILLER, W. G. (1981) *Avian Pathology* **10,** 187–262

THAXTON, J. P., TUNG, H. T. & HAMILTON, P. B. (1974) *Poultry Science* **53,** 721–725

VAN DER MERWE, K. J., STEYN, P. S., FOURIE, L., SCOTT, D. B. & THERON, J. J. (1965) *Nature* **205,** 1112–1113

Received for publication July 6, 1983

Research in Veterinary Science 1984, 36, 104–116

Ultrastructural study of the liver and kidney in ochratoxicosis A in young broiler chicks

P. DWIVEDI, R. B. BURNS, M. H. MAXWELL, *Agricultural Research Council's Poultry Research Centre, Roslin, Midlothian EH25 9PS*

Ultrastructural changes are reported in the kidney and liver of 20-day-old broiler chicks fed ochratoxin A (OA), incorporated in the diet at levels of 2 and 4 ppm. Changes in the kidney included the presence of abnormally shaped mitochondria in the proximal convoluted tubules. There was an increase in the size and number of mitochondrial dense granules and cytoplasmic peroxisomes. Intranuclear and cytoplasmic lipid droplets and electron dense round bodies in the dilated smooth endoplasmic reticulum were also noted. Regional thickening and degeneration of the glomerular basement membrane was observed in some cases. In the liver from OA fed birds there was an increased accumulation of cytoplasmic glycogen in the hepatocytes. Abnormal mitochondrial ring forms in the kidney and the accumulation of glycogen in the liver are considered to be of diagnostic significance in ochratoxicosis of young broiler chicks. The severity of the changes was found to be dose related. These results suggest that the mitochondria in the proximal convoluted tubules of kidney were most sensitive to OA toxicity.

OCHRATOXIN A (OA), one of the most toxic fungal metabolites, chiefly produced by *Aspergillus ochraceus* and *Penicillium viridicatum*, has been shown in several recent outbreaks to be associated with natural disease in poultry (Hamilton et al 1982). Though OA is considered to be the most potent nephrotoxic mycotoxin in the broiler chick (Tucker and Hamilton 1971, Huff et al 1974, 1975), no report is available on renal ultrastructural changes in any species of poultry. However, OA induced ultrastructural changes have been described in the kidney of beagle dogs, pigs and mice (Szczech et al 1974, Elling 1977, Kitchen et al 1977, Shadmi and Nobel 1981) and in the liver of ducklings and rats (Theron et al 1966).

Histological examination of broiler chicks with experimentally induced ochratoxicosis showed marked pathological changes in the liver and the proximal convoluted tubules (PCT) of the kidneys (Dwivedi and Burns 1984). The purpose of the present study was to elucidate the nature of the ultrastructural hepatorenal changes in young broiler chicks fed ochratoxin A.

Materials and methods

Experimental procedure

A starter ration, analytically confirmed to be free from contaminating mycotoxins, was used. Diets containing 0, 2 and 4 ppm OA (Calbiochem or Sigma) were fed to randomly assigned groups of 22, 20 and 10 broiler chicks of both sexes respectively for 20 days from hatch. The birds were kept in an electrically heated brooder with food and water available ad libitum. Crystalline OA was dissolved in chloroform and mixed with the diet to give the desired concentration.

Electron microscopy

Pieces of kidney and liver, removed immediately after the birds had been killed by an overdose of pentobarbitone sodium, were chopped into 1 mm cubes and immersed in the following fixatives: kidney, 1 per cent modified Dalton's osmium tetroxide (3 per cent potassium dichromate, 2·6 per cent sodium chloride) at pH 7·4 and 330 mOsm for one hour at 4°C; liver, 1 per cent osmium tetroxide in 0·2 M sodium cacodylate buffer (pH 7·4 and 375 mOsm) for one hour at 4°C.

Following fixation, the tissues were dehydrated through increasing concentrations of ethanol and embedded in Araldite. Sections, 1 μm thick and stained with toluidine blue, were used for orientation. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined in an electron microscope.

Results

The ultrastructural alterations in the kidney and liver were, in general, dose dependent being more pronounced at 4 ppm OA than at 2 ppm.

Kidneys

Proximal convoluted tubules (PCT). PCT from untreated control birds were characterised by a brush border of microvilli with their associated structures lining the lumen and by epithelial cells containing nuclei and cytoplasmic organelles such as densely packed mitochondria, smooth and rough endoplasmic reticulum, peroxisomes (microbodies), lysosomes, etc. A few lipid droplets were also occasionally seen in the cytoplasm (Fig 1a).

In kidneys from OA treated birds changes were seen in various constituents of the PCT cells, particularly the mitochondria.

Mitochondria. The majority of the mitochondria were abnormal in shape. They were strikingly pleomorphic, being often enlarged, distended or elongated. Many of the elongated forms were bent upon themselves taking on a 'U' or 'ring' form (Fig 1b). Fusion of the membranes was often observed where the ends of the mitochondria met (Figs 2d and 2e). The spaces (vacuoles) within such ring forms were lined by a double membrane and appeared either empty or contained cytoplasmic material, peroxisomes or lipid droplets. They were placed either centrally (Fig 2a) or eccentrically (Figs 1b, 2b and 2f) so making the mitochondrion appear uneven in thickness. Such mitochondria had normal matrices but often tightly packed cristae. In extreme cases, one ring form could be found within another so causing concentricity or a chondriosphere (Fig 1b) and mitochondria were recognisable within membrane bound cytolysosomes (Figs 2e and 2f).

The matrices of the mitochondria contained electron-dense mitochondrial granules which were more prominent and numerous in the affected PCT (Figs 3a and 3b). These granules varied from 45 to 117 nm in size (they measured 30 to 50 nm in sections from normal birds) and could be observed anywhere in the matrix (Fig 3b). They also occurred in the ring form mitochondria (Fig 2c).

The numbers of mitochondria appeared to be reduced in many affected PCTs (Figs 5a and 5b).

Peroxisomes. Peroxisomes were increased in number and size in ochratoxicosis. Their size varied from 0·13 to 0·9 μm (normal: 0·1 to 0·5 μm) and were round, oval or elongated and bound by a single membrane (Fig 3a). Their granular matrix contained a crystalline nucleoid and lacked periodicity (Fig 3a inset).

Lysosomes. The lysosomes of affected PCT were normal in appearance, number and size, which ranged from 0·93 to 1·7 μm.

Endoplasmic reticulum. The smooth endoplasmic reticulum (SER) appeared dilated and contained round electron-dense bodies of variable size (Fig 4).

Cytoplasmic lipid droplets. Numerous lipid droplets ranging in size from 0·3 to 2·0 μm were seen in the cytoplasm. They were usually more numerous towards the basal part of the cells, round, oval or irregular in shape and they had a homogeneous structure with a moderately electron-dense and characteristic striated (wavy) appearance (Figs 4 and 5a). They were enclosed by an irregular dense osmiophilic border with a distinct interface. Some lipid droplets were also found in the basal processes and in the interstitium.

Golgi bodies. These and other cytoplasmic organelles did not appear to be affected in ochratoxicosis.

Nucleus. Nucleoli were prominent in some of the PCT epithelial cells from OA treated birds. Some of the nuclei contained lipid inclusions measuring from 0·03 to 0·76 μm in diameter. Their shapes varied from round or oval to irregular with a dense osmiophilic border (Fig 5b). Usually lipid droplets were seen at the periphery of the nucleus in close proximity to the nuclear membrane (Fig 5b inset).

Distal convoluted tubules. Some distal convoluted tubules were also affected showing a decrease in the number of mitochondria, vacuolation of the cytoplasm and some lipid droplets. Collecting tubules, collecting ducts and medullary loops showed no apparent deviation from the normal.

Glomerulus. In untreated fowls, the centre of the glomerular tuft contained a mass of mesangial cells round which the capillary loops were arranged. The glomerular capillary consisted of epithelial foot processes, capillary basement membrane and endothelial cell. The cytoplasm of the endothelial cell was continuous with the glomerular basement membrane throughout the internal surface of the capillary loops in the form of an uninterrupted delicate, thin, even membrane containing some fibrillary component in the dense central core (Fig 6a).

In kidneys from OA treated fowls, the glomerular basement membrane was unevenly thickened either locally or throughout the capillary loop (Fig 6b). There was extensive degeneration of the glomerular basement membrane. Aggregations of collagen fibres were often seen in the thickened region of the dense central core (Figs 7a and 7b). The endothelial cells were swollen and these together with the thickened glomerular basement membrane sometimes caused

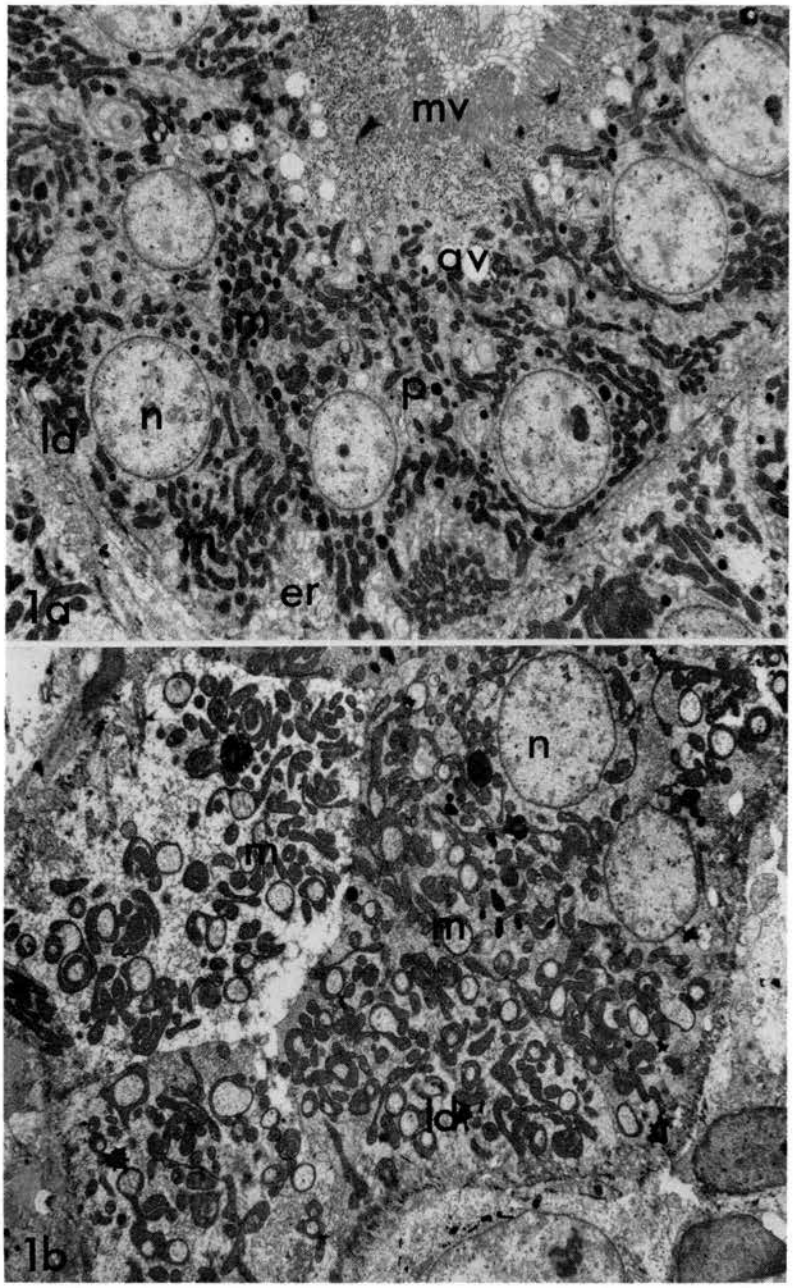


FIG 1a, 1b: Proximal convoluted tubules. (a) PCT from an untreated control fowl showing the normal structure. ×3280. (b) PCT from an OA treated (2 ppm) fowl showing U, C, cup, signet-ring and doughnut mitochondria and lipid droplets. Some mitochondria enclose other intracellular structures. ×3280. mv Microvilli; n Nucleus; m Mitochondria; av Apical vacuoles; ld Lipid droplets; er Endoplasmic reticulum; p Peroxisomes

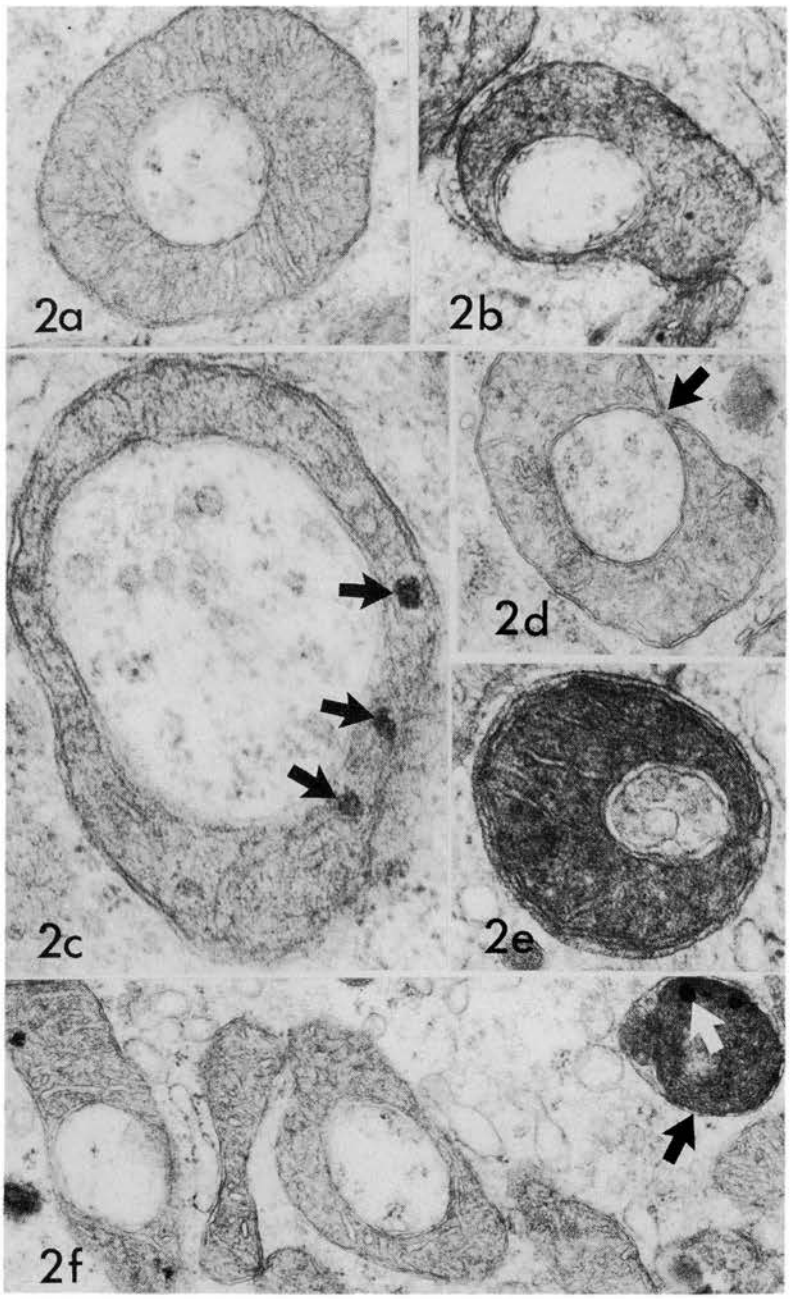


FIG 2a-2f: Pleomorphic mitochondria in PCT epithelial cells from an OA-treated (4 ppm) fowl. (a) Annular mitochondrion of uniform thickness. ×38,880. (b) A 'signet-ring' mitichondrion of uneven thickness. ×29,160. (c) A 'signet-ring' mitochondrion containing three large mitochondrial dense granules (arrows) with large central hole. ×61,560. (d) Fusion of the tips of a curved mitochondrion (arrow) forming a doughnut shape. ×28,560. (e) A double membrane bound doughnut mitochondrion (cytolysosome) enclosed within a double membraned structure and thereby revealing four closely associated membranes. ×61,560. (f) Ring form mitochondria with 'holes' centrally or eccentrically placed. Note the presence of a cytolysosome (arrow) enclosing a doughnut mitochondrion in the process of fusion with two prominent dense granules (white arrow). ×29,160

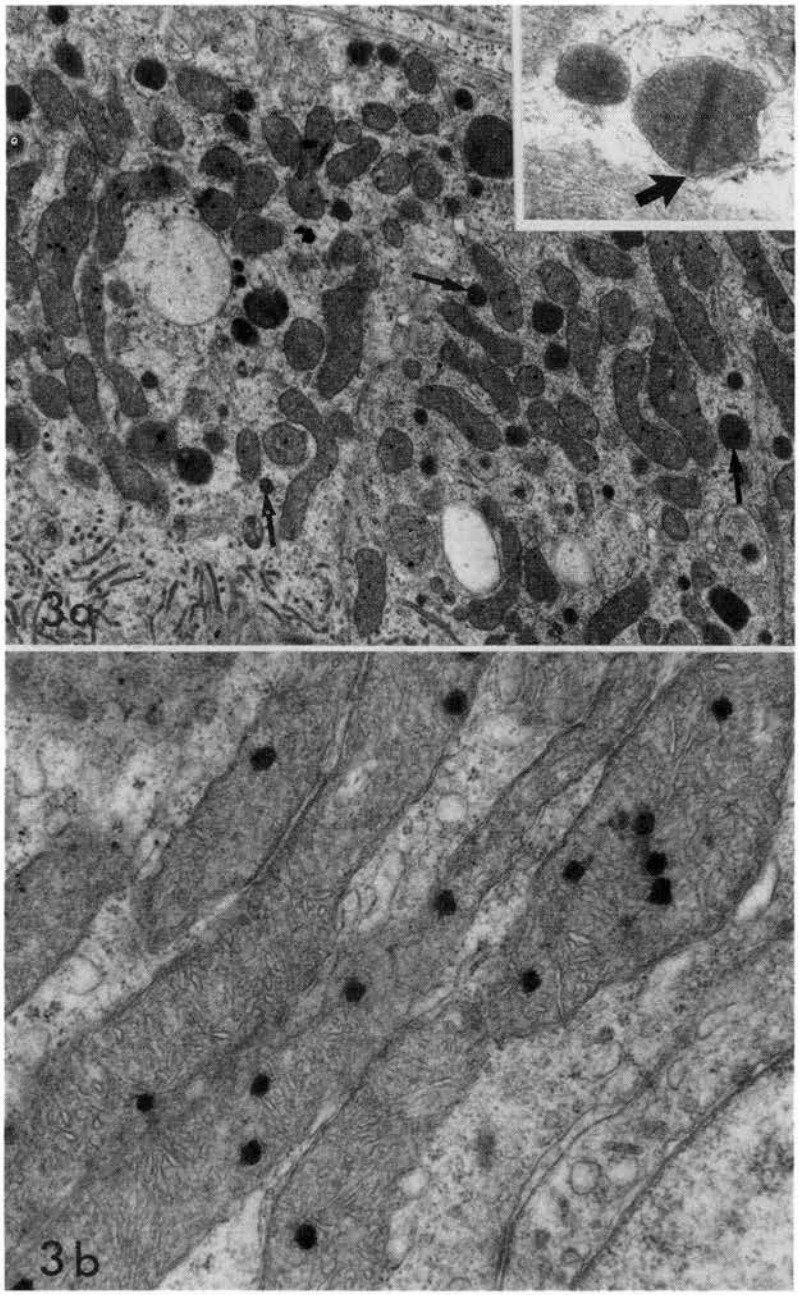


FIG 3a, 3b: (a) Epithelial cells of the PCT from an OA treated fowl. Note the presence of large numbers of peroxisomes (arrow) of varying size and prominent mitochondrial dense granules. ×8748. The inset shows a peroxisome with a crystalline rod-like nucleoid (arrow). ×29,160. (b) Elongated mitochondria from PCT cells from an OA treated fowl. Note the presence of numerous large electron-dense mitochondrial granules. ×29,160

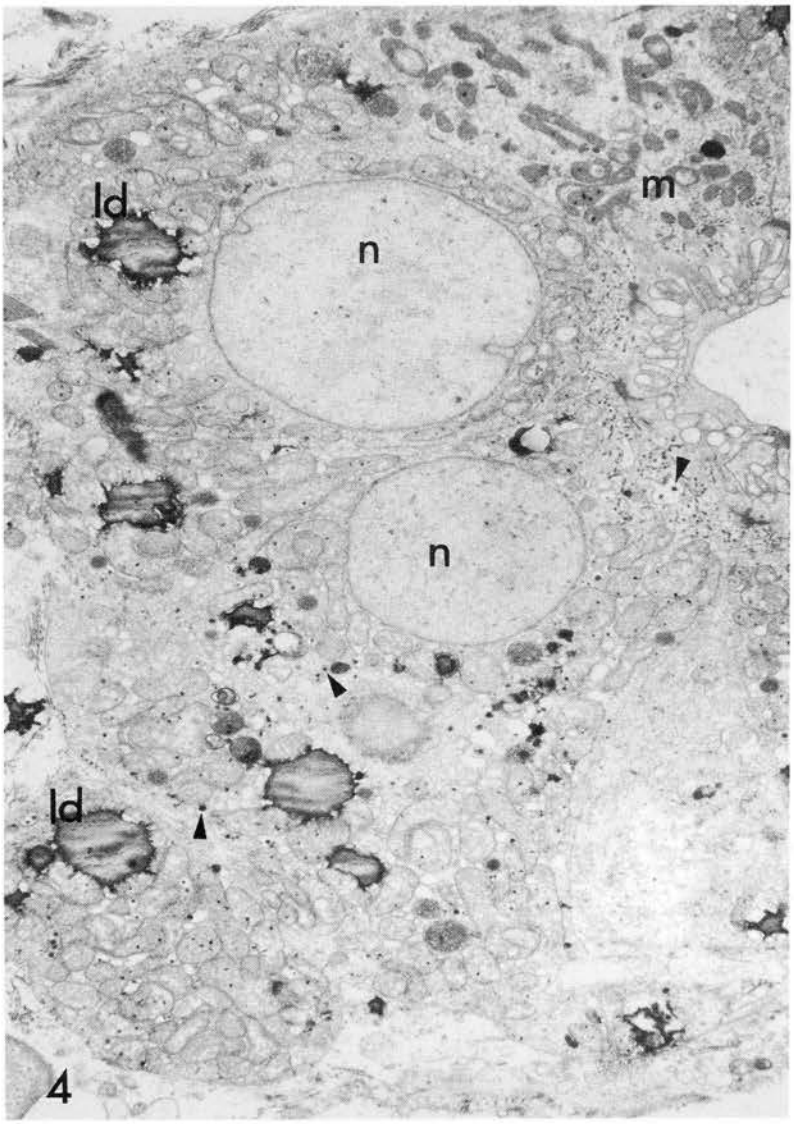


FIG 4: PCT epithelial cells from an OA treated fowl showing numerous cytoplasmic lipid droplets (ld) some of which mask the mitochondria and other organelles. Note the crenated, irregular, dense osmiophilic border of the lipid droplets. A large number of electron-dense small round bodies are in the dilated smooth endoplasmic reticulum profiles (arrowheads). Doughnut mitochondria (m) are also present adjacent to the nucleus (n). ×6156

almost complete obliteration of the capillary lumen (Figs 6b and 7a).

Liver

Small amounts of glycogen scattered unevenly in the cytoplasm in the form of electron-dense granules measuring about 20 nm in diameter was a characteristic feature of the hepatocytes from normal control birds (Fig 8a). In liver sections from OA treated birds, the glycogen content was considerably increased (Fig 8b) and not infrequently the hepatocytes were entirely studded with glycogen. The glycogen appeared as large aggregations of electron-dense granules, either in a monoparticulate form, with individual particles ranging in size from 15 to 30 nm in diameter (Fig 9a) or in the form of rosettes in close association with the SER (Fig 9b). Their size and

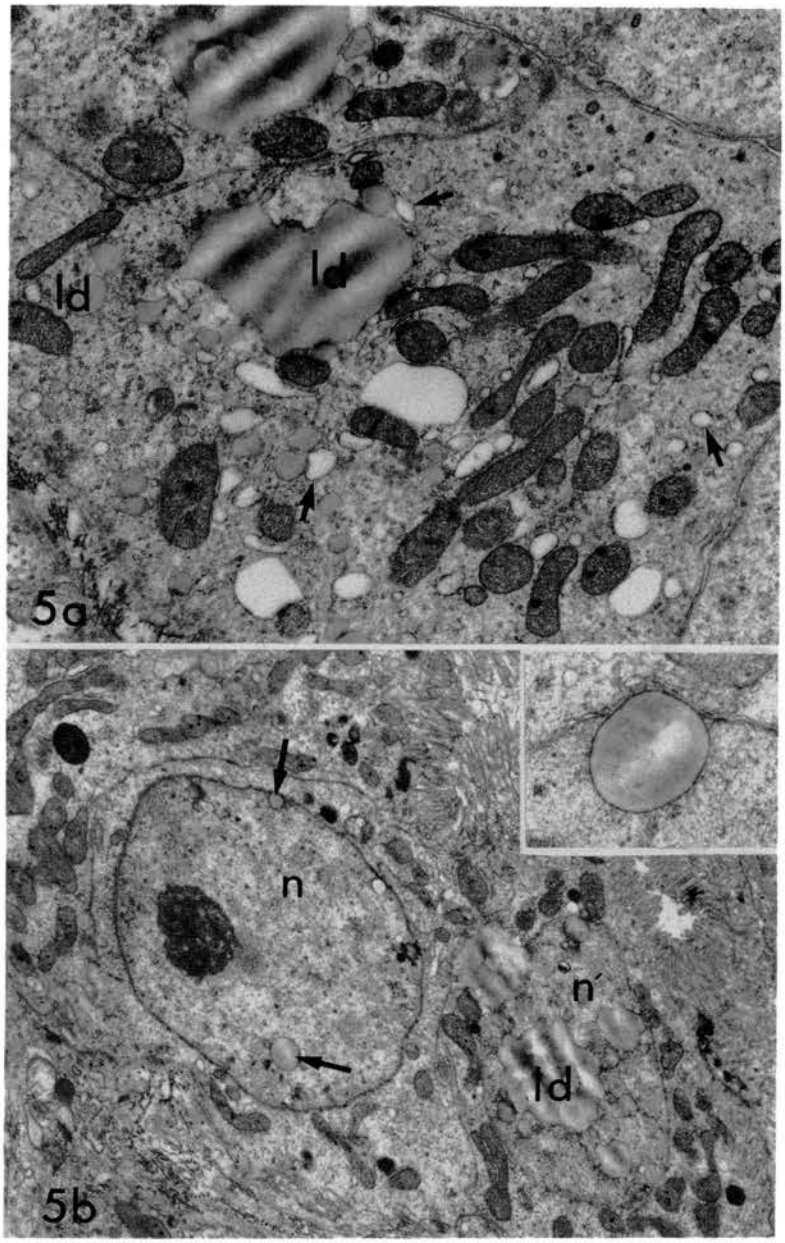


FIG 5a, 5b: (a) An epithelial cell (PCT) from an OA treated fowl showing the presence of characteristic small and large sized lipid droplets (with an irregular outline and a wavy appearance). Note the dilated endoplasmic reticulum (arrows) many of which contain small LD. × 14,094. (b) PCT cells showing several round lipid inclusions (0·03 to 0·06 μm) at the periphery of the larger nucleus (n). The smaller nucleus (n') is almost full of irregular lipid droplets (measuring up to 0·5 μm). Note the scarce mitochondrial population. × 7050. The inset shows a very large lipid droplets (0·76 μm), adjacent to the nuclear membrane. × 18,792

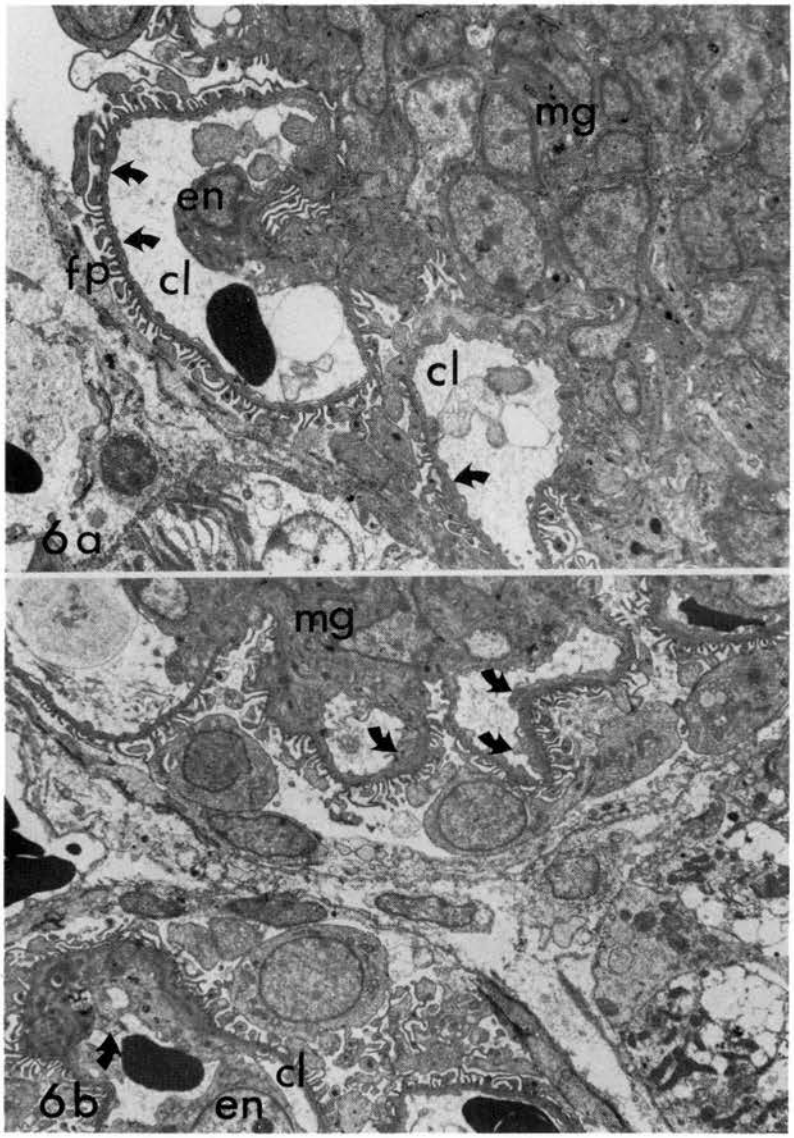


FIG 6a, 6b: Glomeruli showing glomerular basement membrane. (a) Glomerulus from a control fowl. Note the glomerular basement membrane as a thin even membrane (arrows) throughout the capillary loops. ×3280. fp Foot processes; cl Capillary lumen; en Endothelial cell; mg Mesangium. (b) Glomeruli from an OA fed (4 ppm) fowl showing localised thickening of the basal lamina of the glomerular basement membrane (arrows). Note the obliteration of a capillary lumen (cl) by thickened glomerular basement membrane and swollen endothelial cell (en). ×3280

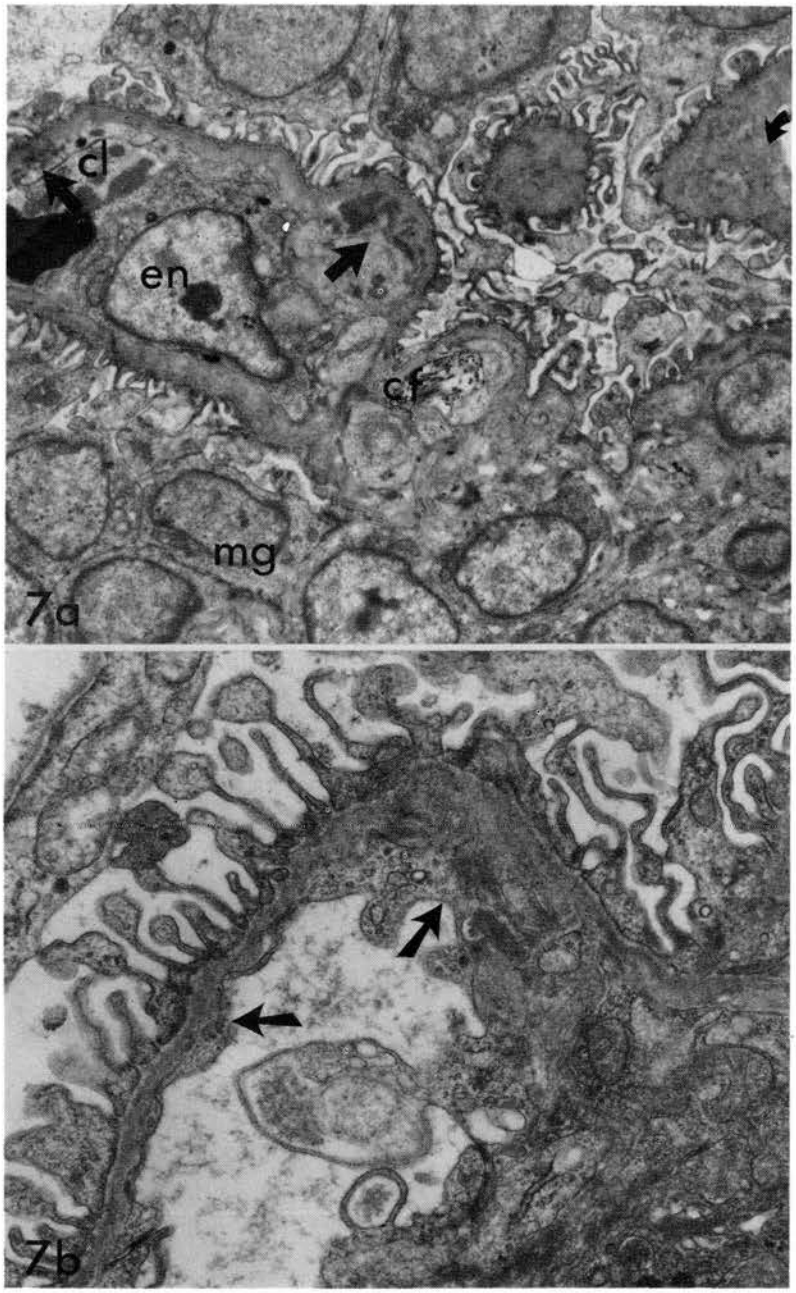


FIG 7a, 7b: Higher magnification of the thickened GBM from an OA treated fowl. (a) Thickened GBM (arrows) showing extensive degeneration and also collagen fibres (cf). The endothelial cell (en) is swollen and the capillary lumen (cl) is reduced. ×5710. (b) GBM showing thickened dense core, extensive degeneration (arrow), and presence of collagen fibres. × 14,095

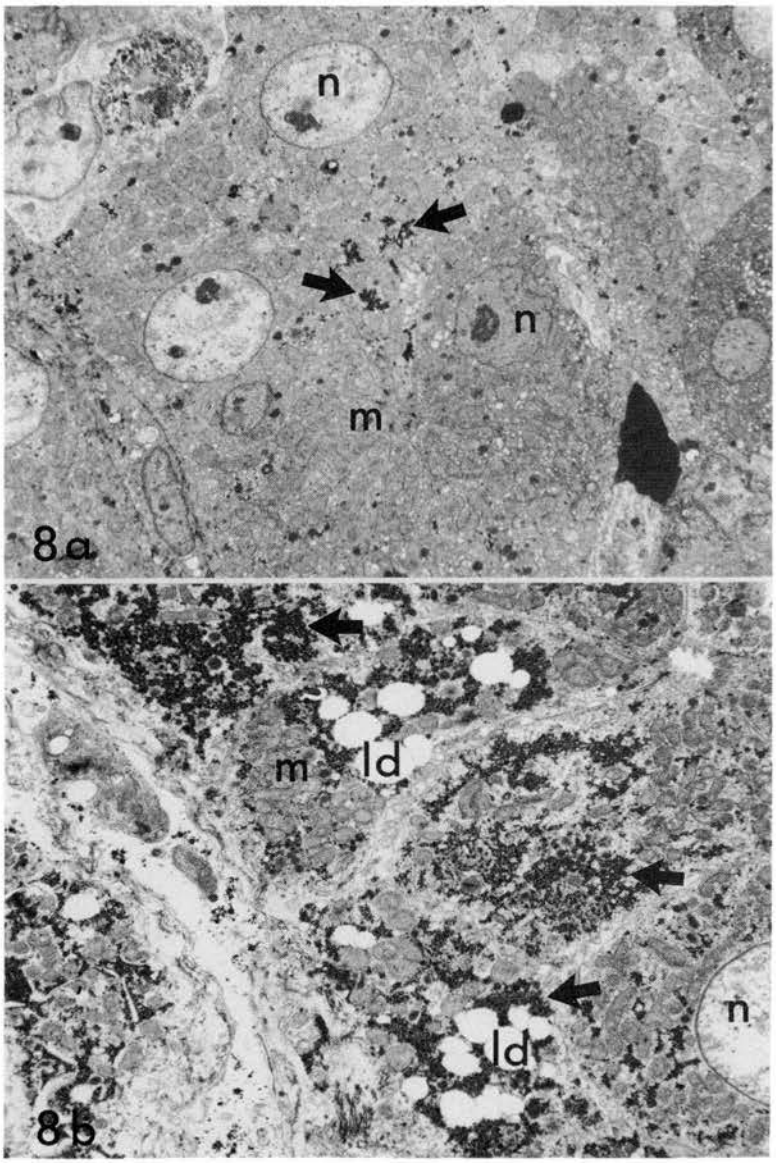


FIG 8a, 8b: (a) Liver from a control fowl. Note the presence of small aggregates of electron-dense glycogen granules (arrows) in the cytoplasm. ×3280. n Nucleus; m Mitochondria. (b) Liver from an OA treated (4 ppm) fowl. Note large aggregates of electron dense glycogen granules (arrows) and fat vacuoles (ld). The mitochondria (m) have been pushed aside in some hepatocytes. ×4620. n Nucleus

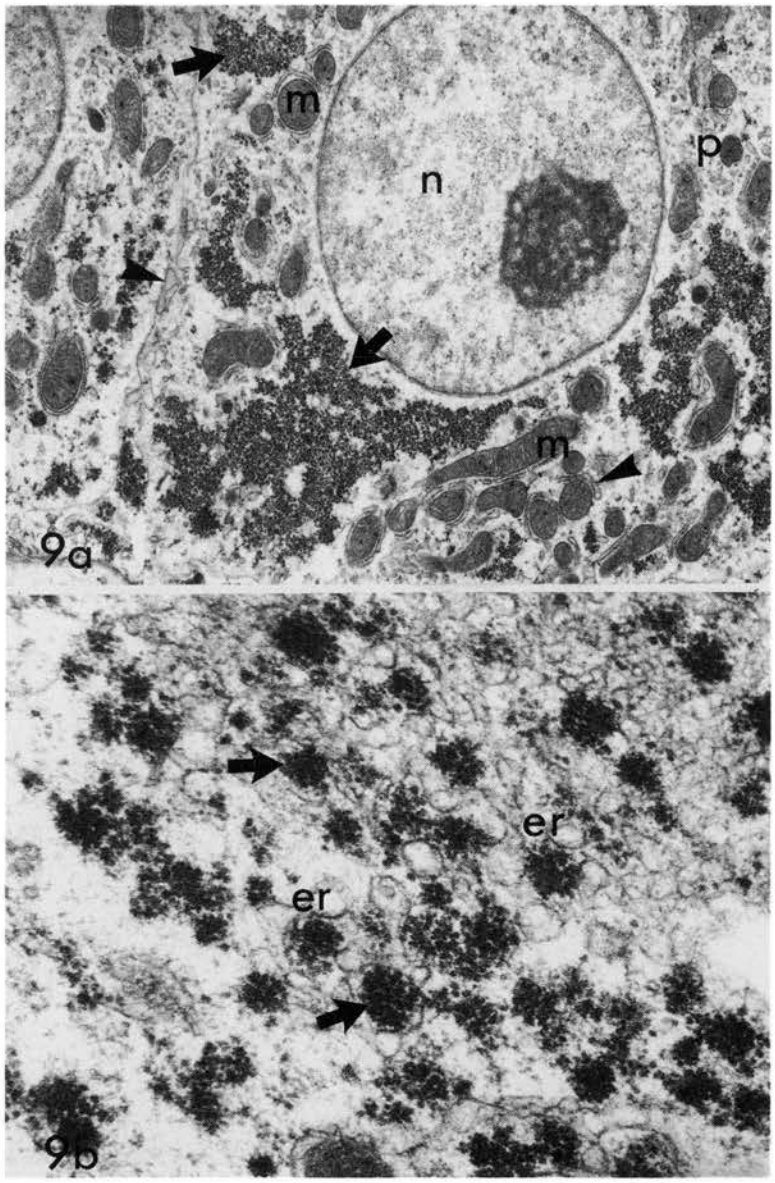


FIG 9a, 9b: Liver from OA treated fowls. (a) Glycogen, as monoparticulate granules (arrows), is increased in the cytoplasm. The mitochondria (m), endoplasmic reticulum (arrowheads) and peroxisomes (p) appear to be largely unaffected. ×8750. (b) Aggregates of glycogen rosettes (arrows) in the cytoplasm of the hepatocytes in close association with the smooth endoplasmic reticulum (er). ×46,170

concentration varied widely. Sometimes these aggregates seemed to mask the cell organelles. Many electron-dense peroxisomes, ranging from 0·15 to 0·42 μm in diameter, were also seen in the cytoplasm of affected cells. Small lipid droplets were seen as clear round or oval vacuoles in the cells (Fig 8b). Lipid droplets and glycogen aggregates sometimes appeared to push the mitochondria and other cell organelles to the periphery of the hepatocytes. However, the nucleus, mitochondrial architecture, endoplasmic reticulum, peroxisomes and lysosomes appeared to be largely unaffected (Fig 9a). Birds fed 4 ppm OA had a higher glycogen content in the liver cells than those fed 2 ppm OA.

Discussion

Ultrastructural examination of kidneys and livers from broiler chicks with experimentally induced ochratoxicosis has confirmed much of the light microscopic findings (Dwivedi and Burns 1984) and demonstrated a possible site of cellular injury.

Abnormal mitochondria were common in the PCT of OA fed fowls (Fig 1b). Less severe changes have been reported in the kidney of pigs (Elling 1977) and mice (Shadmi and Nobel 1981) during ochratoxicosis and of the fowl in phenoxy-acid toxicity (Björklund and Erne 1971). Ring and 'U'-shaped mitochondria have been observed in the liver from fowls with fatty liver and kidney syndrome but not in the kidney (Siller and Wight 1976). Ring shaped mitochondria have been reported in liver from rats treated with carbon tetrachloride (Reynolds 1960), ammonium carbonate (David and Kettler 1961) and alcohol (Koch et al 1978) and in clear cell carcinoma of the kidney (Seljelid and Ericsson 1965). However, the significance of such transformation of mitochondrial morphology is not known (Ghadially 1982). Ring form mitochondria, a more or less constant feature in PCT cells in the present study, appeared to be formed by fusion of the membranes at the tips of curved elongated mitochondria (Fig 2d). The spaces or vacuoles, which could be of varying size and shape, were lined by a double membrane similar to the mitochondrial membrane (Figs 2a to 2f), further supporting the notion of their formation by this process.

Since OA is known to inhibit enzyme activity (Elling 1979) and mitochondrial respiration (Moore and Truelove 1970, Meisner and Chan 1974, Meisner 1976), the mitochondrial changes seen in the present study might account for the impairment in renal function in ochratoxicosis of the fowl, reported by Huff et al (1975) and by Svendsen and Skadhauge (1976).

Mitochondrial dense granules are related to mineral metabolism, representing sites of divalent cation accumulation (Ghadially 1982). Since Huff et al (1975) found plasma and electrolyte changes, particularly a decrease in potassium levels, at higher levels of dietary OA in broiler fowls, it seems to be of considerable relevance that these granules were greatly increased, both in number and in size, in the present study.

Normally there is very little lipid in the PCT of the fowl, but in OA-fed birds there was a moderate deposition of cytoplasmic lipid, similar to that reported in OA-treated beagles (Szczech et al 1974). In the present study, lipid droplets were also found in the nuclei. This occurs under certain pathological conditions and is usually accompanied by lipid accumulation in the cytoplasm (Ghadially 1982). The nature of round electron-dense bodies seen in the SER is unknown but their density is similar to some of the lipid droplets and this could be evidence of lipid synthesis in the endoplasmic reticulum (Stein et al 1972). Similar bodies were demonstrated in the endoplasmic reticulum of fowls with fatty liver and kidney syndrome (Siller and Wight 1976).

The increase in number and size of peroxisomes is interesting, since Ghadially (1982) relates peroxisomes with lipid metabolism and Björklund and Erne (1971) found an increase in the number of peroxisomes (microbodies) in the PCT from phenoxy acid treated broilers.

Thickening of the glomerular basement membrane has not often been reported in fowls. Focal thickening was seen in endotheliosis (Simpson et al 1959) and diffuse thickening and degeneration with extensive collagen deposition was observed in salt toxicity (Siller 1981) in fowls. In the present study, thickened glomerular basement membrane was often found to be accompanied by swelling of the glomerular capillary endothelium and these changes sometimes led to the occlusion of the lumen. This could be associated with the decreased glomerular filtration found in ochratoxicosis of fowls by Huff et al (1975) and Svendsen and Skadhauge (1976).

Huff et al (1979) observed an increase in glycogen at the periphery of the hepatic lobules of fowls fed 4 and 8 ppm OA levels (but not at lower levels), and it was suggested (Warren and Hamilton 1980) that the accumulation of glycogen in ochratoxicosis was due to an inhibition of protein kinase enzyme and glycogenolysis. In the present study, an accumulation of excessive amounts of monoparticulate and rosette forms of glycogen was observed in the cytoplasm of the hepatocytes of all the OA treated birds even at 2 ppm, and throughout the liver. This increase in the amount of glycogen in the liver could perhaps be due to diminished usage and increased storage rather than as a result of altered metabolic activity. This is supported by the absence of appreciable changes in

the mitochondria, endoplasmic reticulum and other cell organelles in the present study. The only other report of ultrastructural studies in birds is that of Theron et al (1966) in ducklings. They did not find any accumulation of glycogen in the liver; however, their findings are based on a single dose of OA (100 μg) over a short period.

On the basis of the present study it can be concluded that in young broiler chicks OA is more nephrotoxic than hepatotoxic and that the mitochondria of the PCT are the organelles which are most sensitive to OA injury. The mitochondrial ring forms in the kidney PCT and the accumulation of glycogen in the liver may be of diagnostic significance.

Acknowledgements

We would like to thank Miss J. Robb, East of Scotland College of Agriculture, Edinburgh, for analysis of the feed samples for mycotoxins. Thanks are also due to the Association of Commonwealth Universities, London for the grant of a Commonwealth Scholarship to P.D.

References

BJÖRKLUND, N.-E. & ERNE, K. (1971) *Acta Veterinaria Scandinavica* **12,** 243–256

DAVID, H. & KETTLER, L. H. (1961) *Zeitschrift für Mikroskopische Anatomie* **53,** 857–866

DWIVEDI, P. & BURNS, R. B. (1984) *Research in Veterinary Science* **36,** 92–103

ELLING, F. (1977) Mycotoxins in Human and Animal Health. Eds J. V. Rodricks, C. W. Hesseltine & M. A. Mehlman. Illinois, Panthotox Publishers. pp 499–506

ELLING, F. (1979) *Acta Pathologica Microbiologica* Sect A **87,** 237–243

GHADIALLY, F. N. (1982) Ultrastructural Pathology of the Cell and Matrix. 2nd edn. London, Butterworths. pp 80–222, 582

HAMILTON, P. B., HUFF, W. E., HARRIS, J. R. & WYATT, R. D. (1982) *Poultry Science* **61,** 1832–1841

HUFF, W. E., DOERR, J. A. & HAMILTON, P. B. (1979) *Applied and Environmental Microbiology* **37,** 122–126

HUFF, W. E., WYATT, R. D. & HAMILTON, P. B. (1975) *Applied Microbiology* **30,** 48–51

HUFF, W. E., WYATT, R. D., TUCKER, T. L. & HAMILTON, P. B. (1974) *Poultry Science* **53,** 1585–1591

KITCHEN, D. N., CARLTON, W. W. & HINSMAN, E. J. (1977) *Veterinary Pathology* **14,** 392–406

KOCH, O. R., ROATTA DE CONTI, L. L., BOLANOS, L. P. & STOPPANI, A. O. M. (1978) *American Journal of Pathology* **90,** 35–44

MEISNER, H. (1976) *Archives of Biochemistry and Biophysics* **173,** 132–140

MEISNER, H. & CHAN, S. (1974) *Biochemistry* **13,** 2795–2800

MOORE, J. H. & TRUELOVE, B. (1970) *Science* **168,** 1102–1103

REYNOLDS, E. S. (1960) *Journal of Histochemistry and Cytochemistry* **8,** 331–332

SELJELID, R. & ERICSSON, J. L. E. (1965) *Journal de Microscopie* **4,** 759

SHADMI, A. & NOBEL, T. N. (1981) *Annales de Recherches Vétérinaires* **12,** 75–83

SILLER, W. G. (1981) *Avian Pathology* **10,** 187–262

SILLER, W. G. & WIGHT, P. A. L. (1976) *Research in Veterinary Science* **21,** 79–89

SIMPSON, C. F., PRITCHARD, W. R. & HARMS, R. H. (1959) *Journal of the American Veterinary Medical Association* **134,** 410–416

STEIN, O., BAR-ON, H. & STEIN, Y. (1972) Progress in Liver Diseases. Volume 4. Eds H. Popper & F. Schaffner. New York and London, Grune and Stratton. p 45

SVENDSEN, C. & SKADHAUGE, E. (1976) *Acta Pharmacologica et Toxicologica* **38,** 186–194

SZCZECH, G. M., CARLTON, W. W. & HINSMAN, E. J. (1974) *Veterinary Pathology* **11,** 385–409

THERON, J. J., VAN DER MERWE, K. J., LIEBENBERG, N., JOUBERT, H. J. B. & NEL, W. (1966) *Journal of Pathology and Bacteriology* **91,** 521–529

TUCKER, T. L. & HAMILTON, P. B. (1971) *Poultry Science* **50,** 1637

WARREN, M. F. & HAMILTON, P. B. (1980) *Applied and Environmental Microbiology* **40,** 522–525

Received for publication July 6, 1983

Proceedings XVII World's Poultry Congress, Helsinki, Aug. 1984, pp. 559-561

Scientific

OCHRATOXICOSIS A IN BROILERS AND TURKEYS : A COMPARATIVE IMMUNOPATHOLOGICAL STUDY. P. Dwivedi and R.B. Burns, AFRC Poultry Research Centre, Roslin, Midlothian EH25 9PS, SCOTLAND.

Ochratoxicosis A has caused heavy morbidity and mortality in natural disease outbreaks in broilers and turkeys (7). We have reported pathological and immunological changes in ochratoxin (OA)-fed broiler chicks (4, 5, 6).

The present work reports the relative effects of OA on growth response and on the pathological and immunological changes of young broilers and turkeys.

Materials and Methods

Experimental Procedures. Broiler chicks and turkey poults were assigned to four groups of eleven birds and fed diets containing 0 and 4 ppm OA for 8 or 10 weeks from hatch. The birds were weighed weekly and clinical signs recorded. From each group, 2 birds were killed at 2, 4, 6, 8 and 10 weeks of age and the liver, kidney, spleen, thymus, bursa of Fabricius, crop, proventriculus and gizzard were weighed. Pieces from the liver, kidney and lymphoid organs were collected at 4 weeks of age for histology (4) and immunofluorescent microscopy (6). The liver and kidney were examined for ultrastructural changes (5).

Delayed Hypersensitivity (DH) Response. For DH studies, groups of 4 or 5 broilers and turkeys were used, which were maintained separately on diets containing 0, 2 and 4 ppm OA. They were sensitised at 7 to 8 weeks of age with Avian Tuberculin (PPD) and killed Mycobacterium avium, and tested 3 weeks later (12) at various skin sites (Fig. 2, 3). Corresponding contralateral areas were used for control saline injections. Skin thickness was measured at 0, 24, 48 and 72 hours post injection. Skin was collected at 48 h for histology.

Statistical Analysis. Data on body weights were analysed by weighted analysis of variance, examining the differences between diets and sexes within weeks. Organ weights were

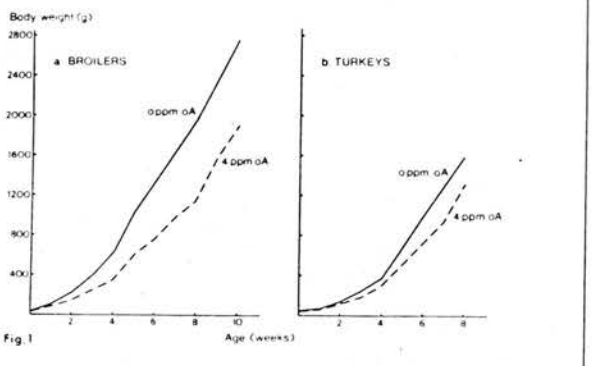


Fig. 1

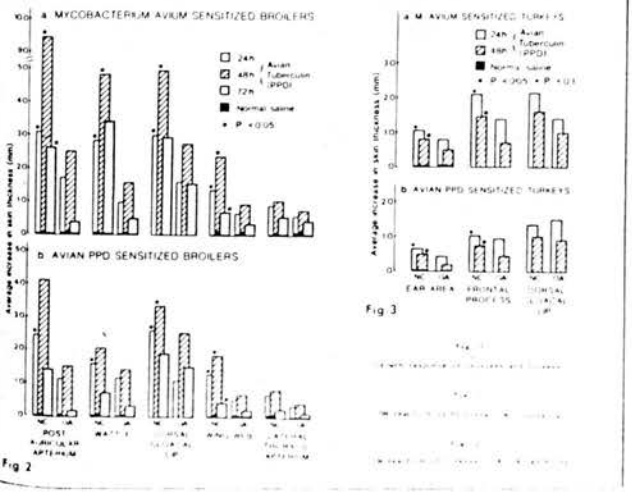


Fig. 2

Fig. 3

also analysed by a weighted analysis of variance, taking absolute weights. Treatment differences were examined by analysis of covariance, taking body weights as a covariate. Comparative changes in the lymphoid organs of broilers and turkeys (corrected for body weight) from week to week between 0 and 4 ppm birds were also examined.

For DH responses, increases in skin thickness were examined at each time interval by analysis of variance, examining the differences between diets and sensitisation. Where appropriate, the corresponding saline swelling was also examined as a covariate.

Results and Discussion

Growth Response. Growth depression in both broilers and turkeys became more marked with age (Fig. 1), becoming detectable at 2 weeks of age in broilers and at 3 weeks in turkeys. The changes in weight differences in both broilers (F10, 117 of 23.23) and turkeys (F8, 110 of 10.78) were consistent with earlier reports (4, 9, 2).

In broilers the most noticeable clinical effect was a loss of yellow pigmentation from the shank, but this was not obvious in turkeys. Loss of carotenoid pigmentation may be correlated with the hypocarotenoidemia observed in experimental ochratoxicosis in broiler chicks (8, 11) but not in turkey poults (2).

Organ Weights. No detectable differences were observed between 0 and 4 ppm OA groups in the weights of the liver, kidney, crop, proventriculus and gizzard. Table 1 shows that the growth and development of the lymphoid organs were depressed significantly with age. The effect of OA on the spleen and bursa was more severe in broilers than in turkeys and a similar effect occurred in the thymus.

Suppressed development and regression of the immunological organs in the present study appears to be one of the most consistent effects of OA (2, 4, 9). Absolute organ weight analysis (14) and analysis of covariance (13) as done in the present experiments, are considered to be superior to analysis of relative organ weight.

TABLE 1. EFFECT OF OA ON IMMUNOLOGICAL ORGANS IN BROILERS AND TURKEYS

WEIGHT (g)	Treatment	AGE (WEEKS) 2	4	6	8	10	F VALUE
A. BROILERS							(F4,11)
Body wt	NC	276.20	470.80	1189.00	2014.25	2750.00	
	OA	133.89	124.35	419.50	1083.25	1886.67	
Spleen	NC	0.28	0.62	3.02	5.79	6.38	62.75
	OA	0.16	0.29	1.32	2.21	4.03	
	(1)[1]	0.16 (0.07)	0.38 (0.12)	-0.04 (0.26)	-0.44 (0.38)	0.25 (0.36)	1.69
Bursa of Fabricius	NC	0.52	1.34	2.78	5.11	6.39	12.27
	OA	0.53	1.08	1.35	1.57	3.57	
	(2)[1]	-0.05 (0.21)	-0.31 (0.35)	-0.45 (0.60)	-1.74 (0.88)	-0.99 (0.83)	1.01
Thymus	NC	1.21	4.71	6.07	6.87	12.61	3.76
	OA	0.27	0.67	1.37	1.96	6.43	
	(3)[1]	-0.75 (0.62)	-1.55 (1.00)	-3.28 (1.46)	-2.45 (2.41)	-4.20 (2.42)	0.62
B. TURKEYS							(F3,11)
Body wt	NC	121.25	266.20	981.60	1582.60		
	OA	140.60	264.15	587.50	1135.26		
Spleen	NC	0.07	0.14	0.89	1.94		7.30
	OA	0.07	0.13	0.50	1.11		
	(1)[1]	-0.01 (0.05)	-0.01 (0.07)	-0.14 (0.19)	-0.61 (0.18)		3.79
Bursa of Fabricius	NC	0.25	0.73	1.70	2.46		18.35
	OA	0.21	0.43	0.84	1.50		
	(2)[1]	-0.07 (0.06)	-0.29 (0.10)	-0.22 (0.16)	-0.56 (0.14)		3.87
Thymus	NC	0.27	0.65	1.50	2.49		10.74
	OA	0.38	0.37	0.54	1.27		
	(3)[1]	-0.10 (0.09)	-0.28 (0.13)	-0.38 (0.21)	-0.75 (0.20)		3.10
C. BROILER-TURKEY							(F3,19)
Spleen	(1)[2]	0.00 (0.13)	0.06 (0.19)	1.10 (0.39)	1.79 (0.54)		5.83
Bursa of Fabricius	(2)[2]	0.00 (0.15)	0.09 (0.23)	0.49 (0.33)	1.50 (0.42)		4.33
Thymus	(3)[2]	0.69 (0.41)	1.38 (0.63)	3.22 (0.88)	2.21 (1.07)		2.55

NC (Normal Control) - 0ppm OA; OA (Contaminated) - 4ppm OA

(1)[1] Estimated difference of the mean organ weights (body weight included as a covariate) between OA and NC birds (and their standard errors).

(2)[2] Estimated difference of the regression of the mean organ weights (body weight included as a covariate) between OA and NC birds, in broilers compared with turkeys (and their standard errors).

Histology, Immunofluorescence and Electron Microscopy. Changes in 4-week-old broilers were similar to those of 3-week-old broilers (4, 5, 6). In general, similar but less marked changes were noted in turkey poults. Changes frequently observed in the kidney were: congestion and haemorrhage, dilatation of the proximal convoluted tubules (PCT), thickening of the glomerular basement membrane (GBM) which fluoresced with anti-IgM and IgG. Abnormal ring-form and swollen mitochondria together with an increased number of peroxisomes in the PCT were seen in both species (Fig. 4). Vacuolation of the hepatocytes and a hepatic accumulation of glycogen were more marked in broilers than in turkeys. In the lymphoid organs the germinal centres were reduced and lymphoid cell depletion was a common feature. Correspondingly, IgA-, IgM- and IgG-cells were markedly reduced. Regression of the immunocompetent organs appeared to be due to lymphoid depletion, resulting in decreased immunoglobulin levels in ochratoxicosis (3, 4, 6).

DH Responses. Classical delayed hypersensitivity, a common measure of CMI, was markedly depressed in OA birds of both species (Fig. 2, 3). Killed M. avium was better for sensitisation than Avian Tuberculin (PPD). The reactions were very strong in broilers. The postauricular apterium of the fowl and the earskin of the turkey were found to be the best sites and they can be recommended for routine use. Our results agree with other workers (12). In the skin, histology showed characteristic granulomatous reactions in both species, though less severe in turkeys. An absence of gross thickening should not be interpreted as a negative PPD (10), without histological confirmation. Our results suggest that OA caused severe depression of CMI in both species, though more markedly in broilers. This could possibly be correlated with the regression of the thymus. The immunosuppression of our experiments might explain the increased incidence of air-sacculitis seen in natural ochratoxicosis A outbreaks (7). Immune responses can be depressed by a synergistic effect of OA and aflatoxin (1).

We conclude that OA not only caused damage to the liver and kidney but also adversely affected the immune system by suppressing the immunocompetent organs. Both humoral and CMI responses were depressed in broilers and turkeys and, though the effects of OA were more severe in broilers, the mechanism of toxicity appeared similar in both species.

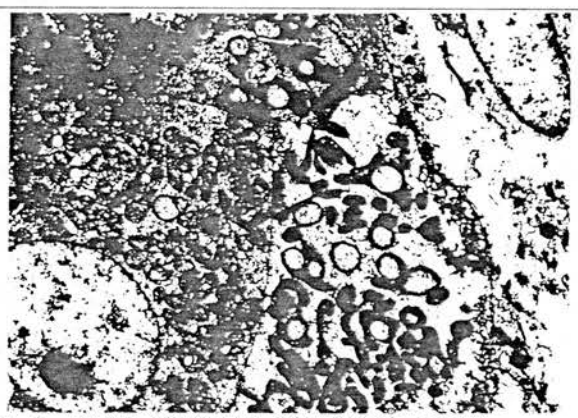


Fig. 4. Turkey kidney PCT. Mitochondria x 7310.

Acknowledgements. We thank Mr D. Waddington, AFRC Poultry Research Centre, for statistical analyses and Blount Memorial fund of the Animal Health Trust, U.K. for financial assistance.

1. Campbell, M.L. Jr., May, J.D., Huff, W.E. and Doerr, J.A. (1983). Poult. Sci. 62: 1138-1144.
2. Chang, C.-F., Doerr, J.A. and Hamilton, P.B. (1981). Poult. Sci. 60: 114-119.
3. Creppy, E.-E., Størmer, F.C., Röschenthaler, R. and Dirheimer, G. (1983). Infect. & Immun. 39: 1015-1018.
4. Dwivedi, P. and Burns, R.B. (1984). Res. Vet. Sci. 36: 92-103.
5. Dwivedi, P., Burns, R.B. and Maxwell, M.H. (1984). Res. Vet. Sci. 36: 104-116.

6. Dwivedi, P. and Burns, R.B. (1984). Res. Vet. Sci. 36: 117-121.
7. Hamilton, P.B., Huff, W.E., Harris, J.R. and Wyatt, R.D. (1982). Poult. Sci. 61: 1832-1841.
8. Huff, W.E. and Hamilton, P.B. (1975). Poult. Sci. 54: 1308-1310.
9. Huff, W.E., Wyatt, R.D., Tucker, T.L. and Hamilton, P.B. (1974). Poult. Sci. 53: 1585-1591.
10. McCorkle, F.M., Luginbuhl, G.H., Simmons, D.G., Morgan, G.W. and Thaxton, J.P. (1983). Dev. Comp. Immunol. 7: 517-524.
11. Osborne, D.J., Huff, W.E., Hamilton, P.B. and Burmeister, H.R. (1981). Poult. Sci. 61: 1646-1652.
12. Rose, M.E. and Bradley, J.W.A. (1977). Avian Pathol. 6: 313-326.
13. Shirley, E. (1977). Toxicol. 8: 13-22.
14. Stevens, M.T. (1976). Toxicol. 5: 311-318.

OCHRATOXIKOSE-A BEI MASTHUHNERN UND PUTEN: EINE VERGLEICHEND IMMUNPATHOLOGISCHE STUDIE. P. Dwivedi und R.B. Burns, AFRC Poultry Research Centre, Roslin, Midlothian EH25 9PS, SCOTLAND.

Masthühner und Puten wurden vom Schlupf an, 10 Wochen hindurch mit einer Mischung gefüttert, die 4 mg Ochratoxin A pro Kg enthielt. Diese Tiere blieben in ihrem Wachstum zurück und bei den Masthühnern, aber nicht den Puten, war ein Verlust des karotinoiden Pigmentes der Beine zu verzeichnen. In den Nieren der Mastküken, und zu einem geringeren Grad der Puten, verursachte die Ochratoxikose eine Verbreiterung der Basalmembranen der Glomeruli und eine Vergrößerung der Hauptstücke mit ringformiger Veränderung ihrer Mitochondrien. In der Leber konnte eine Glykogenansammlung nachgewiesen werden. Der Lymphozytengehalt der lymphatischen Organe, und besonders der Bursa Fabricii, war bedeutend verringert. Auch dieser Befund war bei den Masthühnern stärker ausgeprägt als bei den Puten. Aber bei beiden Geflügelarten waren die antikörperbildenden und immunoglobulinhaltigen Zellen verringert. Die verzögerten Hypersensitivitätsreaktionen der Haut zeigten bei den Tieren die mit Ochratoxin behandelt worden waren, daß die zellvermittelten Immunreaktionen unterdrückt waren.

L'OCHRATOXICOSE A DES POULETS DE CHAIR ET DES DINDES : UNE ÉTUDE IMMUNOPATHOLOGIQUE COMPARATIVE. P. Dwivedi et R.B. Burns, AFRC Poultry Research Centre, (Centre de Recherches sur les Volailles), Roslin, Midlothian EH25 9PS, ECOSSE.

Des poulets de chair et des dindes ont été alimentés avec des regimes contenant 4 ppm d'ochratoxine A (OA) pendant 10 semaines à partir de leur éclosion. Leur croissance a été retardée et une perte de pigmentation caroténoïde a été constatée aux jarrets des poulets de chair, mais non dans le cas des dindes. L'ochratoxicose a provoqué un grossissement des tubules circonvolutionnaires proximaux (TCP) et un épaississement de la membrane glomérulaire de base du rein, ainsi qu'une accumulation de glycogène dans le foie; ces changements étaient plus prononcés chez les poulets de chair que chez les dindes. Il

y a eu une perte de cellules lymphoïdes dans les organes lymphoïdes, notamment la bourse de Fabricius et le thymus, dans le cas des poulets de chair, et en moindre mesure dans le cas des dindes. Des mitochondries annulaires anormales ont été constatées dans les TCP.

Dans le cas des poulets de chair ainsi que des dindes, les cellules contenant des immunoglobulines et constituant des anticorps ont diminué en quantité. Les réactions d'immunité transmises par les cellules, évaluées au moyen des réactions hypersensibles retardées dans la peau, ont été affaiblies chez les oiseaux traités à l'OA.

LA OCRATOXICOSIS A EN GALLINAS TIPO BROILER Y EN PAVOS : UN ESTUDIO INMUNOPATOLOGICO. P. Dwivedi y R.B. Burns, AFRC Poultry Research Centre, Roslin, Midlothian EH25 9PS, ESCOCIA.

Gallinas de tipo broiler y pavos fueron alimentados con dietas de contenido 4 ppm ocratoxína A (OA) para 10 semanas de la nidada. Se retardó la crece, y hubo una perdida de pigmento carotinoide de las patas de las gallinas pero no de los pavos. La ocratoxicosis resultó en un agrandecimiento de los tubulos convolutados proximales y en un espesamiento de la membrana basal de los glomérulos en los riñones junto con una acumulación del glícogeno en el hígado; estos cambios se notarón mas en las gallinas que en los pavos. Las celulas linfoides se reducieron de los organos linfoides, especialmente de la bolsa de Fabricio y del tímo, de las gallinas y a un grado menos de los pavos. Mitocondrías con una forma abnormal de anillo se vieron en los tubulos convolutados proximales.

En las gallinas y en los pavos, celulas conteniendo la inmunoglobulina y formando anticuerpos se reducieron en numero. Las respuestas de la inmunologia celular, veidos por medio de reacciones de la hipersensitividad en la piel, se deprimieron en todas las aves tratadas con la OA.

ОХРАТОКСИКОЗ А БРОЙЛЕРОВ И ИНДЮШЕК: СРАВНИТЕЛЬНОЕ ИММУНОПАТОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ. П. Двиведи и Р.Б. Бернс, АФРЦ, Исследовательский Центр Птицеводства, Рослин, Мидлотиан, EH25 9PS, Шотландия.

Бройлерам и идюшкам скармливали корм, содержащий 4 ппм охратоксина А (ОА) в течение 10 недель со времени выведения. Торможение развития и потеря каротеноидных пигментов на голенях происходило у бройлеров, но этого не наблюдалось у индюшек. Охратоксикоз вызывал увеличение проксимальных извитых канальцев (ПИК) и уплотнение клубочковой базальной мембраны почек и накопление гликогена в печени; такие изменения более выделены у бройлеров. Лимфоидные клетки в лимфоидных органах истощались, особенно в фабрициевой сумке и вилочковой железе бройлеров и в меньшей степени у индюков. Абномальные кольцевидные митохондрии наблюдались в ПИК.

Количество клеток, содержащих иммуноглобулин и формирующих антитела уменьшилось. Иммунологическая реакция посредством клеток, наблюдаемая при замедлении реакции повышенной чувствительности кожи, была понижена у птиц, обработанных ОА.

561