

THE UNIVERSITY OF HULL

**Comparative studies on the structure and
function of teleost lymphoid organs**

**being a Thesis submitted for the Degree of
Doctor of Philosophy
in the University of Hull**

by

Christopher John Secombes, B.Sc. (Leeds)

January, 1981.

TABLE OF CONTENTS

		Page No.
Chapter 1	General Introduction	1
Chapter 2	General Methods	15
Chapter 3	Histological changes in Lymphoid organs following injection of soluble or particulate antigens	
	Introduction	23
	Materials and methods	27
	Results	30
	Discussion	49
Chapter 4	Antigen localization in the Carp <u>Cyprinus carpio</u> L.	
	Introduction	65
	Materials and methods	67
	Results	73
	Discussion	83
Chapter 5	Humoral immune responses of Carp to soluble and cellular Antigens	
	Introduction	95
	Materials and methods	97
	Results	110
	Discussion	126
Chapter 6	Cell-mediated responses in Carp	
	Introduction	147
	Materials and methods	147
	Results	156
	Discussion	169

Chapter 7	The effect of thymectomy and early immunization in Rainbow Trout	
	Introduction	179
	Materials and methods	180
	Results	186
	Discussion	197
Chapter 8	General discussion: The fish immune system in phylogenetic perspective	208
	Summary	219
	Acknowledgements	223
	Bibliography	224
	Plates 1-52	

GENERAL INTRODUCTION

Current concepts in immunology

The bodies of all animals need a defence mechanism to prevent invasion by pathogens and this requires the capacity to recognise self and non-self, so that no damage to self tissues occurs. In its less sophisticated form there is a fairly non-specific recognition of self or invading organisms, particularly microorganisms, which results in phagocytosis, encapsulation or the production of various soluble substances such as bacterial lysins (Manning, & Turner, 1976). The main effector cells of phagocytosis and intracellular destruction are the macrophages and polymorphonuclear leucocytes in vertebrates, or amoebocytes/coelomocytes in invertebrates. Metchnikoff (1905) was the first to study these phagocytic cells in a systematic way, using a wide variety of animal species from simple multicellular organisms to mammals. In all cases cells were seen to migrate to the sites of injury or infection, followed by ingestion of any invading material. These cells do not carry intrinsic receptors with the structural characteristics necessary for antigen recognition, yet play a very important role in defence of the body (Rowley, 1962, 1966).

In vertebrates, however, it has been known since the work of Behring and Kitasato (1890) with diphtheria and tetanus, that specific protection can be obtained. Nowadays it is realised that two broad categories of this specific mechanism can be distinguished operationally. In one case, the ability to respond immunologically can be conferred

upon an animal that has been challenged by a foreign antigen merely by transfer of blood serum from an immune animal. This type of reaction is called humoral immunity and is detected by the presence of specific antibodies in the serum. The second type of immune response can be conferred upon an animal by transfer of living cells, but not by immune serum, and is called cell-mediated immunity. The cells of central importance for both of these types of immune response were established, largely by the work of Gowans and his co-workers (Gowans, 1962; Gowans, McGregor, Cowen & Ford, 1962; McGregor & Gowans, 1963), to be lymphocytes. Upon antigenic stimulation a distinct subpopulation of precursor lymphocytes can be shown to develop into plasma cells, which are known to produce and secrete antibody (Fagraeus, 1948). Lymphocytes have also been shown to be present in the infiltrate of skin grafts in the process of rejection.

The immune system of vertebrates is also characterized by memory and diversity. This adaptive immune response provides a more effective defence in that appropriate immunological cells combat the particular agents infecting the body at any one time. Specific antibodies can also greatly enhance the disposal of antigen by facilitating its adherence to phagocytic cells, an effect called opsonization by Wright and Douglas (1903), as can complement, once activated by antigen-antibody complexes. These responses seem to be superimposed on the more phylogenetically ancient defences, increasing the efficiency of the non-specific immune system to a considerable extent.

This relatively simplistic view of the immune response has been drastically refined in the last fifteen years. It was noted that in children suffering from immunological deficiency disorders there was usually a thymic abnormality (Good, Dalmasso, Martinez, Archer, Pierce & Papermaster, 1962). This was soon clarified by Miller and his colleagues (Miller, Dukor, Grant, Sinclair & Sacquet, 1967; Miller, Mitchell & Weiss, 1967) who showed that thymectomy in mice at birth caused a decrease in the number of circulating lymphocytes, severe impairment of graft rejection and in vitro manifestations of cellular immunity, and a reduced humoral antibody response to some but not all antigens. Normal responsiveness could be restored by an injection of thoracic duct cells from an unoperated donor (Miller, Mitchell & Weiss, 1967). Further, if adult mice were X-irradiated, which destroys the ability of lymphocytes to divide, bone-marrow cell reconstitution restored their immunological responsiveness. However, if they were thymectomized before irradiation they were similar to neonatally thymectomized mice even after bone marrow reconstitution. It was concluded that precursor cells from the bone marrow migrate to the thymus (a primary lymphoid organ) where they differentiate into thymus-derived lymphocytes, T cells. These T cells do not synthesize antibody but divide, after contact with antigen, and differentiate into cells capable of destroying tumours or allografts (killer T cells), or into cells able to collaborate in the production of antibody to a variety of antigens (helper cells). The effect of T cells

upon antibody production can also be suppressive. Following antigen stimulation they develop into large lymphocytes but not plasma cells (Sell & Gell, 1965). Lastly, they have also been found to liberate a variety of non-immunoglobulin factors, lymphokines, which are released specifically but which react in a non-specific way on other cells. Such lymphokines include macrophage migration inhibition factor (MIF), interferon, lymphotoxin, vascular permeability factors and blastogenic factors (Bloom, 1971; David & David, 1972).

Glick, Chang and Jaap (1956) were the first to show that in birds the bursa of Fabricius had a role in antibody production. It has since been found that hormonal bursectomy in ovo, or surgical bursectomy at hatching, particularly when combined with sublethal X-irradiation, has a marked suppressive effect on the ability of the chicken to produce specific antibodies, but without affecting cell-mediated responses (Cooper, Peterson, South & Good, 1966; Alm & Peterson, 1969). It is now generally agreed that the population of cells responsible for antibody biosynthesis is a thymus-independent population.

Present evidence suggests that both T and B cells possess specific receptors for antigen but that their specificity is clonally restricted (Wigzell, 1970). The clonal selection theory postulates that individual immunocompetent cells are committed before their initial contact with antigen to respond to a limited range of antigenic specificities and that this commitment is expressed by the existence of cell surface antigen-binding receptors of single specificity (Burnet, 1957).

Some antigens such as flagellin polymer can elicit antibody production when only B cells are present, however the situation with many other antigens is more complex, requiring interaction between T and B cells for a full antibody response. In irradiated, thymectomized mice reconstitution with either thymus cells or bone marrow cells elicits only weak responses to such antigens. When both cell types are given a normal response is obtained (Miller & Mitchell, 1968; Mitchell & Miller, 1968; Nossal, Cunningham, Mitchell & Miller, 1968), but not if the thymus cells were sonicated or irradiated (Claman, Chaperon & Selner, 1968). In vitro experiments were more difficult to accomplish, but Doria, Martinozzi, Agarossi and Di Pietro (1970) have shown that spleen cells from neonatally or adult thymectomized, irradiated bone marrow chimeras could be restored, by the addition of normal syngeneic thymus cells, to respond to sheep erythrocytes (SRBC's) in vitro. More recently Schimpl and Wecker (1971) showed that thymocytes from cortisone-treated syngeneic donors could restore the primary in vitro anti-SRBC response of anti- θ serum-treated mouse spleen cells. The T cell component appears to become optimally functional after it has peripheralized to secondary lymphoid organs and undergone specific antigenic stimulation. So in general to obtain optimal restoration of in vitro anti-SRBC responses, thymus cells needed to be injected into the irradiated recipients with SRBC's, to "educate" the T cells (Chan, Mishell & Mitchell, 1970; Hartman, 1970). The thymus derived lymphocytes act in a specific way as

"helper" cells, allowing B cells to realize their full specific antibody forming potential.

The effect of T cells is also seen on the type of immunoglobulin molecule produced. T-independent antigens usually induce only IgM antibody responses, whereas with T-dependent antigens there is a switch from IgM to IgG antibody production. However it has recently been shown that appropriate T-independent antigens can induce IGY (IgRAA) as well as IgM antibody in Xenopus laevis (Manning & Jurd, 1981). Nevertheless, there is increasing evidence that the switch from IgM to IgG is largely dependent on the regulatory effect of appropriately activated T cells. The evolutionary advantages of such a mechanism are readily apparent. Since the two lymphocyte types are specific, and need to be activated by antigen for antibody synthesis by B cells to develop optimally, important control mechanisms concerning antigen recognition and tolerization should have evolved primarily in the T cell population.

Antigen induced unresponsiveness, or tolerance, can result from either induced suppression of B cells by T cells or from clonal deletion. Taylor (1968, 1969) showed that T cells of mice made tolerant to bovine serum albumin were unable to co-operate with normal B cells in an adoptive transfer system in irradiated recipients. Miller and Mitchell (1970) induced tolerance to SRBC in mice with cyclophosphamide and observed that recirculating T cells in the thoracic duct lymph (but not the thymus) were specifically tolerant. Chiller, Habicht and Weigle (1970) found that tolerance existed in both B and T cells of mice

rendered tolerant by a single injection of deaggregated human gamma globulin. So both T and B lymphocytes are susceptible to tolerance induction, but the susceptibilities of these two lymphocyte classes differ considerably in both the dose of tolerogen and the time required for tolerance induction. The duration of tolerance is significantly less in B cells than in T cells and the immunological status of the whole animal reflects that of the T cell population in the case of a T-dependent antigen. These complex interactions have received several extensive reviews (Katz & Benacerraf, 1972; Katz, 1977).

The co-operation of T and B cells was initially thought of as a simple bridging between T cells and B cells, to provide a stimulus to the latter by concentrating antigen. However, evidence was soon discovered that responses to T-dependent antigens were to a large extent also macrophage-dependent. It was known a few years before the realisation of T and B cell co-operation that macrophages were a necessary component before lymphocytes would produce antibody (Fishman, 1961; Fishman & Adler, 1963; Mosier, 1967). Animals injected with antigen, bound to live macrophages, developed satisfactory cell-mediated immunity (Unanue & Feldman, 1971). Animals injected with macrophages that had been exposed to antigen gave better secondary responses than animals given equivalent amounts of antigen (Mitchison, 1969). Lastly, macrophages that had been reacted with lysed SRBC's, washed and mixed with thoracic duct lymphocytes, conferred an ability on such lymphocytes to bring about a response

to SRBC's. Macrophage-free preparations injected into irradiated hosts produced antibodies within a few days (Ford, Gowans & McCullagh, 1966). No antibody was detected if the macrophage stage was omitted. Thymectomized animals did not respond to antigen bound to live macrophages (Unanue, 1970). Similarly, X-irradiated or immunologically tolerant recipients did not respond (Mitchison, 1969) even though macrophages from tolerant animals handle immunogens normally (Humphrey & Frank, 1967). Clearly macrophages were unable to synthesize antibody in these experiments. Indeed, tolerance was shown not to be associated with macrophage dysfunction. In fact tolerance such as seen in "high dose" tolerance, may result from direct interaction of excess antigen with lymphocytes, whereas to elicit a positive immune response the antigen must first be processed by macrophages.

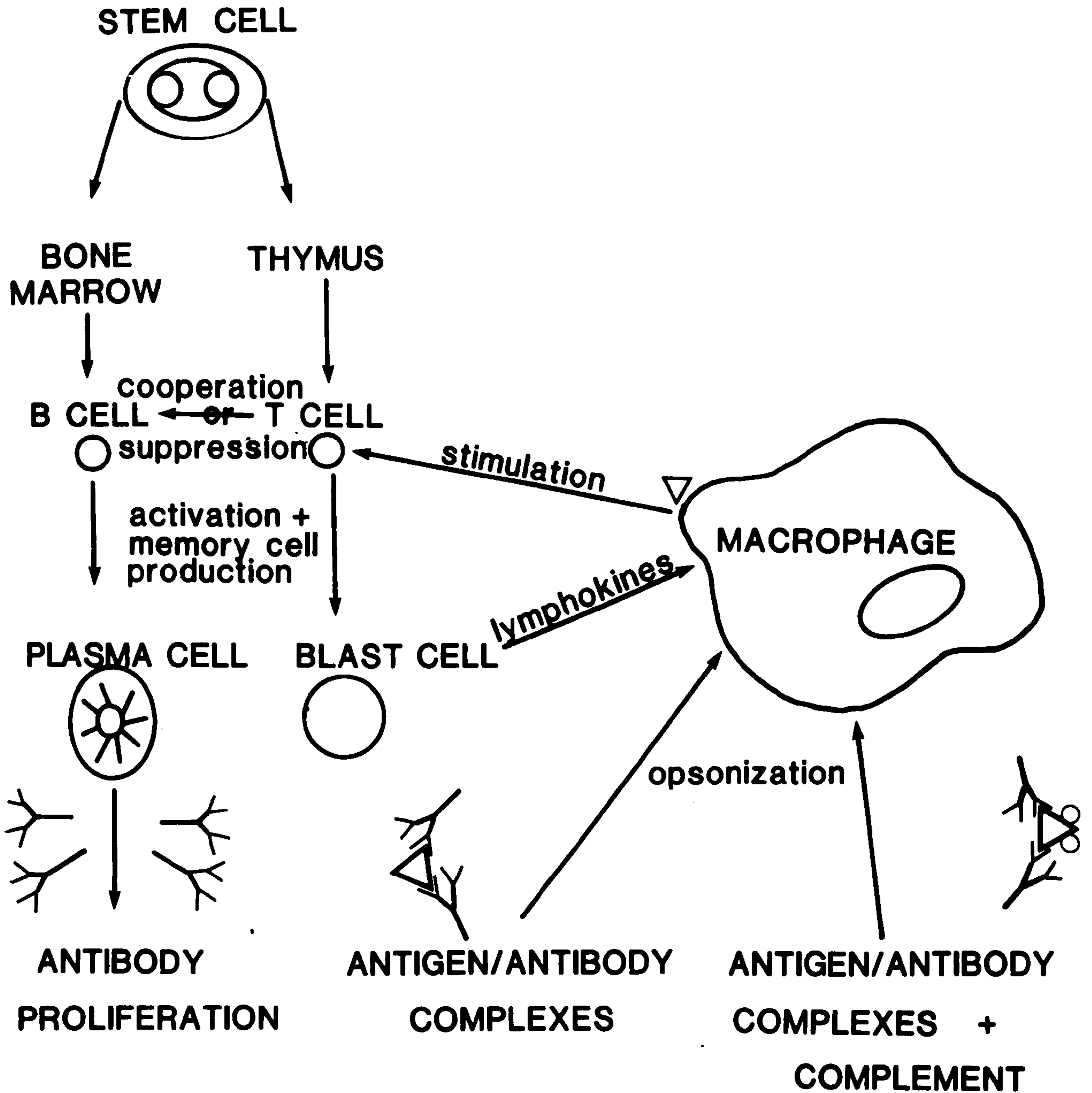
The regulation of lymphocyte functions by macrophages has recently been reviewed by Unanue (1978). The main interrelationships within the lymphoid system and between the lymphoid system and the phagocytic system are summarized in Figure 1. Obviously with such a complex system it is difficult to isolate and work on the various components. Simpler antigen-specific systems which reflect those of higher animals have been sought, and their phylogenetic relationships postulated.

Phylogenetic perspectives

Current concepts of immunity are largely based on studies in homiotherms, birds and mammals. At lower levels

Figure 1.

Diagram of the main interrelationships within the mammalian
immune system.



of phylogeny these mechanisms may be easier to understand and separate out.

Specific cell-mediated immunity arose early in evolution, particularly striking in the graft reactions of coelenterates (Hildemann, Raison, Cheung, Hull, Akaka & Okamoto, 1977) and annelids (Cooper, 1969, 1971), but the appearance of immunoglobulins was a vertebrate innovation. Thus, the basic phenomenon of vertebrate immunity exists at the level of the cyclostomes (Pollara, Finstad & Good, 1966) and appears to be correlated with the earliest appearance of definitive lymphoid tissue (Good & Papermaster, 1964; Papermaster, Condie, Finstad & Good, 1964). However, agnathans lack plasma cells. A well organised thymus and spleen, as well as kidney-associated lymphoid aggregates, appear at the levels of Chondrichthyes and Osteichthyes, and the immunocompetence of these fish has been clearly demonstrated (Hildemann, 1958; Clem & Sigel, 1963; Sigel & Clem, 1966; Ridgway, Hodgins & Klontz, 1966; Botham, Grace & Manning, 1980). Plasma cells have also been found in advanced elasmobranchs and in chondrosteian and teleost fishes (Finstad, Papermaster & Good, 1964; Clawson, Finstad & Good, 1966; Good, Finstad, Pollara & Gabrielsen, 1966). However, only IgM antibodies, composed of light chains and μ -type heavy chains, have been conclusively found in fish (Marchalonis, 1971b). They occur predominantly as high molecular weight tetramers in teleosts (Shelton & Smith, 1970) although low molecular weight, monomer, IgM does exist in some species. A low molecular weight non-IgM antibody of the IgY (IgRAA) class and primitive lympho-

myeloid nodes first appear in the Amphibia and may have arisen in relation to life on land (Manning & Turner, 1976). Urodeles only possess IGM (Marchalonis & Cohen, 1973) whereas anurans have both IGM and IGRAA (Marchalonis, 1971a). Further, urodeles lack lymph-node like structures and many lack lymphopoietic bone marrow and gut-associated lymphoid tissue which are all present in advanced anurans. Nevertheless, Ruben, Van der Hoven and Dutton (1973) have shown that antibody production in the newt involves two or more cell populations and a similar helper activity has been seen in anurans (Ruben, 1975). So the mechanisms of cell collaboration are present at the level of primitive amphibians. Hapten-carrier phenomena have also been shown to occur in fish. However, as yet there is no evidence that these functions are performed by separate cell lines. A dichotomy of the lymphocyte population into T and B cells has been demonstrated in anuran amphibians (Manning, Donnelly & Cohen, 1976). Whether a complete structural and functional dichotomy of T and B cells first occurs at the level of adult anurans, or whether it is present throughout the vertebrates is not known.

Fish are of particular interest in this respect especially since intensive fish culture brings with it the need to prevent and combat disease outbreaks. Vaccines are as yet a new phenomenon to fish farmers and the need to understand the immune capabilities of fish is becoming very important at both the commercial and academic levels.

The effect of a vaccine can be more fully assessed with increasing knowledge of the animals' immune capabilities.

As with all poikilotherms, the immune capabilities of teleosts are temperature dependent (Cushing, 1942; Bisset, 1948; Hildemann & Cooper, 1963; Avtalion, 1969a). Nevertheless, at a constant temperature both humoral and cell-mediated responses exhibit typical anamnestic responses (Hildemann, 1958; Avtalion, 1969b; Trump & Hildemann, 1970; Van Muiswinkel, Rijkers & Van Oosterom, 1978).

The lymphoid organs of fish have been well studied. Lymph nodes are lacking and there is little, if any, bone marrow. The head kidney (pronephros) is of importance as a lymphoid and haemopoietic organ. Antibody producing cells can be detected here and in the spleen using the haemolytic plaque technique (Smith, Potter & Merchant, 1967; Chiller, Hodgins & Weiser, 1969; Rijkers & Van Muiswinkel, 1977); the rosette test (Chiller, Hodgins, Chambers & Weiser, 1969) or by immunofluorescence (Ortiz-Muniz & Sigel, 1971; Emmrich, Richter & Ambrosius, 1975). Indeed, even the thymus has been shown to contain antibody producing cells in some investigations (Ortiz-Muniz & Sigel, 1971; Emmrich, Richter & Ambrosius, 1975; Sailendri & Muthukkaruppan, 1975a). A lymphoid cell population has been seen in the intestine of some fish (Weinberg, 1975; Davina, Rijkers, Rombout, Timmermans & Van Muiswinkel, 1980), again with associated antibody containing cells.

The production of serum antibodies in fish is well documented, with many studies on agglutinating antibodies

(Krantz, Reddecliff & Heist, 1963; Spence, Fryer & Pilcher, 1965; Post, 1966; Hodgins, Weiser & Ridgway, 1967; Trump, 1970; Harris, 1973); precipitating antibodies (Everhart & Shefner, 1966; Everhart, 1972; Harris, 1973) and virus neutralizing antibodies (Uhr, Finkelstein & Franklin, 1962; Sigel & Clem, 1965; Vestergard Jørgensen, 1973; O'Neill, 1978, 1979). Antibodies have also been found in the mucus of fish (Fletcher & Grant, 1969).

Cell-mediated immunity, such as delayed hypersensitivity and allograft rejection, have also been investigated. A positive delayed corneal reaction to PPD has been demonstrated in rainbow trout one month after they were immunized with complete Freund's adjuvant (Ridgway, Hodgins & Klontz, 1966). Macrophage migration inhibition, as an in vitro test for measuring cell-mediated immunity, has been reported by several authors. Similarly, several papers have shown a positive mixed leucocyte reaction (Ellis, 1977; Etlinger, Hodgins & Chiller, 1977) with stimulation indices in carp as high as those in mammals (Ambrosius, personal communication). Many studies of scale transplantation (Hildemann, 1958; Hildemann & Haas, 1960; Sailendri, 1973; Rijkers & Van Muiswinkel, 1977); skin transplantation (Botham, Grace & Manning, 1980) or even whole fin transplantation (Kallman & Gordon, 1958), have also been carried out. The observed graft rejection was similar to that observed in mammals in that lymphocyte invasion commenced soon after grafting, breakdown of the dermal layer of the graft was seen after peak invasion, and melanin pigmentation was progressively lost from the graft. Complete rejection is

assessed by the absence of melanin pigmentation from the graft. There are several reviews of these humoral and cell-mediated responses in fish (Ridgway, Hodgins & Klontz, 1966; Cushing, 1970, Corbel, 1975; Ellis, 1978).

Lymphocyte heterogeneity, using mitogenic responses, have been demonstrated in fish (Etlinger, Hodgins & Chiller, 1976a). So has the co-operation of two cell populations in response to hapten-carrier systems, showing the presence of a helper population of cells and an antibody producing population (Avtalion, Weiss, Moalem & Milgrom, 1975; Stolen & Makela, 1975; Yocum, Cuchens & Clem, 1975; Cuchens, McLean & Clem, 1976). However, the necessary experiments have not yet been performed to demonstrate whether the carrier-dependent population is thymus-dependent. The teleost thymus would seem to be an especially good subject for experimental manipulation and study in connection with its role in the ontogeny of the immune response.

The development of the thymus in fish has been described by several authors (Beard, 1894; Deanesly, 1927-28; Lele, 1933a, b; Hafter, 1952), and the sequence of histogenesis in relation to the other lymphoid organs has also been investigated (Sailendri, 1973; Grace & Manning, 1980). It has been suggested to be a central lymphoid organ (Ellis, 1978), where the traffic of lymphocytes is only outwards (Ellis & De Sousa, 1974). No phagocytosis of carbon is seen to occur in the thymus (Ellis, Munroe & Roberts, 1976), nor has antigen been detected using fluorescein-labelled antibodies (Ellis, 1974, 1980; Secombes & Manning, 1980).

However, surface immunoglobulin has been demonstrated on fish thymocytes (Ellis & Parkhouse, 1975; Fiebig & Ambrosius, 1976; Ellis, 1977) and antibody producing cells can be detected in the thymus of certain species. Although some investigators have shown that rabbit antisera to a fish IgM can react with non-immunoglobulin, carbohydrate determinants on the membrane of fish thymus lymphocytes (Yamaga, Kubo & Etlinger, 1978), placing some doubt on cytofluorescent observations which indicate thymocytes bear surface immunoglobulin, such observations do not refute physicochemical evidence of immunoglobulin molecules isolated from fish thymocytes using anti-IgM antisera (Warr, DeLuca & Marchalonis, 1980). In view of the need to resolve such basic questions it is surprising that thymectomy has been described in only one investigation (Sailendri, 1973). Removal of the only other accessible lymphoid organ, the spleen, seems to have either enhancing or diminishing effects upon antibody production depending upon the species investigated (Ferren, 1967; Yu, Sarot, Filazzola & Perlmutter, 1970).

Other basic immune phenomena, such as the time of onset of immunocompetence, the induction of tolerance, where and how antigen interacts with the immune system and the recirculation of lymphocytes, are areas of research virtually untouched in fish.

The present study has been made to elucidate some of these basic phenomena, to advance our knowledge in these areas both from a phylogenetic viewpoint and in terms of the commercial interests in vaccine development.

Chapter 2GENERAL METHODS

This Chapter describes the animals, the general techniques for immunization and for the collection of blood samples, and animal care. More specialized techniques are described in the relevant subsequent Chapters.

Animals(a) Carp

Mirror carp, Cyprinus carpio L, were obtained from Humberside Fisheries, Skerne, Humberside, U.K. They were kept in tanks containing well aerated, standing (dechlorinated) water at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Aeration was found to be critical. A light regime of 12 h light and 12 h dark was used. These fish hatched 2 days from spawning and were fed on brine shrimp, Artemia nauplii (San Francisco Bay Brand), supplemented with vitamins, for the first 12 days after hatching. They were collected from the fish hatchery and brought to the laboratory as soon as dry food feeding commenced. Table I gives the age from hatching, with the average weight and length of the groups used up to week 8. The youngest group was staged according to Balinsky's Normal Table for cyprinid fish (1948). Due to limited aquarium facilities it was decided to restrict experimental animals to a maximum length of 150 mm.

Carp less than 12 weeks of age were fed 6 days a week on aquarian fish flakes (Thomas's, Halifax, West Yorkshire, U.K.). Older animals were fed on Mainstream Trout Diets Grower Pellets size 4 (BP Nutrition, Stepfield, U.K.),

TABLE IAverage weight and length of carp fry

Age from hatching	Stage	Average Weight (g)	Average length not including the tail (mm)
2 weeks	41	0.03	12 see plate 1
4 weeks		0.12	17 see plate 2
8 weeks		0.55	26

TABLE IIAverage weight and length of rainbow trout fry

Age from hatching	Stage	Average Weight (g)	Average length not including the tail (mm)
1 day	30	0.08	15 see plate 3
1 week	35	0.08	19
2 weeks	36	0.10	21 see plate 4
3 weeks		0.13	25
4 weeks		0.21	25
8 weeks		0.24	31
12 weeks		1.48	48
16 weeks		2.47	57

2 to 3 times a week. Young animals were cleaned twice a week, whereas larger animals were cleaned the day following feeding. Carp are usually sexually mature by one year.

(b) Rainbow trout

Rainbow trout, Salmo gairdneri Rich. 1836, were obtained from Costa Fisheries, Yorkshire Water Authority, Pickering, Yorkshire, U.K., a furunculosis free fish farm. Eyed ova were kept on grids in running dechlorinated tap water, at 14°C. They were sorted twice a day to remove dead eggs (dead eggs turn white due to fungal attack and are removed to prevent fungus attacking the live eggs). The eggs hatched 15 days from spawning. On hatching the fry were transferred to well aerated, dechlorinated water tanks. Feeding did not commence until 14 days after hatching, when the fry were fed 3 times daily on Mainstream Trout Diets Fry Pellets size 01 (BP Nutrition, Stepfield, U.K.). The light regime was the same as for carp and the fry were cleaned 3 times a week. The water temperature was slowly raised during this time to 17°C, due to the lack of running water facilities. Growth of these fry was as good, or better, than on the fish farm. However, after 4 months the fry became limited by the size of the tanks and deaths became more frequent. So fish reared from eggs were only kept up to this time. Table II shows the weights and lengths of the fry used. When the trout hatch they have a very large yolk sac (Plate 3) and are similar to stage 30 of Vernier's Normal Table for trout (1969). The yolk sac is resorbed during the first two weeks to give a stage 36 animal,

and relatively little weight is put on until feeding is well under way.

Larger trout were purchased as required and kept in dustbins of aerated, dechlorinated water. They were fed twice a week on Mainstream Trout Diets Grower Pellets size 4 as for the carp, and cleaned out the following day.

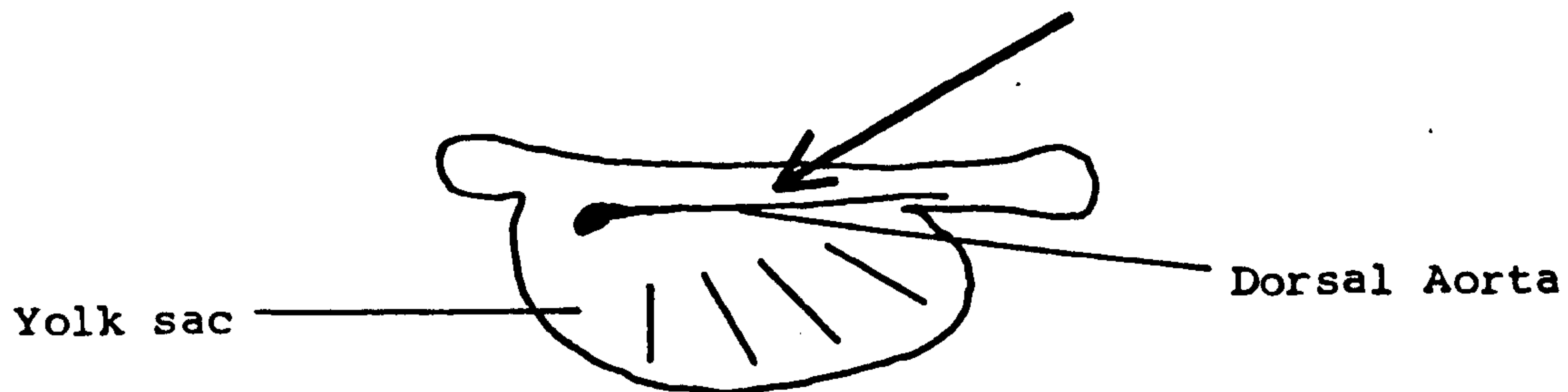
Immunization

(a) Route

1. Young fish

Young carp are extremely small and difficult to inject. The intraperitoneal route was thought to be the best route for immunization owing to the relatively large size of the peritoneal cavity. After anaesthetization in MS222 (tricaine methanesulphonate, Sandoz, Basle, Switzerland), at a concentration of 0.125 g/l, the fish were injected with a 30 gauge sterile needle, mounted on an Agla micrometer syringe (Wellcome, Beckenham, U.K.). A volume of 10 μ l/g fish was the maximum quantity that could be injected.

In trout, the large yolk sac of the fry (Plates 3 and 4) makes immunization via the intraperitoneal route impractical, and in the case of trout the intramuscular route was used. Fish were anaesthetized and injected as for carp, under a binocular microscope with a x10 objective.

Figure 2Route used to immunize rainbow trout fry2. Adult fish

All injections into adult fish were carried out with a 25 gauge sterile needle attached to a 1 ml sterile syringe. In general large carp required no anaesthetic, whereas all trout had to be anaesthetized, to prevent them from making sudden movements.

For intraperitoneal injections the needle was inserted at the posterior edge of the pelvic fin and passed into the abdominal cavity. Again a volume of 10 μ l/g fish was considered to be the maximum quantity that could be injected.

For intramuscular injections the needle was inserted into the dorsal muscles flanking the dorsal fin. The area was massaged immediately the needle was withdrawn to try to minimise leakage from the site of injection. However, even in the largest fish 0.1 ml was the largest volume used because too much antigen escaped with larger volumes.

If Freund's complete adjuvant (FCA) (Difco, Detroit, USA) was used with either of the above routes the antigen

solution was first made up to twice the required concentration in 0.85% saline. It could then be emulsified in an equal volume of FCA to give the desired final concentration.

The intravenous route was used only in the largest fish. Here the needle was inserted into the caudal vein, just posterior to the ventral fin. A little blood was withdrawn to ensure the vein had been located, followed by injection of the test material.

Hyperosmotic vaccination (HI) of fish was carried out according to the 2-step method of Amend and Fender (1976). Here the carp were initially placed in a 5.23% salt solution for 2 minutes before being placed in the antigen solution for 30 seconds. The fish are then quickly placed into well aerated standing tap water.

(b) Bleeding

1. Young fish

Bleeding of small fish, less than 3 g, was performed with a drawn out Pasteur pipette. The animals were first killed in MS222 (1 g/l) and dried with tissue to prevent contamination of the blood sample. The caudal vein was then punctured immediately behind the ventral fin and blood was drawn up by capillary action. This method was found to yield more blood than by simply cutting the tail. Nevertheless, individual fish gave only small quantities of blood, so the blood from 10 fish was usually pooled in approximately equal quantities.

2. Adult fish

Larger fish were bled from the caudal vein. Blood was collected using a sterile 25 gauge needle mounted on a 1 ml sterile syringe. As for immunization, trout had to be anaesthetized beforehand whereas carp did not. The serum obtained was enough to assay the fish individually and bleedings at weekly intervals were possible provided the quantity of blood removed was kept to the minimum required. Approximately 0.2 ml of blood was needed for an agglutination test.

With all samples the blood was allowed to clot overnight at 4°C for clot retraction, and the serum was removed the following day after centrifugation of the blood sample. A drawn out Pasteur pipette was necessary to recover the maximal volume of serum after centrifugation when only small samples of blood were available.

Treatment of Diseases

Diseases such as white spot, fungus and fin rot, occurred occasionally. White spot, caused by the protozoan Ichthyophthirius multifiliis, was the most common and mortalities were high if young fish became heavily infected. If caught at an early stage treatment with methylene blue, as set out by Van Duijn (1967) was usually successful. This involved adding 2 ml of a 0.1% stock solution to every 10 l of water. Fish were kept in this solution for 2 days. In extreme cases the quantity of methylene blue was doubled or the fish were first put into a solution of 0.001% potassium permanganate and 0.7% saline for 1 h.

Fin rot, a bacterial disease, was relatively rare and when it occurred was treated with phenoxethol. Fish were immersed for 3 days in a solution containing 10 ml of a 1% v/v stock solution, in every litre of water.

Fungus infections, caused by many kinds of moulds of the Family Saprolegniaceae, were also relatively rare and were usually preceded by injury. Treatment consisted of immersing the infected fish in a tank containing 13 mg oxytetracycline dihydrate/l for 2 days. The tank had to be aerated as this substance has toxic effects.

Chapter 3HISTOLOGICAL CHANGES IN LYMPHOID ORGANSFOLLOWING INJECTION OF SOLUBLE ORPARTICULATE ANTIGENS

In homiotherms the lymphatic nodules of the spleen, the cortex of the lymph nodes and the Peyer's patches of the intestine have a characteristic histologic picture. There is an aggregated zone of large pale-staining cells and a zone of dark-staining, tightly packed cells, consisting mostly of small and medium sized lymphocytes. The former zone was termed "Keimzentrum", or germinal centre, by Flemming in 1885, who considered the area to be actively forming lymphocytes due to its high mitotic activity. Ringertz and Adamson (1950) supported the idea that the role of these centres in animals exposed to antigens was indeed germinal, but not lymphocytopoietic. They suggested the role of germinal centres was to produce immature lymphocytes which, upon antigenic stimulation, became converted into antibody-producing plasma cells. This, combined with the failure of isotope labelling studies (Fliedner, Kesse, Cronkite & Robertson, 1964) to support the lymphocytopoietic function of germinal centres as proposed by Flemming, made the concept of germinal centres as areas of immunologic activity the major theory.

Studies involving antigenic stimulation, following immunization or skin grafting, revealed marked changes occurring in germinal centres, and even the formation of new germinal centres in response to strong antigenic stimulation (Ringertz & Adamson, 1950; Congdon & Makinodan, 1961;

Thorbecke & Hurlimann, 1965; Simar, Betz & Lejeune, 1967; White, French & Stark, 1967). Using the stain methyl green-pyronin, which stains nucleic acids, histological studies have shown that plasma cell formation and maturation begins in the white pulp. Plasma cells and their precursors are very pyroninophilic (Opstad, 1959), showing brilliant red cytoplasm and dark blue nuclei when stained with methyl green-pyronin. This is due to the presence of a well developed endoplasmic reticulum, polyribosomes, and a high incidence of mitotic activity (Hanna, Swartzendruber & Congdon, 1967; Johnson, Jacobs, Abrams & Merritt, 1967). Initially large pyroninophilic cells appear in the peri-arteriolar sheaths. They accumulate at the border of red and white pulp whilst assuming the appearance of immature plasma cells (Hanna et al., 1967; Simic & Petrovic, 1967). At later stages the red pulp contains considerable numbers of plasma cells, whilst some of the large pyroninophilic cells have migrated back into the germinal centres. The use of adjuvant seems to greatly magnify these effects (Ward, Johnson & Abell, 1959; Langevoort, Asofsky, Jacobson, DeVries & Thorbecke, 1963), but often causes great distortion of germinal centre architecture (Balfour & Humphrey, 1967; White, 1973).

Antibody forming cells can also be demonstrated in germinal centres, using immunofluorescent or localised haemolytic techniques (Ortega & Mellors, 1957; Burtin & Buffe, 1967; Young & Friedman, 1967; Berenbaum & Stringer, 1970), especially after secondary stimulation (Coons, Leduc &

Connolly, 1955; White, 1958, 1963; Mellors & Korngold, 1963). However, not all of the immunoglobulin appears to be intracellular, some could be detected in a reticular fashion at the level of dendritic expansions from macrophages, throughout the whole centre. This and the fact that the immunoglobulins present in germinal centres were shown to consist of a mixture of molecules, of different class and type (Pernis, 1967), led to the conclusion that these extracellular immunoglobulins were not solely produced by cells in the centre, but that at least part were synthesized elsewhere in the body and were bound there secondarily.

Antigen was also detected here (White, 1963; Ada, Nossal & Austin, 1964; Balfour & Humphrey, 1967; White, French & Stark, 1967) and it was suggested that as soon as circulating antibody appeared in immunized animals a fraction became attached to the surface of these dendritic cells in the form of immune complexes.

Germ-free animals were found to lack germinal centres (Thorbecke, Gordon, Wostman, Wagner & Reyniers, 1957), as were foetuses (Silverstein & Lukes, 1962), but they could be induced to produce them by exposure to specific antigen. This suggests that in both cases they were absent due to a lack of antigenic stimulation. However, germinal centre formation is not an essential component of primary antibody formation in mice (Gallily & Feldman, 1967; Grobler, Buerki, Cottier, Hess & Stoner, 1974), and several other roles have now been proposed for these very active areas. They have been suggested to be involved with the 'memory' component of

the immune response (Thorbecke, Jacobson & Asofsky, 1964; Thorbecke, 1969; Grobler, Buerki, Cottier, Hess & Stoner, 1974; White, Henderson, Eslami & Nielsen, 1975), in the switch from 19S to 7S immunoglobulin (Blythman & White, 1977) and in feedback inhibition of the immune response (White & Nielsen, 1975; Stockinger, Botzenhardt & Lemmel, 1979; VanRooijen, 1980). All require the localization of immune complexes on dendritic cells and will be discussed in the following Chapter.

So germinal centres appear to be of prime importance to the central immune mechanism. Yet poikilotherms lack germinal centres (Good & Finstad, 1967; Borysenko, 1976) and most lack any vestige of lymph nodes or Peyer's patches. Numerous studies have shown that anamnestic responses do occur in these lower forms (Evans, Kent, Bryant & Moyer, 1966; Avtalion, 1969b; Trump & Hildemann, 1970; Lin, Caywood & Rowlands, 1971) and the kinetics of the antibody response follow a course typical of a system exhibiting negative feedback on antibody production. Also in the higher amphibians IgRAA as well as IgM is produced (Lykakis, 1969; Marchalonis, Allen & Saarni, 1970), coinciding with the appearance of a more advanced lymphoid apparatus which includes primitive lymphomyeloid nodes (Ranidae, Bufonidae) or a complex splenic architecture (Xenopus laevis). Even where only IgM is produced it has recently been shown that as the antibody response continues antibody affinity is increased (Fiebig & Ambrosius, 1977). Thus the proposed roles of mammalian and avian germinal centres occur in animals which lack these structures. Pyroninophilic cells

and plasma cells are present however (Good, Finstad, Pollara & Gabrielsen, 1966; Cowden, Dyer, Gebhardt & Volpe, 1968) and are similar to those that occur in mammals (Fey, 1967). It has been suggested that the areas where pyroninophilic cells aggregate may be acting as primitive germinal centres (Pollara, Cain, Finstad & Good, 1969).

The spleen, thymus and pronephros have been shown to be the main lymphoid organs in many species of teleosts (Smith, Wivel & Potter, 1970; Sailendri & Muthukkaruppan, 1975a), with the pronephros being the major peripheral organ of lymphocytopoiesis in the carp. The present study examined the appearance of large pyroninophilic cells and other histological changes in the carp, Cyprinus carpio L, after both primary and secondary responses to a soluble protein antigen, human gamma globulin, and a cellular bacterial antigen, Aeromonas salmonicida, as well as the effect of injecting either antigen emulsified in adjuvant.

MATERIALS AND METHODS

Animals

Young carp, aged 6 months to 1 year, were used in all experiments except those described in Section E.

Staining techniques

Animals were sacrificed on the appropriate day of immunization and the spleen, liver, mesonephros, pronephros and thymus were removed, fixed in Carnoy's fluid and embedded in wax. Sections were cut at 8 μ m and stained with methyl green-pyronin. Tissue fixed in Carnoy's fluid was also cut and stained for reticulin fibres using a silver impregnation technique (Gomori, 1937).

Some organs from control and immunized animals were fixed in Bouin's fluid for staining with haematoxylin and eosin, or were fixed in 2.5% glutaraldehyde for electron microscope studies.

An attempt to elucidate the pigment type (or types) present in many of the organs was made, using a variety of staining techniques. These included the Prussian blue reaction for ferric iron, the Schmorl, Sudan Black B and Ziehl Neelsen methods for lipofuscin, the Masson Fontana method for melanin, and the periodic acid-Schiff technique for glycogen. Staining schedules were adopted as described in Bancroft and Stevens (1977) or in Humason (1979).

Immunization

(a) Pilot studies on the primary response

Doses of human gamma globulin (HGG) (Kabi, Stockholm) and Aeromonas salmonicida, known to be immunogenic (see Chapter 5), were used. Lyophilized HGG was dissolved in 0.85% saline to a concentration of 2.5 mg/ml. The carp were injected intraperitoneally in quantities of 0.01 ml/g fish (0.025 mg/g fish) on days 0 and 12, to ensure a good response. Suspensions of A. salmonicida that had been incubated for 3 days in 0.5% formalin were obtained from the Marine Laboratory of the Department of Agriculture and Fisheries for Scotland, at Aberdeen. This stock solution contained 8×10^{10} cells/ml and was stored at 4°C. When required they were washed twice in 0.85% saline and resuspended to a concentration of 1×10^{10} cells/ml of 0.85% saline. These were also injected into carp intraperitoneally in quantities of 0.01 ml/g fish on days 0 and 12, giving a dose of 1×10^8 cells/g fish at each

injection. Animals were killed on days 1 to 7, and thereafter at weekly intervals for 8 weeks. Uninjected and saline injected animals were included at each sampling time.

(b) Effect of adjuvant and route

The effect of adjuvant was examined by injecting HGG or A. salmonicida in Freund's complete adjuvant (FCA) (Difco, Detroit, USA) at the same concentration as in the experiments described in Section (a). Antigen solutions were initially made at twice the required concentration in 0.85% saline, and were then emulsified in an equal volume of FCA. Injection and killing schedules were also followed as in Section (a).

HGG in FCA was injected either intraperitoneally or intramuscularly to compare the effect of different routes of administration. A. salmonicida in FCA was injected via the intraperitoneal route only.

(c) Dosage

The effect of increasing the dose of HGG in FCA was investigated by injecting fish intraperitoneally at 4-fold (0.1 mg/g fish) and 10-fold (0.25 mg/g fish) the normal dose. Immunization and killing schedules were followed as in Section (a)

(d) Secondary Responses

Carp were immunized as for a primary response with the normal dose of HGG in FCA, HGG in saline or with A. salmonicida in saline. They were given a secondary immunization 8 weeks after the first injection and animals were killed on days 1, 7, 14, 21 and 28 after the secondary injection.

(e) Responses of young carp

Carp aged 8 weeks were injected intraperitoneally on

days 0 and 12 with HGG in FCA, HGG in saline or A. salmonicida in saline, at the concentrations used in Section (a).

Fish were killed at weekly intervals for up to 8 weeks. A booster injection was given on day 56 to the remaining fish in this group and animals were killed on days 1, 7, 14 and 21 after this booster injection.

(f) Administration of heat aggregated antigen and of immune complexes

Two different methods of presentation of antigen were investigated to see whether accelerated histological changes resulted.

The presentation of immune complexes was studied in two ways. The first involved injecting antigen and allogeneic antiserum separately, to form immune complexes in vivo. The second involved the injection of immune complexes formed in vitro. Both experiments were carried out using the intraperitoneal and intravascular routes, and are described more fully in Chapter 4.

Similarly, heat aggregated HGG was prepared as described in Chapter 4 and injected into fish after dialysis using the intraperitoneal and intravascular routes. An additional experiment was also carried out in which a 0.25% solution of HGG was simply heated at 60°C in a water bath for 15 min, cooled and injected without prior dialysis, either intraperitoneally or intravascularly.

RESULTS

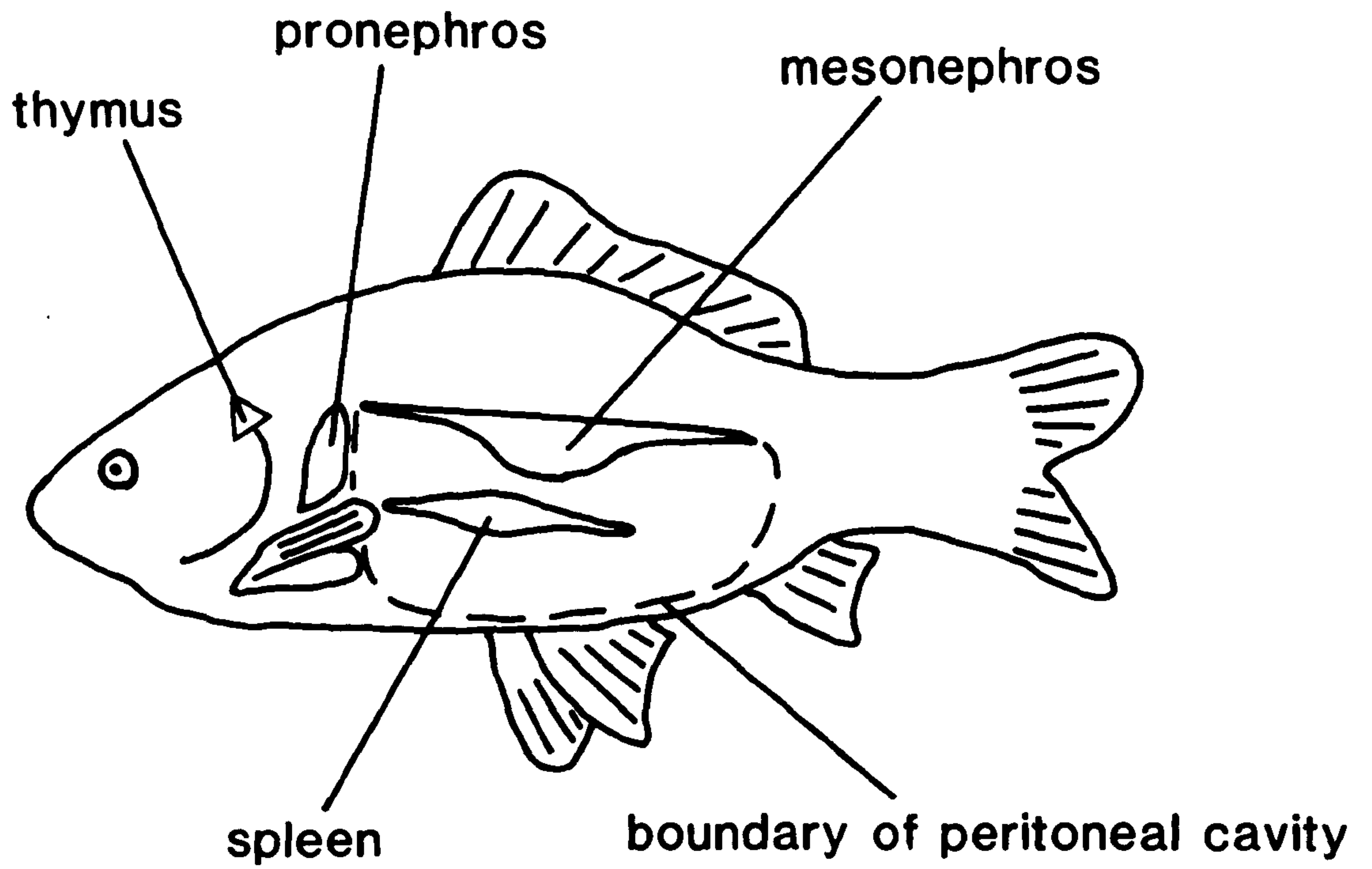
Histology of unimmunized animals

The position of the major lymphoid organs investigated are shown in Figure 3.

Figure 3.

Diagram of the position of the main lymphoid organs

in the carp.



The spleen in carp is situated alongside the intestine and tends to be a rather dark, elongated organ. As in most teleosts, it consists of lymphoid tissue, pulp and ellipsoids (Yoffey, 1929). The ellipsoids consist of a thin endothelial layer surrounded by a cuff of cells, usually a single cell thick and containing macrophages (Plate 5). This cuff is bounded by a sheath and enmeshed within a fibrous reticulum (Plate 6). The lumen is narrow and allows only one or two blood cells to pass at a time. These ellipsoids extend and branch throughout the red pulp and are very similar to those seen in the plaice (Ellis, Munroe & Roberts, 1976) and brown trout (O'Neill, 1978). The red pulp consists of large blood sinuses containing many red cells. There is no organized white pulp, but occasional white cells are seen in the ellipsoid cuff and within the red pulp. On some occasions pigment-containing cells are seen in both the ellipsoids and the red pulp, these have been termed melano-macrophages (Roberts, 1975). These cells tended to aggregate at the axils of branching ellipsoids to form centres similar to those seen in the plaice (Ellis et al., 1976) and the turbot (Ferguson, 1976), but without the associated lymphoid cuff of the latter. Although the exact type of pigment present in these cells is not known, the terms melano-macrophage and melano-macrophage centre, or melanin centre, will be retained to denote such pigment-containing macrophages and aggregations of them. Where relevant, the specific pigment is named.

Only a few leucocytes could be detected in the spleen at the electron microscope level. In animals in which the

pigment-containing cells were numerous cells resembling macrophages and containing electron dense granules were seen.

The kidney is divided into pronephros and mesonephros. The pronephros is situated anterior to the transverse septum, on both left and right sides of the head, and is a densely cellular, pink organ (Smith, Wivel & Potter, 1970). There are many vascular sinuses, some of which have a thick layer of steroidogenic cells (Turner, 1966). The pronephric cells lie between the sinuses and smaller vascular spaces, but the sinuses and vascular spaces are generally devoid of cells (Plates 7 and 8). Erythropoietic, granulocytic and lymphoid cells can all be discerned in the pronephric parenchyma. Lymphoid cells, particularly plasma cells, tend to cluster along the vascular channels, but there appears to be no patterned arrangement.

Using an electron microscope, plasma cells, lymphocytes and granulocytes are all easily distinguished. The lymphocytes and granulocytes are numerous, whilst numbers of plasma cells are few. The lymphocytes are small round cells, about 5 μm , the largest part of which is occupied by the rounded nucleus, with dense chromatin (Plate 9). There is a poorly developed Golgi apparatus and the endoplasmic reticulum is absent. There are on occasions a few small mitochondria and free ribosomes scattered throughout the cytoplasm.

Plasma cells, on the other hand, have a large amount of rough endoplasmic reticulum, an eccentric nucleus, and a centrally situated Golgi apparatus (Plate 10). Mitochondria occur between the cisternae.

Two types of granulocyte were detected, the predominant type resembling an eosinophil. The nucleus seldom appears to be indented or lobulated as in mammalian forms, but the cytoplasmic granules do have rather characteristic crystalline inclusions (Plate 11). The granules are flattened and ovoid in shape, with an electron dense equatorial crystal. This type of granulocyte also has some rough endoplasmic reticulum, a few large mitochondria and multivesicular bodies.

The second type of granulocyte has many large vacuoles, varying enormously in shape and density. The nucleus is eccentric and there appears to be little endoplasmic reticulum (Plate 12). These cells resemble the plasmacytoid cells seen in the pronephros of carp by Smith, Wivel and Potter (1970). These authors proposed that such cells were mature plasma cells that had continued to accumulate secretory products, filling the rough endoplasmic reticulum. However, in the present study the cisternae of the small amount of endoplasmic reticulum seen in this type of cell was not associated with the granules. The cells described here are similar to the type 2 granulocyte in goldfish (Davies & Haynes, 1975), particularly those found in the tunica propria of the intestine. Certainly at the light microscope level two types of granulocyte are visible in carp (Davina, Rijkers, Rombout, Timmermans & Van Muiswinkel, 1980) and the cells described in the present electron microscope study are therefore probably a type of granulocyte.

The mesonephros of carp is situated beneath the vertebral column and enlarges to form a pair of lobes about half way

down the body length, at the position of a constriction in the air sac. It is separated from the body cavity and air sac by connective tissue. Many nephric tubules are present in connection with its excretory function and there is also a haemopoietic parenchyma between the tubules.

The thymus is situated on either side of the head at the level of the gills, just dorsal to the superior corner of the operculum. It is an opaque-whitish organ, partially surrounded by the bones of the operculum. It is well vascularized, with an extensive capillary network. There is little distinction between the cortex and medulla, but the population of lymphocytes is sparse surrounding the capillaries and blood vessels while the bulk of the thymus parenchyma is composed of thymocytes (small, round, dark-staining cells which are morphologically similar to the lymphocytes found elsewhere in the body). Many of the larger thymocytes have a bright red rim of cytoplasm when stained with methyl green-pyronin. Plasma cells can also be identified in the thymus by electron microscopy, although they are very few in number.

The liver is situated predominantly at the anterior end of the peritoneal cavity, but has several loosely connected lobes, closely adherent to the portal veins and concealed by the intestine. The liver is not a lymphoid organ in fish, neither does it play a major role in phagocytosis, although the hepatic sinuses are lined by endothelial cells that have been described by Wislocki (1917) to correspond in appearance and behaviour to Kupffer cells.

Histology of the lymphoid organs of immunized animals

(a) Effect of immunizing with human gamma globulin or
Aeromonas salmonicida.

As can be seen from Table III, after injection of HGG in saline there is an initial increase in the numbers of pyroninophilic cells in the mesonephros, with cells aggregating together to form small groups (Plate 13). There is little activity in the spleen, although the occasional animal does have increased numbers of pyroninophilic cells present in the ellipsoids. A more marked reaction is observed by day 21, especially in the pronephros, with an increase in the numbers of pyroninophilic cells throughout the pronephric parenchyma and on some occasions aggregates of cells were seen around the larger blood vessels. At no time were any pyroninophilic cells found in the liver or thymus. In a few animals, during the first 3 weeks, the mesonephric kidney tubules contained pyroninophilic cells (Plate 14). Pigment-containing cells seem to increase in the long-term and are often found in areas where pyroninophilic cells had been conspicuous a few weeks earlier.

The response to *A. salmonicida* is more sporadic (Table IV). There appears to be an increase in the numbers of pyroninophilic cells during the first week, particularly in the pronephros, thereafter a slightly reduced level was maintained up to the end of the study at week 13. However, comparison with saline injected controls, suggests that the reaction to *A. salmonicida* is not very pronounced. Again, with this antigen, no pyroninophilic cells were observed

TABLE III

Histologic responses to an i.p. injection of HGG in saline administered on days 0 and 12. Each symbol represents one individual

Days	0 (Control fish)	1-11	14-20	21-27	28-34	35-56
Spleen	++ ---	++ ---	+ ---	++ ---	---	+++ ---
Pronephros	++ ---	+++ +++	+++ +	++ ++ ++	+++	+++ ---
Mesonephros	++ ---	⊙⊙⊙ ++	⊕ ++ + -	+++ ---	⊕+++	+++ ---

⊙, Cell clusters; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; ⊕, few pyroninophilic cells; -, no pyroninophilic cells. See Plate 21 for an

illustration of the assessment of pyroninophilia (⊕, +, and ++) as used in this Table and in subsequent Tables.

TABLE IV

Histologic responses to an i.p. injection of A. salmonicida in saline administered on days 0 and 12. Each symbol represents one individual

Days	0 (Control fish)	1-11	14-20	21-27	28-34	35-56
Spleen	++ -----	+++ -----	+ -----	++ -----	+ -----	+ -----
	-----	+ -----	-----	-----	-----	-----
	-----	-----	-----	-----	-----	-----
Pronephros	++ -----	++ -----	++ -----	++ -----	++ -----	+++ -----
	-----	+++ -----	-----	-----	-----	-----
	-----	-----	-----	-----	-----	-----
Mesonephros	++ -----	++ -----	++ -----	++ -----	++ -----	●++ -----
	-----	+++ -----	-----	-----	-----	-----
	-----	-----	-----	-----	-----	-----

●, Cell clusters; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

in the liver or thymus. Pigment-containing cells increased considerably, noticeably in the spleen, but also in the pronephros and mesonephros. On several occasions individual pigment-containing cells were found scattered throughout the blood spaces in the pronephros, during the first week after immunization and by week 6 large aggregates of pigment-containing cells began to form, particularly at the axils of branching ellipsoids. These aggregates appeared to be surrounded by fine extensions from the reticulum of the ellipsoid sheath, as shown in sections impregnated with silver (Plate 15). In sections stained for ferric iron only the spleen was positive. The location of iron corresponded with the location of pigment-containing cells but was present in only about half of these cells, the rest being apparently devoid of ferric iron. This indicates that haemosiderin is present at these sites, possibly as well as melanin and lipofuscin. The question as to whether both melanin and lipofuscin were present in the spleen, and whether they were in the same, or different, cells as the haemosiderin was investigated using reducing methods of staining for melanin and lipofuscin, such as the Masson Fontana technique or Schmorl's reaction. Schmorl's reaction was positive, indicating the presence of lipofuscin. The Masson Fontana technique was inconclusive, the large yellow coloured pigment was not stained, but small black granules were seen intermingled between this yellow pigment. Lastly, the PAS reaction was positive at these pigment-containing sites, suggesting the pigment is contained within macrophages.

Little pigment was found in control saline injected animals. In the few animals where some pigment occurred, it was mainly in the spleen, and did not vary in quantity between animals killed early on and those killed at later timings.

(b) Effect of adjuvant and route

The use of adjuvant seems to have a marked effect on the response to HGG injected intraperitoneally (Table V). During the first week there is an increase in pyroninophilic cells in the pro- and mesonephros, similar to that which occurs with HGG in saline, but the response during the next two weeks is markedly different. There is a dramatic increase in pyroninophilic cells in both the pro- and mesonephros, peaking at 3 weeks after immunization with the formation of large cell clusters, particularly prominent in the pronephros (Plates 17 and 18). These cell clusters are larger in the pronephros than in the limited tissue of the mesonephros, and seem to occur mainly in the pulp between the blood sinuses. At the electron microscope level a large increase in the number of large lymphocytes was seen. These cells are an intermediate type between lymphocytes and plasma cells. They have a large nucleus of loose chromatin structure, much more abundant cytoplasm, more obvious endoplasmic reticulum and many large mitochondria (Plate 16). Lymphocytes were fewer than in control animals, and plasma cells were unchanged.

The elevated levels of pyroninophilic cells and cell clusters were still present in the pro- and mesonephros

TABLE V

Histologic responses to an i.p. injection
of HGG in FCA administered on days 0 and 12.

Each symbol represents one individual

Days	1-11	14-20	21-27	28-34	35-56
Spleen	<u>+</u> --- ---	<u>+++</u> - --	<u>+++</u> <u>+</u> <u>+</u> ---	<u>+++</u> ---	<u>++</u> --- ---
Pronephros	<u>+</u> <u>+++</u> <u>+</u> ---	<u>+</u> <u>+++</u> <u>+</u> -	<u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u>	<u>+</u> <u>+</u> <u>+</u> <u>+</u>	<u>++</u> --- ---
Mesonephros	<u>+</u> <u>+</u> <u>+</u> <u>+</u> ---	<u>+</u> <u>+</u> <u>+</u> <u>+</u> -	<u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u> <u>+</u>	<u>+</u> <u>+</u> <u>+</u>	<u>++</u> --- ---

+, +, Cell clusters; +, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

TABLE VI

Histologic responses to an i.m. injection
of HGG in FCA administered on days 0 and 12.

Each symbol represents one individual

Days	1-11	14-20	21-27	28-34	35-56
Spleen	N.T.	<u>+++</u> - -	<u>++</u> ---	<u>++</u> - ---	---
Pronephros	N.T.	<u>+</u> <u>+++</u>	<u>+</u> <u>+</u> <u>+</u> <u>+</u>	<u>+++</u> ---	<u>++</u> - ---
Mesonephros	N.T.	<u>+++</u> <u>+</u>	<u>+</u> <u>+</u> <u>+</u>	<u>+++</u>	<u>++</u> -

N.T., Not tested; +, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

during week 4, but had completely gone by week 5. The spleen again had a limited role, as was the case when antigen was administered without adjuvant. In general, increased numbers of pyroninophilic cells occurred only in animals where there was a very large response in the pronephros. As in animals injected without adjuvant, an increase in the amount of pigment was seen towards the latter part of the experiment.

Conversely, with A. salmonicida the use of adjuvant had little effect. As with the response in saline, a large increase in the number of pyroninophilic cells occurred in the pro- and mesonephros during the first week, with a less clear response after that. No cell clusters were observed as seen when adjuvant was given with HGG. Pigment-containing cells do appear, particularly in the spleen, again similar to the response in animals injected with A. salmonicida in saline.

Adjuvant injected alone had little effect. After 2 weeks there was a small increase in pyroninophilic cells in the spleen, pro- and mesonephros, but in no animals were large numbers seen or cell clusters formed. Little pigment was found apart from a small amount which was seen early on, darker than in normal fish and restricted to the ellipsoids in the spleen, and may have been melanin. Only a few animals possessed this dark pigment after week 5.

The response to HGG in FCA injected intramuscularly instead of intraperitoneally can be seen in Table VI. Here, although the intensity of the reaction was similar to the intraperitoneal route, no obvious cell clustering

was observed either in the pronephros or in the mesonephros. However, the pyroninophilia in the pronephros during week 4 was so intense that the whole pronephros could have been said to be one very large cell cluster. Again little pigment was found in this group. Some did appear in animals killed at week 3 when both dark and yellow pigment was seen.

(c) Effect of dose

In general, with larger doses of antigen there was a more pronounced effect. Increased numbers of pyroninophilic cells were seen early on in the pro- and mesonephros. However, the cell clusters which form in the pronephros, still do so at week 3 and not earlier, even when using 10 times the normal concentration (Table VII). The response in the mesonephros was variable, but appeared stronger than when the smaller dose was used and more numerous cell clusters were present both early on and later. Quite early in the response, during the first week, pyroninophilic cells were seen in the blood spaces in the pronephros, as well as in the pulp (Plate 19). A marked response in the spleen was noted in two fish, both of which were killed between weeks 1 and 2. The response in the pronephros was more conspicuous with large numbers of pyroninophilic cells present. There was a general increase in cell numbers during the first 5 days, after which time grouping of the cells around large blood vessels, of the type seen in Plate 7, occurred especially where the sheath of steroidogenic cells was lost. The aggregates increased in size and number during the following week, and by week 3 cell clusters had appeared, no longer only adjacent to the large blood vessels, but spread throughout the pulp.

TABLE VII

Histologic responses to an i.p. injection of HGG in FCA at ten-fold the normal dose administered on days 0 and 12. Each symbol represents one individual

Days	1-11	14-20	21-27	28-34	35-56
Spleen	++++ +--- ----	++ ++ -	++- -	N.T.	---
Pronephros	++ ++ ++ ++++ ++++	++ ++ ++ +	⊕⊕ +	N.T.	+++ --
Mesonephros	⊕⊕ ⊕⊕ ⊕⊕ ++ +++ ++++	⊕⊕ ++ ++ +	⊕ ⊕+	N.T.	⊕ ++

N.T., Not tested; ⊕, ⊕, cell clusters; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

TABLE VIII

Histologic responses to an i.p. injection of HGG in FCA administered on days 0, 12 and 56. Each symbol represents one individual.

Days	1-11	14-20	21-27	28-34	35-56
Spleen	++ ++ ++ +++-- --	+ +-- --	++-- --	+ +--	N.T.
Pronephros	++ +++ ++++ ++++	++++ --	++++ --	+++ --	N.T.
Mesonephros	++ ++ ++ ++ ++ +++ --	+++ --	++++ --	+++ --	N.T.

N.T., Not tested; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

The appearance of yellow pigment when fish were immunized with 0.1 mg HGG/g fish, or with 0.25 mg HGG/g fish, was similar to that following HGG in FCA at 0.025 mg/g fish. However, at 0.25 mg HGG/g fish, dark pigment was seen in the spleen confined completely to the ellipsoids (Plate 20). This response was much stronger than that seen with adjuvant alone, and was confined to the first 5 days after immunization. After this time intermediate types of pigment were seen, gradually all becoming a light golden colour.

(d) Effect of a third immunization

After a booster immunization with HGG in FCA there is a rapid increase in pyroninophilic cells in both the pro- and mesonephros (Table VIII), not unlike that occurring during the primary response with high doses of antigen. This response seems to peak at day 7, when the spleen is also in a highly reactive state. Here, the pyroninophilic cells are located within the ellipsoids but seem to accumulate in such large numbers that the sheath bulges outwards to form a nodule. This transition is shown in Plate 21.

In sections stained to show the reticulum, it can be seen that this nodule of cells is still enmeshed by an expanded portion of the reticulum (Plate 22). This acquires spherical proportions resembling, in a rudimentary form, the white pulp reticulum of the tetrapod spleen.

The response in the pro- and mesonephros also showed differences from the primary response. Particularly noticeable was the absence of cell clusters. Even though large numbers of pyroninophilic cells were present in both the

pro- and mesonephric kidneys, in only one animal were small aggregates seen. Indeed, after 3 weeks from immunization the pyroninophilic cells remaining in the pronephros were still predominantly grouped around the major blood vessels, as occurred early on in the primary response. The response in all three organs rapidly decreased after the first week, again in contrast to the primary responses.

No response was seen in the liver or thymus, nor were pyroninophilic tubules seen in the mesonephros.

The secondary response to HGG in saline was much weaker than that given to HGG in FCA, and no effect was seen in the spleen. The response also peaked by day 7 and showed no pyroninophilic cell clusters in the pro- or mesonephros at any time.

Using A. salmonicida the picture following a third injection of antigen was again different from the primary response. There was no effect in the spleen. In the pro- and mesonephros the response rose quickly during the first week and continued up to the end of the third week, when cell clusters appeared. The initial increase in pyroninophilic cells in the pronephros followed a pattern similar to that which occurred in the fish injected with HGG in FCA during the primary response. Pyroninophilic cells collected around the major blood vessels; small aggregates formed during the second week and increased in numbers; finally, discrete cell clusters appeared in the pulp by week 3. These clusters, however, were not as intense as those seen in fish injected with HGG in FCA.

In all animals during the secondary responses, pigment-containing cells were present in the spleen, pronephros and mesonephros, although in varying amounts. In many animals areas of pale staining cells, some containing large vacuoles, became apparent in the pro- and mesonephros. Pigment was often present here. They were of similar size to the cell clusters seen during primary responses, and may have been an end product or intermediate from them.

(e) Responses of young carp

Carp aged 8 weeks at immunization, responded in a similar fashion to adult fish aged 6 months to 1 year. There was an increase in the number of pyroninophilic cells by the second week, in both the pro- and mesonephros, using HGG in saline or HGG in adjuvant, or with A. salmonicida. The response continued up to the end of week 4, and with HGG in FCA cell clusters were seen in the pro- and mesonephros. As with the adult responses, the spleen, liver and thymus played no role in the primary response.

Secondary responses in fish aged 8 weeks at the first immunization, were also similar to adult responses. The response to HGG in FCA was accelerated, but without the splenic involvement. Possibly the spleen was too immature in these animals. As in the adults, no cell clusters were seen. Responses to A. salmonicida were obviously accelerated only in the mesonephros.

(f) Effect of modified antigen

1. Immune complexes

Injecting antigen in saline and homologous antibody

separately showed no marked effect on the spleen or mesonephros over and above that expected for the injection of antigen alone. However, cell clusters were seen in the pronephros, which were not seen at all with HGG alone, and they appeared at an earlier time than with HGG in FCA.

Using complexes in antigen excess the effect was also largely upon the pronephros. Here the response was very similar to an injection of HGG in FCA at 0.25 mg HGG/g animal. There was a large increase in the number of pyroninophilic cells seen during the first week, leading to the formation of cell clusters by week 3 (Table IX).

No obvious difference between the intraperitoneal and intravascular routes was noticed.

2. Heat aggregated HGG

Using heat aggregated HGG prepared as described in Chapter 4, a much accelerated response was seen which rapidly terminated by the end of week 3. Using the intraperitoneal route cell clusters were seen in the pronephros earlier than with HGG in FCA (Table X). The intravascular route also gave an increase in the number of pyroninophilic cells, but without clustering of cells. Pyroninophilic cells were also seen in the ellipsoids of the spleen, similar to a secondary response. Pigment was prominent by week 3 when most of the pyroninophilic cells had gone. Also by week 3 cells were noted in the pronephros that had a smaller rim of pyroninophilic cytoplasm, and were possibly of the plasma cell series.

Using the non-dialysed preparation of heat aggregated HGG also gave accelerated and enhanced responses in the

TABLE IX

Histologic responses to an i.p. injection of HGG-anti-HGG immune complexes, in antigen excess administered on day 0. Each symbol represents one individual

Days	1-11	14-20	21-27	28-34	35-56
Spleen	<u>++++</u> +- -	<u>+++</u> -	<u>+++</u>	<u>+++</u>	N.T.
Pronephros	++ ++ ++ + +- -	<u>+++</u>	⊕⊕+	<u>+++</u>	N.T.
Mesonephros	⊕⊕ ++ + ++ -	<u>+++</u>	⊕⊕⊕	<u>+++</u>	N.T.

N.T., Not tested; ⊕, cell clusters; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

TABLE X

Histologic responses to an i.p. injection of heat aggregated HGG administered on day 0. Each symbol represents one individual.

Days	1-11	14-20	21-27	28-34	35-56
Spleen	<u>+++</u> - --	++ ++	----	--	N.T.
Pronephros	++ ++ ++ + ++	⊕⊕⊕ ++	<u>+++</u>	<u>++</u>	N.T.
Mesonephros	⊕⊕⊕+ ++ -	⊕⊕⊕ ++	<u>+</u> ---	<u>++</u>	N.T.

N.T., Not tested; ⊕, ⊕, cell clusters; ++, large numbers of pyroninophilic cells; +, many pyroninophilic cells; +, few pyroninophilic cells; -, no pyroninophilic cells.

pronephros, with cell clusters forming by week 2. It was of interest that using these unrefined aggregates every animal killed between days 1 to 5 contained large amounts of dark coloured pigment in the ellipsoids, very similar to the response with 0.25 mg HGG/g animal in adjuvant. On the last day that this was observed, dark pigment-containing cells were seen throughout the pulp, as well as in the ellipsoids (Plate 23), possibly migrating to some other location.

DISCUSSION

The role of pyroninophilic cells

It is clear that even with a limited structural complexity in the spleen and kidney of fish, marked cellular changes occur after antigenic challenge. The increase in pyroninophilic cells in the pro- and mesonephros roughly coincides with the first appearance of circulating antibody (see Chapter 5), and the peak cellular reaction just precedes the peak antibody response. This would seem to indicate their involvement in antibody production in a similar fashion to that seen in mammals. The transformation of lymphocytes to plasma cells, containing demonstrable intracellular immunoglobulin is now well established in mammals (Amos, 1962; Thorbecke & Benacerraf, 1962; Langevoort, 1963; Feldman, 1964). Certainly both of these cell types are present in the pronephros of the carp, as demonstrated by the electron micrographs (Plates 9 and 10) and have been shown to be homologous to the corresponding cell forms of mammals (Fey, 1967). However, by far the most

prominent cell type is the intermediate pyroninophilic cell. Whether this cell type represents the large lymphocyte or plasmacytoid cell is uncertain. It is worth noting, however, that in other poikilotherms, such as the toad Xenopus laevis (Turner & Manning, 1973) or Bufo marinus (Diener & Nossal, 1966; Evans, Kent, Bryant & Moyer, 1966), a large increase in pyroninophilic cells, but not plasma cells, was noted after immunization. Primitive pyroninophilic cells with a high nucleo-cytoplasmic ratio were first noted 5 days after antigen injection in B. marinus, comparable to the first appearance of large pyroninophilic cells in the rat lymph node 2 to 3 days after a similar antigenic stimulus. However, in the rat, proliferation of such cells leads to the formation of germinal centres and to plasmacytopoiesis in well defined areas of lymphatic tissue (Nossal, Ada & Austin, 1964). In the toad the numbers of pyroninophilic cells increased for about 4 weeks, during which time some loose clusters of cells were formed and some differentiation towards more mature cells was seen. Similarly, in X. laevis (Turner & Manning, 1973) and in Cyprinus carpio (in the present study), large numbers of pyroninophilic cells were seen by the third week after immunization, with very obvious cell clusters in the pronephros and mesonephros of C. carpio. The question arises as to the function of these pyroninophilic cells. Do they act purely as a proliferative cell line, an intermediate cell type in antibody formation, or do they act as both a proliferative and an antibody producing cell as suggested by Turner and Manning (1973)?

Cowden, Dyer, Gebhardt and Volpe (1968) demonstrated the presence of plasma cells in long-term bovine serum albumin (BSA) stimulated B. marinus, using electron microscope and fluorescent techniques. The plasma cells were found mainly in the mesonephros and in the margins of granulomas formed at the sites of injection of antigen. They argued that the paucity of plasma cells in the lymph nodes and spleen of immunized poikilotherms was simply because it was the mesonephros that was the principal site of antibody formation. However, the mesonephros of X. laevis was found to have few proliferative cell types, even after splenectomy (Turner, 1973), and only large pyroninophilic cells were prevalent in the mesonephros of C. carpio in the present study. Furthermore, large pyroninophilic cells capable of synthesizing anti-BSA antibody have been demonstrated in B. marinus (Evans, Kent, Bryant & Moyer, 1966), whilst in X. laevis lymphocytes have been tentatively identified as splenic antibody-forming cells using immunocyto-adherence tests (Amirante, 1968) or plaque forming cell assays (Auerbach & Ruben, 1970). A large morphological heterogeneity of specific antibody forming cells in B. marinus was observed by Diener and Marchalonis (1970) after injection of Salmonella adelaide. In the early phase of antibody production small and medium lymphocytes played an important role in antibody synthesis, while at later stages large cells resembling immature plasma cells were the predominant producers of antibody. Similar findings on the mouse (Russell & Diener, 1970) have shown that the

presence of a large number of antibody forming small lymphocytes is characteristic of the early primary response to S. adelaide. These small cells may have been the precursors of the subsequent population of antibody forming cells.

So it would appear that the large pyroninophilic cells seen in the present study may be just a proliferative phase before migration and differentiation elsewhere, or they may be able to synthesize antibody without the production of typical plasma cells. The possibility of intermediate cell stages on the way to plasma cells being able to secrete antibodies is very interesting. Perhaps these large pyroninophilic cells are able to secrete antibody in the primary response, when few plasma cells are present, whereas in hyperimmunized animals larger numbers of plasma cells have matured and become the predominant antibody producer. This would explain the plasma cells seen in the paddlefish after multiple injections of killed Brucella cells (Pollara, Finstad & Good, 1969), those seen in B. marinus (Cowden et al., 1968) and the findings of Evans, Kent, Bryant and Moyer (1966), that pyroninophilic cells with a nuclear-cytoplasmic ratio, similar to that in mammals, were seen more frequently in anamnestic responses than in normal or primary response animals. Obviously studies involving the identification of cells containing intracellular antibody during different stages of the immune response would help elucidate the role of the large pyroninophilic cells and the apparent dearth of plasma cells in the spleen and kidney of C. carpio.

It is clear that adjuvant is having a marked effect on the response to HGG in the pronephros, and to a lesser extent in the mesonephros, during the primary response; also on the spleen during a secondary immunization. The effect of adjuvant on the histological changes leading to antibody production, has been demonstrated in many animals. Ward, Johnson and Abell (1959) showed that in rabbits injected with bovine gamma globulin (BGG) and endotoxin, there was a profound augmentation of events observed on a much smaller order of magnitude following a single injection of BGG alone. No new cell types were incited by endotoxin and rabbits receiving only endotoxin lacked the normal sequence of events. Similarly, Langevoort, Asofsky, Jacobson, DeVries and Thorbecke (1963) showed that rabbits injected with BGG and endotoxin had increased numbers of haemocyto blasts and immature plasma cells early in the response; elevated numbers of plasma cells during the whole period of observation and germinal centres which appeared slightly earlier, were larger, showed more mitotic figures, and remained for a longer period of time. Since only slight antibody production was observed in the white pulp in the later phase of the response, the effect of endotoxin seemed mediated through its effect on plasma cell proliferation rather than through stimulation of the germinal centres.

The effect of adjuvant on the cellular responses leading to antibody formation seems clear, and the observations on the appearance of pyroninophilic cells in the spleen and kidney of carp, correlated to antibody titres (see Chapter 5), would

seem to fully support this. The significance of the larger and more active germinal centres, which result from adjuvant stimulation is not fully understood. If they are involved in the generation of memory cells, one would expect a good secondary response on subsequent immunizations. White (1973), whilst postulating the role of germinal centres in antibody feedback mechanisms in chickens, proposed the effect of adjuvant to be one of disorganization of germinal centre function. He found that in birds receiving antigen in adjuvant, after a slight fall from the initial primary response peak, there was a second rise in antibody production which was very prolonged and between 10- and 100-fold higher than the primary peak level. Interference with the normal negative feedback inhibition of the antibody response, due to the presence of the adjuvant, seemed to be occurring and it has been suggested that in birds the effect is involved more with regulation of the primary response than with the production of memory cells.

Fish, like all poikilotherms, lack germinal centres. However, the pyroninophilic cell clusters seen in the mesonephros, with HGG in saline or in adjuvant, and those seen in the pronephros, with HGG in adjuvant or during a secondary immunization with A. salmonicida, appear to be sites of extreme activity. They do not occur until after the time when antibody can first be detected in the serum and in some cases they do not appear at all, even though antibody production is certainly taking place. So they are not the sole producers of antibody and may not be involved in antibody production at all.

Pollara, Cain, Finstad and Good (1969) have suggested that the pyroninophilic cell clusters seen in the spleen of B. marinus, although lacking a true collar of small lymphocytes, may represent primitive germinal centres. The clusters of pyroninophilic cells in the pronephros and mesonephros of C. carpio in the present study could perhaps also be interpreted in this way. Several authors working with fish have already proposed that melano-macrophage centres in the spleen and kidney may be acting as a primitive germinal centre (Ellis, 1974, 1980; Ferguson, 1976; Agius, 1979). Both carbon-containing macrophages (Ellis, Munroe & Roberts, 1976) and small circulating lymphocytes (Ellis & DeSousa, 1974) have been shown to migrate towards these centres. It was speculated that aggregations of phagocytes in the kidney and spleen of fish could provide sites of concentrated antigen through which circulating lymphocytes percolate and where competent lymphocytes would be stimulated to respond immunogenically. These centres have also been shown to contain large quantities of iron (Agius, 1979).

In the present experiments, after most of the pyroninophilic cells have disappeared in the pro- and mesonephros, areas of very faintly staining cells are seen, not dissimilar to the degenerating macrolymphocytes described in X. laevis (Turner, 1970). In Xenopus it was suggested they were effete large pyroninophilic cells which had discharged their antibody, because of the similarity in structure and location of the two cell types. However, it now seems more likely that in Xenopus they are an active dendritic type of cell involved

in the transport, and possible retention, of antigen (Baldwin & Cohen, 1981). The cells in C. carpio containing pigment occur in the areas previously occupied by large pyroninophilic cells. It may be that these areas gradually develop into melano-macrophage centres, able to influence future lymphocyte populations. Also, in the spleen, when large pyroninophilic cell aggregates occur in the ellipsoid walls, the reticulum bears many resemblances to that seen in germinal centres of homiotherms, or even in the white pulp of X. laevis (Plate 39). These aggregates may also develop into melano-macrophage centres, often seen in spleens at the axils of branching ellipsoids. Indeed, Ferguson (1976) has described changes occurring in the spleen of turbot infected with intracellular coccidial protozoa, where the phagocytic sheath cells of the ellipsoids collect at the periphery as clusters of macrophages. These clusters eventually became very large and indistinguishable from normal discrete melano-macrophage centres. Certainly in the present study the spleen appears to have a much larger involvement during secondary responses. If these areas do represent primitive germinal centres, apart from being able to attract lymphocytes and macrophages how do they fit in with other possible functions of germinal centres?

It has been proposed that the initiation of germinal centre formation depends upon the primary attachment of an antigen-antibody complex to white pulp dendritic cells, which subsequently associate specifically with B cells (Blythman & White, 1977). In the carp, the cell clusters do not appear until after the first detectable antibody,

so presumably antigen-antibody complexes could be present. Also, the lymphocytes migrating through melano-macrophage centres in the plaice were small lymphocytes (Ellis & DeSousa, 1974), as are B lymphocytes in mammals. Further, in mice, a primary injection with complexes of tetanus toxoid and isologous antitoxin at equivalence, leads to an earlier and more pronounced germinal centre development than a primary injection of antigen alone (Laisue, Cottier, Hess & Stoner, 1971). Although injection of immune complexes of HGG and homologous anti-HGG gave enhanced responses compared to HGG alone, with the formation of cell clusters in the pronephros, it did not accelerate the timing of the response. On the other hand when carp were given antigen and antibody separately, or were given heat aggregated HGG, there was a degree of accelerated formation of pronounced cell clusters, as is the case with the germinal centres of homiotherms.

Germinal centre formation has been implicated in the induction of B memory cells in mammals (Thorbecke, Jacobson & Asofsky, 1964; Grobler, Buerki, Cottier, Hess & Stoner, 1974). In the present studies it appears that some form of immunological memory exists for HGG given in saline or adjuvant, and for A. salmonicida given in saline (see Chapter 5). However, relatively few cell clusters were observed in animals not given adjuvant. When HGG was administered in saline, the antibodies formed during the secondary response could be detected earlier than in the primary response but the titres were no higher than during the primary response, so few memory cells would appear to be present. This contrasts with HGG given in adjuvant,

where a secondary immunization gives both higher and faster responses, presumably due to abundant B memory cells. This function possibly being taken over by melano-macrophage centres in later responses. The response to A. salmonicida also appeared to be higher and faster, but few cell clusters were seen on the primary response, although they did become more frequent on the secondary response. This might at first seem contrary to the explanation that pyroninophilic cell clusters could be acting as possible sites of memory cells. However, in chickens it has been found that injection of Salmonella adelaide O antigen, a thymus-independent antigen, leads to the formation of 19S antibody, but that only very late in the response is there the normal switch to 7S antibody (White & Nielsen, 1975). During this delay there were repeated excursions of 19S antibodies instead of the normal rapid decline, and no germinal centre formation until 7S antibody was increasing. So it would appear that thymus-independent antigens may cause a different form of response, and A. salmonicida is probably such an antigen (see Chapter 5).

Although in homiotherms germinal centres are not necessary for 19S immunoglobulin formation, they do seem to be required for B cell segregation and multiplication to give rise to 7S immunoglobulin. Obviously in animals lacking IgG no system is needed to switch over the class of immunoglobulin produced during the immune response. This still leaves the possibility that the production of high affinity antibody, even if IgM, as well as negative feedback, would be

a function of a primitive germinal centre. The appearance of obvious pyroninophilic cell clusters in the pronephros seems to precede peak antibody titres, rather than coinciding with the decline of the antibody response. However, it has been proposed by Van Rooijen (1980) that immune complexes trapped in germinal centres may be responsible for feedback inhibition, and that these complexes only arrive after the onset of germinal centre formation. So it should not be too surprising that the pyroninophilic cell clusters would be active at a time before inhibition occurs. The possible functioning of trapped antigen in feedback inhibition will be discussed in Chapter 4.

Overall, it would appear that the pyroninophilic cell clusters of carp may well be functionally analogous to the homiotherm germinal centre, in many of its proposed roles.

The role of melanins

The appearance of large quantities of pigment, seen late in the immune response in the present investigation, and in abundance in the melano-macrophage centres throughout teleosts, has attracted numerous theories about its possible role. Edelstein (1971), in a review on melanin pigments, discussed the possible significance of melanins in defence mechanisms against disease and in tissue damage in a variety of organisms. He used the term melanins to include "all biologic golden-brown to black organic substances that are polycyclic polymers of high molecular weight", such as true melanin and visceral melanin, lipofuscin. Of his many suggestions, one of particular interest was that melanins

may be employed with the hydrogen peroxide-peroxidase system for killing bacteria by iodination of the bacterial cell wall. It has been shown that melanins can oxidise NADH with the generation of hydrogen peroxide, which could then be used with cellular peroxidase and iodide to bring about a cell wall iodinating system analogous to that of the polymorphonuclear leucocytes. Certainly in various diseased states of fish, such as furunculosis and longstanding bacterial ulceration, melanin containing cells are known to increase (Thorpe & Roberts, 1972; McQueen, MacKenzie, Roberts & Young, 1973; Roberts, 1975; Ellis & Wootten, 1978). In certain ulcerative conditions, such as ulcerative dermal necrosis of salmon, melanocytes grow into the epithelialized scar tissue as it heals and develop into melanophores (Roberts, 1975). Although initially they are a lighter brown than the mature melanophores nearby, they often become extremely numerous so that the healing lesion appears black. The function of these melanocytes, that migrate over the surface of fresh cutaneous wounds, may be to destroy surface microorganisms via the hydrogen peroxide-peroxidase bacterial cell wall iodinating system, prior to or during the phase of re-epithelialization.

In chronic skin lesions associated with bacteria, there is extensive granulation and macrophages with foamy cytoplasm and dark melanin pigment are often seen. They are morphologically the same as the melanin containing macrophages of the spleen and kidney, and are probably such cells that have passed out via the circulation to the site of the

lesion. The melanin granules are probably not synthesized in these cells, but are simply granules that have been phagocytosed from ruptured melanophores (Roberts, 1975; Agius, 1979).

In certain diseases, such as Vibriosis, where extreme anaemia occurs (Anderson & Conroy, 1970), or during starvation (Agius, 1979), large increases in the number of melanomacrophages are found, particularly in the spleen, with large deposits of haemosiderin resulting from the breakdown of haemoglobin from effete erythrocytes. The haemosiderin is detected by the presence of ferric iron, and was found in the present study in the splenic centres of long-term A. salmonicida immunized carp. It did not appear to be present in all of the pigment-containing cells, and as most of the stains for melanin appeared negative in these splenic centres, the other abundant pigment here was possibly the golden-brown lipofuscin. The presence of haemosiderin may have been related to the stress of immunization, although many other explanations are possible. Agius (1979) found that immunization with BSA gave no significantly increased pigment-containing cells in trout, however, BSA is not a very good immunogen in contrast with the antigens used in the present study. The increase of haemosiderin would not seem to be due to starvation in the present study, as all fish seemed to feed normally and little pigment was accumulated by the saline injected controls during the course of the experiments.

Whether the different types of pigment, in a melano-macrophage centre, reside in the same cell or in different cells, is difficult to assess. Roberts (1975), believes both melanins and haemosiderin can reside within one cell. Unfortunately, electron microscope studies in the present study gave no clear indication, so there may indeed be 'siderophages' as well as melano-macrophages, or simply cells with a mixture of both. Interestingly, Ellis and DeSousa (1974) found that small lymphocytes migrate through melano-macrophage centres, which probably contain haemosiderin; and in mammals it has been shown that haemosiderin containing macrophages in the spleen of normal mice, bind both antigen and antigen-antibody complexes (Van Rooijen, 1978). In the context of the melano-macrophage centres in fish, this would give an area of concentrated antigen towards which lymphocytes are attracted and may be influenced immunogenically.

It has also been shown that melano-macrophage centres are homing sites for carbon-laden macrophages (Mackmull & Michels, 1932; Ellis, Munroe & Roberts, 1976; Ferguson, 1976). This, and their accumulation of haemosiderin in haemolytic anaemias, suggests that another function of these centres is as a repository for metabolically inert materials, or those required for recycling. It may even be that the intracellular digestive processes of fish macrophages are not well developed on the evolutionary scale (Corbel, 1975; Bach, Chen & Chapman, 1978; Agius, 1979), giving rise to very obvious areas of phagocytic cells heavily laden with indigestible materials.

A further possible function of melanins may be related to their ability to absorb free radicals (Agius, 1979). Fatty acids, of living organisms, shift towards greater unsaturation under lower environmental temperatures, as a means of maintaining protoplasmic viscosity. Thus fish, because of their poikilothermic nature, have high levels of unsaturated fatty acids in their bodies and are more prone to lipofuscin formation. Certainly, the yellow lipofuscin pigments appear to be the most abundant pigment in splenic melano-macrophage centres. Black melanin is another component, with haemosiderin present in considerable quantities under certain conditions. Since lipid peroxidation and recycling of iron compounds lead to the formation of free radicals and cations, these potentially toxic entities may well arise spontaneously in melano-macrophage centres, where they can then be taken up by the melano-macrophages.

So melanins should not be thought of as static inert biopolymeric pigments, but as reactive end products with reactive intermediates, in reference to the functions of tissues and organs containing melanins. Further, it must be realised that 'melanin' centres may contain more than one type of pigment.

In fry of rainbow trout (Salmo gairdneri), Scophthalmus maximus and Tilapia zilli, melano-macrophage centres are first seen 1 to 2 weeks after feeding has commenced (Agius, 1979). In salmon fry, both surface immunoglobulins on lymphocytes and mixed leucocyte reactivity appear simultaneously and coincide with the onset of feeding (Ellis,

1977). Phagocytosis of carbon can occur at an age before feeding commences in rainbow trout and carp (Grace, Botham & Manning, 1981), even with the formation of centres in the same position as melano-macrophage centres, but which lack pigment.

The paucity of melanin centres in normal animals may be due to a lack of antigenic stimulation. In humans, it has been shown that the normal foetus lacks germinal centres until after birth, even though the foetus is immunologically competent (Silverstein & Lukes, 1962). However, if a foetus is stimulated with a congenital infection, both enlargement of the splenic lymphoid nodules and germinal centre formation result. In carp, few melano-macrophage centres are seen in young fish, but the proliferative response giving rise to pyroninophilic cells seems to be well developed even by 8 weeks after hatching. Rainbow trout fry do not produce many pyroninophilic cell clusters in the primary response; in trout there appears to be more emphasis on the melano-macrophage centres. Indeed, Mitsuhashi, Kurashige, Mishima, Yamagushi and Fukai (1971), found so few pyroninophilic cells in adult rainbow trout that they proposed that macrophages may be involved in antibody production.

Certainly there would appear to be a structural and functional relationship between melano-macrophage centres and lymphoid tissues in teleosts, perhaps providing a site where primitive immuno-regulatory mechanisms can operate.

Chapter 4ANTIGEN LOCALIZATION IN THE CARPCYPRINUS CARPIO L.

The phagocytic properties of the lympho-reticular tissues of fish have been studied using vital dyes (Wislocki, 1917; Hoskins & Hoskins, 1918) or colloidal carbon (Mackmull & Michels, 1932; Ellis, Munroe & Roberts, 1976). Non-antigenic particulate material injected intraperitoneally in the plaice, Pleuronectes platessa L, apparently gains access to the circulation as free particles which are then phagocytosed at three major sites - the ellipsoids of the spleen; the network of reticulo-endothelial cells of the haemopoietic tissue of the kidney; and the macrophages of the intermuscular spaces of the atrium of the heart. Cells containing carbon then migrate to the melano-macrophage aggregations of the kidney and spleen (Ellis et al., 1976). A similar pattern occurs in brown trout, Salmo trutta L., and carp, Cyprinus carpio L, following the injection of carbon (O'Neill, 1978). In amphibians, the spleen, the kidneys, the jugular bodies of higher anurans, the liver and the free macrophages of the body cavities can be involved in particle uptake (Diener & Nossal, 1966; Turner, 1969). In the clawed toad, Xenopus laevis (Daudin), blood- and lymph-borne carbon is removed mainly by phagocytic cells in the spleen and liver (Turner, 1969).

It is well known that a marked difference exists between the localization of inert particles and the trapping of antigens. The specialized way in which the immune system

handles antigenic material has been demonstrated in homoiotherms, for example, using aggregated human gamma globulin (HGG) both in mice (Brown, De Jesus, Holborow & Harris, 1970; Brown, Harris, Papamichail, Slijivic & Holborow, 1973) and chickens (White, Henderson, Eslami & Nielsen, 1975); ^{125}I -bovine gamma globulin (BGG) in mice (Van Rooijen, 1973a) and ^{125}I -HGG and [^3H]dinitrophenol-BGG in rabbits (Van Rooijen, 1973b); ^{125}I -flagellin polymer in rats (Nossal, Austin, Pye & Mitchell, 1966) and human serum albumin in chickens (White, 1963; White, French & Stark, 1967). The studies show that, unlike inert particles, antigenic material is retained for only a short time intracellularly but persists for periods of several weeks in the form of antigen-antibody complexes held extracellularly on the surface of dendritic cells in the lymphoid follicles of the spleen and lymph nodes. Indeed, this dendritic trapping of immune complexes is the only mechanism by which small amounts of antigen are retained in the body for a long time (Nossal & Ada, 1971). A similar form of antigen-trapping occurs in the jugular bodies of the marine toad, Bufo marinus, injected with [^{125}I]Salmonella flagella (Diener & Marchalonis, 1970) and in the spleen of Xenopus laevis injected with HGG (Collie, 1974, 1976; Horton & Manning, 1974).

Much less has been discovered about the fate of antigen injected into fish, although the work of Ellis (1974, 1980) suggests that a primitive type of dendritic localization of a soluble protein antigen (bovine serum albumin) occurs in

the plaice. Experiments were carried out on the carp where antigen-trapping of a soluble antigen, human gamma globulin, and a cellular antigen, the bacterium Aeromonas salmonicida, was investigated and compared to the way antigen is localized in the lymphoid tissues of a tetrapod, the toad, X. laevis.

MATERIALS AND METHODS

Animals

Young carp, aged 6 months to 1 year, were used in all experiments, unless otherwise stated.

Immunization

The immunization schedules used were those already known to be highly immunogenic (see Chapter 3).

(a) Primary responses to antigen

1. Human gamma globulin

Lyophilized human gamma globulin (HGG) (Kabi, Stockholm) was dissolved in 0.85% saline to a concentration of 5 mg/ml and was then emulsified with an equal volume of Freund's complete adjuvant (FCA) (Difco, Detroit) to give a final concentration of 2.5 mg/ml. Carp were injected intraperitoneally in quantities of 0.01 mg/g fish (0.025 mg HGG/g fish) on days 0 and 12.

Some additional experiments were carried out at the above concentration but without adjuvant; at ten times the above concentration with adjuvant; and at the above concentration intramuscularly in adjuvant.

2. Aeromonas salmonicida

Formalin-killed preparations of A. salmonicida that had been resuspended to a concentration of 1×10^{10} cells/ml of

0.85% saline were also injected into carp intraperitoneally in quantities of 0.01 ml/g fish on days 0 and 12, giving a dose of 1×10^8 cells/g fish at each injection. One group was injected with A. salmonicida that had been resuspended to a concentration of 2×10^{10} cells/ml and then emulsified with an equal volume of FCA. These animals were also injected intraperitoneally on days 0 and 12, at 1×10^8 cells/g fish at each injection.

Both HGG in FCA and A. salmonicida in saline were also used to investigate antigen-trapping in 8 week carp, by injecting the standard doses intraperitoneally into these animals.

Animals were killed at various time intervals after antigen administration. The first group was killed before the second injection and thereafter fish were killed weekly for up to 8 weeks after the first injection.

(b) Modified antigen and secondary immunization

Various procedures that might be expected to alter the time course of antigen-trapping include the injection of immune complexes, the injection of heat aggregated HGG, or even a further injection of antigen whilst antibody titres are still high. These procedures were carried out following the methods of White, Henderson, Eslami and Nielsen (1975) for immune complexes and Brown, Schwab and Holborow (1970) for heat aggregated HGG, in an attempt to shed light on the mechanisms involved in this phenomenon.

1. Immune complexes

(a) Formation 'in vitro'

The ring test was employed to determine the optimal proportions of HGG and anti-HGG antiserum. Fish that had received multiple injections of HGG in FCA were bled and the serum pooled. Aliquots (0.1 ml) of serum were put into small glass tubes and 0.1 ml of antigen solution was carefully layered on top in serial dilutions. A milky white precipitate gradually forms at the interface, and appears most rapidly at the concentration of antigen that gives optimal proportions with this antiserum. The optimal concentration of antigen and the remainder of the antiserum were mixed together in equal volumes and left at room temperature for 2 h. The precipitate was centrifuged at 1700 rpm (250 g) for 5 min, the supernatant discarded and 0.85% saline added to wash the precipitate. After centrifuging the precipitate was mixed with a solution of HGG at ten times the concentration used for optimal proportions. This was left overnight at 4°C to dissolve.

The immune complexes obtained were injected intraperitoneally or intravascularly into fish, in a volume of the solution that contained the normal immunogenic dose, i.e. if a 1 mg/ml HGG solution gives optimal proportions with the antiserum, a 10 mg/ml solution is used to dissolve the precipitate and injected into fish at a rate of 0.025 ml/10 g fish. It is known that after passive immunization of serum antibody in rainbow trout, agglutinins can be detected in the serum as early as 10 min after an intraperitoneal injection (Harrell, Etlinger & Hodgins, 1975). So an

intraperitoneal injection of immune complexes would be expected to reach the circulation. However, the intravascular route was used as well, to ensure this did occur. One group was injected intraperitoneally with immune complexes emulsified in an equal volume of adjuvant. These animals received twice the previous volume to keep the concentration constant.

(b) Formation 'in vivo'

Antigen and antibody solutions were injected separately into fish to form immune complexes in vivo. The optimal concentration of antigen was injected to give the normal immunogenic dose (0.025 mg HGG/g fish), followed by the injection of an equal volume of antiserum. Again this was carried out both intraperitoneally and intravascularly.

2. Heat aggregated HGG

A 1% solution (10 mg/ml) of HGG was made up in phosphate buffered saline (PBS) pH 8.0. This was heated at 60°C in a water bath for 15 min, cooled to room temperature and centrifuged at 1,000 g for 15 min, to remove insoluble aggregates. This supernatant was then ultracentrifuged at 140,000 g for 90 min in an MSE Superspeed 50, at 20°C. The supernatant was discarded and the pellet was redissolved in PBS. It was then precipitated with 0.62 M sodium sulphate. The precipitate was removed by centrifugation, dissolved in PBS and dialysed against the same until sulphate free. Following dialysis large particles were removed by centrifugation at 1,000 g for 20 min. This pellet is referred to as aggregated HGG. Its protein

content was determined at 280 nm in an SP800A ultraviolet spectrophotometer (Pye-Unicam, Cambridge, U.K.) and was injected either intraperitoneally or intravascularly into fish at the normal concentration (0.025 mg/g fish).

3. Booster injections

After the initial period of fluorescence had subsided, but whilst antibody titres were still relatively high, a third injection of HGG in FCA, HGG in saline or A. salmonicida in saline, was given. This was carried out in both 8 week and adult fish, to see if the presence of specific antibodies altered the time course of antigen-trapping.

Killing and staining techniques

All animals were killed by placing them in 1g/l MS222 (tricaine methanesulphonate, Sandoz, Basle, Switzerland), followed by removal of the spleen, pronephros, mesonephros, liver and thymus. These organs were mounted in liver and quick-frozen in liquid nitrogen. Tissue was allowed to equilibrate to -20°C for approximately one hour before cutting on a cryostat. Sections were cut at 8 μm , placed on acid alcohol-cleaned slides, and stored at 4°C until testing.

The presence of HGG was detected using the direct single layer immunofluorescence technique (Horton & Manning, 1974) utilising fluorescein-labelled sheep anti-human immunoglobulin antiserum (Wellcome, Beckenham, U.K.), and the presence of A. salmonicida by means of a double layer indirect test with rabbit anti-A. salmonicida antiserum (DAFS, Aberdeen, U.K.) and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum (Wellcome, Beckenham, U.K.).

Sections were dried on to the slides before incubation with antiserum. Fluorescein-labelled antisera were diluted 1:10 with 0.85% saline, whilst unlabelled antisera were used neat. Sections for use in the single layer test were incubated for 30 min with the antiserum, in a moist chamber. They were then washed in PBS pH 7.6 for one hour to remove excess material, mounted in glycerol/PBS and viewed with a Leitz u.v. incident light microscope. Sections used in the double layer test were incubated for a further 30 min with the second antiserum and washed again in PBS, before mounting and viewing. Care was taken to ensure cleanliness throughout this procedure in order to minimise non-specific fluorescence associated with dust particles. Sections were also cut from frozen tissue and stained with methyl green-pyronin.

Control animals were included for each group investigated (uninjected, saline injected and FCA plus saline injected animals), as was control staining for specificity following the methods used by Horton and Manning (1974). Sections were incubated with antiserum after they had been absorbed with optimal proportions of the antigen in question, or a similar non-cross-reacting antigen. For HGG the supernatant after centrifugation from a 1 mg/ml HGG solution or a 1 mg/ml BSA solution was incubated with the fluorescein-labelled anti-HGG solution for 30 min before diluting in PBS. Similarly, some sections were incubated with unlabelled sheep anti-human immunoglobulin antiserum (Wellcome, Beckenham, U.K.) prior to incubating with fluorescein-labelled antiserum.

Cryostat sections from the spleen and pronephros of several of the control and immunized fish were also tested for carp immunoglobulin with rabbit anti-carp immunoglobulin antiserum (obtained from the Agricultural University, Wageningen, The Netherlands) followed by fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum (Wellcome, Beckenham, U.K.).

RESULTS

(a) Antigen-trapping during primary responses

At no time was antigen detected within the liver or the thymus, although in the first few days after injection of HGG or A. salmonicida a little fluorescence was seen in the hepatic blood vessels and in the extra-thymic connective tissue.

1. Responses to HGG

The results are shown in Tables XI, XII and XIII. In the spleen of fish injected intraperitoneally with HGG at 0.025 mg/g (Table XI), an initial fluorescence indicating the presence of antigen, was seen in the blood vessels and sometimes a diffuse fluorescence occurred throughout the pulp. However, this had usually gone before the second injection was given. After 3 weeks a very bright fluorescence was seen in the blood vessel walls of the spleen, throughout the inner and outer layer (Plate 24). This fluorescence has been shown to occur in the same areas as the reticulin fibres (Plate 6). Carp immunoglobulin could also be demonstrated in this same area (Plate 25),

TABLE XI

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with HGG in FCA on days 0 and 12. Each symbol represents one individual

		Days						
		2-11	14-20	21-27	28-34	35-56		
Concentration of HGG (mg/ml):		0.025	0.025	0.025	0.025	0.025	0.25	0.25
Spleen		++++	++++	+++	+-	N.T.	+---	-
		----	----	----			-	

		--						
Pronephros		+---	++-	***	-	N.T.	---	N.T.

Mesonephros		++---	----	----	---	---	---	-

N.T., Not tested; *, bright ellipsoidal or pronephric fluorescence; +, generalized tissue fluorescence; --, no fluorescence.

and was detectable in both control and immunized animals. There did not appear to be a middle layer in the blood vessel walls as in the plaice. Antigen could be detected up to 5 weeks after the first injection before it disappeared. In fish injected with higher doses of HGG the fluorescence was seen earlier, but did not last longer.

Similarly, in the pronephros, an initial fluorescence in blood vessels was seen, with a very bright fluorescence after 3 weeks. This was not in the blood vessel walls or associated with reticulin fibres as in the spleen, but appeared to be cell associated (Plate 26). The peripheral location of the antigen around the edge of the cell suggested it to be extracellular. This fluorescence lasted for about 1-2 weeks before disappearing and was associated with pyroninophilic cell clusters in sections stained with methyl green-pyronin.

The mesonephros showed very little fluorescence after that initially seen in the first 2 weeks. There was, however, on occasions a small intertubular fluorescence of varying intensities after week 3 (Plate 27). As with the pronephros, sections of mesonephros stained with methyl green-pyronin showed correspondence between fluorescent and pyroninophilic areas.

Using either HGG in saline, injected intraperitoneally (Table XII), or HGG in adjuvant injected intramuscularly (Table XIII), gave no response in the spleen, possibly related to the mode of presentation of antigen or local responses. The response in the pronephros and mesonephros

TABLE XII

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with HGG in saline on days 0 and 12. Each symbol represents one individual.

Days	2-11	14-20	21-27	28-34	35-56
Spleen	---	---	---	--	----
Pronephros	---	*--	***	++	----
Mesonephros	---	+--	*--	--	----

*, Bright pronephric fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

TABLE XIII

Immunofluorescence indicating antigen localization in carp injected intramuscularly with HGG in FCA on days 0 and 12. Each symbol represents one individual.

Days	2-11	14-20	21-27	28-34	35-56
Spleen	---	--	--	--	----
Pronephros	---	+--	++	**	***
Mesonephros	---	--	+--	**	***

*, Bright pronephric fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

was similar to the above. However, when using the intramuscular route the fluorescence occurred much later and appeared to be more widespread throughout the pronephros. With HGG in saline, fluorescence was present in the pronephros at the original timing, but was not as bright as with adjuvant.

The response to HGG in FCA by the 8 week carp was in general much poorer, with only a diffuse fluorescence occurring in the pronephros after week 3.

2. Responses to *A. salmonicida*

The results are shown in Tables XIV and XV. In the spleen localization of the bacterial antigen followed a similar pattern to that of HGG. With *A. salmonicida* injected in saline (Table XIV) there was a faint initial fluorescence for the first 2 days but the intensity increased and became localized in large cells within the next 3 days. Fluorescence was then absent and reappeared 2 weeks later (3 weeks from the first injection), when it was very bright and present in large cells in the ellipsoids. Pigment was often present at these later times and seemed to be associated with some of the fluorescent cells (Plate 28). Fluorescence was particularly obvious around the periphery of such melanin centres (Plate 29) and may have been in association with the reticular extensions from the ellipsoids (Plate 15). The fluorescence continued into the eighth week after injection.

In the pronephros the fluorescent pattern using *A. salmonicida* was markedly different from that using HGG.

TABLE XIV

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with *A. salmonicida* on days 0 and 12. Each symbol represents one individual.

Days	2-11	14-20	21-27	28-34	35-56
Spleen	⊙⊙⊙+ ++++ ---- --	-----	⊙-	⊙-	⊙⊙⊙-
Pronephros	⊙+++ +--	---	⊙⊙	⊙	⊙⊙⊙⊙
Mesonephros	⊙+-- ---- ---	-	--	⊙-	⊙⊙⊙-

⊙, Cellular fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

TABLE XV

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with *A. salmonicida* in FCA on days 0 and 12. Each symbol represents one individual.

Days	2-11	14-20	21-27	28-34	35-56
Spleen	+++	⊙⊙⊙	⊙⊙	⊙⊙⊙	N.T.
Pronephros	+++	⊙⊙⊙	⊙⊙	⊙⊙⊙	N.T.
Mesonephros	+++	⊙++	⊙⊙	⊙⊙⊙	N.T.

N.T., Not tested; ⊙, cellular fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

The fluorescence was again seen at an early stage but only in individual cells that were scattered throughout the pulp. This picture reappeared after 3 weeks (Plate 30) and also continued into the eighth week.

In the mesonephros the picture also contrasted strongly with that seen with HGG, the fluorescence was much brighter and more widespread both early and later (Plate 31). The early fluorescence was not seen in every animal and only appeared after 4 weeks from the first injection.

The responses in 8 week carp were almost identical to those above for adults, but with little early fluorescence.

In adults the use of adjuvant did have an effect, by accelerating the whole process (Table XV).

In both the spleen and the pro- and mesonephros, the intense fluorescence due to A. salmonicida remained for a longer period than that due to HGG.

(b) Responses to complexes or modified antigen

1. Immune complexes

(a) Immune complexes formed 'in vitro'

This part of the study concentrated on the early timings after immunization since the possibility of accelerated localization was the main point of interest. Following the injection of immune complexes intravascularly a faint fluorescence could be detected in the ellipsoids of the spleen and large spots of fluorescence occurred throughout the pulp in the pro- and mesonephros (Plates 32 and 33). The fluorescence appeared to diffuse out from the spots and may simply have been antigen that had been sequestered

by reticuloendothelial cells. However, the injection of antigen via the intravascular route failed to elicit the later immunofluorescent picture found when antigen was injected intraperitoneally or intramuscularly. Using the intraperitoneal route no early fluorescence was seen and the picture was very similar to that seen with HGG alone. The bulk of the trapping occurring during weeks 3 to 4, in the pro- and mesonephros.

When immune complexes were injected intraperitoneally in adjuvant a different picture emerged. Fluorescence was found to appear very quickly in the pro- and mesonephros during the first week after immunization, and had disappeared by the second week (Table XVI).

TABLE XVI

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with immune complexes in ten-fold antigen excess, emulsified in FCA.

Each symbol represents one individual.

Day	1	5	7	14	21
Spleen	--	--	-	--	--
Pronephros	+--	**	*	--	--
Mesonephros	--	*--	*	--	--

*, Bright pronephric or mesonephric fluorescence;

+, generalized tissue fluorescence; -, no fluorescence

(b) 'In vivo'

Using antigen and antibody injected simultaneously into fish, only negative results were found early on.

Fluorescence did occur in the pronephros at the normal timing, as with complexes formed in vitro.

2. Heat aggregated HGG

The experiments again concentrated on the early times after immunization. The timing of the response using either the intraperitoneal or the intravascular route was the same, although the fluorescence was fainter in the animals immunized by the intravascular route. No fluorescence was seen during the first week, but appeared during the second week in both the pro- and mesonephros. It continued until the beginning of the third week, when the response to unaggregated HGG normally begins. So an earlier trapping was seen using heat aggregated HGG, but not as early as that seen using immune complexes in FCA.

3. Booster injections

As can be seen in Table XVII, after a third injection of HGG in FCA, antigen is trapped very rapidly in the splenic ellipsoids. This occurs within 1 day of injection and lasts up to day 7. The lack of a response in the pronephros is quite noticeable until late into the first week, when trapping begins to occur here. In young animals the timing of trapping in the pronephros is the same as in adults, however, the spleen seems immature in that it does not trap HGG in the splenic ellipsoids.

Using HGG injected in saline (Table XVIII) a similar effect was seen, with trapping occurring early on in the splenic ellipsoids after a third injection in the adults, but not in 8 week carp. Again the pronephros only starts

TABLE XVII

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with HGG in FCA on days 0, 12 and 56. Each symbol represents one individual.

	Days							
	1-11		14-20		21-27		28-34	
Age at first injection (weeks)	8	52	8	52	8	52	8	52
Spleen	+--	***-	---	+--	---	---	---	---
Pronephros	*++	*+-	++	---	---	---	---	---
Mesonephros	---	*+-	---	+--	---	---	---	---

*, Bright ellipsoidal or pronephric fluorescence;
+, generalized tissue fluorescence; -, no fluorescence.

TABLE XVIII

Immunofluorescence indicating antigen localization in carp injected intraperitoneally with HGG in saline on days 0, 12 and 56. Each symbol represents one individual.

	Days							
	1-11		14-20		21-27		28-34	
Age at first injection (weeks)	8	52	8	52	8	52	8	52
Spleen	---	***	---	**	---	---	N.T.	N.T.
Pronephros	*++	*++	+--	+--	---	+--	N.T.	N.T.
Mesonephros	---	*++	---	*+-	---	---	N.T.	N.T.

N.T., Not tested; *, Bright ellipsoidal or pronephric fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

to trap late in the first week. The response seems to persist for longer without adjuvant and splenic trapping occurs during the second week as well.

With A. salmonicida almost no effect of a third injection is seen in any of the animals, young or adult. The cellular fluorescence, which is still present at the time of the third injection, continues without increasing or decreasing intensity throughout the period of study.

DISCUSSION

The significance of the long-term retention of antigen in the spleen and lymph nodes of homoiotherms has received various interpretations. Early workers believed that the function of antigen-trapping was to induce antibody production (Diener & Marchalonis, 1970). It was also suggested that it provides a means of selection of lymphocytes with the highest avidity and that subsequent proliferation of the selected cells increases the general avidity of the antibodies synthesized late in the immune response (White, French & Stark, 1970). However, it was later shown, after a single injection of labelled antigen, that the time when antigen begins to concentrate at the periphery of the spleen follicle is well after the onset of the germinal centre reaction (Van Rooijen, 1972b). Indeed, it has been concluded by several authors that complexing of antigen with specific antibody is a major condition for follicular antigen localization (Humphrey & Frank, 1967; Lang & Ada, 1967). Furthermore, after injection of labelled immune

complexes, antigen can be detected immediately in the follicle centres but has no effect on the time taken to initiate the germinal centre reaction (Van Rooijen, 1980). So it would appear that antigen-trapping cannot be responsible for either the germinal centre reaction or the initiation of antibody production.

It is now well established that antigen-trapping starts immediately after injection of antigen-antibody complexes (Van Rooijen, 1972b; White, Henderson, Eslami & Nielsen, 1975) or as soon as specific antibody is produced during the immune response. Both the Fc fragment of immunoglobulin (Herd & Ada, 1969) and complement (Bianco, Dukor & Nussenzweig, 1971; Papamichail, Gutierrez, Embling, Johnson, Holborow & Pepys, 1975) are required for this localization. Rapid localization is also seen after injection of heat aggregated HGG (Brown, Schwab & Holborow, 1970; White, et al., 1975), possibly by means of the Fc portion of its molecule which has an affinity for complement. So substances which themselves activate complement undergo rapid antibody-independent localization. Brown et al. (1970) speculated that such a trapping mechanism could provide a normal means of disposal of toxic aggregated or complexed immunoglobulin.

Other authors suggested antigen localization may be involved in the induction of tolerance (Parish & Ada, 1971), but recently it has been shown to have a role in immunological memory and feedback inhibition of the immune response. After injection of immune complexes into mice in antigen

excess or at equivalence, an enhanced antibody response was seen when compared to animals injected with antigen alone (Terres, Morrison, Habicht & Stoner, 1972; Terres, Habicht & Stoner, 1974). An early primed state seemed to have occurred, dependent both on antibody and on free antigenic determinants. Similarly, Klaus (1978) has shown that immune complexes in antigen excess or at equivalence were far more effective than antigen alone in generating memory in mice. Optimal priming by complexes required the integrity of the Fc portion of the antibody, as $F(ab')_2$ antibody fragments were less effective. This capacity to prime B memory cells could be abrogated by depriving the animals of C₃. So the same conditions exist for the localization of immune complexes in follicle centres as for the generation of B memory cells. Klaus concluded that the generation of B memory cells involves the C₃-dependent localization of antigen-antibody complexes within lymphoid follicles, the free antigenic determinants being needed to select the right antigen specific precursors of B memory cells, whilst the Fc portions of antibodies induce B cell proliferation (Berman & Weigle, 1977).

It has been known since the work of Chan and Sinclair (1971) that the Fc fragment on antibody molecules mediates a feedback inhibition of the immune response. Later work showed that treatment of murine spleen cells with immune complexes, in vitro, specifically suppressed their ability to transfer an adoptive immune response into syngeneic, lethally irradiated recipients (Sinclair, Lees, Abrahams, Chan, Fagan & Stiller, 1974). At low concentrations of

antigen-antibody complexes, this was also dependent on the Fc portion of antibody and on the possession of free antigenic groups to bind antigen specific receptors on immunocompetent cells. It has also been shown that complete, antigen-specific immune inhibition could be obtained by the transfer of 7S antibody or 7S plasma cells into unimmunized recipients (Stockinger, Botzenhardt & Lemmel, 1979). This phenomenon was thought to be identical to antibody mediated immune suppression. So the immune complexes which were formed at the peak of an immune response with 7S antibody in excess, would provide a negative feedback inhibition mechanism for the control of antibody production.

It seems remarkable that both immune complex-mediated specific immunosuppression and immune complex-mediated specific B memory cell priming have the same characteristics of intact Fc determinants and free antigenic determinants. The dual function of immune complex localization has been proposed by Van Rooijen (1980) and Ellis (1980). Antigen may stimulate directly 19S antibody production. The 19S complexes thus formed may localize to dendritic cells and activate potential 7S producers. With the release of 7S antibody large scale trapping would result with the formation of germinal centres. Early in the response, whilst antigen is in excess, potential 7S producing B cells may be stimulated to mature into 7S plasma cells and memory cells. With increasing titres these immune complexes would be gradually replaced by new immune complexes, antibody would become in excess, and there would be a rapid decrease in the number of free antigenic determinants. Remaining

free determinants would be occupied by antibody molecules which show an increase in avidity, migrating through the germinal centres during the immune response. The changing characteristics of the trapped immune complexes would result in no further stimulation of potential 7S producers. B memory cell priming would cease and antibody titres would fall.

These processes are thought to occur in germinal centres because they are the only sites in the body where immobilized immune complexes are retained, undegraded, long after the onset of antibody production. The majority of the complexes are degraded, by macrophages, soon after their formation.

In teleosts, the spleen is an important organ in respect to antigen-trapping. After an initial delay subsequent to injection, soluble antigen can be detected in the ellipsoid sheaths surrounding the splenic capillaries in both carp (Plate 24) and plaice (Ellis, 1974, 1980). This fluorescence is of a fine dendritic nature and appears to be associated with the reticulin fibres in the ellipsoid walls (Plate 6) suggesting the trapping of antigen to be extracellular. Carp immunoglobulin was also detected at these areas, using rabbit anti-carp immunoglobulin antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum. This was found in both control and immunized animals indicating that receptors for homologous immunoglobulin may be present at these sites. Also the peak antibody titre to HGG occurs during the period of positive immunofluorescence (Chapter 5). The delay in trapping,

the correlation with antibody titres and the fact that homologous antibody can be detected in these same areas, suggests that the trapping of antigen is in the form of immune complexes. Further, if a third injection of HGG is given, in adjuvant or saline, after the usual period of positive immunofluorescence has subsided but when antibody titres are still moderately high, antigen can be detected the following day. Cyprinid fibroblasts have also been shown to possess receptors for human and mouse C_3 , in vitro, (Ueki, Fukushima, Hyodoh & Kimoto, 1978). This is of interest because in homiotherms it has been postulated that the sole structural requirement for germinal centre localization of immunoglobulin is the ability to fix C_3 (Embling, Evans, Guttierrez, Holborow, Johns, Johnson, Papamichail & Stanworth, 1978).

The fluorescent picture in the spleen is similar with a cellular antigen, A. salmonicida (Plate 28), but the initial trapping bears resemblances to that which occurs with non-antigenic particulate material, such as carbon (Plate 34). The fluorescence is seen within large cells similar to macrophages, situated in the ellipsoid walls. Some fluorescent macrophages are located in the pulp of the spleen as well as in the ellipsoids, but the fluorescence in the ellipsoids continues for longer than carbon in the plaice (Ellis, Munroe & Roberts, 1976) and appears to be brightest between 3 and 5 weeks after immunization when antibody titres are high (Chapter 5). A further injection of A. salmonicida had little effect on the fluorescent picture.

The response by young fish to HGG, even after a third injection, showed little splenic involvement in antigen-trapping, although with A. salmonicida it was very similar to that seen in the older fish. Possibly in this respect these young carp are not fully immunocompetent towards murine T-dependent antigens.

The amphibian, Xenopus laevis, also shows positive extracellular immunofluorescence in the spleen following immunization with HGG (Plate 35) or with A. salmonicida (Plate 36). In X. laevis the fluorescent area lies within a more architecturally advanced structure. The spleen in X. laevis has distinct white pulp areas (Turner & Manning, 1973) each with a central arteriole, a large conspicuous cuff of lymphocytes and a marginal zone (Plate 37). The central arterioles have branches which enter the red pulp and this is possibly the first route of entry of antigen into the spleen where it is taken up by red pulp macrophages (Collie, 1974; Manning, 1975). Near the point of entry into the red pulp the sheaths of these branches contribute to the formation of the Grenzschichtmembran of Sterba (1951), a double layer of cells which forms a boundary delineating the white pulp. The antigen-trapping zone lies in the white pulp just within the boundary layer. The structure of the X. laevis spleen is therefore more highly organized than that of carp or plaice. This structure bears many resemblances to the mammalian spleen, including gaps in the boundary layer which form bridges between white and red pulp (Van Rooijen, 1972a; Mitchell, 1973). However the

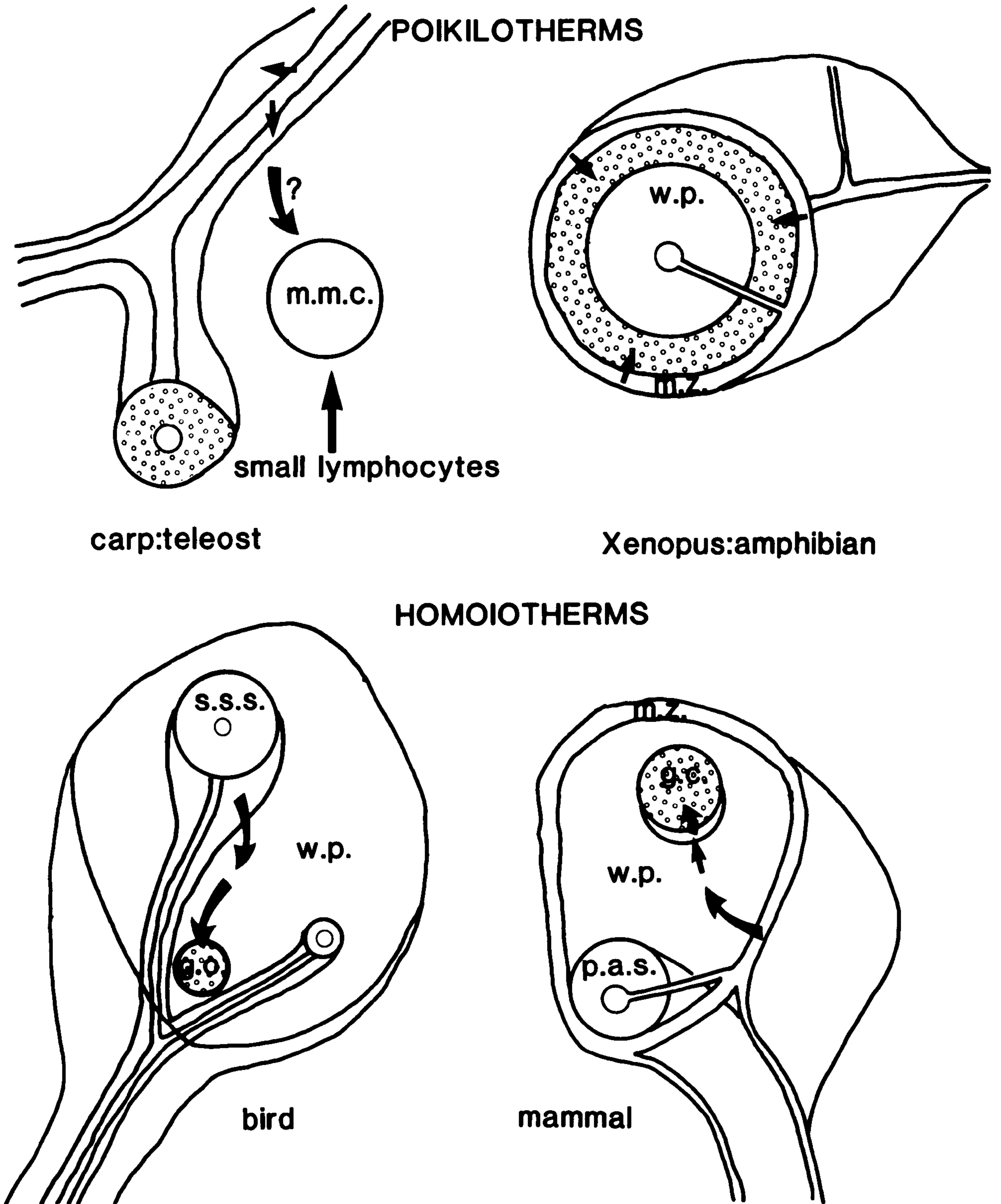
X. laevis spleen, as in all poikilotherms (Turner & Manning, 1973; Borysenko, 1976), lacks the germinal centres which are typical of homiotherms.

Another similarity between X. laevis and homiotherms is the migration of trapped antigen from the capillary terminals to the site of long-term retention. In homiotherms localization occurs initially in the marginal zone (mammals) or Schweigger-Seidel sheaths (birds). In the few hours after localization these complexes migrate towards the follicles and end up in the follicle centres on the processes of the dendritic cells (see Figure 4). Although there is evidence for a role of B lymphocytes in the transport of the immune complexes in mammals (Mitchell, 1972; Brown, Harris, Papamichail, Sljivic & Holborow, 1973; Van Rooijen, 1973a), it is becoming increasingly clear that lymphocytes only bind a very small part of the complexes (Van Rooijen, 1980). The bulk of the immune complexes are transported to the follicle centre bound to dendritic cells in a manner very similar to that which occurs in birds (White, French & Stark, 1970). Whether the immune complexes move along the cell membranes of the processes of non-migrating dendritic cells, or whether the dendritic cells themselves move, is still not clear.

In X. laevis such a migration of antigen is seen, from the marginal zone into the white pulp (Collie, 1974) and more recently a primitive dendritic splenocyte has been discovered which may be responsible for this transfer (Baldwin & Cohen, 1981). These dendritic splenocytes have long cytoplasmic processes that are in intimate contact with

Figure 4.

Diagram of the progressive increase in splenic white pulp architecture in the vertebrates.



➔, migration of immune complexes; ⬤⬤⬤⬤, antigen-trapping area; g.c., germinal centre; m.m.c., melano-macrophage centre; m.z., marginal zone; p.a.s., periarteriolar lymphocytic sheath; s.s.s., Schweigger-Seidel sheath; w.p., white pulp.

adjacent lymphocytes. Some of these processes extend into the red pulp and appear to trap and transport foreign material. Such a cell has not been discovered in fish, and this may be the reason why migration of trapped antigen is not usually seen. Nevertheless, fluorescence is sometimes associated with macrophages, which can themselves aggregate at melano-macrophage centres, and may represent the beginnings of such a system. Indeed, dendritic cells specialized for trapping antigen in the form of immune complexes may be developmentally related in evolution to melanocytes, which are themselves dendritic (Ellis, 1974).

The origins of the tetrapod spleen and the structural advances in the immune system, together with improvements in antigen-trapping, may be related to the more efficient circulatory systems which evolved in relation to life on land (Manning & Turner, 1976; Manning, 1978). The major difference between the spleen in fish and amphibians is the appearance in the amphibians of a definite and extensive lymphocytic cuff between the central arteriole and the boundary layer. The boundary layer could perhaps represent the outer layer of the ellipsoid sheath seen in the fish spleen, to the extent that in tetrapods the ellipsoid is now expanded to include a permanent lymphocyte population (white pulp) as well as macrophages. Interestingly, the fluorescent germinal centres in mammals, the fluorescent white pulp areas in amphibians and the fluorescent ellipsoids in fish, are all associated with metalophilic reticulin fibres and, in X. laevis, immunized animals appear to have a more expanded reticular pattern in the white pulp (Plate 39) than unimmunized ones

(Plate 38). This interpretation is perhaps supported by the finding that, on occasions in carp, particularly during booster responses, the reticulum of the ellipsoids acquires spherical proportions, resembling the white pulp reticulum of tetrapods (Plate 22). The increasing complexity of the splenic white pulp, from the rather simple situation seen in teleosts, to the highly specialized and departmentalized white pulp in homiotherms, is represented diagrammatically in Figure 4. In homiotherms antigen is trapped in discrete areas, the germinal centres; also, in mammals there is a distinct T-dependent periarteriolar lymphocytic sheath.

The antigen-trapping which occurs in the pronephros of carp and plaice (Ellis, 1974, 1980), is not as discrete as in the spleen. In the carp with a soluble antigen, areas of fluorescence are seen associated with pyroninophilic cells, but not with the reticulin fibres. This less discrete pattern is reminiscent of that seen in the jugular bodies of Bufo marinus (Diener & Nossal, 1966) where antigen-trapping occurs in a random fashion with nothing resembling a follicular arrangement. The pattern with a cellular antigen was similar to that observed in plaice, the fluorescence being found in individual cells scattered throughout the pulp. The mesonephros did on occasions show small fluorescent areas between the tubules, mostly with A. salmonicida. This fluorescence was again associated with pyroninophilic areas but not with reticulin fibres, and is probably similar to the areas in the pronephros. In the pronephros, both immune complexes and heat aggregated HGG are trapped more quickly

than antigen alone, indicating that the mechanism of trapping in fish is similar to that in homiotherms, which requires the Fc region of an immunoglobulin molecule to mediate complement fixation. Fluorescence has been detected in the kidney of X. laevis, but only briefly in association with the nephrosomes and glomeruli.

The evolution of the kidney is also of interest in this respect. In hagfish, Myxine glutinosa L, the kidney contains haemopoietic tissue and can trap substances reaching it from the coelom (Holmgren, 1950). The kidneys of fish and of anuran amphibians also contain haemopoietic tissue and form organs where lymphoid cells meet antigens. Substances may gain access to the inter-tubular renal tissue from the blood, especially where extensive renal portal sinusoids provide an efficient filter bed. Localization of antigen was restricted to these haemopoietic areas in fish, mainly in the pronephros where few tubules occur. In X. laevis, where there is only a mesonephric kidney, only transitory or sporadic fluorescence was seen. During vertebrate evolution the kidney evolved in the amniotes to become exclusively excretory, a metanephros and the spleen and lymph nodes specialized in the trapping of immune complexes. However, although the fluorescent pattern in the pronephros has similarities to the jugular bodies in B. marinus, evolutionary trends to both lymph nodes (Smith, Potter & Merchant, 1967; Ellis, 1974) and bone marrow (Zapata, 1979) have been put forward for this organ. It may even be possible that cells migrated to the spleen from the pronephros, as is

suggested to occur to certain leucocytes in the goldfish, Carassius auratus (L), (Neale & Chavin, 1971) and became located within the boundary layer of the ellipsoid sheath to give both fluorescent and pyroninophilic cells as seen in X. laevis.

Whatever the possible evolutionary trends of antigen-trapping leading to germinal centre formation, it is clear that the trapping of immune complexes in fish seems poorly organized in comparison with germinal centre antigen-trapping in homoiotherms or with the concentrated localization of antigen in the splenic white pulp of X. laevis. In some teleost species antigen is localized in melano-macrophage centres (Ellis, 1974, 1980) through which small lymphocytes percolate and may be influenced immunogenically. The evolution of germinal centres can either be seen as an extension of this melano-macrophage system or as a divergence from it whereby the system for extracellular trapping of immune complexes on dendritic cells is further elaborated.

Chapter 5HUMORAL IMMUNE RESPONSES OF CARP TO
SOLUBLE AND CELLULAR ANTIGENS

It has been known since the times of Babes and Riegler (1903) that fish exhibit immune responses. Yet we still have insufficient knowledge about fish immunological capabilities despite the interest both from a phylogenetic viewpoint in the mid-sixties (Papermaster, Condie, Finstad & Good, 1964; Smith, Miescher & Good, 1966) and from the numerous vaccination trials brought about by the recent surge in aquaculture.

The vaccination trials have shown that the route of administration has a marked effect on antibody responses, but these are also dependent on the antigen used, its dose and the fish species looked at. The possibility of immunizing large numbers of fish, probably at the fry stage, would seem to favour administration orally, via hyperosmotic infiltration (Antipa & Amend, 1977; Croy & Amend, 1977) or flush exposure (Anderson, Roberson & Dixon 1979a; Anderson & Dixon, 1980). However, although young fish have been shown to be capable of producing antibodies (Dorson, 1974; Paterson & Fryer, 1974; Khalifa & Post, 1976), the exact age at which immunocompetence is reached has not yet been determined, and the possibility of inducing tolerance instead of immunization has received almost no attention at all. It has recently been shown that the thymus is in a superficial position under the operculum in salmonid species, without a specialized epithelium to protect it from outside influence (Grace, Botham & Manning, 1981). The shock of

hyperosmotic infiltration, which has been shown to allow antigen to enter via the gills (Bowers, 1979), could well be enough to get antigen across the thymic epithelium and induce tolerance in young fish. The thymic route has been shown to be a particularly good route to induce tolerance in rabbits and mice (Ohara, Shimizu, Kakinuma, Kimura & Okada, 1979), where 100% of animals were affected.

Certainly injecting individual fish is time consuming, and possibly only of value to vaccinate brood stock. Here, most studies have involved investigation only of the primary response, yet after immunization it would be the anamnestic response that is all important. A good priming dose or route may not be optimal for memory formation (Benner, Meima, Van der Meulen & Van Ewijk, 1974; Grantham & Fitch, 1975; Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a).

It has been stated that teleost fish possess only antibody of the IgM class. However, controversy exists even about this. In hyperimmunized goldfish a small immunoglobulin molecule with IgG-like activity has been described (Uhr, Finkelstein & Franklin, 1962), and two types of immunoglobulin were reported in the serum of the salmon Salmo salar L, (Alexander, Wilson & Kershaw, 1970). These workers, however, did not claim to have found an antibody type homologous to mammalian IgG, or to any other known immunoglobulin class.

Lastly, the properties of fish lymphocytes themselves deserve closer inspection. It would be of particular interest to determine whether the cell-mediated and antibody-mediated immune processes are the functions of distinct

lymphocyte populations, the T and B cells of mammals and birds.

In the present investigation a number of avenues have been explored to try to help elucidate some of these unknown areas. The antigens chosen include soluble protein antigens, and a cellular bacterial antigen Aeromonas salmonicida, the causative agent of one of the most widespread diseases of salmonids, furunculosis. This Chapter comprises an investigation into the humoral responses of carp, whilst Chapter 6 concentrates on cell-mediated responses.

MATERIALS AND METHODS

1. Antibody determinations

A preliminary study was carried out to examine the agglutinating and precipitating antibody responses in one-year-old carp to a small range of antigens. The effect of using different routes, adjuvant and booster injections to some of these antigens was then investigated. In general 6 fish were used per group.

Immunization

(a) Route of administration

1. Intraperitoneal injection of soluble protein antigens. The immunization schedules were based on previous experience in the Hull Laboratory (Horton & Manning, 1974; Collie & Turner, 1975). In these first experiments soluble protein antigens were injected in adjuvant. Lyophilized human gamma globulin (HGG) (Kabi, Stockholm) was dissolved in 0.85% saline to a concentration of 5 mg/ml and was then emulsified with an equal volume of Freund's complete adjuvant (FCA)

(Difco, Detroit, USA) to give a final concentration of 2.5 mg/ml, as used in the work reported in the two previous Chapters. The carp were injected intraperitoneally with doses of 0.01 ml/g fish (0.025 mg/g fish) on day 0. Lyophilized bovine serum albumin Cohn fraction V (BSA) (Sigma, St. Louis, USA) and ovalbumin grade III (OVA) (Sigma, St. Louis, USA) were prepared in the same manner and injected into the carp.

2. Intraperitoneal injection of cellular antigen.

Suspensions of Aeromonas salmonicida containing 1×10^{10} formalin-killed cells/ml of 0.85% saline were also injected into carp intraperitoneally in quantities of 0.01 ml/g fish on day 0, giving a dose of 1×10^8 cells/g fish. A second group of fish was injected with A. salmonicida emulsified in adjuvant. Here, the concentration of the A. salmonicida suspension before adding an equal volume of FCA was 2×10^{10} cells/ml.

3. Intramuscular and intravascular injection of

HGG. Animals were injected with HGG in FCA intramuscularly at the same concentration as in the above study using the intraperitoneal route. The intravascular route does not lend itself to the use of adjuvant, therefore in the group injected intravascularly the antigen was administered without adjuvant.

Animals were also injected intraperitoneally and intramuscularly with HGG without adjuvant, using 2.5 mg HGG/ml 0.85% saline.

4. Hyperosmotic administration of Aeromonas

salmonicida. With A. salmonicida the hyperosmotic route was the only alternative to the intraperitoneal route used

in this study. Here the method outlined in the general methods was used, with the fish being immersed for 30 s in a solution of 1×10^9 A. salmonicida cells in the second step. A quantity of approximately 10 ml/g fish of the antigen solution was required.

(b) Booster injections

1. Human gamma globulin. A booster injection of HGG in FCA, administered intraperitoneally, was given on day 12 to one group. Other groups, injected with HGG in FCA intraperitoneally or intramuscularly, or injected with HGG in saline intraperitoneally, were given a booster on day 56.

2. Aeromonas salmonicida. A booster injection of A. salmonicida in saline, injected intraperitoneally, was given to one group on day 12 and to a second on day 56, as for HGG.

Testing for antibody production

All sera were used fresh or after storage at 4°C for 1 day. No samples were deep frozen as it has been shown that such samples lose their antibody activity in goldfish (Rio & Recco, 1971).

I. Agglutinating antibodies

It has been shown that heat-labile complement exists in carp (Cushing, 1945), so all control and immunized sera were heat inactivated at 56°C for 30 min before use.

1. Soluble protein antigens. Fish were bled at weekly intervals for 2 months and their antibody titres were determined using the passive haemagglutination method (Stavitsky, 1954). The method was identical for HGG, BSA and

OVA. All buffers were prepared fresh before each assay. So was the serum diluent, 1% pooled, heat inactivated and sheep erythrocyte absorbed, normal carp serum, used to stabilize tanned sheep red blood cells (SRBC's). After heat inactivation all sera were cooled before absorbing with an equal volume of thrice washed, packed, SRBC's. This removes any natural agglutinins to the SRBC's before use. The centrifugation of SRBC's was done routinely at 1,100 g for 5 min in an MSE Minor centrifuge.

The sheep erythrocytes used here for testing, and in all subsequent experiments were from the same source (Gibco, Glasgow, UK). They were stored at 4°C in Alsever's solution, and when required were washed 4 times in 0.85% saline. After the final washing they were resuspended in phosphate buffered saline (PBS) pH 7.2, to a 2.5% solution, and mixed in equal volumes with 0.005% tannic acid for 10 min at 37°C. After centrifugation the cells were washed in PBS pH 7.2 and resuspended in PBS pH 7.2 to a 2.5% solution. The cells were then coated with the appropriate antigen solution, using a 1 mg/ml solution of the antigen. A ratio of 4 volumes PBS pH 6.4:1 volume antigen solution:1 volume tanned SRBC's, was used for coating cells, or using 0.85% saline instead of the antigen solution for control, uncoated cells. They were mixed and left to stand for 10 min. at room temperature. They were then centrifuged, washed in serum diluent and resuspended in diluent to give a 0.25% solution. Antibody determinations were carried out in microtiter plates (Sterilin, Teddington, UK), consisting of 8 rows of

12 U-shaped wells. In all wells 50 μ l of diluent was added. Using 2 rows per sample, serum samples of 50 μ l were serially diluted up to well 20, and again from well 21-24. In wells 1-20 were added 50 μ l of coated cells, whilst in wells 21-24 were added uncoated cells. Using this regime 2 mls of coated cells were required per tray. Appropriate controls using serum from saline injected animals, serum from uninjected animals and diluent alone, were carried out on separate rows. The trays were then gently agitated, and sealed with sellotape to prevent desiccation. They were left at room temperature for 3 h, and then at 4°C overnight before reading. The last well to show complete agglutination was taken as the end point (Plate 40). If a large non-specific effect was seen using the uncoated cells, the residual serum was absorbed for a second time and retitrated. A photograph was often taken of the trays, and a quick method was to place the whole tray in an enlarger and use it to produce a negative print.

Cross-reactivity of anti-HGG and anti-OVA antisera was also investigated. SRBC's were coated with a variety of antigens, such as BSA, bovine gamma globulin (BGG), chicken gamma globulin (CGG), OVA, and HGG, and the amount of agglutination recorded in the normal fashion.

2. Cellular antigen. Fish were bled at weekly intervals for 2 months. Antibody titres were determined using a tube agglutination technique adapted from Krantz, Reddecliff and Heist (1963). Decomplemented anti-A. salmonicida antisera were serially diluted in a microtiter plate (Sterilin) in

0.85% saline, starting with 50 μ l of each, up to well 20. An equal volume of 1×10^9 cells of A. salmonicida/ml 0.85% saline was added to each well. These trays were also gently agitated, sealed with sellotape, left for 2 to 3 h at room temperature and then overnight at 4°C, before reading. The end point was determined under a microscope at x25 magnification. This technique gave similar results to the slide agglutination test described by many authors (Conroy & Withnell, 1974), but the end point was much easier to define (Plate 41).

II. Precipitating antibodies

In many of the groups, precipitating antibodies to HGG were detected alongside the agglutinating antibodies, using double diffusion in agar. Ouchterlony plates were made using I.D. Agar Tablets (Oxoid, London, UK) and Oxoid Barbitone Acetate Buffer (Oxoid, London, UK). One tablet was added to 12.5 ml oxoid barbitone acetate buffer and made up to 50 ml with distilled water. The agar was dissolved by boiling, cooled to 55-60°C and left for 1 h to remove air bubbles. Agar (2.7 ml) was then pipetted on to each acid alcohol cleaned plate from a wide ended pipette. When the agar had set, wells were cut using a punch and template. Patterns consisted of a central well and 6 outer ones. Neat serum (25 μ l) was placed in the central well, and the same volume of the appropriate antigen dilution in 0.85% saline into the outer ones. Normally two wells were filled at 5 mg antigen/ml, two at 1 mg/ml and two at 0.5 mg/ml. If all concentrations produced strong precipitin lines the

result was scored as ++; if there were strong lines only to the lower dilutions it was scored as +; if only weak lines appeared, whether to all or to only some antigen dilutions it was scored as \pm . The plates were left in petri dishes containing filter paper, moistened with 0.5% phenol, for 1 week at 4°C before reading.

Cross-reactivity was investigated using double diffusion in agar. Anti-HGG antisera, which formed strong precipitation lines to HGG, were put into the central wells of several plates and solutions of other antigens were put in the outer wells at the same concentrations as the HGG. The antigens tested were BSA, OVA, BGG and OGG. These were also left for a week at 4°C and scored as for HGG.

To stain Ouchterlony plates the gels were washed for 2 days in 0.85% saline, with several changes, and for 1 day in distilled water. They were then dried at 37°C and stained with Azo-carmin for 20 min. Destaining in 2% aqueous acetic acid was carried out until the background was clear. They were then dried again and mounted.

To ensure that the precipitate was not due to C-reactive protein, several plates were immersed in 5% sodium citrate for up to a week.

2. Mercaptoethanol sensitivity

Several of the anti-HGG immune sera and some pooled hyperimmune anti-HGG sera, were tested for their sensitivity to 2-mercaptoethanol (2ME) (Sigma, St. Louis, USA) at different stages during an immune response. The response of agglutinating and precipitating antibodies to a dose range was tested. Solutions (0.1, 0.2, 0.4 and 0.6 M) of 2ME

were prepared in a fume cupboard. They were added to an equal volume of serum to give a final concentration of 0.05, 0.1, 0.2 and 0.3 M 2ME, and incubated for 1 h at 36°C.

The sera were allowed to cool and were tested without prior dialysis. Hyperimmune sera were needed when looking at the effect of 2 ME on precipitating antibodies. Control sera consisted of immune sera that were diluted 50% with 0.85% saline, before undergoing the same treatment as above.

Similarly anti-A. salmonicida antiserum was tested for 2ME-sensitivity of agglutinating antibodies, as above.

3. Column chromatography

Serum proteins of hyperimmunized animals were investigated to see if more than one immunoglobulin class could be detected under such conditions, as suggested to occur in the goldfish (Uhr, Finkelstein & Franklin, 1962).

Carp were given an intraperitoneal injection of HGG in FCA on days 0, 12 and 56, followed by an intramuscular booster on day 84. They were bled on day 112 and in all cases gave very strong precipitating antibody responses. The serum was fractionated on three types of gel, at room temperature. One of the gels used was Ultragel ACA 34, which has a linear fractionation range of 20,000 to 350,000 daltons. The column measured 80 cm in height and 1.5 cm in diameter. Tris-HCl buffer, pH 8.0, was used consisting of 150 mM sodium chloride, 50 mM Tris-HCl and 0.02% sodium azide. Pooled serum samples were fractionated at a flow rate of 5.6 ml/h and collected in 2 ml aliquots. Absorbance was measured at 280 nm on an LKB Uvicord u.v. detector, using absorption and auxiliary output fed into an LKB 2210 two

channel potentiometric recorder. The light path length was 0.25 cm. The sample size was 1ml.

Ultragel ACA 22 was also used on a column with the same dimensions, buffer and detector, but with a flow rate of 4.8 ml/h. This gel has a linear fractionation range of 100,000 to 1,200,000 daltons. Lastly, Sephacryl S-300 was used, which has a fractionation range of 10,000 to 1,000,000 daltons. The column measured 88.5 cm in height and 1.6 cm in diameter. Again the same buffer and detector were used, with a flow rate now of 8 ml/h. The sample size was now 0.2ml.

Pooled hyperimmune anti-HGG antiserum was fractionated on all 3 gels, and the passive haemagglutination titre determined on the aliquots after concentrating up. This concentration was done by centrifuging the 2 ml fraction from the column at 1,000 rpm for 30 min in an MSE Mistral 4 L centrifuge, in an Amicon CF 25 centriflow cone. These cones retain molecules larger than 25,000 daltons, anything smaller passes through. The remaining sample, usually 50 μ l, was made up to 100 μ l with 0.85% saline, in order to carry out the antibody titration. The peak fractions from the Sephacryl column were also tested for precipitating antibody after concentrating, by using Ouchterlony plates as described in Section 1. Normal pooled control serum was also fractionated on the Sephacryl column.

The Sephacryl column was used to determine the sedimentation coefficient of the high molecular weight antibody molecule of a second group of hyperimmune animals. It was not possible to get an accurate measurement of the

molecular weight of IgM using column chromatography, because of the discoid shape of the molecule (the column is calibrated for globular proteins, and so gives an overestimation).

The sedimentation coefficient is an obvious choice, relating the molecule in terms of its particle size. Pooled serum (2 ml) was fractionated on the column and the four aliquots of the first serum protein peak were pooled and concentrated to approximately 1 mg protein/ml. Sedimentation was carried out in an MSE Analytical Ultracentrifuge using the Schlieren optic technique. The temperature was maintained at 25°C, the rotor speed was 48,100 rpm, and the cells were scanned at 200 s intervals. For average g see Plate 44.

4. Maturation of humoral responses with age

A study was carried out to see whether the antibody response to HGG and to A. salmonicida in carp varied quantitatively during the course of a year, from hatching to sexual maturity, and to see whether tolerance was induced in the youngest, least mature fish. The youngest age at which carp were able to be injected was 2 weeks (Plate 1), and even then they were very susceptible to damage from injection. Nevertheless, one group was injected with HGG in FCA, one with HGG in saline and one with A. salmonicida in saline, at 2, 4 and 8 weeks after hatching. The standard dose was 0.025 mg HGG/g fish and 10^8 A. salmonicida cells/g fish. A further group of the 8 week carp was injected with HGG in saline at 0.25 mg/g fish, to see whether it was possible to induce a type of high dose tolerance in these

slightly older fish. All groups of carp injected at 2 or 4 weeks after hatching died before they were large enough to bleed. This part of the experiment was therefore unsuccessful and will have to be repeated at a later date.

Also, carp aged 16, 24, 32, 40 and 48 weeks, were injected with HGG in FCA or with A. salmonicida, at the standard dose. In each case, a second group was given a booster of the same antigen 8 weeks later, at 24, 32, 40, 48 and 56 weeks. All groups injected at 8 weeks were also injected with HGG in FCA or with A. salmonicida at week 16. For carp aged 16 weeks and older, six animals per group were examined but many more were needed for younger groups because pooling of blood was necessary to obtain enough serum for the assay. Antibody titres were determined using passive haemagglutination for HGG injected fish and the tube agglutination technique for A. salmonicida injected fish. For small samples of pooled sera a microagglutination technique was used, with only 10 μ l of diluent, serum and cells. To improve the visibility of button formation at the bottom of the wells with such small samples, V-shaped wells were required.

5. Effect of dose

Some adult animals, 56 weeks old, were injected with HGG at 4-(0.1 mg/g fish) and 40-(1 mg/g fish) times the standard dose to see if high dose tolerance could be induced, as described by Marchalonis and Germain (1971) for Bufo marinus. The HGG was injected intraperitoneally with and without adjuvant, and intramuscularly with adjuvant, at 1 mg/g fish, to see whether adjuvant had an effect

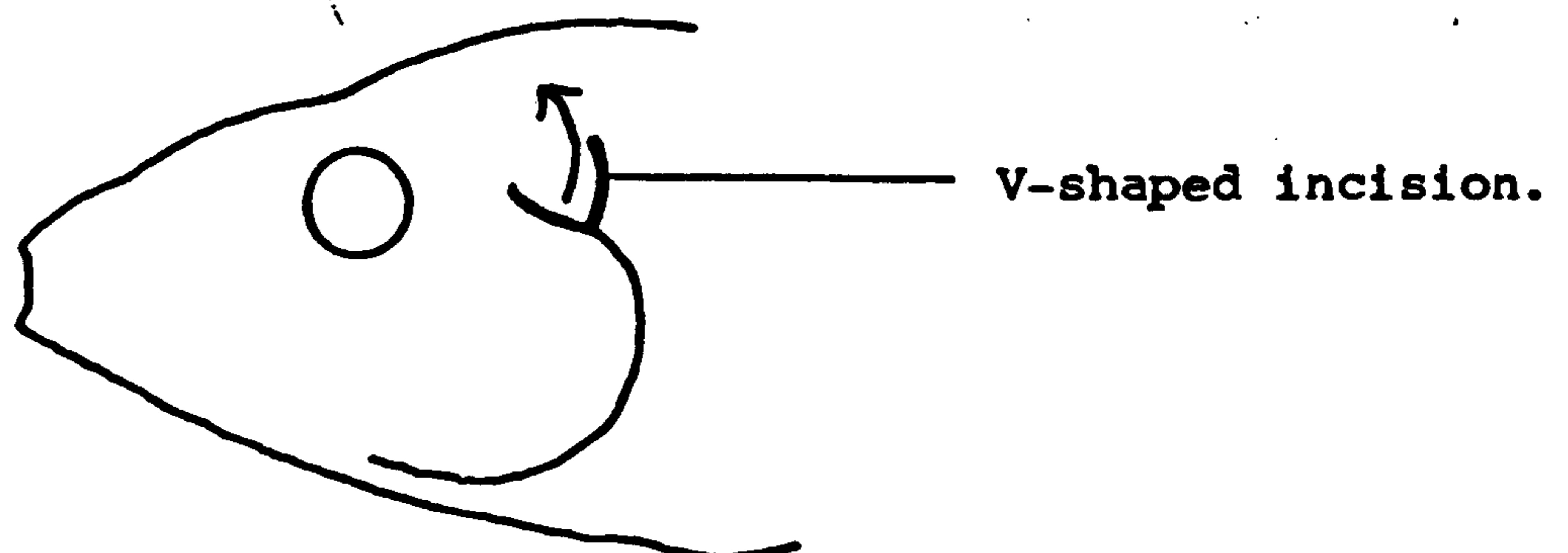
depending on the route used. These animals were tested 4 and 8 weeks after immunization and challenged at this second testing with an immunogenic dose of HGG in FCA injected intraperitoneally. They were also injected with OVA in FCA at this same time to see the response to a non-cross-reacting antigen. A further testing was carried out at 4 and 8 weeks after the second immunization. Also an immunofluorescence test was performed on day 1, to see if antigen was trapped immediately after the booster injection (see Chapter 4: Tables XVII and XVIII).

6. Adult thymectomy

The effect of adult thymectomy on antibody production was investigated in adult carp. The thymus in carp is partially surrounded by the bones of the opercular region and is situated adjacent to the ear, making this operation quite difficult. The carp were anaesthetized in MS222 (0.125 g/l) and the side of the head was swabbed with alcohol. Sterile scissors were used to make a V-shaped incision at the dorsal edge of the operculum. This enabled part of the bone and skin to be folded back allowing access to the thymus (Figure 5). The thymus was removed with sterile fine forceps and the area swabbed with cotton wool. The flap of bone and skin was then replaced and sealed with cyanoacrylate adhesive (Radio Spares, Stockport, UK).

Figure 5

Site of incision made for adult thymectomy



The animals were then placed into well aerated standing water to recover. The water was replaced daily with clean water in an attempt to keep contamination of the wound to a minimum. This operation was initially done unilaterally, and the head region sectioned to ensure that the thymus was being completely removed. Also the tissue removed was sectioned to ensure that only the thymus was being taken. In all cases the tissue removed was found to be composed of densely packed lymphocytes. After this was confirmed bilateral thymectomy operations were performed.

Sham operations were also carried out. The incisions were made and the bone lifted up and replaced, but without the removal of the thymus.

These animals were given 3 weeks to recover from the stress of the operation before intraperitoneal injection with either HGG in FCA or A. salmonicida, at the normal doses. Weekly bleedings were performed and antibody titres determined using the passive haemagglutination and tube agglutination techniques previously described. The thymic region was serially sectioned to ensure that thymectomy had been successful in these fish, either at the time of death during the experiment or at the end of the experiment when the fish were killed.

7. Antibody response to Aeromonas salmonicida in the toad Xenopus laevis, using thymectomized and unoperated animals

It has been well documented that HGG is a T-dependent antigen in poikilotherms, such as in the toad Xenopus laevis (Turner & Manning, 1974). However, A. salmonicida used in

the above experiments has had no such previous testing. As a preliminary to the use of this antigen, X. laevis toads, that had been thymectomized at 8 days from fertilization and checked by visual examination under a binocular microscope in vivo before metamorphosis to ensure no thymic remnants remained, were injected intraperitoneally with A. salmonicida at the standard dose of 10^8 cells/g animal. Unoperated animals were similarly injected. Sham operated animals were not available, however, they do not seem to differ in their immune responses from unoperated animals (see Turner & Manning, 1974). These animals were bled from the sciatic vein, after swabbing the leg with alcohol, and a tube agglutination assay was performed at weeks 2, 4, 6 and 8 after immunization. The results can be seen in Table XIX.

Clearly both thymectomized toads responded to A. salmonicida as well as unoperated animals. So A. salmonicida can certainly be considered a T-independent antigen in the Xenopus model.

RESULTS

1.I. Agglutinating antibody determinations

(a) Route of administration

1. Intraperitoneal injection of soluble protein antigens. The antibody responses to HGG in FCA and OVA in FCA are shown in Figure 6. With HGG there is a latent period of about one week before serum antibodies begin to appear. They then steadily increase up to the fourth week and level off at around 1:1,024 (10). With OVA the latent period is longer, about two weeks, but again there is a

TABLE XIX

Serum antibody titres of adult X. laevis toads injected with

A. salmonicida, expressed as $-log_2$ titre. Each figure

represents one individual.

	Week tested			
	2	4	6	8
Thymectomized	6 (animal a)	7 (animal b)	8 (animal a)	8 (animal b)
Unoperated	4, 6, 6	7, 7, 8	8, 9, 9	7, 7

Figure 6

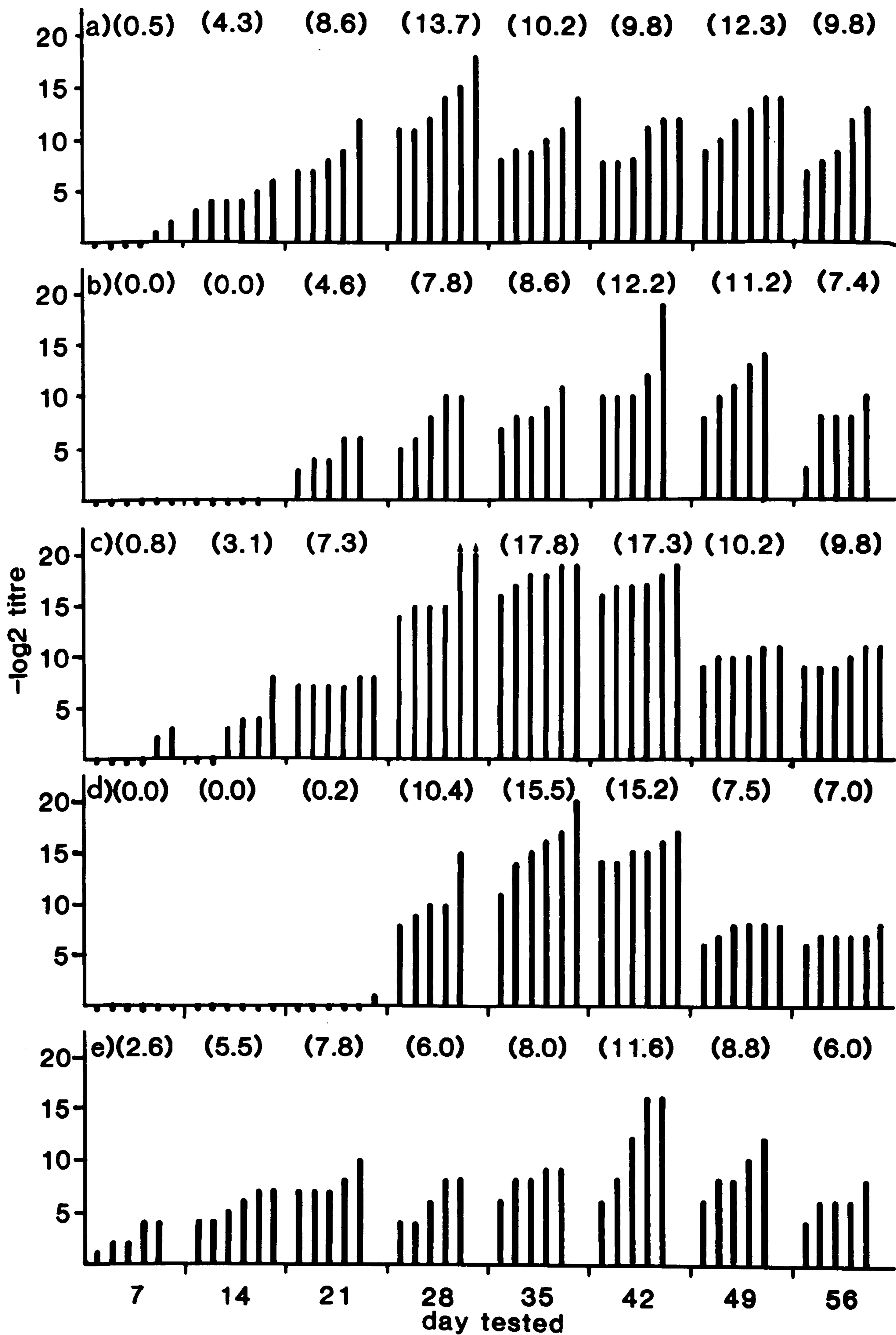
Anti-HGG passive haemagglutination titres of sera from animals immunized with:

- (a) HGG in FCA administered intraperitoneally on day 0.
- (b) OVA in FCA administered intraperitoneally on day 0.
- (c) HGG in FCA administered intramuscularly on day 0.
- (d) HGG in saline administered intraperitoneally on day 0.
- (e) HGG in saline administered intramuscularly on day 0.

Each column represents one individual. Means in parentheses.

Statistical analysis of results.

A comparison of animals immunized intraperitoneally with HGG in FCA (Figure 6a) with animals immunized intraperitoneally with HGG in saline (Figure 6d) showed no consistent significant differences between the groups, using the t-test corrected for small samples (Parker, 1973). However, a comparison of animals that had been immunized intramuscularly with HGG in FCA (Figure 6c) with animals that had been immunized intramuscularly with HGG in saline (Figure 6e), showed a significantly higher titre in animals immunized with HGG in FCA on days 28($p < 0.001$), 35($p < 0.001$), 42($p < 0.02$) and 56($p < 0.001$).



†, Titre exceeded well 20.

steady increase which levels off after about 4 weeks.

With BSA no antibody titres were detected, even when varying the concentration of antigen used to coat the SRBC's from 0.25 mg/ml to 40 mg/ml.

The results of using potentially cross-reacting antigens are shown in Table XX. Clearly anti-HGG antisera

TABLE XX

Serum antibody titres of carp injected intraperitoneally with HGG in FCA or OVA in FCA, expressed as $-\log_2$ titre, against a variety of test antigens. Each row represents one individual.

<u>Antigen used to coat tanned SRBC's</u>	HGG	BGG	CGG	BSA	OVA
Anti-HGG antiserum	20	7	2	0	0
	16	4	1	0	0
Anti-OVA antiserum	4	N.T.	N.T.	0	10
	3	N.T.	N.T.	0	8

N.T., Not tested

cross-react to a moderate extent with BGG, to a small extent to CGG and not to BSA or OVA. Conversely, anti-OVA antisera seem to cross-react with HGG, but not BSA.

Fully absorbed serum from uninjected animals never yielded any detectable antibody titre, and serum from saline injected animals did not exceed a titre of 1:4.

2. Intraperitoneal injection of cellular antigen.

The antibody titres to A. salmonicida are shown in Figure 7. With A. salmonicida injected in saline there is a rapid appearance of serum antibody, which seems to plateau very

Figure 7

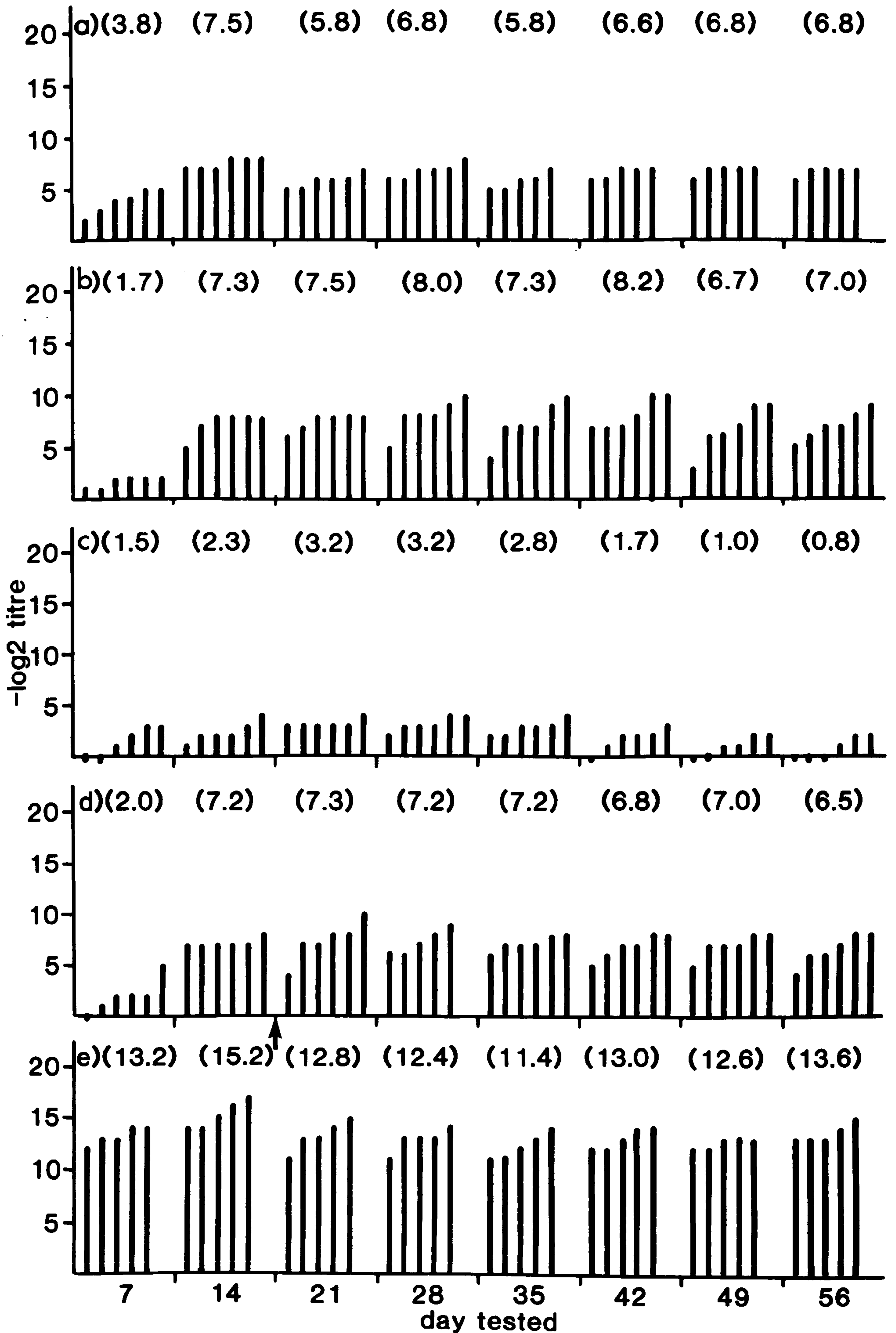
Tube agglutination titres of sera from animals immunized with A. salmonicida:

- (a) administered intraperitoneally in saline on day 0.
- (b) administered intraperitoneally in FCA on day 0.
- (c) administered hyperosmotically.
- (d) administered intraperitoneally in saline on days 0 and 12.
- (e) administered intraperitoneally in saline on days 0 and 56.

Each column represents one individual. Means in parentheses.

Statistical analysis of results.

Apart from a possible slight delay in the attainment of good antibody titres at day 7 (a comparison of Figure 7a with Figure 7b shows $p < 0.01$) there were no consistent significant differences at any time after immunization between animals immunized with A. salmonicida in saline (Figure 7a), compared to animals immunized with A. salmonicida in FCA (Figure 7b), or compared to animals immunized with A. salmonicida in saline on days 0 and 12 (Figure 7d). However, animals immunized with A. salmonicida in saline on days 0 and 56 (Figure 7e) had significantly higher titres ($p < 0.001$) at every time after immunization when compared to animals given a single injection only (Figure 7a). Also, animals immunized with A. salmonicida hyperosmotically (Figure 7c) showed significantly lower titres at every time after immunization when compared to animals immunized intraperitoneally with A. salmonicida in saline (Figure 7a).



↑, booster given.

quickly at about 1:128 (7). The use of adjuvant has no obvious effect on the primary response. Overall, titres to A. salmonicida are lower when compared with those of HGG or OVA injected animals. This may simply reflect a dose effect difference, or a true difference between soluble and cellular antigens, or may be a result of differences in sensitivity of the assay used.

3. Intramuscular and intravascular injection of HGG. Using the intramuscular route the rise in titre to HGG follows that for the intraperitoneal route very closely (Figure 6c). It reaches a peak at about the same time, but persists for longer at this level before declining to the level of 1:1,024 seen with the intraperitoneal route.

The intravascular route (Figure 8a) seems to produce only a poor response and titres rarely exceed 1:32. The lack of adjuvant probably has marked effects, but it is interesting that most fish respond within the first week, presumably because the antigen is distributed to the organs involved in antibody production more rapidly using this route.

The lack of adjuvant also seems to have pronounced effects when either the intraperitoneal or the intramuscular route is used (Figure 6). The intraperitoneal route yields a very long delay before serum antibodies can be detected. However, once present they seem comparable to those seen when adjuvant is used. In contrast, the lack of adjuvant has no effect on the time taken to produce serum antibodies when antigen is administered by the intramuscular route, but it does effect the magnitude of the response.

Only by week 6 do the mean titres exceed 1:256 (8) and then for only one week; in contrast to the very high titres seen between weeks 4 and 6 with adjuvant.

4. Hyperosmotic administration of *Aeromonas salmonicida*. Using *A. salmonicida* administered by hyperosmotic infiltration only a poor serum antibody response was obtained (Figure 7c). Titres did not exceed 1:8 at any time, and were certainly less than the titres seen using the intraperitoneal route.

(b) The results of booster injections

1. Human gamma globulin. The effects of giving an intraperitoneal booster injection of HGG in FCA are shown in Figure 8. When given on day 12 (Figure 8b) the result is a general, but relatively small, increase in titres throughout the period tested. When given on day 56 (Figure 8c) the effect seems to act on the existing titre, increasing it by almost exactly the amount that would be expected to appear if an injection had been given on day 56 only. Titres reach very high levels during weeks 5 and 6, but do not start to decline until week 8. When the booster of HGG in FCA is given intramuscularly on day 56, the result is very high titres for almost the entire period tested, with animals having titres greater than 1:1,048,576 (Figure 8d). This route certainly seems much more effective than the intraperitoneal route, but care must be used in drawing conclusions from such small sample sizes.

It is difficult to interpret these increases as true anamnestic responses. For the intraperitoneal route, the

Figure 8

Anti-HGG passive haemagglutination titres of sera from animals immunized with:

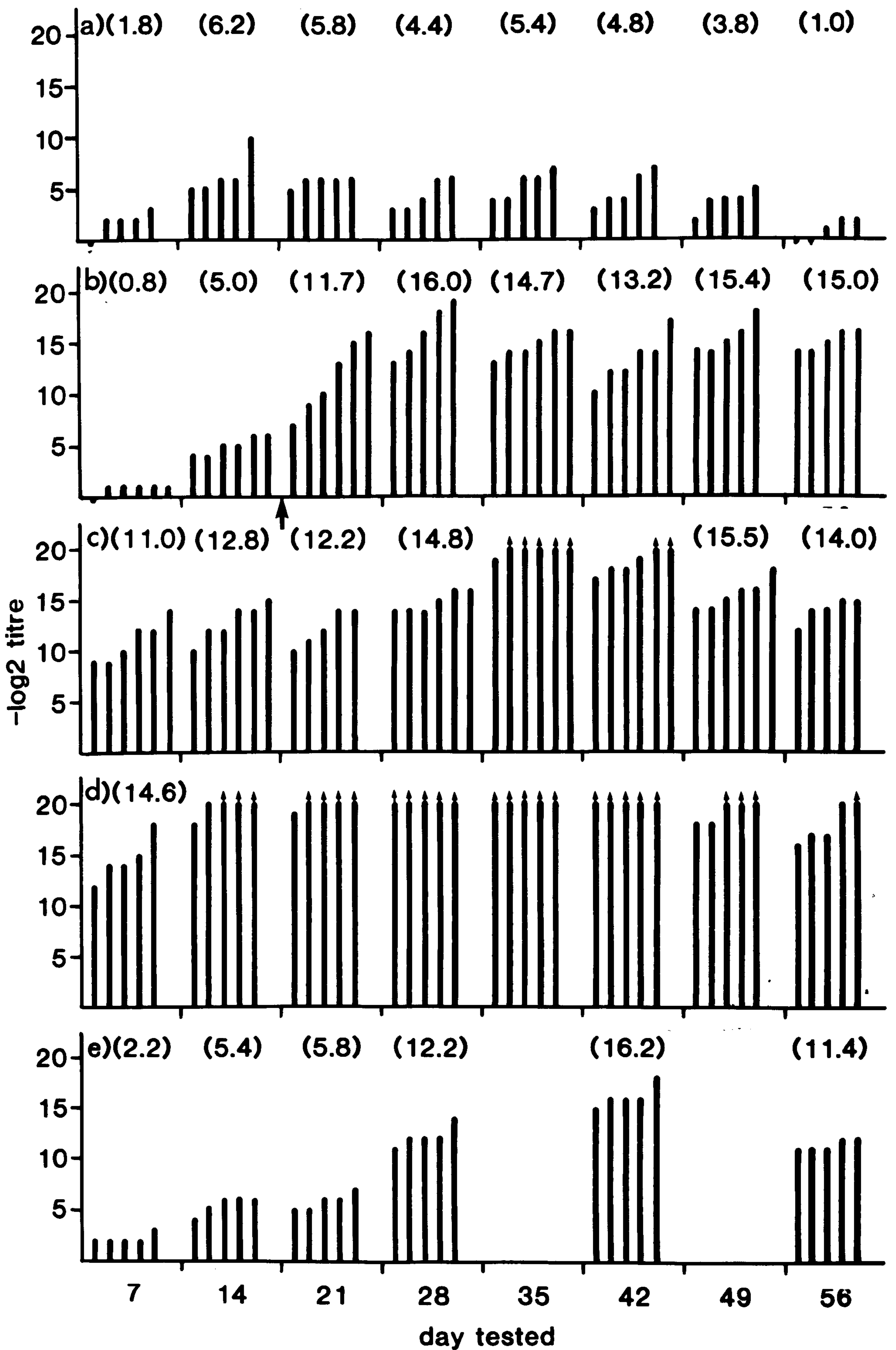
- (a) HGG in saline administered intravascularly on day 0.
- (b) HGG in FCA administered intraperitoneally on days 0 and 12.
- (c) HGG in FCA administered intraperitoneally on days 0 and 56.
- (d) HGG in FCA administered intramuscularly on days 0 and 56.
- (e) HGG in saline administered intraperitoneally on days 0 and 56.

Each column represents one individual. Means in parentheses.

Statistical analysis of results.

A comparison of Figure 8b with Figure 6a showed that animals immunized intraperitoneally with HGG in FCA on days 0 and 12 (Figure 8b) had significantly higher titres than animals immunized on day 0 only (Figure 6a), on days 35 to 56. Using the t-test corrected for small samples $p < 0.01$ for all comparisons. Similarly, in animals immunized intraperitoneally with HGG in FCA on days 0 and 56 (Figure 8c) titres were significantly higher than in animals immunized on day 0 only (Figure 6a) at every time after immunization.

A comparison of Figure 8e with Figure 6d, however, showed that when animals were immunized with HGG in saline on days 0 and 56 (Figure 8e) only on the last day of testing were titres increased ($p < 0.001$) compared to animals given one injection only (Figure 6d).



†, Titre exceeded well 20. ↑, booster given.

rate of increase is only the same as in the primary response and the picture is further confused by the fact that the primary response was still persisting at the time when these fish were given the secondary immunization.

Giving a booster of HGG in saline intraperitoneally results in titres very similar to those obtained during the primary response (Figure 8e). The long delay before detectable serum antibody no longer occurs, but the magnitude of the titres appears to be unaffected.

2. Aeromonas salmonicida. A booster injection given on day 12 seems to have little effect on the primary response to A. salmonicida (Figure 7d). However, if given on day 56, the titres almost double in size during the first 2 weeks, and level off at around 1:4,096 (12) (Figure 7e). In contrast the primary response levelled off at about 1:128. The effect of the booster is very similar to the effect of a booster injection of HGG, in that the existing titre is increased by almost exactly the amount of antibody that would be expected to appear during a primary response. Again, it is difficult to say a true anamnestic response is seen. The titres are higher, but the rate of antibody production only parallels that seen in the primary response.

1. II. Precipitating antibody responses

The results from the Ouchterlony plates are presented in Table XXI. In no instances did the precipitin lines dissolve in sodium citrate, indicating that they were due to antigen-antibody precipitates and not C-reactive protein.

TABLE XXI

Precipitating antibody responses of sera from animals injected with HGG in saline or FCA. Each symbol represents one individual.

<u>Time and route of injection</u>	<u>Week tested</u>							
	1	2	3	4	5	6	7	8
Day 0 in FCA i.p.	---	---	---	---	---	+++	++ ++ +	++ ++ ++
Day 0 in FCA i.m.	---	---	---	+++	+++	+++	+++	+++
Day 0 in saline i.p.	---	---	---	+-	+-	---	---	---
Day 0 in saline i.m.	---	---	---	---	---	---	---	---
Day 0 in saline i.v.	---	---	---	---	---	---	---	---
Days 0 and 56 in FCA i.p.	+++	+++	+++	+++	+++	+++	+++	+++
Days 0 and 56 in FCA i.m.	+++	+++	+++	+++	+++	+++	+++	+++
Days 0 and 56 in saline i.p.	---	---	---	---	---	---	---	---

i.p., intraperitoneally; i.m., intramuscularly; i.v., intravascularly; ++, very strong response; +, strong response; +, weak response; -, no response; Day 0, the day the first injection was given.

In general it seems that precipitating antibodies are detected only when antibody titres are very high. Only on a few occasions was a weak precipitating antibody response seen, it usually appears to be a strong response (Plate 42), or no response. During the primary response precipitating antibodies are mostly detected in the groups injected with antigen in adjuvant. The intramuscular route yielded precipitating antibodies about 2 weeks before the intraperitoneal route, correlated with higher agglutinating antibody titres. Similarly, with the animals injected on days 0 and 56, only the adjuvant injected groups respond by producing precipitating antibodies, and there is a slightly stronger response in the intramuscularly injected group. However again caution must be exercised when using such small groups.

Cross-reactivity of anti-HGG antiserum to BGG, CGG, BSA and OVA, was not seen in the precipitating antibody response in any of the animals tested (Plate 43).

2. Mercuraptoethanol sensitivity

The results of the sensitivity of anti-HGG antisera are shown in Table XXII. Clearly the agglutinating antibody response is not abolished by treatment with 2ME. Even early immune sera show 2ME-resistant components. Late antisera still have titres of 1:512 after treatment with a final concentration of 0.3 M 2ME. As a percentage of the whole titre it appears that the 2ME-resistant antibody increases as the immune response progresses.

Only during the peak response can precipitating antibodies be detected. These antibodies appear to be

TABLE XXII

Passive haemagglutination titres and precipitation responses of sera from carp immunized intraperitoneally with HGG in FCA, after treatment with 2ME.

Each row represents the serum from one sample. Titres are expressed as $-\log_2$ titre.

Sera	+ Saline	+0.1 M 2ME	+0.2 M 2ME	+0.4 M 2ME	+0.6 M 2ME
<u>Passive haemagglutination titres</u>					
Early response	8	N.T.	5	N.T.	N.T.
Peak response	24	N.T.	16	N.T.	N.T.
Late response	32	N.T.	27	17	12
	>20	>20	19	19	16
	18	15	15	12	9

Precipitating antibody

Peak response

++

++

+

-

-

N.T., Not tested; ++, very strong precipitation response; +, strong precipitation response;

-, no precipitation response.

sensitive to 2ME treatment, in line with mammalian studies on IgM precipitins. Also, all anti-A. salmonicida agglutinating antibody sera tested were 2ME-sensitive.

3. Column chromatography

The trace of hyperimmune serum fractionated on the Ultragel ACA 34 column is shown in Figure 9. The antibody titres of selected aliquots are also shown. It is clear that only the first high molecular weight protein peak shows antibody activity. Also it should be noted that the peak antibody titre corresponds to the peak protein values.

Similar results were obtained with the Ultragel ACA 22 column (Figure 9). However, the fractionation range caused the middle protein peaks to come together giving a less clear picture overall.

By far the best fractionation was obtained using Sephacryl S-300 (Figure 10). Four very obvious protein peaks can be seen, with a possible fifth appearing as a shoulder on the fourth. Again only the first, high molecular weight peak contains detectable antibody and peak antibody titres correspond with peak protein values. Once concentrated all peak fractions were tested for precipitating antibodies, and again only in the first peak could they be detected. The two aliquots at the very tip of the first peak gave strong precipitin responses, so here also the peak protein value corresponds with the peak precipitating antibody response, as well as the peak agglutinating antibody response.

Figure 9

Serum fractionation traces from carp hyperimmunized with HGG in FCA. Sera separated on (a) AcA34 and (b) AcA22. The dotted lines represent the anti-HGG antibody titre.

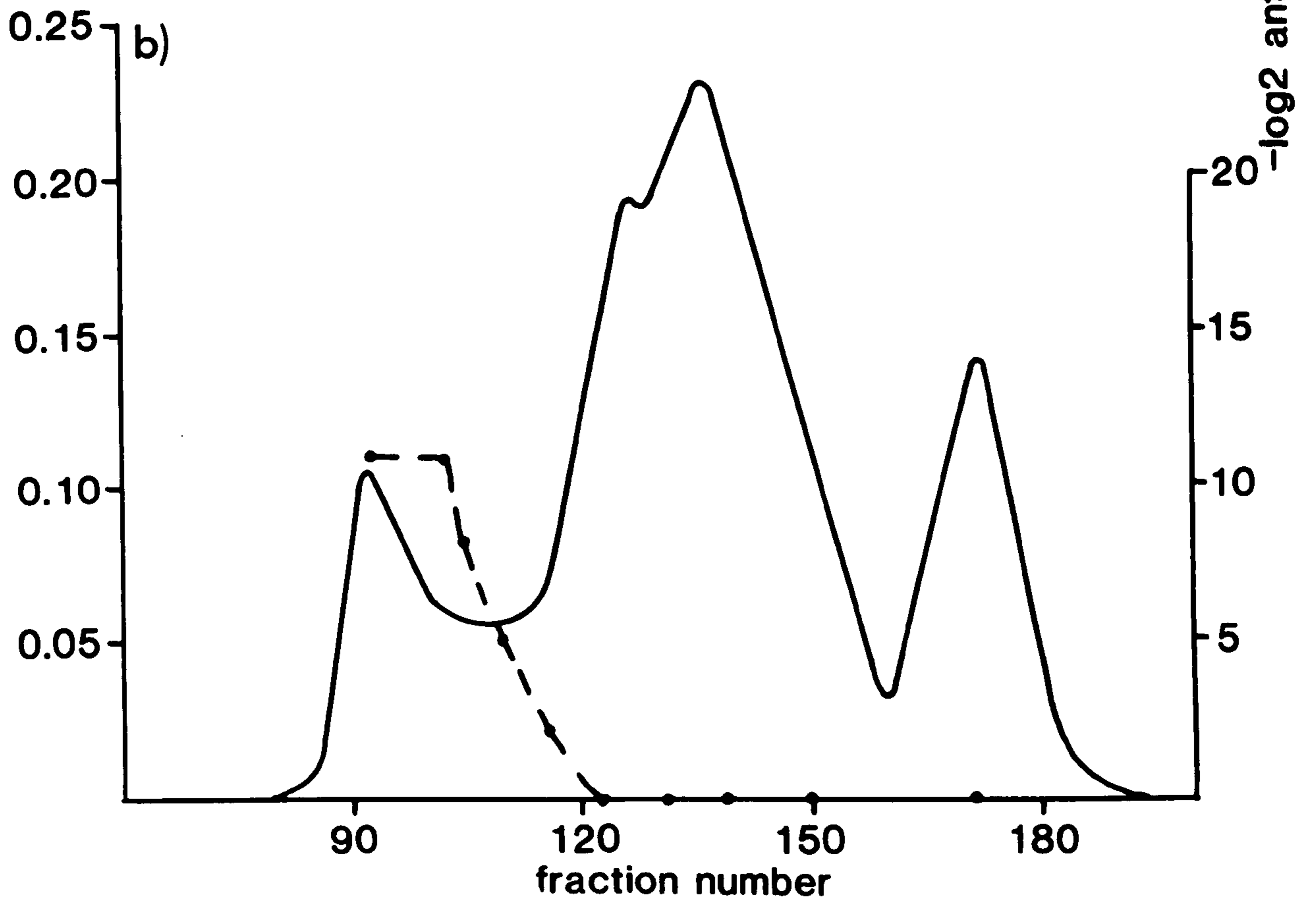
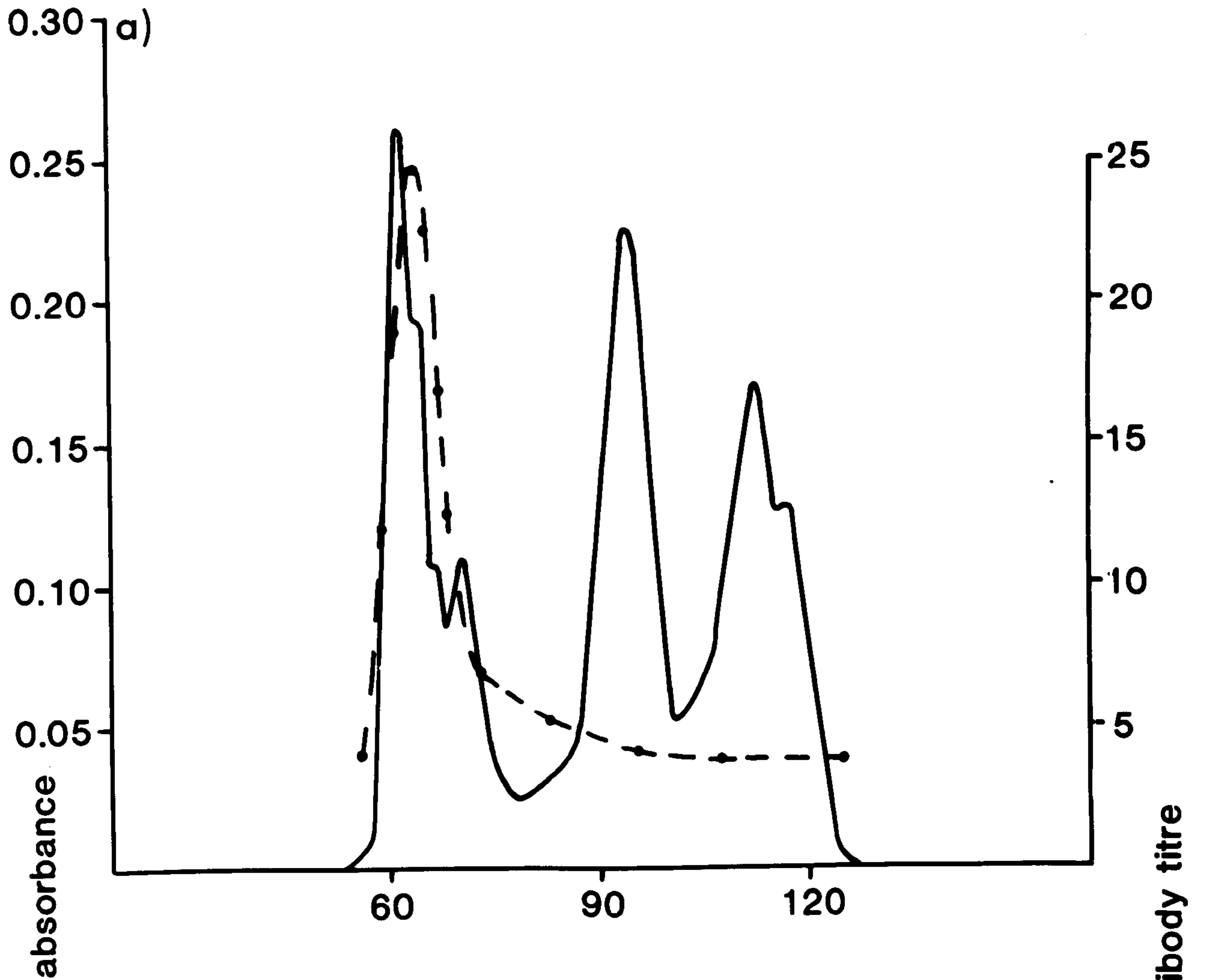
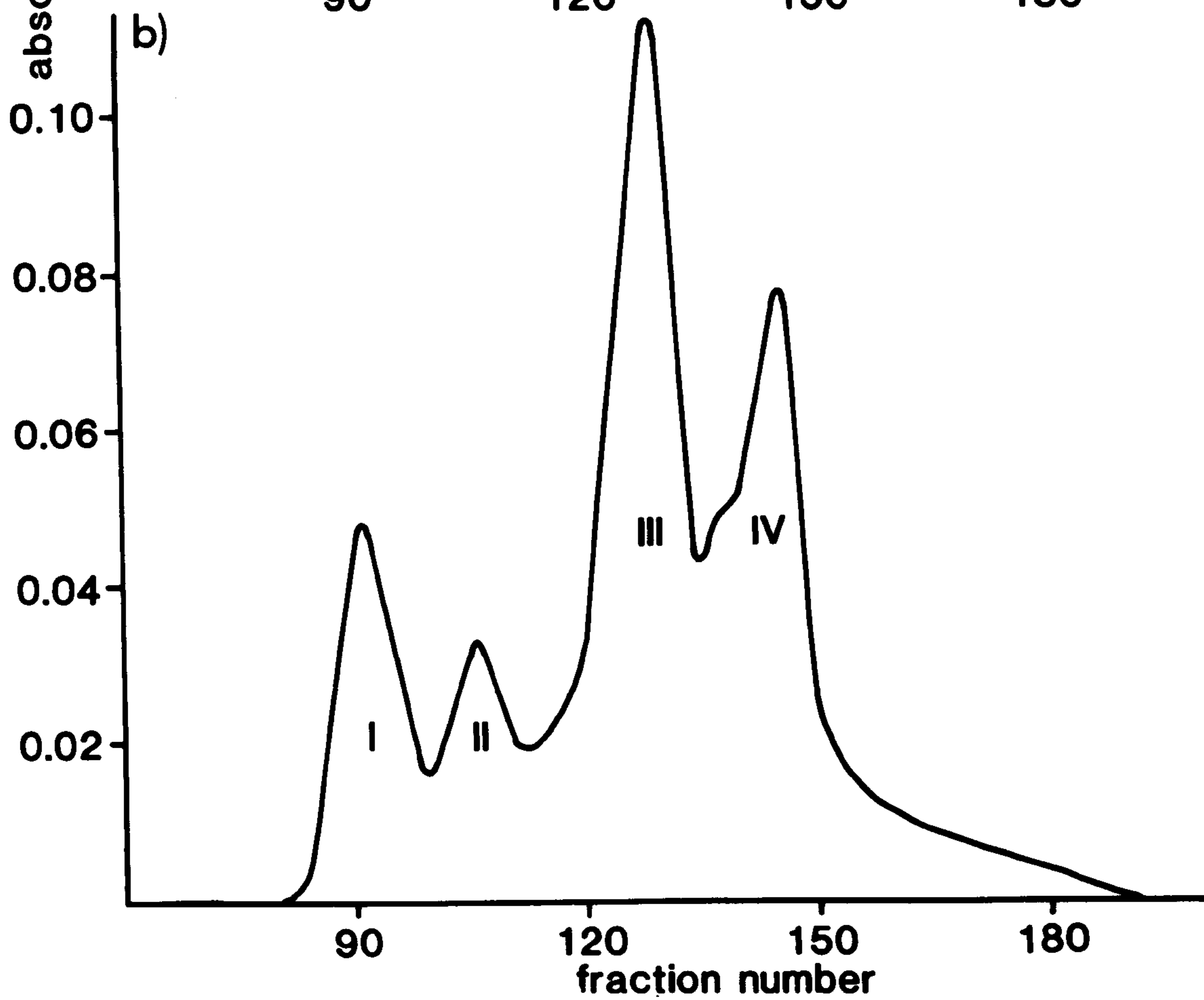
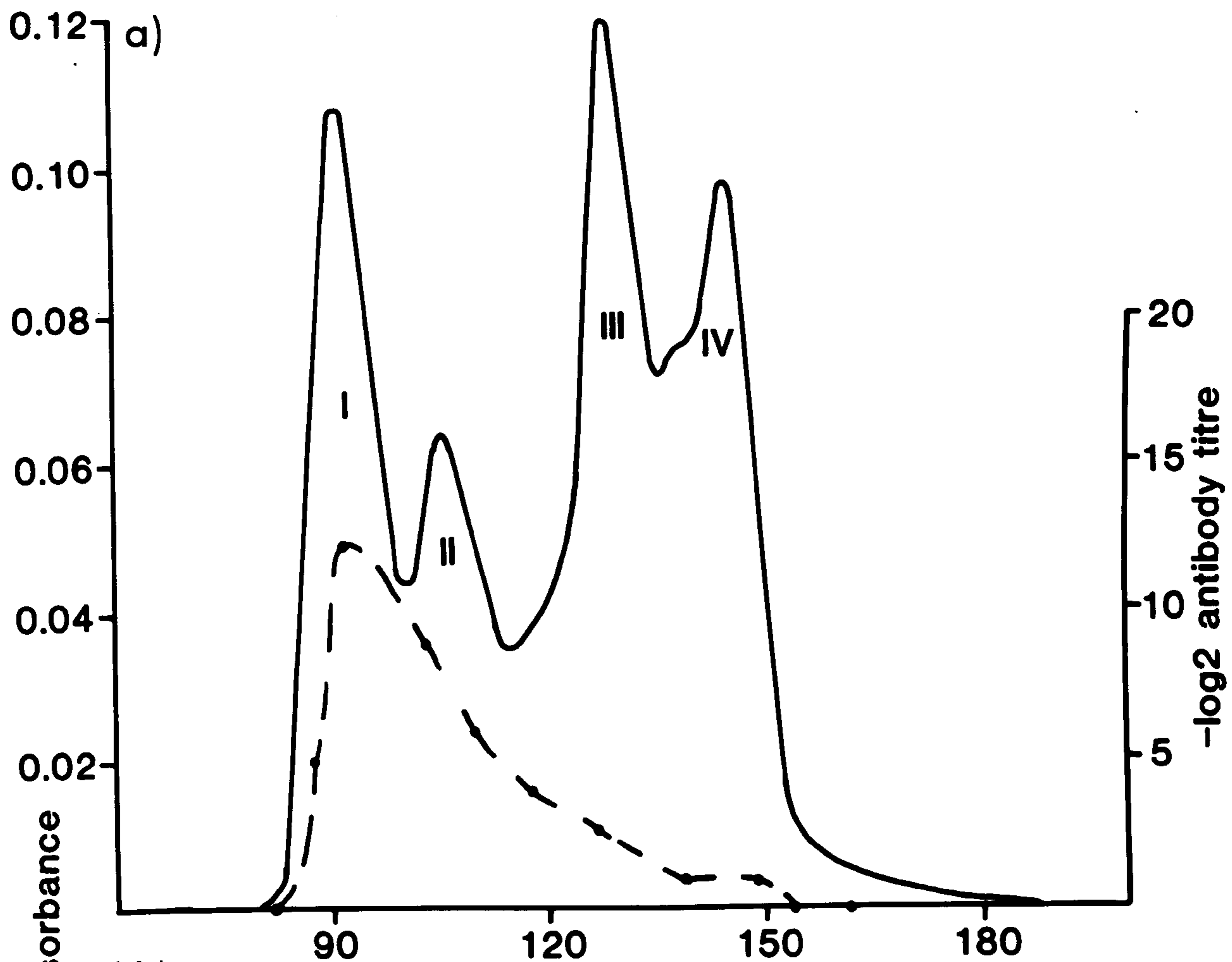


Figure 10

Serum fractionation traces using Sephacryl-300 from (a) carp hyperimmunized with HGG in FCA and (b) uninjected control animals. The dotted lines represent the anti-HGG antibody titre.



Antibody titres were consistently zero in serum from unimmunized fish.

I am grateful to Dr. I. G. Jones of the Department of Biochemistry who performed this analysis for me. It was a pilot study carried out on a single sample only.

The trace of normal serum fractionation, from uninjected animals, can be seen in Figure 10. Again all the protein peaks are visible, but with no detectable antibody. The first peak can be seen to be about half the size of the first peak in the hyperimmune serum, whilst the other peaks are not affected. Clearly during immunization, as specific antibody synthesis occurs, the high molecular weight protein peak increases in amount and specific antibody can be demonstrated within it.

The sedimentation coefficient of the protein from this first peak, corrected to 20°C, was calculated to be about 18.2. This was calculated from the migration of the protein peak, whilst undergoing ultracentrifugation (Plate 44), during set time intervals.

4. Antibody responses to HGG and *Aeromonas salmonicida* by carp of different ages

Carp injected at 8 weeks after hatching with HGG in FCA or *A. salmonicida* were bled at 2 week intervals by pooling blood from 10 fish. Duplicate samples were obtained for each group. All groups were re-injected on day 56 with a normal immunizing dose of antigen and the results can be seen in Table XXIII. Clearly the results show that 8 week carp respond to a primary injection of HGG in FCA, or to *A. salmonicida* in a fashion similar to that of year old adult fish. The response to a booster injection of HGG in FCA is also similar to that of the adult but the boosted response to *A. salmonicida* does not increase to the levels seen in adult fish. The responses of 8 week carp initially injected with HGG in saline and then reinjected with the normal immunizing dose in adjuvant are more difficult to

TABLE XXIII

Serum antibody titres of carp injected intraperitoneally 8 weeks after hatching, expressed as $-log_2$ titre. Each figure represents a pooled sample from 10 fish.

Immunization schedule	Weeks after injection			
	2	4	6	8
Injected on Day 0 with HGG in FCA.	6,6	6,7	14,14	17,18
Injected on Day 0 with <u>A. salmonicida</u> in saline.	4,6	6,6	8,8	8,8
Injected on Days 0 and 56 with HGG in FCA	6,17	>20, >20	15,18	14,16
Injected on Days 0 and 56 with <u>A. salmonicida</u> in saline.	5,7	8,9	9,10	8,8
Injected on Day 0 with HGG in saline at 0.025 mg/g fish and Day 56 with HGG in FCA.	N.T.	10,12	N.T.	7,8
Injected on Day 0 with HGG in saline at 0.25 mg/g fish and Day 56 with HGG in FCA.	N.T.	10,11	N.T.	4,6

N.T., Not tested

interpret, but definitely show positive antibody responses, i.e. no tolerogenic effects were observed. Certainly, both groups of fish initially injected with HGG in saline have lower titres in the booster response than in fish initially injected with HGG in FCA. This is particularly noticeable in the late booster response of the fish initially injected with HGG in saline at 0.25 mg/g fish. Unfortunately, due to a shortage of animals, serum from this group could not be tested against a non-cross-reacting antigen, to see if this was due to a partially suppressed response.

The results obtained from fish injected in the intermediate age groups, injected at 16, 24, 32, 40 and 48 weeks after hatching, with either HGG in FCA or with A. salmonicida, are shown in Figures 11 and 12. With HGG in FCA, the primary response appears to be relatively unaffected by any increase in age at the time of injection. Animals injected at 16 weeks respond just as well as those injected at 48 or 56 weeks. The largest titres are usually obtained by the fourth week after immunization (in comparison with the group injected at 8 weeks where the peak may occur somewhat later). When groups of individuals are pooled so that the mean antibody titre produced in the groups injected before week 40 is compared with that in the groups injected on or after week 40, only small differences occur. Also, with A. salmonicida, little difference with increasing age is observed. There is no sharp peak to the antibody response and a titre of 1:128 (7) is very common. Pooling of data from the groups above the age of 40 weeks and from those below the age of 40 weeks also shows only small differences (see Figure 12).

Figure 11

Anti-HGG passive haemagglutination titres of sera from animals injected with HGG in FCA:

- (a) 16 weeks after hatching.
- (b) 24 weeks after hatching.
- (c) 32 weeks after hatching.
- (d) 40 weeks after hatching.
- (e) 48 weeks after hatching.

Each column represents one individual. Means in parentheses.

Statistical analysis of results.

A comparison of pooled data from the groups above the age of 40 weeks (Figures 11d, e and 6a) with pooled data from groups below the age of 40 weeks (Figures 11a, b and c) showed significantly higher titres in the older groups only at day 7 and 14 after immunization. Possibly older fish begin to respond a little earlier after immunization than the younger fish.

		'p' values.						
Day	7	14	21	28	35	42	49	56
	p<0.01	p<0.01	ns	ns	ns	ns	ns	ns

ns, not significant.

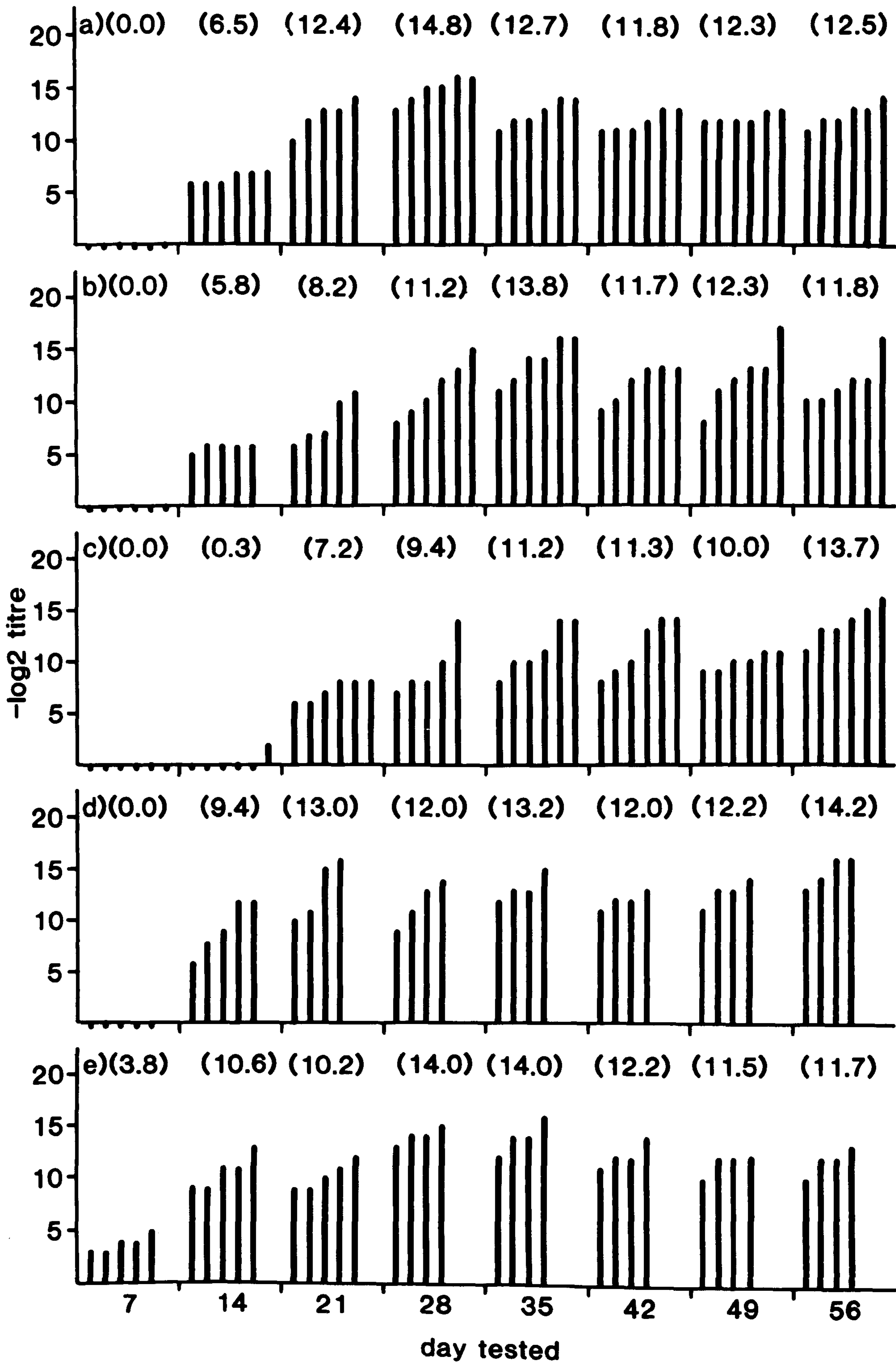


Figure 12

Tube agglutination titres of sera from animals injected with A. salmonicida in saline:

- (a) 16 weeks after hatching.
- (b) 24 weeks after hatching.
- (c) 32 weeks after hatching.
- (d) 40 weeks after hatching.
- (e) 48 weeks after hatching.

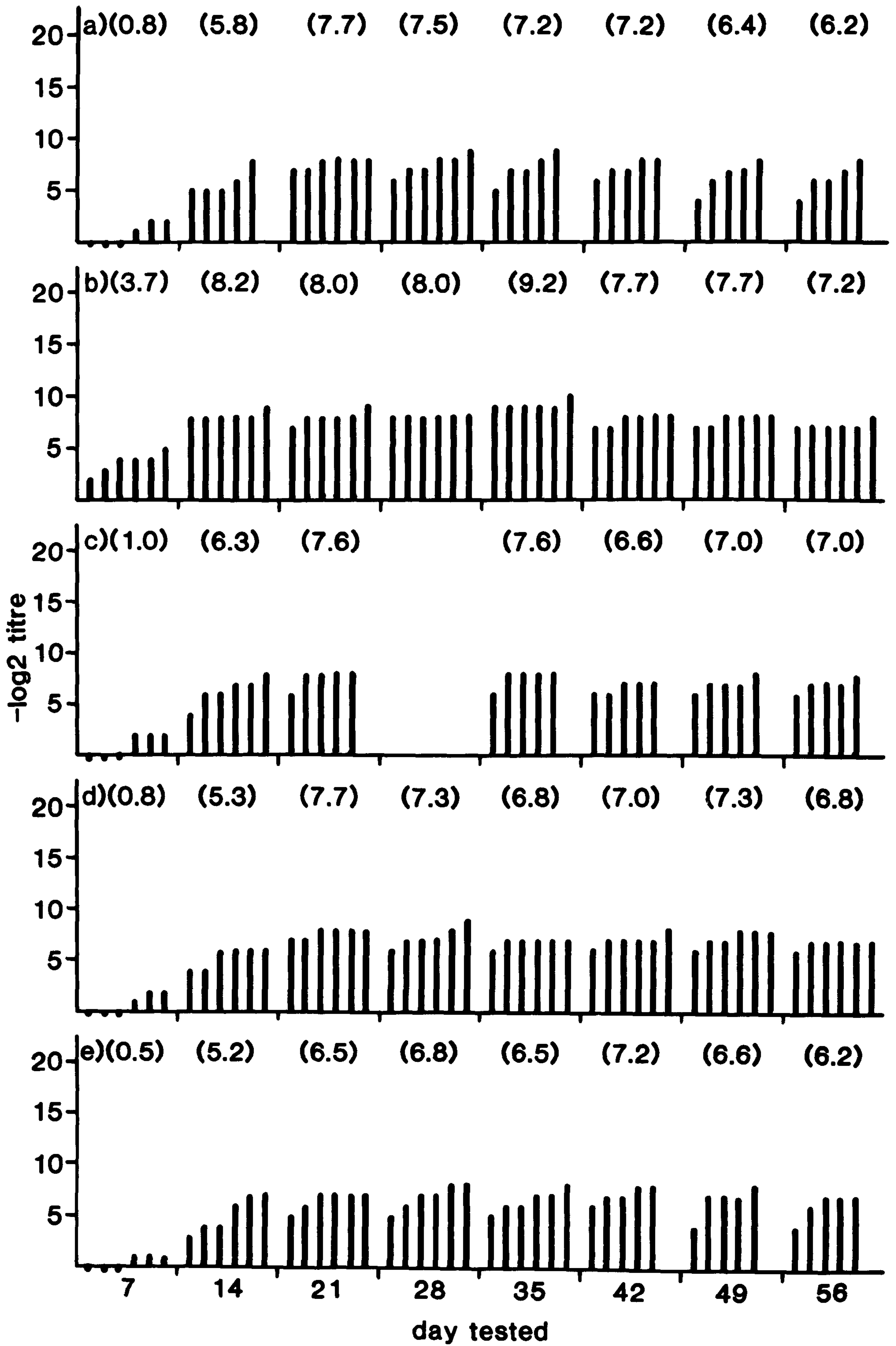
Each column represents one individual. Means in parentheses.

Statistical analysis of results.

A comparison of pooled data from the groups above the age of 40 weeks (Figures 12d, e and 7a) with pooled data from groups below the age of 40 weeks (Figures 12a, b and c) showed no significantly increased titres in the older fish at any time after immunization. There was, however, a significantly larger response in younger animals on days 21 and 35.

		'p' values.						
Day	7	14	21	28	35	42	49	56
	ns	ns	p<0.01	ns	p<0.001	ns	ns	ns

ns, not significant.



The results following a booster injection are shown in Figures 13 and 14. Again with HGG in FCA there is no apparent difference between any groups. With A. salmonicida, as with HGG, little difference between groups was observed following a booster immunization. After a booster injection the responses are generally higher, often with a maximum titre at week 2. The groups injected at the ages of 8 weeks and 16 weeks however, showed no heightened response to a booster injection.

It is interesting that older fish were able to give enhanced titres to T-independent (in tetrapods) antigens. It is also interesting that for this antigen but not for the T-dependent antigen (HGG) the boosted response occurred in fish of the older age groups but not in fish aged 8 weeks at the primary injection.

5. Effect of dose

The results obtained from injecting high doses of antigen into mature animals and subsequently challenging with immunogenic doses injected intraperitoneally are shown in Table XXIV.

The animals injected with 0.1 mg/g fish responded normally to both agglutinating and precipitating antibodies. They were not tested any further. Animals injected with 1.0 mg HGG/g animal only responded to the primary injection if this was given in adjuvant intramuscularly. However, even this response was smaller in magnitude than any of the groups previously investigated. No titre was detected using the intraperitoneal route and no precipitating antibodies were observed at any time using either route.

Figure 13

Anti-HGG passive haemagglutination titres of sera from animals injected with HGG in FCA:

- (a) 16 and 24 weeks after hatching
- (b) 24 and 32 weeks after hatching
- (c) 32 and 40 weeks after hatching
- (d) 40 and 48 weeks after hatching
- (e) 48 and 56 weeks after hatching

Each column represents one individual. Means in parentheses.

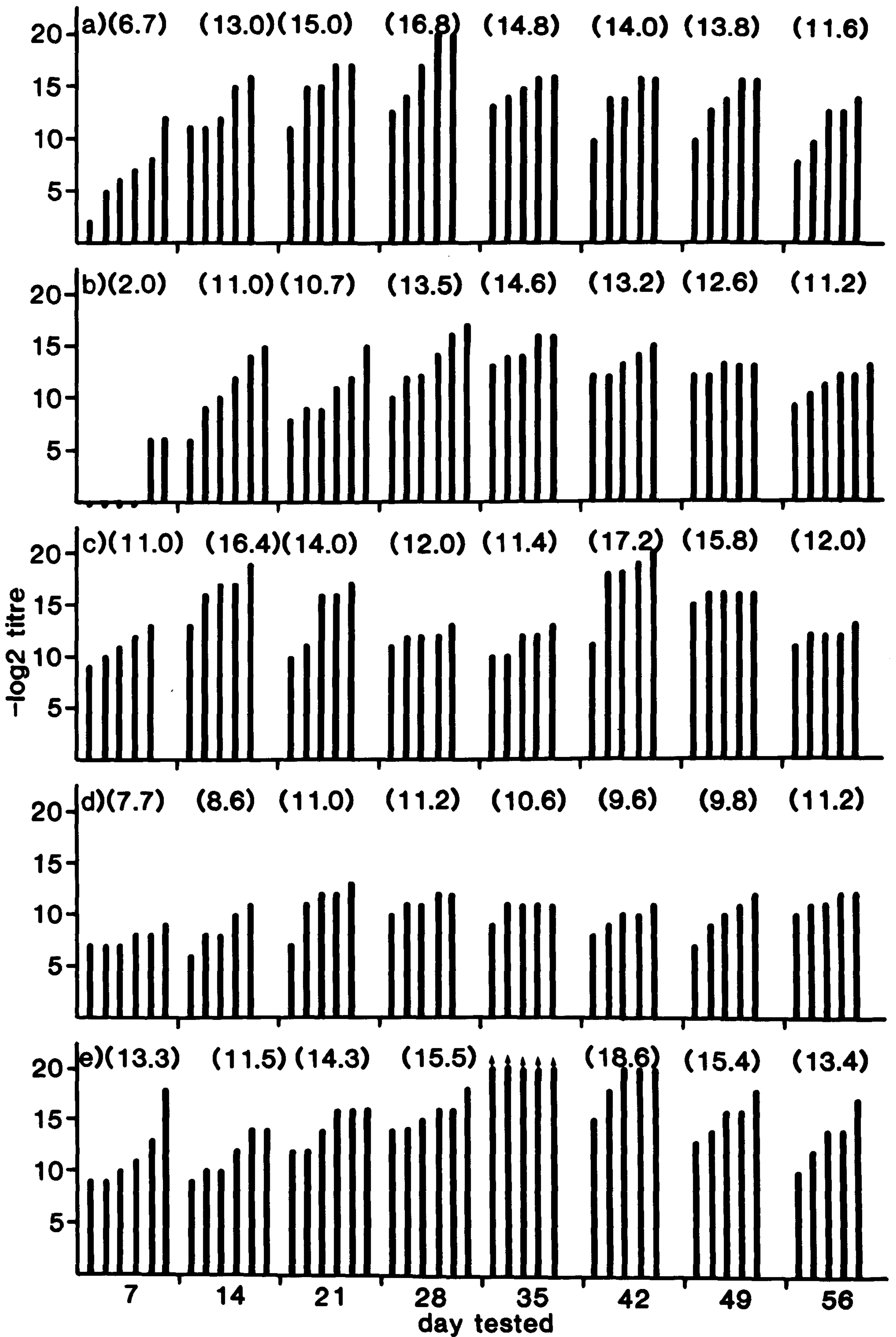
Statistical analysis of results.

From Table XXIII (page 121) it can be seen that for animals immunized at an even younger stage, i.e. at weeks 8 and 16 after hatching, the mean titres were:

Day	14	28	42	56
$-\log_2$ titre	11.5	>20	16.5	15

For animals immunized at an older age, i.e. at weeks 52 and 60 after hatching, see Figure 8c.

Taking these figures into account it would seem that both younger and older fish are capable of producing antibody titres greater than 1:1,048,576 (well 20) 4 to 5 weeks after immunization. Only in one group, injected 24 and 32 weeks after hatching, is a latent period observed before serum antibody titres begin to rise. When pooled data from the groups above the age of 40 weeks (Figures 13d, e and 8c) is compared to pooled data from groups below the age of 40 weeks (Figures 13a, b and c) the differences were not significant except at day 7 after immunization when older animals had a significantly higher titre ($p < 0.01$).



†, Titre exceeded well 20.

Figure 14

Tube agglutination titres of sera from animals injected with A. salmonicida in saline:

- (a) 16 and 24 weeks after hatching
- (b) 24 and 32 weeks after hatching
- (c) 32 and 40 weeks after hatching
- (d) 40 and 48 weeks after hatching
- (e) 48 and 56 weeks after hatching.

Each column represents one individual. Means in parentheses.

Statistical analysis of results.

A comparison of animals receiving a secondary immunization (Figures 14a, b, c, d and e) with those only receiving a single injection (Figures 12a, b, c, d and e) showed a significantly greater increase in the animals injected on both days 0 and 56 as compared to those receiving a single dose only. The only exception being on days 14 and 21 in Figure 14c, when no significant difference was seen.

A comparison of pooled data from the groups above the age of 40 weeks (Figures 14d, e and 7e) with pooled data from groups below the age of 40 weeks (Figures 14a, b and c) showed no significant differences except during the first 2 weeks after immunization ($p < 0.01$), when the older animals have significantly higher titres.

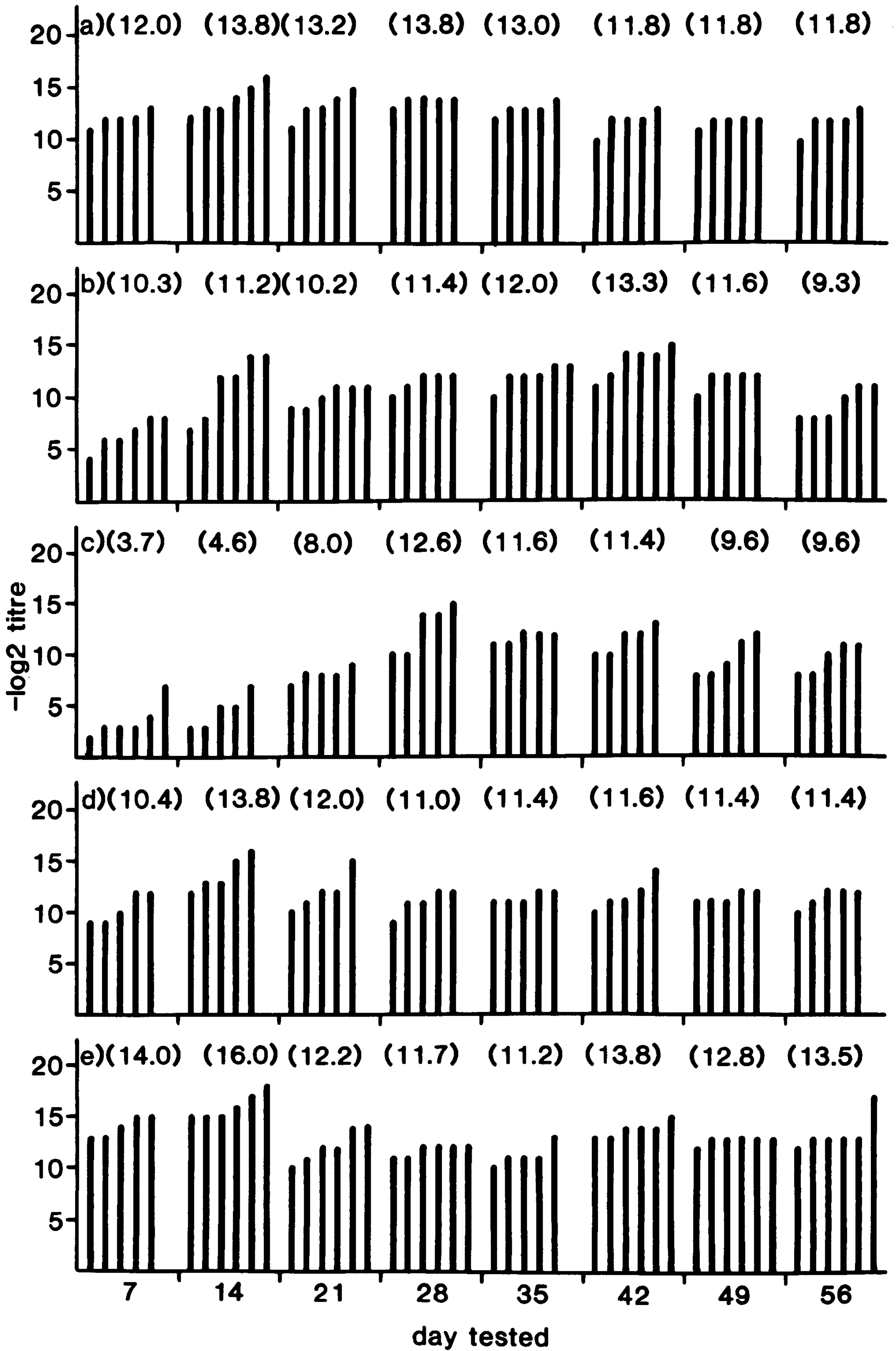


TABLE XXIV

Serum antibody titres and antigen-trapping responses of adult carp injected with high dose ranges of HGG. Each figure represents one individual.

Titres are expressed as $-\log_2$ titre.

<u>Antibody titres</u>	<u>Dose</u>			
	0.1 mg/g i.p. in FCA	1.0 mg/g i.p. in FCA	1.0 mg/g i.p. in saline	1.0 mg/g i.m. in FCA
<u>Primary response</u>				
Day 28	11, 13, 14, 15, 15, 16	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	2, 2, 2, 3, 3, 3
Day 56	11, 11, 12, 12, 13	0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0, 0	5, 7, 7, 8, 8, 10
<u>Secondary response to HGG</u>				
Day 28	N.T.	18, 20, 20, 20, 20	16, 19, 20, 20, 20	13, 14, 20, 20, 20
Day 56	N.T.	19, 20, 20, 20, 20	20, 20, 20, 20, 20	20, 20, 20, 20, 20
<u>Secondary response to OVA</u>				
Day 28	N.T.	N.T.	N.T.	N.T.
Day 56	N.T.	12, 12, 14, 20	11, 14, 15, 17	10, 12, 13, 13
<u>Antigen-trapping on Day 1 after booster.</u>				
Spleen	N.T.	-	-	*
Pronephros	N.T.	+	+	+
Mesonephros	N.T.	-	*	*

N.T., not tested; * bright ellipsoidal or mesonephric fluorescence; +, generalized tissue fluorescence; -, no fluorescence.

After a booster injection of HGG in adjuvant, the usual bright fluorescence in the ellipsoids of the spleen was detected only in the animals injected intramuscularly in the first instance and not in those injected by other routes. This phenomenon, discussed in Chapter 4, is considered to be antigen trapped extracellularly in the form of immune complexes, on the dendritic reticulum of the ellipsoid sheaths. The apparent absence in the animals injected intraperitoneally suggests that no antibody is present to form the immune complexes in these animals which have been injected in the first instance with a high dose.

6. The effect of adult thymectomy on antibody production.

The position of the thymus in an unoperated animal can be seen in Plate 45. The experiment in which the fish were unilaterally thymectomized was considered to be successful to the extent that in most of the animals only a small vestige of the thymus, or none at all, remained on the operated side.

The survival from bilateral thymectomy was approximately 80%. Unfortunately, the operation was very severe and often affected the ear, around which the thymus curves. Affected fish could not swim properly due to a loss of balance, and were obviously greatly stressed. During the 3 weeks of recovery few fish died. However, after immunization, but before testing, there were very high mortalities. In two trials using HGG in FCA, all animals, both thymectomized and sham operated, had died before antibody could be detected in the serum. In the group injected with

A. salmonicida, some preliminary results were obtained before the animals died and are shown in Figure 15. The titres obtained from both groups of operated animals are comparable to the results from unoperated animals. However, a statistical analysis carried out between thymectomized and sham-thymectomized animals shows that although there is no significant difference between the thymectomized and sham operated groups at the late timings, there is a significant difference at day 7 where $p < 0.01$, using the t-test corrected for small samples (Parker, 1973). The thymectomized animals have enhanced titres, suggesting a possible elimination of suppressor cells. These thymectomized animals had at least 95% of the thymus missing when sectioned afterwards and about 40% were considered to be completely thymectomized.

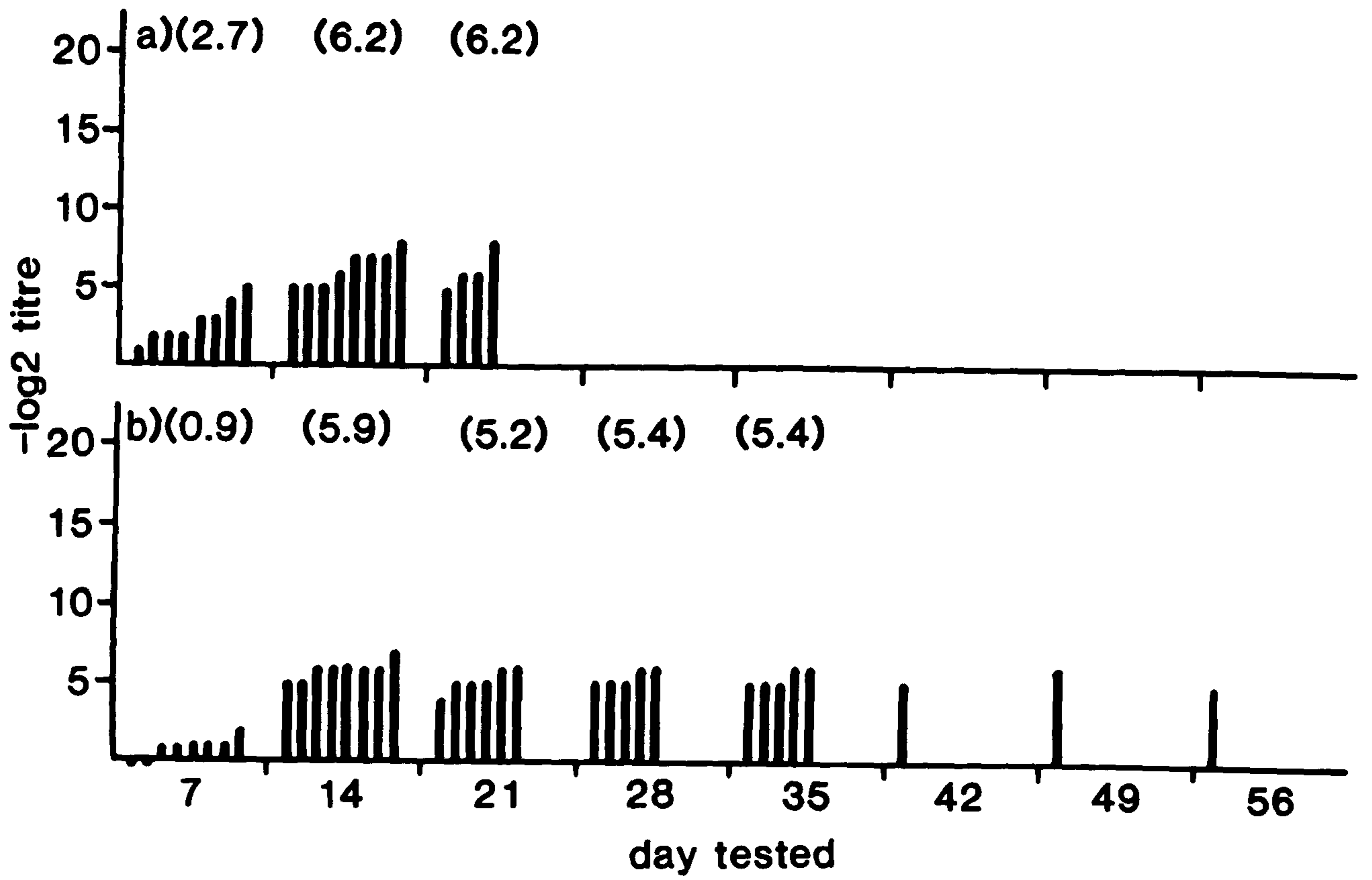
DISCUSSION

It is clear that the carp, Cyprinus carpio L., is well able to respond to soluble protein antigens, and cellular antigens. Both precipitating and agglutinating antibodies were detected. Other authors using C. carpio have also shown good antibody responses to both soluble protein antigens (Avtalion 1969a,b; Richter & Ambrosius, 1972; Weiss & Avtalion, 1977) and cellular antigens (Smith, 1940; Goncharov, 1971; Schaperclaus, 1972; Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a,b). The time of onset and the magnitude of these responses show that these animals can produce antibodies in a manner comparable to other species of fish, to other classes of poikilotherms and even to mammals (if the effect of temperature on poikilotherms

Figure 15

Tube agglutination titres of sera from (a) adult thymectomized carp injected with A. salmonicida in saline and (b) sham-thymectomized carp injected with A. salmonicida in saline. Each column represents one individual.

Means in parentheses.



is taken into account). There is also good evidence to suggest that in fish, like mammals, the collaboration of two types of antigen-recognizing cells is needed in order to produce a strong antibody response (Frenzel & Ambrosius, 1971; Yocum, Cuchens & Clem, 1975; Stolen & Makela, 1980). However, a major difference between the production of antibodies in fish and in mammals is the lack of a 7S immunoglobulin, even during the secondary response of fish. It could be argued that teleosts only have T-independent antibody responses. This argument is weakened by the finding that 19S antibody responses in mammals are enhanced by helper cells (Schirrmacher & Rajewsky, 1970) and that thymectomy at 2 months in Tilapia totally suppresses the plaque forming cell response to SRBC's (Sailendri, 1973).

The apparent inability of the fish in the present study to respond to BSA in FCA contrasts markedly with the results of Avtalion and co-workers (Avtalion, 1969a,b; Avtalion, Malik, Lefler & Katz, 1970) who regularly use BSA with carp. However, only the intraperitoneal route was tried in the present study, whereas the intramuscular route was used by the above authors. The results presented here using HGG in FCA injected intramuscularly are almost identical with the results by Avtalion with BSA in FCA. HGG and OVA seem much better antigens, as both produce good responses using the intraperitoneal route as well as the intramuscular route, at doses comparable to doses of soluble protein antigens given to elicit antibody responses in mammals (Richter & Haurowitz, 1960; Dixon, Maurer & Deichmiller, 1954). Also HGG was shown to elicit responses

when given in saline, either intraperitoneally, intramuscularly or intravenously. With adjuvant there was little difference in the primary responses between the intramuscular and intraperitoneal routes. Without adjuvant the intraperitoneally injected group showed a delay in the onset of antibody formation but higher titres when compared with the group injected intramuscularly. With a booster injection on day 56 the differences between intraperitoneal and intramuscular routes becomes more marked, with titres being much higher after intramuscular administration. Whether this is due to the effect of memory cells or in vivo formation of immune complexes which may be more immunogenic than antigen alone, is not clear, but that some routes are better than others for good booster responses is apparent. If the higher titres are due to the development of memory cells from the priming injection, it would seem that the intramuscular route is more effective in generating memory cells. Other workers with fish have also found that different routes affect the antibody response, with the best secondary response being obtained using the intramuscular route (Ingram & Alexander, 1976, 1979; Michel, 1979; Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a). However, the needs of aquaculturists to vaccinate thousands of fish in the field has led to extensive trials for easier, less expensive immunization routes. The vigorous procedures and testing required to develop fish vaccines have recently been described by Brown (1978) and Antipa and Croy (1979). Oral immunization, first tried by Duff (1942), has yielded some degree of success (reviewed by Klontz & Anderson, 1970 and

Snieszko, 1970). In the early 1960's much work was carried out on oral immunization (Post, 1966; Krantz, Reddecliff & Heist, 1963; Klontz, 1967). However, the low level of protection obtained under laboratory experiments was generally inadequate to resist infection under field trials, when stress and unfavourable conditions were prevalent. It has received some new interest from Fletcher and White (1973), who have shown that the result of oral immunization is to cause high levels of antibody in the intestinal mucus, but not in the cutaneous mucus of the same fish. The degree of protection offered by this local antibody synthesis is not known, and obviously further research in this field is warranted.

Selective breeding of trout for resistance to furunculosis has been attempted (Ehlinger, 1977), but again only limited success was obtained in the field. A more recent development involves dipping fish into antigen solutions. This method was first described by Amend and Fender (1976). It consisted of dipping fish into a hyperosmotic solution containing the antigen in a one-step method, or, in a two-step method, by first immersing the fish in the hyperosmotic solution and then in the antigen solution. This technique is not limited by fish size and would be available to mass immunization. Certainly BSA can cross into the blood stream using this technique (Amend & Fender, 1976; Fender & Amend, 1978) but there are no reports on subsequent antibody titres to this particular antigen. Using this technique to immunize juvenile sockeye salmon with killed Vibrio anguillarum, Croy and Amend (1977)

obtained excellent survival after challenge with a virulent culture, although no agglutinating antibodies could be detected. Similar results were obtained by Lannan (1978) using chum salmon fry vaccinated with V. anguillarum and Antipa and Amend (1977) using coho salmon and chinook salmon vaccinated with either V. anguillarum or A. salmonicida. In the latter case antibodies were significantly increased to V. anguillarum, but not to A. salmonicida, whereas intra-peritoneal injection, which also provided protection on challenge, resulted in the production of serum antibodies to both antigens. The present experiment shows that serum antibody production does occur in carp after hyperosmotic infiltration with A. salmonicida, but that it is certainly only a low level response. This does not mean that a low secondary response would result. Indeed in mammals priming of low doses of antigen, which give low primary responses, paradoxically gives the best secondary responses to subsequent antigen challenge (Sterzl, 1966; Grantham, 1972; Grantham & Fitch, 1975). Another alternative is that cellular immunity, or even locally secreted antibodies, particularly in the external mucus, may be playing a more important role than has been previously realised. The effects of secondary challenge after primary immunization by hyperosmotic infiltration has yet to be tested in our fish.

The hyperosmotic dip is very stressful to fish, both handling and from the osmotic shock itself. Two related techniques have been tried which reduce this stress. The obvious improvement was to simply dip the fish in the antigen

solution alone. Again excellent survival after secondary challenge was obtained with sockeye salmon when using bivalent V. anguillarum vaccines (Gould, Antipa & Amend, 1979). Even spraying fish with V. anguillarum vaccines conferred high levels of immunity (Gould, O'Leary, Garrison, Rohovec & Fryer, 1978). Similarly, Anderson, Roberson and Dixon (1979b) found no difference in plaque forming cells and humoral antibody when using the O-antigen of Yersinia ruckeri by injection, hyperosmotic infiltration or simple immersion with rainbow trout. These workers further elaborated this technique to eliminate handling of the fish by simply adding antigen solution to the ambient water for a flush exposure (Anderson, Dixon & Roberson, 1979; Anderson & Dixon, 1980). Again plaque forming cells could be detected as frequently as in fish given either Y. ruckeri O-antigen or A. salmonicida O-antigen by injection. With low antigen doses however nonresponders began to appear in the groups treated by flush exposure.

It is clear that to optimise infiltration of various vaccines into fish, consideration must be given to dose and to immunization schedule since these can be as important as the route. The effects of adjuvant are also to be taken into consideration. However, the necrotic lesions induced in fish muscle by injection of adjuvant may preclude their use in fish reared for human consumption.

The present study used booster injections given on day 12 or day 56. The study by Goncharov (1971) on the dynamics of the accumulation of antibodies in carp, in

relation to the intervals of injection, showed that intraperitoneal injection of Aeromonas punctata on days 0 and 10, and between days 30 and 60, completely ensured the effectiveness of the vaccination schedule. Obviously different antigens and species will vary, but the results with HGG in adjuvant and with A. salmonicida, in carp would seem to agree with these earlier studies. Perhaps the result of HGG in saline, where no secondary effect was seen, could be accounted for by the timing. Rijkers, Frederix-Wolters and Van Muiswinkel (1980a), found no anamnestic response to low doses of SRBC's if the booster was given one month later. However, a second injection six months later resulted in a clear secondary response.

The effect of varying the dose of HGG in the present study, showed little difference until 1.0 mg/g fish was used. Here the primary response was abolished using the intraperitoneal route with or without adjuvant, but the effect of immunization by the intramuscular route, although diminished, did show detectable serum antibody levels. Secondary responses seemed unaffected, so presumably some priming occurred in all cases. High dose tolerance, as demonstrated in mammals (Mitchison, 1965) and in amphibians (Marchalonis & Germain, 1971) has still to be shown conclusively in fish. In snappers, comparable quantities of ultracentrifuged soluble proteins such as BSA or BGG, injected intravenously, did not even yield reduced responses (Sigel, Russell, Williams, Gaines & Lucas, 1968). However, specific suppression for BSA was obtained using a hapten carrier system (Serero & Avtalion, 1978). Carp primed with native or

biologically filtered BSA intracardially did not respond to any of the antigen doses used in the primary immunization, including a high dose of 2.3 mg/g animal. Furthermore, they proved to be tolerant to a secondary challenge with a known immunizing dose. This suppression to the carrier did not affect the anti-hapten titre, nor an anti-rabbit gamma globulin response. No tolerance could be induced if the carrier was injected outside the circulation, nor if acetylated BSA was used. It was concluded that tolerance was dependent primarily on both the route of injection and the physicochemical nature of the antigen, rather than the antigen dosage or biological filtration.

Other work using the intravenous route has not shown tolerance to SRBC's (Jayaraman, Mohan & Muthukkaruppan, 1979; Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a). Indeed, increasing the antigen dose caused increased primary or secondary responses. Similarly, using the intraperitoneal route, primary and secondary responses to MS2 bacteriophage in the brown trout, increased significantly with increasing concentrations, with no immuno-incompetent animals (O'Neill, 1979). In contrast, no concentration dependence, either positive or negative, was found using the intraperitoneal route with rainbow trout using FH5 bacteriophage (Dorson, 1972) or when using BSA in adjuvant injected into carp in doses from 0.1 to 1 mg (Avtalion, Wojdani, Malik, Shahrabani & Duczyminer, 1973). However, doses of 20 to 50 mg (approximately 0.04 to 0.1 mg/g fish) of soluble BSA were found to be tolerogenic. The high dose tolerance was

expressed in both primary and secondary responses. At low temperatures, 12°C, low doses of BSA (0.04-0.2 mg/k) were tolerogenic also. These results are similar to the ones in the present study, with small increases in antigen concentration not affecting the magnitude of the immune response, but large increases resulting in much reduced primary responses and partial tolerance to a secondary challenge.

A more unusual situation was found using the intramuscular route in carp. Priming with 10^7 SRBC's was found to be optimal for memory formation (Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a). Injection of high antigen doses, 10^9 SRBC's, gave high primary but poor secondary responses. Whereas injection of low doses, 10^5 SRBC's, gave a poor primary but high secondary response. This was not seen in animals injected intravascularly, where the normal increased primary and secondary responses with increasing antigen doses was seen. It was speculated that an antibody feedback inhibition might be responsible for this priming dose-secondary response phenomenon, similar to that occurring in mammals using the intravascular route (Moller & Wigzell, 1965; Benner, Meima, Van der Meulen & Van Ewijk, 1974; Grantham & Fitch, 1975). Antibody produced during the primary response may act specifically to limit the magnitude of the secondary response to subsequent challenge. It has also been observed in mammals that high molecular weight antibody is more susceptible to suppression than low molecular weight antibody (Uhr & Moller, 1968), and may help account for this suppression seen after secondary challenge

using the intramuscular route in teleosts. It may also explain the initial suppression often seen before enhancement using this and other routes (Avtalion, 1969a; O'Neill, 1979). In the present study an initial suppression after the booster injection was seen only occasionally, and may simply have been due to the formation of immune complexes in vivo. The good responses seen after the primary and booster intramuscular injections of HGG, suggest that the optimal concentration was being used.

The enhancing effects of adjuvant on antibody production has also been shown by many authors (Ambrosius & Lehmann, 1965; Post, 1966; Evelyn, 1971; O'Neill, 1979). However, adjuvant does not always have an effect (Muroga & Egusa, 1969) and may even cause a delay in the onset of the immune response (Post, 1966). The effect of adjuvant injected intraperitoneally or intramuscularly with HGG in carp, is clearly one of enhancement. Yet with A. salmonicida there is little or no effect. This may reflect the size of the antigen, as adjuvants certainly help to stimulate antigen-reactive cells to small molecular weight proteins, possibly resulting through lengthened contact of the antigen with effector cells, or even aggregation of the protein in the preparation of the emulsion (White, 1972). However, it may also reflect the ability of the antigen to stimulate B cells directly without cell to cell interaction. In mammals the effects of FCA seem to be directed towards T cells (Taub, Krantz & Dresser, 1970; Allison & Davies, 1971), phagocytes (Halpern, Prevot, Biozzi, Stiffel, Mouton,

Morard, Bouthillier & Decreusefond, 1963; Nicol, Quantock & Vernon-Roberts, 1966), and lymphocyte recirculation (Frost & Lance, 1978), encouraging optimal contact between antigen and antigen reactive cells. If a similar situation occurs in the carp, HGG would be expected to act in a typical T-dependent manner, whilst A. salmonicida, probably possessing lipopolysaccharide on its cell wall, a classical B cell activator (Coutinho & Moller, 1973) could easily act as a T-independent antigen and would not be affected by the use of adjuvant.

After a booster injection the effect of adjuvant on the response to HGG seems to be in two forms. It causes larger titres than are seen during the primary response, and the titres are of a longer duration. The high titres observed after a booster may be related to the formation of memory cells during the primary response, thought to occur in germinal centres in homoiotherms. Certainly, using adjuvant, areas of intense activity are seen in the pronephros after the onset of antibody formation (Plate 17) and may represent primitive germinal centres (Chapter 3). However, higher titres are seen after a booster with A. salmonicida in saline without the appearance of such areas during the primary response. So memory cells would not seem to be exclusively confined to such areas of pyroninophilic cells. Interestingly, only with the very high anti-HGG titres late in the immune response were precipitating antibodies seen, and they were almost exclusively confined to animals receiving adjuvant. These precipitating antibodies were found in the first, high molecular weight, peak on the column chromatography traces,

and seem to be 2ME-sensitive. Precipitating antibodies have been found in the high molecular weight fraction of mammals and anurans (Coe & Peel, 1970) but only when IgM levels are very high. They are more usually found in association with low molecular weight antibody in both mammals (Shulman, Hubler & Witebsky, 1964; Rubin, 1971) and anurans (Lykakis & Cox, 1968; Hadji-Azimi, 1971), where they occur at much lower antibody levels. One of the possible roles of germinal centres has been proposed to be the conversion from 19S immunoglobulin to 7S immunoglobulin production during the immune response (Blythman & White, 1977). Perhaps these active areas in the carp may be responsible for the conversion to precipitating antibody seen in the response to HGG.

The prolonged booster response with adjuvant may relate to homeostatic functions. White (1973) has proposed that one of the effects of adjuvant is to interfere with normal negative feedback of the antibody response, mediated through the trapping of immune complexes at germinal centres, resulting in prolonged unchecked antibody production. The booster responses in carp certainly cause less antigen-trapping and produce fewer pyroninophilic cell clusters in the pronephros than occur in the primary response, but these sophisticated control processes may not have evolved at the level of the teleosts in which only high molecular weight antibodies are formed.

The precipitin responses were all 2ME-sensitive, typical of an IgM antibody response. However, the agglutinating

responses were more resistant. Even after treatment with 0.3 M 2ME for 1 h at 37°C, titres in the region of 1:1,000 still remained. 2ME-resistant antibodies have also been seen in other species of fish, such as in the lemon shark (Clem & Small, 1967), perch (Ambrosius, Richter & König, 1967), channel catfish (Heartwell III, 1975) and brown trout (O'Neill, 1979). In sharks the resistant antibody was only seen late in the response and had a low sedimentation coefficient. Since the heavy chains of the lemon shark 19S and 7S immunoglobulins showed similar peptide maps, disc patterns, molecular weights, and antigenic properties, these two proteins were felt to be of the same class, the latter being a monomer of the former. 2ME-resistant activity was not seen to increase during the immune response in the channel catfish (Heartwell III, 1975). In the present study the 2ME-resistant activity did increase as the immune response continued, and in perch where only 2ME-resistant antibodies are normally present, some 2ME-sensitive antibodies could be detected very early in the immune response (Ambrosius, Richter & König, 1967). 2ME-resistant IgM has also been detected in anuran amphibians, using Salmonella flagella (Yamaguchi, Kurashige & Mitsuhashi, 1973) or HGG administered in the adjuvant Corynebacterium parvum (Collie, 1976). Treatment of amphibian antisera with 2ME by other workers has shown diminished antibody activity in the high molecular weight peak, but not complete reduction (Hadji-Azimi, 1971). Phylogenetically the anurans are the most primitive class to secrete a non-IgM immunoglobulin with certain IgG-like characteristics (Marchalonis & Edelman, 1966;

Marchalonis, 1971a). As in mammals, the first antibody class to appear after antigenic stimulation is 19S, followed by the appearance of the activity in the 7S immunoglobulin fraction at a later stage of immunization. The simple picture of 2ME-sensitive 19S immunoglobulin and 2ME-resistant 7S immunoglobulin has even more discrepancies when there are two antibody classes. In anurans, the early low molecular weight haemagglutinins were found to be 2ME-sensitive (Marchalonis & Edelman, 1966; Coe & Peel, 1970) and a similar situation has been observed in mammals (Adler, 1965a,b) and birds (Grey, 1963a), whilst in turtles all the 7S antibody appeared to be 2ME-sensitive (Grey, 1963b, 1966). The appearance of 2ME-resistant IgG antibody is likely to be associated with an antibody of greater avidity, whose activity is less susceptible to partial dissociation. In fish only IgM is present, but an increase in antibody affinity has been observed during the immune response in carp (Fiebig & Ambrosius, 1977). Perhaps with this change in antibody affinity, possibly correlated with the appearance of precipitating antibodies in the present study, a change in molecular class of antibody is not required to obtain an increase in more stable, 2ME-resistant antibody. Clearly many factors are important in rendering an antibody susceptible to reduction, and that reduction in itself is not a reliable criterion for distinguishing between types of immunoglobulin.

An interesting aspect of the phylogeny of immunoglobulins was seen in the HGG antibody experiments, when using gamma

globulins from other species to look at cross-reactivity. Cross-reactivity of anti-HGG antisera was seen with both BGG and CGG, but only at the level of agglutinating antibodies. The cross-reactivity to BGG was higher than that seen to CGG and may reflect the evolutionary differences in the heavy chains of these immunoglobulins. In mammals, cleavage of the heavy chain to release the C-terminal stretch has allowed amino acid sequencing to be carried out (Grey, 1969). The degree of homology of gamma globulin heavy chains between species has been shown to be in the order of 90% or greater, and allowed the postulate that gamma globulin subclasses arose after divergence of these species from a common ancestor. So it should not be too surprising that some cross-reactivity to BGG is seen. Indeed, it has been shown that carp immunized with BGG, when given a subsequent immunization with HGG, gave a strongly suppressed immune response to the second antigen (Richter & Ambrosius, 1972). It was suggested that multipotent antigen-sensitive cells may be present. The small amount of cross-reactivity to CGG may again be caused by reactions against the heavy chain, which is now more distantly related. The 7S immunoglobulin heavy chains from birds, in common with amphibians and reptiles, differs from the mammalian gamma chain in a number of properties (Atwell & Marchalonis, 1976) and has caused several workers to call it IgRAA, 7S immunoglobulin class of reptiles, aves and amphibians. These differences of the heavy chain are probably all important in the recognition of determinants by the carp anti-HGG antiserum, and so a smaller degree of cross-reactivity results than with BGG.

The results from the column chromatography of carp serum show only one protein peak that increases greatly on immunization and this is associated with both agglutinating and precipitating antibodies. The S coefficient of 18.2 found in the present study is high compared to the value of 14S given for carp by Richter, Frenzel, Hadge, Kopperschlager and Ambrosius (1973). Clearly, however, this immunoglobulin has a high molecular weight and is similar to the IgM of mammals (Marchalonis, 1971a; Richter, Frenzel, Hadge, Kopperschlager & Ambrosius, 1973). In most vertebrates, IgM is an 18-19S pentameric molecule, and in fish such a molecule has been found in the Chondrichthyes (Frommel, Litman, Finstad & Good, 1971; Johnston, Acton, Weinheimer, Niedermeier, Evans, Shelton & Bennett, 1971) and Sarcopterygii (Marchalonis, 1969). However, in the Actinopterygii it usually has a lower S value of about 14-17S, and is a tetrameric molecule (Shelton & Smith, 1970; Acton, Weinheimer, Hall, Niedermeier, Shelton & Bennett, 1971; Cisar & Fryer, 1974). 7S monomer IgM has also been reported in fish, giving the appearance of an IgG-like molecule, but without the gamma heavy chain. It occurs predominantly in elasmobranchs, but has also been reported in several species of marine fish (Clem & McLean, 1975). However, some authors have reported that two antigenically distinct immunoglobulin populations are present in fish (Uhr, Finkelstein & Franklin, 1962; Alexander, Wilson & Kershaw, 1970; Trump, 1970; Trump & Hildemann, 1970; Evelyn, 1971; Everhart, 1972; Heartwell III, 1975).

Most of these reports were based on differences in immunoelectrophoretic behaviour between the antibody populations. The work by Trump and colleagues on the goldfish (Trump, 1970; Everhart, 1972) showed these populations to have similar S values, 16.4S and 15.3S, and could account for the apparent shift towards 2ME-resistant antibodies seen in the present study, without the appearance of an obvious second antibody peak in chromatography traces.

It is unfortunate that carp do not lend themselves to either early injection or thymectomy. All carp injected before day 56 after hatching did not survive longer than 3 weeks. The earliest stage at which carp fry in the present study were able to withstand injection was well after immunological maturation, so what had been intended to be a study of tolerogenesis was unsuccessful. The thymic anlagen is first seen 2 days after hatching in this species (Botham, personal communication) and by 8 days the majority of the cells are of the small densely staining type. It is at this same time that mature lymphocytes first become visible in the spleen, pronephros and peripheral blood. Fry of 16 days post-hatch have been shown to demonstrate alloimmune reactivity (Botham, Grace & Manning, 1980), although fewer lymphocytes invade the allograft and graft breakdown is delayed when compared to the adult response. Fry of 30 days post-hatch show an intermediate stage between this and adult responsiveness, possibly related to an increase in lymphocyte numbers in the pro- and mesonephros at this time. So it would appear that in carp there is a

rapidly maturing T cell equivalent population. Cells possessing surface immunoglobulin and cells containing cytoplasmic immunoglobulin have also been observed very early in carp (Van Loon, Van Oosterom & Van Muiswinkel, 1981). The first surface immunoglobulins on cells were detected in the thymus by day 12 post-hatch, and both surface immunoglobulin and cytoplasmic immunoglobulin containing cells were seen in the pronephros after day 18. However, only at 49 days of age did the relative numbers of lymphoid cells reach adult levels. These workers also showed that injection of SRBC's into carp at 4 weeks of age elicited no plaque forming cell response. A booster injection 3 months later also gave a negligible response, in contrast to the good response seen in control animals after primary immunization at this age, and it was thought tolerance had been induced.

The results of the present investigation have shown that the primary or booster agglutinating antibody response to both HGG in FCA and A. salmonicida is almost identical whether the animals are 8, 16, 24, 32, 40, 48 or 56 weeks of age. There is a slight delay in the primary response to HGG in FCA, and no apparent enhancing effect of a booster in the A. salmonicida injected animals, when carp are injected at 8 weeks of age. Even so, these differences were comparatively small. This contrasts with the results in the rosy barb, where the plaque forming cell and haemagglutinating response to SRBC's, although present, is markedly reduced in animals of 3 and 4 months compared to animals of 9 months (Rijkers & Van Muiswinkel, 1977). An explanation

may lie in the nature and presentation of the antigens used. In mice the ontogenetic capacity to respond to T-independent antigens precedes the ability to respond to T-dependent antigens (Etlinger & Chiller, 1979; Rabinowitz, 1976). A. salmonicida is probably acting as a T-independent antigen, at least it would appear such in Xenopus laevis, whereas both HGG and SRBC's are classical T-dependent antigens. Thus the ability to respond fully to A. salmonicida probably occurs before that for HGG or SRBC's. However, the good response to HGG seen in the present investigation, in contrast to the tolerizing response to SRBC's seen in 4 week old carp (Van Loon, Van Oosterom & Van Muiswinkel, 1981), or the diminished response of 3 and 4 month rosy barbs (Rijkers & Van Muiswinkel, 1977) is probably due to the adjuvant. In mice, adjuvants prevent the induction of tolerance to aggregate free BGG (Dresser, 1968). The low level of response to a booster injection of HGG in young (8 week) carp following a primary injection in saline, particularly at 0.25 mg/g fish, may reflect a degree of tolerance. Unfortunately, no data on primary responses, or booster responses to non-cross-reacting antigens is available so that the specificity of this reduced response has yet to be established.

It is interesting that in salmon the thymus becomes lymphoid at 22 days pre-hatch, followed by the appearance of lymphocytes in the kidney and vasculature at 14 days pre-hatch. Yet surface immunoglobulin and mixed leucocyte reactivity do not appear until the onset of feeding at 45 days post-hatch (Ellis, 1977). It would seem that the prefeeding salmon is immunologically immature, and that the coincidence of

immunological maturity and feeding may be due to the larvae being subjected to a large antigenic challenge from the diet at this time. In carp, and most coarse fish, hatching occurs very soon after spawning and feeding commences almost immediately. So to investigate the induction of tolerance or immunity at an age before feeding begins in this species is very difficult, because of the small size and food reserves of the hatching fry. In contrast, salmonid fish have very long intervals between spawning and hatching, giving large fry at hatching. Also these fry still possess large yolk sacs at hatching (Plate 3) and feeding does not commence for several weeks. Obviously they are a better choice for such an experiment.

Thymectomy was only feasible in mature carp, and then only short-term studies were possible. The deaths of all animals injected with HGG, a classic T-dependent antigen, prevented the investigation of the role of the thymus in adult responses. The response to A. salmonicida was not diminished in thymectomized animals, as would be expected for a T-independent antigen. In mammals, short-term adult thymectomy has no effect on primary responses to antigens. However, secondary responses are substantially reduced (Simpson & Cantor, 1975), as are primary responses if antigenic challenge does not take place for at least 10 weeks after thymectomy (Metcalf, 1965; Miller, 1965; Taylor, 1965). Further, adult thymectomy increases the ability of T cells to generate primary cytotoxic responses (Simpson & Cantor, 1975) and prevents mice becoming fully tolerant to deaggregated HGG (Basten, Miller & Johnson, 1975), suggesting a loss of

T suppressor cells. So it appears that although there is a long lived recirculating T-lymphocyte pool, the specialized sub-sets involved in regulatory functions, such as helper T-memory and suppression of T cell differentiation to effector cells, are not so long lived (Kappler, Hunter, Jacobs & Lord, 1974). Long-term studies were not possible with adult thymectomized carp, but possibly A. salmonicida may tentatively be classified as a T-independent antigen in this model, as no diminished effect was seen from the theoretical elimination of specialized regulatory T cell sub-sets after adult thymectomy.

Chapter 6CELL-MEDIATED RESPONSES IN CARP

Cell-mediated responses, as well as antibody production, may play a large role in the immune response after antigenic challenge. One effect of this, delayed hypersensitivity, was investigated using the leucocyte migration inhibition test. Specific inhibition of migration of macrophages by antigens has been found to be an in vitro expression of delayed hypersensitivity (George & Vaughan, 1962). Inhibition of migration has been shown to be due to the liberation, by specific antigen, of a migration inhibition factor (MIF) from sensitized lymphocytes (Bloom & Bennett, 1966; David, 1966). Little is known about the relationship between lymphocytes and macrophages in fish, or whether cells produce soluble, non-immunoglobulin mediators, such as lymphokines or transfer factor. The interaction of cell-mediated and humoral immunity deserves closer inspection, and may be important when looking at the protection afforded by vaccines.

MATERIALS AND METHODS1. Migration of unsensitized cells

A pilot study was carried out to see which organs provided sufficient cells for assay and how many cells were required for the production of a normal fan of cells in the standard migration inhibition test used. Spleen, pronephros and peripheral blood were used.

Adult carp, weighing 30 to 40 g, were heavily anaesthetized in MS222 (1g/l). Peripheral blood was collected from the caudal vein sterilely in a microflow chamber, after swabbing

the site of entry of the needle with 95% alcohol. The blood was collected in a heparinised 1 ml syringe, and was added to heparinised medium in the ratio of 1:2. The medium used throughout the migration inhibition experiments consisted of 87.5% sterile L-15 (Leibowitz) Medium x 1 with L-Glutamine (Gibco, Glasgow, UK), 10% foetal calf serum (FCS) (Gibco, Glasgow, UK), 2% Penicillin/Streptomycin (10,000 u/ml Penicillin and Streptomycin) (Gibco, Glasgow, UK) and 0.5% heparin (5,000 i.u./ml) (Sigma, St. Louis, USA).

This blood-medium mixture was carefully layered on top of sterile lymphocyte separation medium (Flow Laboratories, Irvine, UK) with a specific gravity of 1.077, in sterile glass centrifuge tubes. Alternatively, a lymphocyte separation medium, with the same specific gravity, was prepared in the laboratory using 9.24 g Ficoll 400 (Pharmacia, Uppsala, Sweden), a 25 ml ampoule of Urografin 325 (Schering Ag Berlin/Bergkamen, Germany) and 119 ml distilled water. This was stirred at room temperature until dissolved, filtered through a 0.22 μ Millex millipore filter (Millipore, Molsheim, France) and stored in a dark bottle. The ratio of the blood-medium mixture to separation medium was 1:2. This was centrifuged for 12 min at 110 g in an MSE minor centrifuge. The red blood cells descend to the bottom of the tube, whilst the leucocytes form an opaque ring about half way up. This ring of cells was collected with a sterile, drawn out, heparinised Pasteur pipette, placed into a sterile centrifuge tube and centrifuged at 700 g for 5 min. The supernatant was discarded and the cells were resuspended in 0.1 ml of heparinised medium. A small sample was used in a haemocytometer to determine the number of cells present, and

the concentration was adjusted to 4×10^6 cells/ml. The cell suspension was drawn up into a heparinised 25 μ l microcap tube (Drummond, USA), giving 1×10^5 cells/tube, and one end was sealed with Critoseal (Sherwood, St. Louis, USA). The filled tubes were placed with the sealed ends outermost, into a haematocrit centrifuge connected to a rheostat, and centrifuged at 850 g for 5 min. At this speed any erythrocytes which slightly contaminated the sample went to the bottom of the tube, and the white cells collected on top as a well defined buffy coat. It was found to be important to remove the tubes from the centrifuge soon after it had stopped spinning, and to place them vertically whilst waiting to be cut. This prevented cells migrating along the tube before being placed in the migration chambers. The tubes were cut at the cell-medium interface with a needle file, and placed flat on the bottom of sterile migration chambers (Sterilin, Teddington, UK). Each chamber had the periphery of the well coated with silicone grease (Dow Corning, Midland, USA) prior to use. Silicone grease was also used to keep the tubes in place. The chambers were dealt with one at a time to ensure a minimal amount of desiccation of the cells at the end of the cut tube. As soon as a tube was in place 0.5 ml of medium was added quickly, but carefully, to the chamber, so that cells were not dislodged and air bubbles were not allowed to collect at the end of the tube. Once the medium had been added, a sterile coverslip was placed on top of the chamber, being sealed by the silicone grease. The cut tubes and coverslips

were handled only with sterile fine forceps. At least 4 replicate tubes were used per sample. The trays were incubated for 24 h at 26°C before reading with a Leitz Projection Microscope. The fans projected on to graph paper were drawn at x 20 and the areas calculated.

The same technique was followed for the spleen and pronephros. Here the organs were removed in a microflow chamber, with sterile instruments, and placed in 0.5 ml heparinised medium. The organs were finely minced and transferred to a sterile test tube. After the debris had settled to the bottom the supernatant was removed and layered on top of lymphocyte separation medium as for blood. The only difference between the pronephros or spleen and the blood, was the speed at which the tubes were centrifuged in the haematocrit centrifuge. For pronephros or spleen a lower speed of 120 g was used for 5 min, because it was found that higher speeds packed the cells too tightly and no migration occurred. The spleen produced very few leucocytes and normally 4 animals had to be pooled to get enough. In contrast, both the blood and pronephros of a 30 to 40 g fish, yielded enough cells to assay the animals individually.

Peritoneal exudate cells were also used. A 2% starch solution was injected into the peritoneal cavity at a volume of 50 µl/g fish, to increase the yield of peritoneal cells. Three days later the fish were killed and 3 ml of sterile medium was injected into the body cavity. The abdomen was massaged, an incision was made to allow entry of a Pasteur

pipette, and cells were withdrawn. They were then washed 3 times for 5 min at 2,000 rpm, and each time resuspended in heparinised medium. On the final wash they were resuspended in 0.1 ml of medium and a cell count performed with a haemocytometer. The cells were also adjusted to 4×10^6 cells/ml, but as with the spleen this usually required 4 to 5 animals to get enough cells. The rest of the method was identical to that for the spleen or pronephros cell suspensions.

For the blood and pronephros leucocytes the number of cells was varied between 1×10^5 to 1×10^7 cells/ml medium. The fan areas were calculated at x20 and an area in the region of 20 to 40 sq.cms was considered optimal. Too small a control fan area would make visualization of inhibition difficult, whereas enormous fan areas would seem to be a waste of valuable cells.

Smears were made of the cells in fans obtained from the blood and pronephros and stained using MacNeal's Tetrachrome Stain for differential leucocyte counts. Counts of macrophages, lymphocytes, granulocytes and thrombocytes, were made of 20 fields of view with a x40 objective, and the averages were calculated.

2. Immunized Animals

(a) Sensitization with human gamma globulin in adjuvant

Adult carp were immunized intraperitoneally with HGG in FCA, as in the previous Chapter, at a concentration of 0.025 mg HGG/g fish. Animals were killed at varying times after injection and the peripheral blood, pronephros and occasionally spleen, leucocytes were assayed using the migration

inhibition technique described above, using 4×10^6 cells/ml medium. One group of animals was killed on days 1, 3, 7, 14, 21 and 28, and the tubes were incubated with medium alone and medium containing 2.5 mg HGG/ml. One group was killed on days 3, 7, 14, 21, 28, 42 and 56, and incubated with medium alone and medium containing 0.25 mg HGG/ml. Lastly, one group was killed on days 3, 14, 21, 28, 42 and 56 and incubated with medium alone and medium containing Purified Protein Derivative of Tuberculin (PPD) (Evans Medical Ltd, Speke, UK) at 3,000 i.u./ml (approximately 60 μ g/ml). In all cases as many tubes were incubated with antigen in the medium as were incubated without antigen. Again a minimum of 4 replicate tubes were made of each sample, and 5 to 6 fish were used on each day of sacrifice. Cells from unstimulated animals were also incubated with and without antigen, to see whether the antigen caused any nonspecific toxic effects on cell migration.

The inhibition ratio was calculated using the formula,

$$I = \frac{\text{area with antigen}}{\text{area without antigen}} \cdot$$

A ratio of less than one indicates that inhibition of migration has occurred. The t-test, corrected for small samples (Parker, 1973), was employed to see whether there was a significant difference between the immunized and unimmunized groups.

(b) Sensitization with adjuvant alone

One group of animals was injected daily for 7 days, intramuscularly with 0.1 ml of FCA. Animals were killed on days 3, 7, 14, 21 and 35, and peripheral blood and pronephros leucocytes were tested in the same manner as

above with 3,000 i.u. PPD/ml medium. Controls were also as above, with the additional control of incubating sensitized cells with 0.25 mg HGG/ml medium as a test for specificity.

In all experiments cell viabilities were calculated using Trypan blue dye exclusion. Cell suspension (0.1 ml) was mixed with an equal volume of 0.5% Trypan blue in PBS for 2 min. The viable cells were then counted in a haemocytometer, excluding dye laden dead cells, and expressed as a percentage of the original number used. Cell viabilities for the blood, pronephros and spleen leucocytes were calculated after 24 h of incubation in medium, medium containing HGG and medium containing PPD.

Also, selected trays were everted immediately after recording the fan areas, or after a further 72 h, and read again 6 h later, to see if the adherent cell fan area differed from the whole cell population fan.

3. Mitogen stimulation

(a) Pilot study

An attempt was made to nonspecifically stimulate the in vitro release of lymphokine factors using T cell mitogens such as Phytohemagglutinin M (PHA) (Difco, Detroit, USA) or Concanavalin A (Con A) (Difco, Detroit, USA). Uninjected fish were killed and the blood and pronephros leucocytes obtained in the normal manner. They were incubated in migration trays with medium containing a recommended dose of 200 μ g PHA/ml medium (Smith, MAFF, Weymouth, personal communication) or 10-20 μ g of Con A/ml medium, the dose range found to be stimulatory to rainbow trout leucocytes

(Etlinger, Hodgins & Chiller, 1976a, 1978). Again, as many tubes as were incubated with FHA or Con A in the medium were incubated with medium alone for each of the fish.

(b) Response to released factors after removal of Con A.

A further experiment was carried out, to try to cause inhibition of migration of unstimulated cells by addition to the medium of soluble factors, produced in a similar fashion to the above. Con A was used, since this can be removed from the medium with Sephadex G-200.

Leucocytes from the blood and pronephros of uninjected animals were obtained in the usual manner, and resuspended to a concentration of 10^6 cells/ml. These were plated out in 0.1 ml aliquots into sterile microtiter tray wells (Sterilin, Teddington, UK). Medium containing 20 μ g or 40 μ g Con A/ml was then added in 0.1 ml aliquots, a sterile lid was placed over the wells and the tray was incubated at 26°C for 1, 2 or 3 days. Whilst the incubation was proceeding Sephadex G-200 (Pharmacia, Uppsala, Sweden) beads were swollen as for a column, using a 30 to 40 ml bed volume per g dry gel. Once swollen the slurry was centrifuged at 170 g for 10 min to remove excess water. The cells from the trays were pooled together in rows of ten, centrifuged for 5 min at 700 g and the supernatants added to the Sephadex in the ratio of 1:5 by volume. This mixture was placed in a flat tissue culture flask (Nunc, UK) to give extra surface area, and incubated at 37°C for 1 h, shaking every 10 min. It was then poured back into the centrifuge tubes and centrifuged at 170 g for 10 min,

to remove both the Sephadex and Con A. The supernatant was concentrated, as described under the column chromatography section (page 104), and added to either 2.5 or 5 ml of medium. Using Sephadex gel filtration or sucrose gradient centrifugation, peak MIF activity has been found to occur in fractions with a molecular weight range of 35,000 to 55,000 daltons (Remold, Katz, Haber & David, 1970), so concentrating the supernatant should not lose activity. This was then filtered through a 0.22 μ Millex millipore filter and a normal migration inhibition test performed on the blood and pronephros leucocytes from 4 unstimulated animals. Incubation for 1 day at 26°C was carried out as normal and the migration of cells in medium alone was compared to that of cells in medium plus the supernatant prepared as above and possibly containing lymphokine factors. Controls consisted of incubating medium alone with Con A, followed by the same procedure to remove Con A and testing for inhibition in unstimulated animals.

In both experiments (Sections (a) and (b)), after incubation with PHA or Con A, the cells were looked at for possible transformation into blast cells. This was carried out using an agar-drop technique (Pacha & Kingsbury, 1962), followed by staining with methyl green-pyronin. Blast cells take up the pyronin stain and appear bright red. Thin agar plates were made using 1.5% agar and 0.5% sucrose. They were allowed to harden, inverted and left at 37°C overnight. The cells were harvested and 10 μ l aliquots were placed on to the agar. Once the water was absorbed by the agar, giving the spot a dull appearance, the agar was cut

around the spot and placed cells down on to an alcohol cleaned slide. The slide was immersed in Carnoy's fixative for 25 min. It was then removed, the agar taken off and discarded, and the slide air dried. The slide was then dehydrated and stained in the normal fashion. Untreated and stimulated cells were looked at to see if the number of blast cells increased in the presence of PHA or Con A.

RESULTS

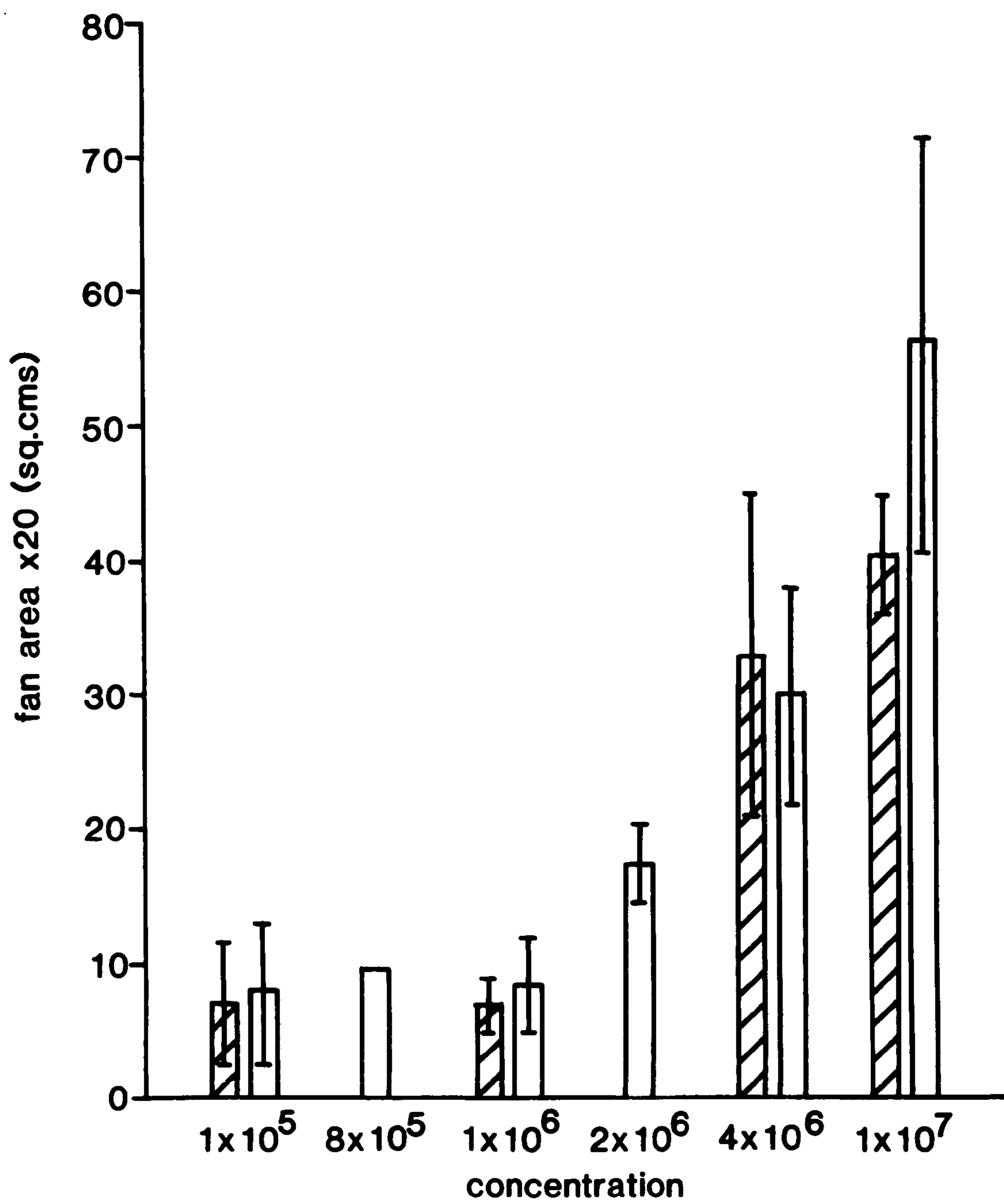
1. Migration of unsensitized cells



The peripheral blood and pronephros alone provided enough cells to assay individual animals. The spleen required on average 4 to 5 animals to get enough cells, as did the peritoneal exudate cells. In both cases the migration was very sporadic and often did not occur at all. In contrast, the blood and pronephros leucocytes always produced a good fan of cells (Plates 46 and 47), although it was quite variable in size from animal to animal, even when using the same concentration of cells. The effect of varying the number of cells present was investigated to see how this influenced the fan size and the results are presented in Figure 16. It is clear that concentrations less than 4×10^6 gave quite small fan areas, whilst concentrations greater than this gave areas that were larger than needed, particularly in the case of blood leucocytes. So 4×10^6 cells/ml was chosen as the standard concentration to use.

It was noted that if the capillary tubes were cut a little way from the cell-medium interface, that the fan area

Figure 16

Average fan areas of blood and pronephros leucocytes resuspended to varying concentrations. Each column represents the mean fan area, and vertical lines represent the standard deviation.



-  blood leucocytes
-  pronephros leucocytes

was reduced. Even though the tubes are cut as near as possible to the cells, variation does occur. The minimum tube length was 1 mm, the area occupied by the cells. Longer lengths of tube presumably have to be travelled over before a fan can appear, and so should be included when calculating the area considered to be the area of the fan. This effect seemed most obvious with pronephros leucocytes. Fan areas from both blood and pronephros were therefore calculated with and without the tube length occupied by cells in all experiments, in case differences in inhibition indices resulted from such variations (see legend to Plate 46).

Differential leucocyte counts were made on smears of blood and pronephros cell suspensions, after treatment with lymphocyte separation medium. Cell types identified are shown in Plate 48, and the percentages of each are shown in Table XXV.

TABLE XXV

Mean percentages of leucocyte types present
in blood and pronephros cell suspensions used
in cell migration studies

Cell types	Macrophages	Lymphocytes	Granulo- cytes	Thrombo- cytes
Blood leucocytes	1.0 ± 0.1	77.4 ± 10.6	6.9 ± 4.0	14.7 ± 6.7
Pronephros leucocytes	8.8 ± 1.1	67.9 ± 1.6	19.6 ± 0.2	3.7 ± 0.7

The blood leucocytes seem to have far fewer macrophages and granulocytes, but larger numbers of thrombocytes, compared to the pronephros leucocytes. The granulocytes of fish are considered to be neutrophils by many authors (Hines &

Yashouv, 1970; Ellis, 1976), however two types of granulocytes have been described by other authors (Davies & Haynes, 1975; Davina, Rijkers, Rombout, Timmermans & Van Muiswinkel, 1980), which differ at the electron microscope level. Certainly in the present study two types of granulocyte have been seen at both the light (Plate 48) and electron microscope level (Plates 11 and 12), one of which resembles a mammalian eosinophil at the electron microscope level (Plate 11). However, without the proper staining identification it is not possible to be sure that some of these cells were not neutrophils.

2. Migration of cells from immunized animals

(a) Responses of animals injected with HGG in FCA

The results of adding antigen to the medium, into which are put antigen-sensitized cells, are shown in Tables XXVI to XXIX. HGG in FCA was injected at a standard dose. Testing for inhibition was carried out at two dose ranges for HGG and one for PPD. The mean inhibition ratio was calculated for each group and compared to responses from unstimulated animals (Table XXIX) using the t-test for small samples.

As can be seen in Tables XXVI and XXVII, with the antigen (HGG) in the medium there does appear to be a limited inhibition of migration of the blood leucocytes taken from immunized animals early on in the immune response. It is confined to just a short time period, varying with the antigen concentration used for testing. The pronephros cells show rather unexpected results with a significant increase in migration very early in the response to both

TABLE XXVI

Mean inhibition ratio using cells from animals injected with HGG in FCA,
migrating in medium containing 2.5 mg HGG/ml

Day Tested	Blood	Blood + tube	Pronephros	Pronephros + tube
1	0.76 ± 0.39	0.76 ± 0.38	1.34 ± 0.12**	1.22 ± 0.54.
3	0.78 ± 0.03**	0.80 ± 0.05**	0.94 ± 0.02	0.98 ± 0.11
7	1.45 ± 0.82	1.30 ± 0.54	1.02 ± 0.30	1.00 ± 0.28
14	1.28 ± 0.59	1.27 ± 0.56	0.60 ± 0.34	0.65 ± 0.28
21	0.94 ± 0.39	0.94 ± 0.31	0.79 ± 0.22	0.84 ± 0.14
28	0.94 ± 0.19	0.96 ± 0.18	0.66 ± 0.01*	0.96 ± 0.37

****, Significantly different from unimmunized animals at the 1% probability level; ***, significantly different from unimmunized animals at the 5% probability level. Absence of asterisk indicates no significant difference.

TABLE XXVII

Mean inhibition ratio using cells from animals injected with HGG in FCA,
migrating in medium containing 0.25 mg HGG/ml.

Day Tested	Blood	Blood + tube	Pronephros	Pronephros + tube
3	0.92 ± 0.13	0.94 ± 0.11	1.21 ± 0.17 **	1.15 ± 0.18*
7	1.17 ± 0.50	1.13 ± 0.42	0.95 ± 0.27	0.95 ± 0.25
14	0.70 ± 0.01**	0.73 ± 0.01**	0.79 ± 0.21	0.89 ± 0.23
21	1.15 ± 0.25	1.12 ± 0.23	0.89 ± 0.23	0.88 ± 0.22
28	0.93 ± 0.33	0.94 ± 0.31	1.04 ± 0.17	1.03 ± 0.14
42	0.94 ± 0.13	0.97 ± 0.11	0.97 ± 0.14	0.98 ± 0.12
56	0.90 ± 0.38	0.89 ± 0.33	0.77 ± 0.09	0.77 ± 0.10

****, Significantly different from unimmunized animals at the 1% probability level; *, significantly different from unimmunized animals at the 5% probability level. Absence of asterisk indicates no significant difference.**

HGG concentrations. The inhibition shown by one group later in the response was significant only for the fan area and lost its significance if the tube area was included, so this inhibition may not be truly representative. Clearly, during the peak antibody response occurring after day 21, there is little effect on cell migration.

Controls for these animals consisted of uninjected animals with HGG in the medium to see whether the HGG itself had inhibitory effects, also of HGG injected animals with PPD in the medium to see if the effect was HGG-specific (Table XXVIII). No significant inhibition was seen in either case, so the HGG does not appear to have any effects on unsensitized cells and the inhibition observed was not simply due to an increased protein concentration affecting sensitized cells non-specifically. Also, the negative results from stimulated cells in medium containing PPD at all times, compared to unstimulated cells with PPD in the medium, show that the inhibition was not directed against the killed Mycobacterium in the adjuvant, ruling out any suggestion of antigenic competition occurring between the HGG and the FCA. Indeed, unstimulated cells on the whole had a smaller fan area in the presence of PPD than stimulated ones. This inhibition in control animals was not due to cell deaths, as 95% of the cells were still viable after incubation with PPD (Table XXXI). It may have been due to some nonspecific stimulation of inhibition factor from these cells.

TABLE XXVIII

Mean inhibition ratio using cells from animals injected with HGG in FCA, migrating in medium containing 3,000 i.u. PPD/ml

Day Tested	Blood	Blood + tube	Pronephros	Pronephros + tube
3	N.T.	N.T.	0.73 ± 0.26	0.75 ± 0.24
14	0.87 ± 0.12	0.89 ± 0.12	0.62 ± 0.23	0.74 ± 0.17
21	0.81 ± 0.20	0.85 ± 0.15	0.75 ± 0.07	0.79 ± 0.08
28	0.81 ± 0.04	0.83 ± 0.05	0.80 ± 0.20	0.83 ± 0.21
42	0.67 ± 0.19	0.71 ± 0.17	0.73 ± 0.13	0.78 ± 0.09
56	0.73 ± 0.38	0.74 ± 0.34	0.87 ± 0.12	0.88 ± 0.11

N.T., Not tested. Absence of asterisk indicates no significant difference.

TABLE XXIX

Mean inhibition ratio using cells from uninjected animals

Antigen in medium	Blood	Blood + tube	Pronephros	Pronephros + tube
HGG at 2.5 mg/ml	1.00 ± 0.05	0.99 ± 0.05	0.84 ± 0.16	0.86 ± 0.14
HGG at 0.25 mg/ml	1.00 ± 0.05	0.99 ± 0.06	0.88 ± 0.15	0.87 ± 0.14
PPD at 3,000 i.u./ml	0.75 ± 0.06	0.77 ± 0.06	0.79 ± 0.07	0.82 ± 0.06
PHA at 0.2 mg/ml	0.20 ± 0.05	0.29 ± 0.04	0.06 ± 0.09	0.20 ± 0.16
Con A at 0.02 mg/ml	0.37 ± 0.02	0.45 ± 0.04	0.20 ± 0.10	0.31 ± 0.08

TABLE XXX

Mean inhibition ratio using cells from animals injected with FCA,
migrating in medium containing 3,000 i.u. PPD/ml.

Day tested	Blood	Blood + tube	Pronephros	Pronephros + tube
3	0.68 ± 0.19	0.70 ± 0.20	0.63 ± 0.27	0.70 ± 0.26
7	0.52 ± 0.18*	0.58 ± 0.15*	0.37 ± 0.18**	0.59 ± 0.21*
14	0.43 ± 0.11**	0.45 ± 0.10**	0.67 ± 0.12	0.72 ± 0.11
21	0.77 ± 0.13	N.T.	0.82 ± 0.50	N.T.
35	0.77 ± 0.14	N.T.	0.50 ± 0.36	0.73 ± 0.06*
14+	0.83 ± 0.24	N.T.	0.87 ± 0.06	0.88 ± 0.04

N.T., Not tested; +, medium contained 0.25 mg HGG/ml; **, significantly different from unimmunized animals at the 1% probability level; *, significantly different from unimmunized animals at the 5% probability level. Absence of asterisk indicates no significant difference.

(b) Responses of animals injected with FCA alone

Previous authors have shown migration inhibition factor production in response to Freund's complete adjuvant. An experiment was therefore set up following a similar immunization schedule to Tahan and Jurd (1979), to see whether intensive immunization with FCA produced a similar effect in carp.

In animals injected with FCA daily for 7 days a much larger significant inhibition was seen than in animals receiving only a single injection of HGG in FCA (Table XXX). Again this occurred relatively early on, on days 7 and 14, and occurred with both blood and pronephros leucocytes (Plates 46 and 47). The inhibition had to be in the order of 50% or more to be significantly greater than that seen with unstimulated cells. Cells tested with HGG in the medium showed no significant inhibition when compared to unstimulated cells with PPD or HGG. So again the inhibition appears to be antigen specific.

Cell viabilities were calculated for each cell suspension, after incubation in medium alone or containing antigen. The results are shown in Table XXXI.

TABLE XXXI

Cell viabilities of cell suspensions after
incubation for 24 h at 26°C, in medium
alone or containing antigen

Cell suspension	Medium alone	Medium + HGG	Medium + PPD
Blood	86%	94%	95%
Pronephros	98%	94%	94%
Spleen	80%	N.T.	N.T.
N.T., Not tested			

Clearly very good survival was obtained in L15 plus FCS, with or without antigen. This was not surprising as other workers have also found L15 very good for fish cell culture (Etlinger, Hodgins & Chiller, 1976b), and that the addition of FCS gives better leucocyte growth than when homologous serum is added.

Eversion of trays gave no difference in size of the fan area for either blood or pronephros leucocytes. The adherent cells were present in every well looked at, at the periphery of the original fan. Often the centre of the fan was devoid of cell though. The adherent cells were still present even if the well was everted after 4 days of incubation, a technique used by some workers to separate polymorphonuclear leucocytes and monocytes (Nordqvist & Rorsman, 1970). Both of these cell types were found to adhere to glass or plastic, but only the latter were still alive and were thus still adherent, if the chamber was everted after 4 days. Possibly the granulocytes are still alive in the fish system after 4 days, or they do not have a large role in the size of the fan formed.

3. Mitogen stimulation

(a) Direct addition to medium

The effect of adding PHA to the medium was quite dramatic. Very high inhibition occurred in every animal, with virtually 100% inhibition in some pronephros cell suspensions (Plate 49). Similar results were obtained using Con A, although the degree of inhibition was a little less (Table XXIX). Unfortunately it is not possible to say

whether this inhibition is due solely to the release of lymphokine factors, or is due to an agglutination effect of PHA on the cells at the end of the tube. One possibility is to try to separate the inhibition factor from the cells and mitogen. This was done for the experiment using Con A.

(b) Response of Con A-free supernatant following Con A stimulation

Using Con A to attempt to nonspecifically stimulate the release of migration inhibition factor and to use the medium freed from Con A to test unstimulated cells, gave two interesting results (Table XXXII). In both cases only pronephros cells were affected, the factors apparently not causing significant differences between blood leucocytes in leucocyte and Con A freed medium compared to Con A freed medium. With pronephros leucocytes in medium containing factors from blood leucocytes stimulated with Con A for 1 or 2 days at 10 $\mu\text{g/ml}$, significant inhibition is seen. At higher concentrations this effect is totally lost. Conversely, with pronephros leucocytes in medium containing factors from pronephros leucocytes incubated for 3 days with 20 $\mu\text{g Con A/ml}$, a significant stimulation is seen. With only 1 or 2 days incubation either no effect or inhibition is seen. This is interesting when compared to the result with HGG. With strong stimulation, as presumably occurs whilst antigen concentrations are still high after injection, a stimulatory, and not an inhibitory, factor seems to be released from pronephros leucocytes. With a weaker stimulation, as may occur a little later after an injection of antigen, an inhibitory factor seems to be released from blood leucocytes.

TABLE XXXII

Mean inhibition ratio using cells from uninjected animals, migrating in medium

freed from Con A and leucocytes

Days of incubation	Source of leucocytes used for the initial Con A stimulation.	Concentration of Con A μ g	Volume suspended in, ml	Blood	Blood + tube	Pronephros	Pronephros + tube
1	Blood	10	5.0	0.96 \pm 0.30	0.96 \pm 0.30	0.68 \pm 0.22*	1.00 \pm 0.25
1	Pronephros	10	5.0	1.00 \pm 0.30	1.00 \pm 0.27	3.39 \pm 3.73	1.63 \pm 1.00
1	Pronephros	10	2.5	N.T.	N.T.	0.98 \pm 0.03	1.01 \pm 0.15
2	Blood	10	2.5	N.T.	N.T.	0.56 \pm 0.23**	N.T.
2	Pronephros	10	2.5	0.90 \pm 0.11	N.T.	1.16 \pm 0.17	N.T.
2	Blood	20	2.5	0.52 \pm 0.34	N.T.	1.03 \pm 0.21	N.T.
2	Pronephros	20	2.5	0.60 \pm 0.16	N.T.	0.80 \pm 0.08**	N.T.
3	Blood	20	2.5	1.00 \pm 0.20	0.99 \pm 0.08	0.97 \pm 0.17	1.04 \pm 0.24
3	Pronephros	20	2.5	1.53 \pm 0.39	1.45 \pm 0.32	1.24 \pm 0.06**	1.24 \pm 0.01**
2	None	20	2.5	1.01 \pm 0.26	1.01 \pm 0.22	0.99 \pm 0.02	0.97 \pm 0.02
3	None	20	2.5	1.00 \pm 0.31	1.01 \pm 0.24	1.00 \pm 0.11	0.98 \pm 0.13

N.T., Not tested; **, significantly different from control at the 1% probability level;

*, significantly different from the control at the 5% probability level. Absence of asterisk indicates

no significant difference.

Why the factors have no effect on blood leucocytes is not known. No significant inhibition was observed in any of the controls. Obviously there is much more work that could be carried out to see if this inhibition is in fact caused by a soluble lymphokine factor secreted by the lymphocytes in culture with Con A. However, this was only a pilot experiment. There are many variables in this system and much work needs to be carried out to elucidate the mechanisms involved.

When using either PHA or ConA, after 1 day of incubation blast cells were looked for. The results were rather unsatisfactory, and there did not appear to be an increase in the numbers of blast cells present after incubation with either mitogen. Other work, in the rainbow trout, has shown that a concentration of 10 μ g Con A/ml medium causes a peak response in the number of blast cells present in vitro on day 3 (Etlinger, Hodgins & Chiller, 1978). No transformation occurred to plasma cells, which was seen with LPS or PPD. Also such cultures lacked plaque forming cells, suggesting that the B cells were unaffected by Con A. Perhaps a longer incubation is required in the present study, or factors can be released without the transformation into blast cells.

DISCUSSION

Most investigations of cell-mediated responses in teleosts have been centred around the rejection of grafts (Hildemann & Haas, 1960; Rijkers & Van Muiswinkel, 1977; Botham, Grace & Manning, 1980). However, cellular immunity also plays a major role in delayed hypersensitivity. The discovery

by Rich and Lewis (1932) that migration of cells from splenic explants from hypersensitive guinea pigs was specifically inhibited by antigen, has served as the basis for a quantitative in vitro assay of delayed hypersensitivity. The technique of placing cells in capillary tubes and observing the effect of a test material on their migration out of the tube on to glass was introduced by George and Vaughan (1962). They found consistent inhibition of migration of sensitized peritoneal exudate cells in the presence of antigen, but rather inconsistent responses from spleen cells. This technique was further refined (David, Al-Askari, Lawrence & Thomas, 1964; Bloom & Bennett, 1966) and enabled intense research into the in vitro study of cellular hypersensitivity. These experiments showed that in mammals, T lymphocytes bearing specific receptors on their surface, are stimulated by contact with antigen to release factors which mediate delayed-type hypersensitivity. Many of these factors act predominantly on macrophages, such as macrophage migration inhibition factor, macrophage activating factor, macrophage aggregation factor and chemotactic factors.

Peritoneal exudate cells are not always easily obtainable, for example in human studies and in the present investigation. Sjøborg and Bendixen (1967) have described a method for detection of cellular hypersensitivity in man using leucocytes from the peripheral blood, and this technique, of in vitro leucocyte migration inhibition, has shown excellent correlation with delayed hypersensitivity to tuberculosis using soluble PPD (Rosenberg & David, 1970).

In poikilotherms the ease with which peritoneal exudate cells are obtained appears to vary from class to class. Peritoneal exudate cells have been used to show migration inhibition in anuran amphibians (Ambrosius & Drossler, 1972; Drossler & Ambrosius, 1972; Rimmer & Gearing, 1980) and elasmobranchs (Morrow, 1978). Other workers using reptiles (Jayaraman & Muthukkaruppan, 1977, 1978_{a,b}), anurans (Ambrosius & Drossler, 1972; Drossler & Ambrosius, 1972) and urodeles (Tahan & Jurd, 1979) have used spleen cell suspensions. Whilst workers using fish have in general had to use pronephros cell suspensions (Jayaraman, Mohan & Muthukkaruppan, 1979) or peripheral blood (McKinney, Ortiz, Lee, Sigel, Lopez, Epstein & McLeod, 1976; O'Neill, 1978).

One problem encountered using human peripheral leucocytes (Sjoberg, 1968) or spleen fragments from guinea pigs (Svejcar, Johanovsky & Pekarek, 1966), was that if low antigen concentrations were used, particularly if the cells were of a low to moderate degree of sensitivity, a stimulation of cell migration was seen. However, higher antigen concentrations and cells of high sensitivity, led in all cases to inhibition of cell migration. Perhaps this may be one explanation for the large individual variations seen in the present study. Animals with varying degrees of sensitivity would show varying degrees of inhibition, and possibly even stimulation. Unfortunately little is known about delayed hypersensitivity in fish and most of this work involves a delayed hypersensitivity manifested as cutaneous erythema. Only in the paddlefish (Finstad & Good, 1966), in rainbow trout (Ridgway,

Hodgins & Klontz, 1966) and in the later stage of granulomatous diseases in the plaice (Timur, Roberts & McQueen, 1977) has delayed hypersensitivity been demonstrated, coinciding with the development of a positive macrophage migration inhibition in the plaice (Timur, 1975). In Ambystoma, where delayed cutaneous hypersensitivity has been demonstrated, only two-thirds of axolotls were shown to be sensitive to PPD after identical injection schedules with FCA (Tahan & Jurd, 1979). Although this variation of sensitivity may be one explanation for high individual variation, it should be remembered that relatively high antigen concentrations were used in the medium in the present study, which eliminated the effects due to weakly sensitized cells.

A major criticism of using cell suspensions containing relatively few macrophages has been that white cells other than macrophages migrate from capillaries purely by gravity, or are inhibited in their migration by factors other than migration inhibition factor, such as antigen-antibody complexes (Kaltreider, Soghor, Taylor & Decker, 1969; Nordqvist & Rorsman, 1970). A modification to measure only the macrophage fan area of mixed cell suspensions was designed by Nordqvist and Rorsman (1970). After 96 h of incubation the migration chambers are everted to free non-sticky cells from the surface and the migration of the adherent cells is then measured after a further 6 h. This showed that the outline of the migration area of human peripheral blood was normally formed by polymorphonuclear leucocytes, but that after 96 h these had died and were

removed when the chamber was everted. They concluded that leucocyte migration and peritoneal exudate cell migration were not strictly comparable. However, even if one measures the migration of only macrophages due to macrophage migration inhibition factor, whilst the other measures the migration of a mixture of leucocyte types due to a leucocyte migration inhibition factor, if both show correlation with delayed hypersensitivity, which they appear to, they are comparable.

The cell suspensions in the present investigation contain few macrophages, yet inhibition was obtained and in all cases the cells at the periphery of the fan were still adherent after 96 h of incubation.

The possibility of inhibition induced by antigen-antibody complexes, caused by either direct inhibition of neutrophils or indirect physical obstruction of cells migrating out by agglutinated cell complexes, cannot be excluded. This is particularly a problem when using antigens which give good antibody responses, such as HGG or SRBC's, to look for in vitro delayed hypersensitivity of mixed leucocyte cell populations. A strong argument against immune complex inhibition is the obvious inverse relationship of antibody titre to migration inhibition. In the present study, injection of HGG in FCA causes rising antibody titres between weeks 2 and 3, with maximum titres usually occurring at week 4. Immune complexes would seem to be most prevalent between weeks 3 and 4 when trapping of antigen can be demonstrated (Chapter 4). The only detectable inhibition

of leucocyte migration using HGG occurred within the first 2 weeks after immunization, before antibody titres have really started to increase. Other workers with poikilotherms have also shown an inverse relationship between antibody levels and leucocyte migration inhibition (Jayaraman & Muthukkaruppan, 1978a,b; Jayaraman, Mohan & Muthukkaruppan, 1979). These authors also noted that both plaque forming cell and migration inhibition responses were inversely proportional to the amount of antigen injected, such that low antigenic stimuli gave high levels of migration inhibition with an insignificant number of plaque forming cells, whilst higher doses gave increasing numbers of plaque forming cells and a suppression of the migration inhibition level. This is very interesting when considered with the antibody responses mentioned in Chapter 5. Small doses of antigen given intramuscularly in fish show good migration inhibition responses, but low plaque forming cell responses (Rijkers, Frederix-Wolters & Van Muiswinkel, 1980a). Yet secondary immunization results in levels of plaque forming cells as high as the secondary responses from optimal antigen dose primed animals. Similarly, in mammals, when minute doses of antigen are given as a primary stimulus, often delayed hypersensitivity alone develops. This, nevertheless, serves as a primary stimulus for a secondary antibody response when a larger antigenic challenge follows (Salvin & Smith, 1960). It is as if the development of cell-mediated immunity can elicit T-helper memory cells, which are then able to affect an anamnestic response. It is even possible that both helper activity and delayed hypersensitivity

is expressed by the same cell (Liew & Parish, 1974). Indeed, the low level of migration inhibition at the peak of plaque forming cell production to SRBC's has been attributed to the nonavailability of T cells for cell-mediated immunity, since substantial numbers of T cells are involved in the helper function for antibody synthesis to the same antigen (Gordon & Yu, 1973).

In animals injected with FCA alone, significant inhibition of blood and pronephros leucocytes was similarly seen early on, on days 7 and 14. Studies on allograft rejection carried out on carp held at the same temperature in our laboratory (Botham, Grace & Manning, 1980) show that graft rejection, as visualised by lymphocyte invasions, begins at 4 days postgrafting; breakdown of the dermal layer of the graft is seen by day 8 and by day 14 the end point of the reaction is seen and the graft is now totally white. Taken together with the migration inhibition data, it would seem that the duration of cell-mediated immunity is in the region of 7 to 14 days, and that a latent period of about 3 days occurs after a primary antigenic exposure. Further exposure to antigen results in accelerated responses, which in the case of delayed hypersensitivity peak at about 18 to 24 h if contact with antigen occurs during the reactive period, presumably related to the degree of sensitization of the cells. If only low antigen concentrations occur after a certain time period, this probably results in cells of low sensitization. It would be of interest to know if a second immunization schedule results in accelerated responses

and a shorter 'sensitized' time period, as occurs after second set grafting.

The inhibition caused by incubation with either PHA or Con A was quite substantial. It is well known that such mitogenic agents have the ability to stimulate lymphocytes nonspecifically, and many soluble lymphocyte products have been detected after incubation with PHA or Con A in mammals, such as migration inhibition factor (Pick, Brostoff, Krejci & Turk, 1970; Lamelin & Vassalli, 1971), skin-reactive factor (Pick, Krejci & Turk, 1970; Schwartz, Leon & Pelley, 1970), lymphotoxin (Granger & Williams, 1968) and a substance resembling interferon (Pick & Turk, 1972). Only one such study has been carried out in poikilotherms (McKinney, Ortiz, Lee, Sigel, Lopez, Epstein & McLeod, 1976), where migration inhibition in gar cells and cytotoxicity in catfish lymphocytes were induced by PHA and Con A. However, although such lymphocytes produce active factors, it is not known if these are identical with the factors resulting from antigen-stimulated lymphocytes. The release of soluble lymphocytic materials, by non-antigenic stimulants, do appear to be the result of a synthetic process, and thymectomy followed by a course of anti-lymphocyte serum suppresses the release of migration inhibition factor in vitro by guinea pig lymph node cells incubated with either specific antigen or Con A (Pick & Turk, 1972). So it is probable that T-lymphocytes are involved in some step leading to the secretion, or possibly in the actual secretion, of both antigen and mitogen-induced migration inhibition factor.

In the present experiment, the possibility of cell agglutination at the end of the tube preventing the migration of cells, could not be excluded. So the experiment to nonspecifically stimulate the cells, to remove both the cells and the mitogen, and to see if the supernatant obtained possessed migration inhibition factor, was designed. It has been shown by several authors that Con A can be removed from supernatants before in vivo testing, by passing them through Sephadex which binds Con A (Pick, Brostoff, Krejci & Turk, 1970; Schwartz, Leon & Pelley, 1970). This method was adopted and some supernatants, particularly from stimulated blood leucocytes, did seem to contain a factor capable of inhibiting leucocyte migration. So the inhibition seen when PHA or Con A are added directly to the medium may be due to the release of a migration inhibition factor, rather than cell agglutination. A stimulatory effect was also seen with some supernatants, and may reflect a sequential release of several factors from cells in different parts of the body, depending on the strength of stimulation.

The correlation between the inhibition of migration of leucocytes or peritoneal exudate cells and cell-mediated immunity, in the species of poikilotherms investigated, seems very good. In both fish and urodeles migration inhibition has been shown to occur in animals exhibiting delayed hypersensitivity (Timur, 1975; Tahan & Jurd, 1979). Similarly, in mammals, migration inhibition has also been shown to be a good in vitro measurement of transplantation immunity (Falk, Collste & Moller, 1969; Al-Askari & Lawrence,

1972, 1973; Kuramochi, 1974) and the same is true in poikilotherms, where migration inhibition has been shown to be a valid measurement of alloantigen recognition (Jayaraman & Muthukkaruppan, 1977).

So even though few studies have been undertaken in the various classes of poikilotherms, it would appear that cellular hypersensitivity, as exhibited by migration inhibition studies and delayed hypersensitivity, is present even in the fishes. The presence of migration inhibition responses in fish, amphibians and reptiles suggests that such a factor arose in the ancestors of these vertebrate classes. Indeed cellular immunity in vertebrates may well have arisen from pre-existing capabilities already present in the invertebrates, since graft rejection in sea stars (Karp & Hildemann, 1976) and sea squirts (Reddy & Bryan, 1974) is very similar to that in vertebrates. Further, in sea stars, a purified protein with a molecular mass of 32,000 daltons, can cause agglutination of coelomocytes of the sea star and also inhibit migration of guinea pig macrophages (Prendergast & Suzuki, 1970). It would seem quite likely that the basic immune apparatus of vertebrates, as exhibited in fish, arose from these more ancient defence mechanisms, supplemented by nonspecific phagocytosis and non-immunoglobulin factors.

Chapter 7THE EFFECT OF THYMECTOMY AND EARLY
IMMUNIZATION IN RAINBOW TROUT

It will be apparent from the work described in Chapter 5 that carp are not an ideal species on which to study the effect of thymectomy, neither is it easy to immunize the fry before they begin to feed. A species with a relatively accessible thymus and reasonably large-size fry was found in the rainbow trout Salmo gairdneri Rich. 1836. Here the thymus lies underneath the operculum on its dorsal edge, and can be easily reached by simply lifting up the operculum. As so little is known about thymectomy in fish, the effect of both adult and young thymectomy is of interest.

The fry hatch after 15 days at 14°C, and although a thymic primordium has appeared before hatching (Grace & Manning, 1980), the fish do not begin to feed for a further 14 days and may not be immunologically competent until then. This coupled with the large size of the fry at hatching thus make them suitable to study the onset of immunocompetence, and to see whether tolerance can be induced by injection of antigens before some critical stage of development.

A Normal Table is also available for this species (Vernier, 1969), as is a study on the histogenesis of the lymphoid organs (Grace & Manning, 1980), enabling the exact state of maturity of the animal at the time of operation or immunization to be known.

Owing to a limitation on holding facilities fish could not be reared long enough to be able to collect enough

serum to sample from individual fish. Thus sera from early immunized animals were, by necessity, pooled. However, this requires large numbers of animals, and as these were not available with young thymectomized trout, an alternative test was sought. Autoradiography was chosen, where individual organs could be tested for their proliferative activity after immunization, allowing smaller groups to be investigated.

Adult thymectomized trout were tested using agglutination techniques on sera from individual fish. The migration inhibition test, using PHA to stimulate non-specifically blood leucocytes, was also used, to see if adult thymectomy affected the degree of inhibition. The inhibition observed using this test has not yet been shown conclusively to be due to the release of lymphokines from stimulated T cells, or functionally equivalent cells. However, one of the possible controls to help to elucidate the mechanisms involved is to use thymectomized animals. If adult thymectomy has an effect on this response a decreased inhibition of migration would be expected.

MATERIALS AND METHODS

A. Effect of thymectomy

1. Operational procedure

(a) Adults

Adult rainbow trout, aged 1 year, were anaesthetized in MS222 (0.125 g/l) and placed under a foot focusing binocular microscope. The operculum was lifted and the thymus cauterized using a Martin-Elektrotom 60 high frequency cautery apparatus,

in which the positive electrode was a fine tungsten needle. Once the needle is positioned the apparatus is turned on. The high frequency current causes coagulation of the tissue in contact with the positive electrode, as witnessed by a whitening of the destroyed cells. The dead thymic tissue can then be scraped away and the operation repeated on the opposite side. Once the operation is complete, the animals are returned to clean, well aerated, standing water to recover.

A pilot experiment was first carried out attempting unilateral thymectomy. Such animals were killed and the head region sectioned and stained, to ensure the thymus was being removed. Sham-thymectomies were also performed, where instead of the thymus a small portion of tissue on the underside of the operculum was cauterized. Bilaterally thymectomized and sham-thymectomized animals were left for 4 weeks before immunizing or testing in any manner. Once the experiments were completed, thymectomized animals were sectioned through the thymic region for staining with methyl green-pyronin, to see if any regeneration had occurred.

(b) Fry

Thymectomy was attempted in fry in a similar manner to the above, at various ages after hatching. The main difference was the setting of the cautery apparatus, which had to be at a somewhat lower setting than for adult thymectomy and in fact was set at minimum on the Elecktrotom 60 apparatus. Also, sham-thymectomy could not be performed under the operculum because in such small animals the thymus would be affected. So equal areas of skin, at the same level as

the thymus, were cauterized further down the body.

2. Testing

(a) Adults: agglutination and migration inhibition tests

All groups contained 6-8 fish.

1. Immunization with *Aeromonas salmonicida*. Four weeks after the operations thymectomized, sham-thymectomized and unoperated animals were injected intraperitoneally with formalin-killed *A. salmonicida*. The dose was the same as for the carp, 1×10^8 cells/g fish. *A. salmonicida* can be a serious disease of salmonids and was chosen for investigation to determine its thymus-dependency in these fish. The animals were bled at weekly intervals for 8 weeks and the serum assayed using the tube agglutination technique described in Chapter 5.

2. Immunization with HGG in FCA. Four weeks after the operations thymectomized and sham-thymectomized animals were injected intraperitoneally with HGG in FCA. Again the dose used was the same as for the carp, 0.025mg HGG/g fish. HGG is a classic T-dependent antigen, and in mammals it has been shown that such antigens have reduced secondary responses after adult thymectomy (Simpson & Cantor, 1975). Thus it was decided to investigate the secondary responses to HGG in FCA in these adult thymectomized trout, to see if they too were diminished. Animals were bled at fortnightly intervals for 8 weeks, when they were given a booster injection at the same dose and tested for a further 8 weeks. All sera were assayed using the passive haemagglutination technique and by double diffusion in agar, as previously

described, using 1% normal trout serum as the diluent instead of the normal carp serum used in the passive haemagglutination tests described in Chapter 5.

3. Effect of mitogen on cell migration.

Four, eight and twelve weeks after the operations thymectomized, sham-thymectomized and unoperated animals were bled, using sterile heparinised syringes. These samples were used to separate off the leucocytes for migration inhibition tests, employing PHA to nonspecifically stimulate these cells. The method used was identical to that described for carp in Chapter 5, using 5 to 6 animals per group. The inhibition ratios were also calculated in the same manner, and compared to both unoperated and sham-operated groups using the t-test corrected for small samples.

(b) Fry: autoradiography

Depending on the age at which thymectomy was performed, fry were immunized intraperitoneally with HGG in FCA 4 to 6 weeks after the operations. These animals, with unoperated controls, were used for autoradiography.

In carp kept at 22°C, the peak proliferative response after a single injection of HGG in FCA, as seen in methyl green-pyronin stained sections, occurs at about week 3. The trout were kept at lower temperatures, 14°C to 17°C, and so would be expected to respond more slowly. Thus weeks 4 and 6 after immunization were chosen as the times at which to kill the animals.

The use of tritiated thymidine as a valid marker for biosynthesizing cells is well established. This label is incorporated specifically into DNA and the very low energy

and low penetrating power of the tritium beta radiation permits precise localization on autoradiographs, to within less than 1μ , of the atom that disintegrates (Evans, 1966). The dose of the radioisotope appears to be critical. It must not be so large that it causes radiation damage or disturbs normal metabolism. On the other hand, enough must be injected to produce an autoradiographic image. The quantity chosen for injection was based upon previous experience in this laboratory using the amphibian Xenopus laevis (Turner, 1970). Toads injected at a rate of $1 \mu\text{c/g}$ body weight showed no cell abnormalities or damaging effects at this dosage.

A stock solution of tritiated thymidine (Radiochemical Centre, Amersham, UK) containing a specific activity of 2 Ci/mM (1 mCi/ml) was stored at 4°C . Shortly before use it was diluted to $100 \mu\text{c/ml}$ with 0.85% saline and was injected intraperitoneally into the fish at a rate of $10 \mu\text{l/g}$ fish ($1 \mu\text{Ci/g}$ fish) 18 h prior to sacrifice.

Once the fish was killed the spleen, mesonephros, pronephros (if large enough) and thymic area were removed and fixed in Carnoy's fluid. These were embedded in paraffin wax, sectioned and mounted on gelatin-coated slides. The dipping technique using liquid emulsion was employed, following the general recommendations of Rogers (1967). This technique facilitates large numbers of samples, so that only two batches had to be run. Thus differences in labelling resulting from different conditions of exposure and processing, were kept to a minimum. Control animals

which had not received tritiated thymidine were also included in both batches. Prior to dipping slides were dewaxed in xylene and hydrated. Under safelight conditions (Ilford S902) the Ilford K5 emulsion was melted in a 43°C water bath, diluted 1:1 with distilled water and 1% v/v glycerine added. Slides were dipped individually into emulsion for a few seconds, withdrawn slowly and the backs wiped. They were then placed horizontally on stainless steel trays, over ice baths, in a stream of cold air for the emulsion to solidify. Once dry the slides were stored in the dark at 4°C for 4 weeks, in boxes containing sachets of calcium chloride. After exposure the slides were placed into distilled water, again under safelight conditions, developed in Kodak D19 at 20°C for 4 min, rinsed quickly in distilled water and fixed in 1 + 5 Amfix high speed fixer (May and Baker Ltd., Dagenham, UK). They were then washed thoroughly in running tap water before staining in methyl green-pyronin and mounting in Depex (Searle, High Wycombe, UK). Methyl green-pyronin staining was carried out as for normal slides. For scoring each organ had the labelled cells counted from 10 fields of view under a x40 objective. The results were analysed using the Student's t-test, corrected for small samples.

B. Early immunizations

Trout fry were injected intramuscularly (the only route possible) on day 1, 7, 14 or 21, with HGG in saline, HGG in FCA, BSA in saline, BSA in FCA, A. salmonicida in saline, or just saline. A minimum of 100 animals per batch were

injected. Eight weeks after the immunization pooled serum samples were tested for agglutinating antibodies using the micro-passive haemagglutination and tube agglutination techniques employing V-shaped microtiter wells (see Chapter 5). The remaining fish were injected with A. salmonicida (if injected with A. salmonicida initially) or with both BSA in FCA and HGG in FCA (if initially injected with one of the remaining combinations). At the time this experiment was carried out CGG was not available, and BGG was known to cross-react with HGG. Equine gamma globulin (EGG) is known to display cross-tolerance to HGG (Chiller, Louis, Skidmore & Weigle, 1974), so it would not be surprising if the same held true for BGG. So a choice of BSA or OVA was available from our laboratory stocks as an alternative soluble protein antigen to HGG and of these BSA was chosen because of the extensive studies using this antigen for tolerization in both poikilotherms and mammals (Mitchison, 1965; Marchalonis & Germain, 1971; Avtalion, Wojdani, Malik, Shahrabani & Duczyminer, 1973). These fish were tested after a further 8 weeks as before, and their antibody titres to both HGG and BSA, or to A. salmonicida, determined.

RESULTS

A. Effect of thymectomy

(a) Adult thymectomy

Adult rainbow trout thymectomized at 1 year of age showed very good survival, usually 80 to 100%. The pilot experiments using unilateral thymectomy were encouraging, almost no remnants being discernible on the cauterized side (see Plate 50).

1. Immunization with Aeromonas salmonicida

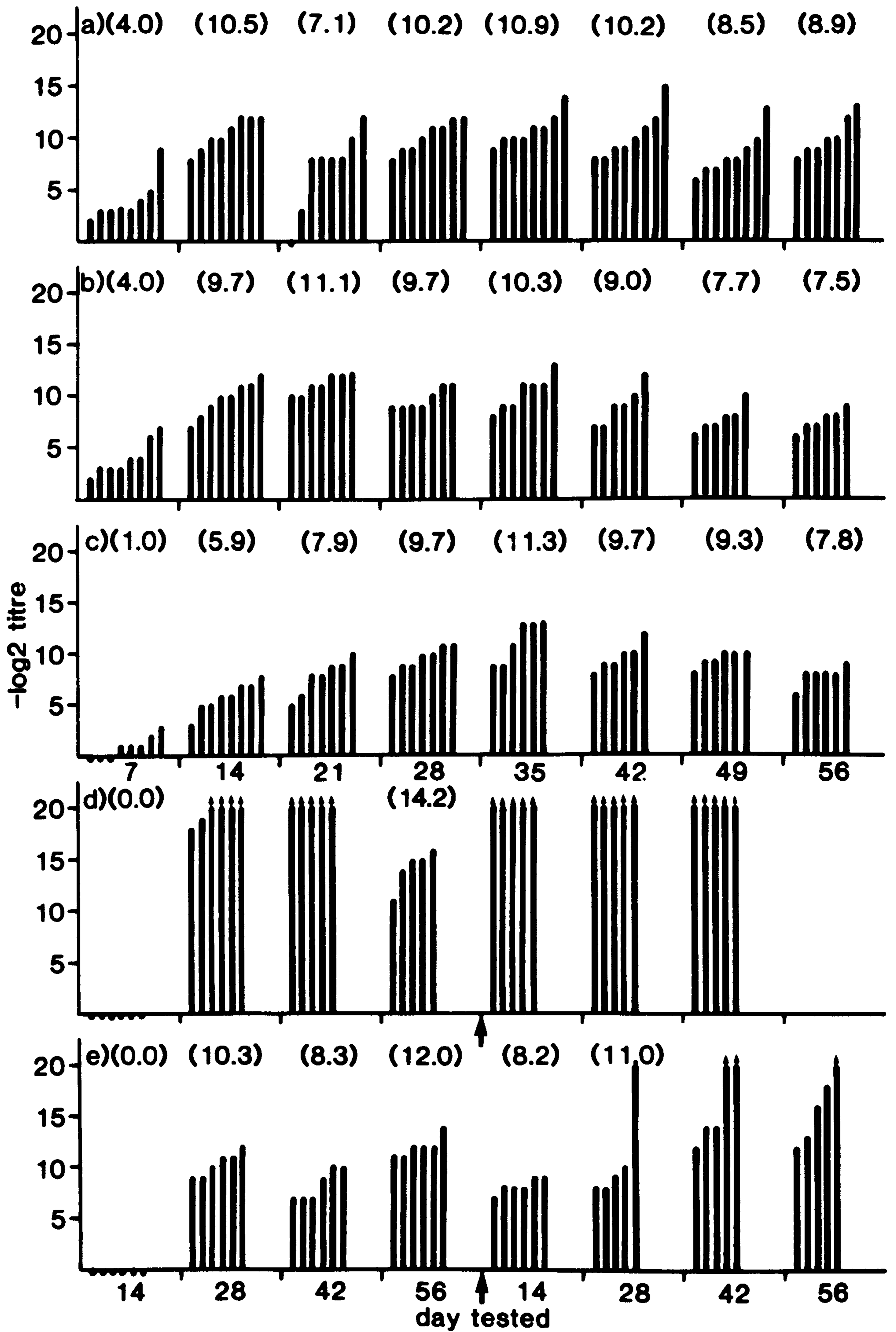
The agglutinating response to a single injection of A. salmonicida (Figure 17) appears to be almost identical in

Figure 17

Agglutinating antibody titres of sera from adult thymectomized and sham-thymectomized trout.

- (a) Tube agglutination titres of sera from adult thymectomized rainbow trout injected with A. salmonicida in saline.
- (b) Tube agglutination titres of sera from sham-thymectomized rainbow trout injected with A. salmonicida in saline.
- (c) Tube agglutination titres of sera from unoperated rainbow trout injected with A. salmonicida in saline.
- (d) Passive haemagglutination titres of sera from adult thymectomized rainbow trout injected with HGG in FCA.
- (e) Passive haemagglutination titres of sera from sham-thymectomized rainbow trout injected with HGG in FCA.

Each column represents one individual. Means in parentheses.



†, Titre exceeded well 20; ↑, booster given.

the thymectomized, sham-thymectomized and unoperated groups. This would either indicate that adult thymectomy has no effect on the primary response in trout, or that A. salmonicida is a T-independent antigen in such animals. Unlike the carp experiment there was no enhanced response.

Sections through the head region of the thymectomized animals revealed that only in one individual was a small thymic remnant present, on the right side only. In all animals the pronephros was very pyroninophilic, and contained pigment.

It is clear from this experiment that sham-thymectomy has no effect on the agglutinating response when compared to unoperated animals.

2. Immunization with HGG in FCA

The responses to HGG in FCA (Figure 17 and Table XXXIII) do seem to show differences between thymectomized and sham-operated animals.

TABLE XXXIII

Precipitating antibody responses of sera
from animals injected with HGG in FCA.
Each symbol represents one individual.

Day tested	PRIMARY RESPONSE				SECONDARY RESPONSE			
	14	28	42	56	14	28	42	56
Thymec- tomized	---	---	††††	††††	††††	††††	††††	†††
Sham- Thymec- tomized	---	---	---	---	+++	---	---	---

†, very strong response; +, strong response; † weak response;
-, no response.

Thymectomized animals appear to have enhanced responses in both the agglutinating and precipitating antibody response. It is realised that with such small numbers of individuals care should be taken in drawing such conclusions, however, this enhanced response does occur during the primary as well as the secondary response, giving a total of four instances where it can be seen. It is of interest to note that the response in the sham-thymectomized animals occurs much later than that seen in response to A. salmonicida. Possibly this reflects a difference between soluble and cellular antigens, or alternatively it is possible that the fish may have already had contact with A. salmonicida. These fish were bought in and were not reared in the laboratory from A. salmonicida-free eggs. Another point of interest with respect to this, is that in nearly every fish tested for anti-A. salmonicida antibodies false negatives were found in the first few wells. This is probably caused by the stabilizing effects of high protein concentration on the particles, the protein coating the particles increasing their net charge and so bringing about increased electrostatic repulsion between individual particles, thus opposing the efforts of the antibody molecules to link the particles together. This is known as the prozone phenomenon (Weir, 1973). Once the protein concentration is reduced by dilution the antibody molecules can then exert their aggregating effect and bring about agglutination. This phenomenon was not seen during the responses by carp to A. salmonicida, so possibly the trout have responded in a much stronger manner to this antigen, or they may have already been primed at some

earlier time. Whether this explains the lack of an enhanced effect in trout compared to carp to this thymus-independent antigen is not clear.

3. Effect of mitogen on cell migration

As can be seen in Table XXXIV, all groups investigated gave marked inhibition in the presence of PHA, comparable to that seen with carp peripheral blood leucocytes.

TABLE XXXIV

Mean inhibition ratio using peripheral blood leucocytes from thymectomized and sham-thymectomized animals, migrating in medium containing 200 μ g PHA/ml

Week tested	Tx	Tx + tube	Sham	Sham + tube
4	0.09 \pm 0.04 \oplus	0.17 \pm 0.05*	0.16 \pm 0.02+	0.21 \pm 0.06*
8	0.06 \pm 0.04*	0.14 \pm 0.03*	0.15 \pm 0.12+	0.18 \pm 0.15
12	0.09 \pm 0.05*	0.22 \pm 0.03*	0.18 \pm 0.15	0.27 \pm 0.14

Unoperated animals 0.27 \pm 0.01

+ tube 0.31 \pm 0.04

Absence of asterisk indicates no significant difference.
 *, Significantly different from unoperated animals at the 1% probability level; +, significantly different from unoperated animals at the 5% level; \oplus , significantly different from sham-thymectomized animals at the 1% probability level.

There seems to be little difference between the groups tested at 4, 8 and 12 weeks after the operations. However, both thymectomized and sham-operated animals show significantly greater inhibition than seen in unoperated animals, particularly at week 4 in the sham-operated group. Only in

one group does there appear to be a significant difference between thymectomized and sham-thymectomized animals, also at week 4. Possibly stress from the operation is having an effect, but why it should cause greater inhibition is not known.

(b) Thymectomy of young fish

1. Survival

Thymectomy was attempted on rainbow trout fry for two consecutive breeding seasons, 1978/79 and 1979/80. The 1978/79 batch of thymectomized trout died from white spot Ichthyophthirius multifiliis. Thymectomy was tried again in the 1979/80 breeding season, when thymectomy was performed at day 14 and at day 56. Survival was poor for the group thymectomized at day 56, all animals were dead within 6 weeks, so all the data in the results refers to animals thymectomized on day 14. These animals were injected intraperitoneally with HGG in FCA on day 56, 6 weeks later. Mortalities due to the operation were mostly due to the immediate trauma or to the rupture of blood vessels adjacent to the thymus. Mortalities after the operation were no greater than with unoperated fish. No runting was seen and the fish appeared to be very healthy. Fish were tested on weeks 4 and 6 after immunization, but due to the small numbers of thymectomized fish available no controls injected with FCA only were included. Nevertheless, any differences between the thymectomized and sham-thymectomized animals would be due to T-dependent phenomena.

2. Autoradiography

The results of this experiment are summarized in Table XXV. In small fish, less than 1 g, it was not always technically possible to remove the pronephros, so results for this organ are rather sporadic. The thymectomy operations were, in general, quite effective, with only very small portions of tissue (if any) regenerating. The results for the thymectomized animals were calculated in two groups, A for all the animals initially thymectomized, and B for animals with no thymic remnants at all. Cells containing silver grains were easily distinguished from cells containing pigment in all organs (Plates 51 and 52).

At 4 weeks after immunization both the sham-thymectomized and thymectomized animals show a significant increase above the non-immunized control values in the number of actively dividing cells in the spleen. This effect is also seen in the mesonephros of the sham-thymectomized animals and the thymectomized group as a whole. By week 6 this effect has waned in both sham-thymectomized and thymectomized groups. In the sham-thymectomized and thymectomized group A at week 6, the pronephros was obtained from several animals, and was smaller in the latter in the number of dividing cells compared to the sham-thymectomized group. However, although not significantly different from control animals, both groups had far smaller mean values. This effect has also been seen in carp (Botham, personal communication), and may be influenced by the intense lymphopoietic, granulopoietic and haemopoietic functions of this organ. The spleen and mesonephros of the control animals at this age are more

TABLE XXXV

Mean autoradiography counts from rainbow trout fry thymectomized on day 14 and injected with HGG in FCA on day 56. Numbers in parentheses indicate the number of individuals in that group

	Day after immunization		Combined data
<u>Control</u>	(1)	(11)	(1 + 11)
	28	42	
Spleen	24.8 ± 18.6 (5)	54.2 ± 28.7 (5)	39.5 ± 26.6 (10)
Pronephros	N.T.	233.5 ± 208.6 (2)	233.5 ± 208.6 (2)
Mesonephros	12.0 ± 4.8 (5)	43.2 ± 19.1 (5)	27.6 ± 21.0 (10)
Thymus	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (10)
<u>Sham-thymectomized</u>			
Spleen	111.7 ± 57.1* (4)	124.2 ± 93.8 (5)	118.7 ± 75.3* (9)
Pronephros	N.T.	98.0 ± 30.6 (3)	98.0 ± 30.6 (3)
Mesonephros	51.0 ± 13.0* (5)	60.8 ± 35.6 (5)	55.9 ± 25.8+ (10)
Thymus	0.2 ± 0.4 (5)	0.0 ± 0.0 (5)	0.1 ± 0.3 (10)
<u>All animals initially Thymectomized: A</u>			
Spleen	77.4 ± 23.3* (5)	56.0 ± 26.8 (5)	66.7 ± 26.3+ (10)
Pronephros	N.T.	40.0 ± 2.8 (2)	40.0 ± 2.8 (2)
Mesonephros	32.8 ± 12.3* (5)	32.4 ± 10.8 (5)	32.6 ± 10.9 0 (10)
Thymus	1.0 ± 1.7 (3)	0.0 ± 0.0 (2)	0.2 ± 0.4 (5)

Completely
Thymectomized
animals: B

Spleen	73.5 ± 10.6 (2)	43.0 ± 7.2 (3)	55.2 ± 18.2 [●] (5)
Pronephros	N.T.	N.T.	N.T.
Mesonephros	35.0 ± 18.4 (2)	32.0 ± 9.5 (3)	33.2 ± 11.5 [●] (5)

N.T., Not tested, too small to remove; *, significantly different from unoperated animals at the 1% probability level; +, significantly different from unoperated animals at the 5% probability level; ●, significantly different from sham-thymectomized animals at the 5% probability level. Absence of asterisk indicates no significant difference.

active compared to the numbers 2 weeks earlier, so perhaps the fish start to increase rapidly in size or in cell numbers at this stage. Taking the results overall, it appears that after immunization there is a significant increase in the numbers of dividing cells in the spleen, and mesonephros of sham-thymectomized animals. This increase is also seen in the spleen of the thymectomized group A, but the mesonephros gave significantly lower counts in the thymectomized than in the sham-thymectomized animals. When looking only at animals with no thymic remnant at all (group B), no significant increases compared to non-immunized control animals occur at all, indeed the spleen and mesonephros are significantly smaller than in the sham-thymectomized animals. By combining the data for the two times after immunization (to give larger replicates) the decreased response in the thymectomized group becomes significant, indicating that thymectomy at 2 weeks of age impairs rainbow trout responsiveness to HGG in FCA.

B. Early immunizations

Survival of the delicate fry from immunization until testing was in general very good, ranging from 50% if injected on day 1, to 90% if injected on day 21. Mortality soon after inoculation was usually due to rupture of the dorsal aorta, where the needle had been inserted too close to it.

The results of immunizing at different ages after hatching and testing 8 weeks later are shown in Table XXXVI. No responses to soluble antigens were obtained even when injected 21 days after hatching, in adjuvant. However,

TABLE XXXVI

Serum antibody titres of rainbow trout fry, tested 8 weeks from immunization, expressed as $-\log_2$ titre. Each figure represents a pooled sample from 10 fish

Antigen injected	Day injected after hatching			
	1	7	14	21
HGG in saline	0,0	0,0	0,0	N.T.
HGG in FCA	0,0	0,0	0,0	0,0
BSA in saline	0,0	0,0	0,0	N.T.
BSA in FCA	0,0	0,0	0,0	N.T.
<u>A. salmonicida</u>	0,0	0,0	0,0	3,3

N.T., Not tested.

TABLE XXXVII

Serum antibody titres of rainbow trout fry given a booster injection of HGG in FCA and BSA in FCA 8 weeks after the first injection, and tested 8 weeks later. Titres expressed as $-\log_2$ titre. Each figure represents a pooled sample from 10 fish

Antigen initially injected	Day initially injected after hatching					
	1		7		14	
	HGG	BSA	HGG	BSA	HGG	BSA
HGG in saline	0	0	0	0	0	0
HGG in FCA	0	0	0	0	0	0
BSA in saline	2	0	3	0	2	0
BSA in FCA	4	0	6	0	6	0
Saline	ALL DEAD		ALL DEAD		ALL DEAD	
<u>A. salmonicida*</u>	3,3		3,3		3,3	
Saline*	3,4		ALL DEAD		ALL DEAD	

*, Animals given a booster injection of A. salmonicida.

responses at this timing could be detected against the cellular antigen, A. salmonicida, although the titres were somewhat lower than those observed in 1 year fish. Responses after the second immunization are given in Table XXXVII. No enhanced effect is seen after a second injection of A. salmonicida, the titre remaining very constant at 1:8. Unfortunately, every animal injected with saline died. A similar phenomenon has been observed in brown trout by Ingram and Alexander (1980), who proposed that deaths could be due to a pyrogen which was inhibited in the presence of the antigen being investigated. Without these controls only tentative comments can be made about the remaining results. It would appear that animals injected with BSA, whether in adjuvant or saline, and whether given in the first and second injection or only in the second, do not respond. This is not too surprising as being a relatively low molecular weight protein, unless the animal is fully immunologically competent it may well not be able to respond to it. In fact rainbow trout in general have been shown to be poor responders to murine T-dependent antigens, such as SRBC's and DNP conjugated to KLH, HGG or BSA (Chiller, Hodgins, Chambers & Weiser, 1969; Etlinger, Chiller & Hodgins, 1979). It is possible that these animals have become tolerant to BSA, even those injected at 10 weeks after hatching. This could have been tested at a later date had some of the saline injected animals survived.

In contrast to the results using BSA, some antibodies against HGG were detected. Animals initially injected with

HGG gave no response on subsequent challenge, but animals initially injected with BSA, whether in adjuvant or saline, did give a titre when challenged with HGG. In as much as the BSA injected animals are serving as controls for the HGG injected animals, a system where tolerance has been induced in the animals initially injected with HGG could be envisaged. The slightly higher titres in animals injected with adjuvant initially may be due to some kind of activation, where upon subsequent antigen challenge has led to a larger response. Without the controls to show the response to HGG in previously unimmunized animals it is not fully justifiable to say that tolerance has been induced instead of some helper effect of BSA on the HGG response. However, it should be remembered that a significant increase in actively dividing cells was observed in the kidney and spleen of thymectomized and sham-thymectomized rainbow trout fry after immunization with HGG in FCA at 8 weeks after hatching, the same time that the first group received their second injections.

DISCUSSION

Adult thymectomy would seem to have an effect in trout, primarily on a type of T cell involved in the regulation of the immune response rather than on the cells involved directly in mounting a cellular response. This is demonstrated by the enhanced antibody responses to HGG after adult thymectomy, whereas the cellular responses, seen in the migration inhibition studies, remain relatively unaffected compared to sham-operated animals. Adult thymectomy also has

no effect on first or second set allograft rejection times in trout (Botham, Grace & Manning, 1980). So it would appear that only a suppressor T cell function has been lost. Adult thymectomy does not decrease the antibody response to A. salmonicida and so in trout, as well as in carp and in Xenopus laevis, it can be considered to be a thymus-independent antigen.

In the only other teleost species where thymectomy has been investigated, Tilapia mossambica, relatively long-term adult thymectomy abrogates both the primary and secondary haemolytic response to SRBC's, but not the responses to polyvinyl pyrrolidone (Jayaraman, Mohan & Muthukkaruppan, 1979) and if young thymectomy was performed the response to SRBC's was totally suppressed and allograft survival prolonged (Sailendri, 1973). These studies would indicate a helper function of T cells, which is lost after thymectomy.

This apparently conflicting data can be explained in two possible ways. Firstly, it may be that in the present experiments short-term adult thymectomy is only affecting suppressor T cells, whereas helper T cell function is eliminated only after more vigorous procedures such as early thymectomy or long-term studies. However, even 4 months after adult thymectomy there are no significantly decreased numbers of lymphocytes in the major lymphoid organs of rainbow trout (Grace, personal communication). Secondly, in mammals it has been shown that helper function can be correlated with the presence of some T cell markers originating in the thymic medulla (Cohen & Claman, 1971). Another T cell population, responsible for suppressive

properties, is of cortical origin. In carp the thymus has no distinct cortical and medullary zones. It may be that in the absence of a well defined medulla in carp, the helper T cell function is less well developed than in mammals.

Tilapia, on the other hand, show three well developed zones in the thymus, perhaps including a cortex and medulla (Sailendri & Muthukkaruppan, 1975b), so possibly helper T cells have a relatively greater role in this fish species.

Immunoregulatory mechanisms mediated through helper and/or suppressor T cells have been demonstrated in reptiles (Muthukkaruppan, Pitchappan & Ramila, 1976; Pitchappan & Muthukkaruppan, 1977) and are well known in anuran amphibians (see for example Horton, Edwards, Ruben & Mette, 1979), although in urodeles immunoregulation appears to be largely effected by suppressor T cells (Charlemagne & Tournefier, 1977). So in poikilotherms as well as in mammals, the thymus would seem to play an important role in regulating the immune response in adult life. In mammals, T cell functions have been roughly divided into two subpopulations, one whose activity is relatively long lived and one relatively short lived (Kappler, Hunter, Jacobs & Lord, 1974). These authors found that helper function in primary responses after adult thymectomy resided in the long lived class, whereas priming for the secondary demonstration of helper activity and delayed hypersensitivity required both classes. Other work has shown the presence of a short lived subpopulation of 'inhibitory' thymus-derived cells (Kerbel & Eidinger, 1972). It would seem that the detection of

200

suppressor T cells after relatively short-term adult thymectomy in trout, and the detection of helper T cells after long-term adult thymectomy in Tilapia, instead of being contradictory, actually agree with the adult thymectomy data of other animal groups.

In trout thymectomized at an early age, two weeks from hatching, a decreased response to HGG in FCA was seen in sections of spleen and mesonephros used for autoradiography, when compared to sham-operated animals. However, although the response was reduced in thymectomized trout one could be detected when compared to unimmunized control animals at 4 weeks after immunization.

The results of Sailendri (1973) showed that the anti-SRBC response was totally suppressed in Tilapia thymectomized at 2 months old. This contrasts with the present results, where a response to HGG in FCA is seen, although reduced. Differences in the antigen being investigated probably account for the difference in the responses observed, as both species have a fully lymphoid thymus and kidney at thymectomy. In X. laevis, thymectomized at different larval stages of immune maturation, antibodies to HGG can be detected in animals thymectomized at 3 weeks of age and later. However, a full response to SRBC's can only be detected in animals thymectomized at 7 weeks of age or later. The thymus appears to be necessary up to this stage in order to establish and maintain a good anti-SRBC reaction. So such differences in thymectomized fish are quite likely to reflect real differences in the arrival of T cell subpopulations at the periphery. The fact that allograft

reactivity still occurs in thymectomized Tilapia whilst the SRBC response is completely abrogated, further demonstrates a heterogeneity in the ontogeny of T cell functions.

The results of early immunization in the present experiments have also proved quite interesting. In the groups tested before given a second injection it is very clear that no response has been obtained to murine thymus-dependent antigens, whereas immunization with A. salmonicida, a thymus-independent antigen, does produce a response when given on day 21 after hatching. It has previously been shown that juvenile coho salmon Oncorhynchus kisutch as small as 1.2 g can respond to A. salmonicida (Paterson & Fryer, 1974). Similarly, rainbow trout fry that had been taking food for 23 days were found to be immunocompetent when tested with A. salmonicida (Khalifa & Post, 1976). Younger rainbow trout, one month old, were used by Dorson (1974) using DNP keyhole limpet hemocyanin. These fish had again responded when tested 3 months later. However Dorson was not sure if these fish were immunocompetent at testing or had acquired immunocompetence at a later stage and were stimulated by persisting antigen. Still no-one had tried even younger animals, before feeding had begun, to see if these animals were immunocompetent, or if the onset of feeding was in some way acting as a trigger. In fact virtually nothing is known about the ontogenetic appearance of immunoglobulin in fish, in contrast to the well studied amphibian groups (Du Pasquier, 1973). Only the work of Ellis (1977) and Van Loon, Van Oosterom and Van Muiswinkel (1981), have given an indication of the time of appearance

of surface immunoglobulin on lymphoid cells.

In the present study it would seem that rainbow trout fry are immunocompetent, at least with respect to thymus-independent responses, when immunized soon after the onset of feeding. It is thought unlikely that these animals became immunocompetent later, between immunization and testing, for two reasons. Firstly, the negative results obtained by animals injected on days 1, 7 and 14. All groups will have passed through an age when immunocompetence has been acquired, as shown by Khalifa and Post (1976), yet only those animals injected on day 21 gave a response. Secondly, it has been shown that rainbow trout fry as young as 4 days after hatching, have a reticuloendothelial system as efficient as that seen in adults (Grace, Botham & Manning, 1981). So it seems unlikely that antigen would be able to circulate for any length of time without being taken up by macrophages and degraded.

It is uncertain whether all individuals were capable of responding to antigen at this time, because so little blood was available that it had to be pooled. However, the work of Paterson and Fryer (1974) does show that coho salmon fry behave as a homogenous population with respect to immunological competence, and a similar situation may well exist in other salmonids.

The appearance of a response to a thymus-independent antigen, before that to a thymus-dependent antigen is also in agreement with other workers. In mice the ontogenetic capacity to respond to thymus-independent antigens precedes the ability to respond to T-dependent antigens (Rabinowitz,

1976; Etlinger & Chiller, 1979). Similarly, in juvenile rainbow trout responses to T-independent antigens have been shown to be as intense as those seen in adult fish, whereas responses to T-dependent antigens were only meagre (Etlinger, Chiller & Hodgins, 1979). The results here help to confirm this difference between thymus-dependent and thymus-independent antigens, but show that when immunized at an even earlier age, there is no response to thymus-dependent antigens and a diminished, but readily discernible, response to thymus-independent antigens as compared to adults.

When a second injection is given to such animals the response to A. salmonicida is as before. However, responses to the soluble protein antigens now become very interesting. Animals primed with BSA give no response to BSA, but do respond to HGG. Animals primed with HGG do not respond to either antigen, even though they were administered in adjuvant. There are two possible explanations for the lack of response to HGG in HGG primed animals, and three possible explanations for the unresponsiveness to BSA by all animals. The most obvious explanation for the unresponsiveness to HGG, is that tolerance was induced in these animals before competence, so that on further challenge no response is mounted, whereas animals that received BSA first are not tolerant to HGG, and are capable of responding to an injection of this antigen. As the controls showing the response of saline injected animals to a subsequent injection of HGG are lacking, it could also be argued that the initial injection of BSA in some way primes these animals to HGG, so that an enhanced

response is seen after the second injection, compared to a primary response in the HGG injected animals which may only elicit cell proliferation and not detectable serum antibody. This effect could certainly only act in one direction, because HGG does not prime for BSA.

The lack of response to BSA could have been due to immune competition with HGG, tolerance induction at both injections, or simply no response. Both antigens were injected in FCA, which in mice has been shown to enable albumins to compete successfully against globulins, giving an antibody response containing 40% anti-globulin and 60% anti-albumin (Taussig, 1974). So this explanation seems unlikely. It is possible that tolerance was induced to BSA, even when given to animals 10 weeks old, but it is equally possible that the animals were simply immunologically incompetent to this weak immunogen. Preference cannot be made for either explanation until further experiments are carried out.

The only other experiment to show unresponsiveness in fish after early immunization has been carried out in carp (Van Loon, Van Oosterom & Van Muiswinkel, 1981). However, the specificity of these responses, and the mechanisms involved have still to be elucidated. In view of the desirability in fish culture to immunize fry as young as possible, it is surprising that more experiments of this kind have not been carried out. It is well established in other animal groups that the antibody response of the newborn can usually be paralysed more easily than the adult (Smith,

1961). On the whole this can be ascribed simply to the relative lack of responsiveness in young animals, presumably the result of deficiency of immunologically competent cells. Species that are least immunologically mature at birth are most susceptible to tolerance induction (Weigle, 1973). Provision of additional competent cells by adoptive transfer impedes the induction of paralysis in newborn mice (Cohen & Thorbecke, 1963; Friedman, 1965). Early work on immunological paralysis suggested that there was a critical period of susceptibility during which paralysis but not immunity could be induced, possibly by the deletion of appropriate cell clones. This concept was abandoned when it was discovered that adults could also be made tolerant provided the antigen was administered under nonimmunizing conditions; also, that the ability of the foetus to produce antibodies to certain antigens upon appropriate stimulation was not uncommon. However, the mechanisms involved are still unclear. In adults, the classic work of Dresser (1962) and Mitchison (1965) showed that there were two zones of dosage capable of inducing immunological paralysis, high and low. There seems little to suggest that the eventual state of paralysis achieved under either of these conditions differs radically from the other. Administration of antigens to the newborn may be a special case of low-zone paralysis (Dresser & Mitchison, 1968). Two divergent interpretations have been put forward to account for such tolerant states (Weigle, Sieckmann, Doyle & Chiller, 1975). On one hand, the kinetic pattern of induction, maintenance and termination of tolerance

could reflect specific clonal deletion and subsequent de novo emergence of clones of immunocompetent cells. On the other hand, it is equally feasible to explain the same data within the context of an active suppressive mechanism, a putative suppressor population of lymphocytes induced by tolerogen treatment. Convincing evidence for the presence of specific suppressor T cells in mice tolerant to thymus-dependent antigens has been presented using adoptive cell transfer studies (Basten, Miller, Sprent & Cheers, 1974; Benjamin, 1975). However, there exists a body of data which raises the question as to whether suppression is the sole mechanism responsible for this experimentally induced unresponsiveness or whether suppression exists as an ancillary parameter of the tolerant state. For example, it has been shown that the elicitation of suppressor cells and the induction of tolerance can be independent of each other (Parks, Doyle & Weigle, 1978), suggesting that the generation of suppressor T cells is not critical for tolerance induction. More recently it has been claimed that immune suppression and the induction of unresponsiveness are two forms of immunologic tolerance (Borel, Kilham, Kurtz & Reinisch, 1980). These authors showed that adult thymectomy prevented the induction of suppression but concomitantly, failed to influence the induction of immunologic tolerance to the same antigenic determinant. Tolerance induction of helper T cells without suppressor T cells was indicated for the first time, probably by the mechanism of receptor blockade (Aldo-Benson & Borel, 1974) which might involve helper T cells as well as B cells. So it would appear that the

cellular mechanisms underlying immune suppression and immune tolerance are different.

Both high zone tolerance (Marchalonis & Germain, 1971) and low zone tolerance (Baskar & Muthukkaruppan, 1980) have been demonstrated in poikilotherms, as well as the preliminary studies on tolerance induction in immature animals presented here. Although much work is required to elucidate the underlying mechanisms, indications that tolerance can be induced as far back phylogenetically as the teleosts is of interest and the experiments presented here suggest that caution should be exerted before mass immunization of fish fry is attempted.

Chapter 8GENERAL DISCUSSION: THE FISH IMMUNE SYSTEM
IN PHYLOGENETIC PERSPECTIVE

Secondary lymphoid tissues, where encounter with antigen takes place and where lymphocytes proliferate in diffuse microenvironments in response to antigenic stimulation, seem poorly organised in teleosts. Yet all the fundamental cell types of the immune system in mammals have equivalents in fish.

Macrophages are readily demonstrated, particularly after an injection of colloidal carbon (Ellis, Munroe & Roberts, 1976; Grace, Botham & Manning, 1981), as are granulocytes, thrombocytes and lymphocytes. In response to immunization pyroninophilic cells and plasma cells can be detected, although only small numbers of the latter. At the electron microscope level these resemble their mammalian counterparts very closely. After a latent period immunoglobulins can be detected in both the serum and the mucus, and these molecules are found even in the most primitive of vertebrates, the agnathans, so cells functionally equivalent to B lymphocytes are present. After the onset of antibody production antigen is trapped extracellularly on cells, probably as immune complexes, and may be responsible for the generation of memory cells and/or feedback inhibition of the immune response as proposed for this phenomenon in mammals. Cellular immunity, as exhibited by allograft rejection, the mixed leucocyte reaction and delayed hypersensitivity, is also present and suggestive of a T equivalent cell population. Leucocyte migration inhibition has been observed in response to both specific antigens and T cell mitogens, and in one investigation the migrating

cells were identified as macrophages (McKinney, Ortiz, Lee, Sigel, Lopez, Epstein & McLeod, 1976). These same workers have shown catfish leucocytes become cytotoxic after activation by PHA or Con A. Similarly, McKinney, Pettey, Lopez and Sigel (1977) have shown that nurse shark peripheral blood leucocytes become cytotoxic after exposure to PHA. So factors comparable to mammalian lymphokines have also been demonstrated, although it has not yet been shown if these factors are released from fish lymphocytes.

Whether these functions are performed by separate cell populations, or multipotential cells, at the level of teleosts has received much speculation. There is now increasing evidence that fish lymphocytes can be divided into T-like and B-like subsets, although this is not yet firmly established and is not accepted by all authors (see for example McKinney, Ortiz, Lee, Sigel, Lopez, Epstein & McLeod, 1976). It would seem clear from the numerous studies on the hapten-carrier system in fish that cell to cell co-operation does exist. Using the nylon wool column adherence method, hapten-reactive and carrier-reactive cells could be physically separated indicating that at least two different lymphoid cell populations exist (Ruben, Warr, Decker & Marchalonis, 1977; Warr, De Luca, Decker, Marchalonis & Ruben, 1977). However, specific enhancement by carrier priming although firmly established in teleosts could not be demonstrated in more primitive jawless vertebrates, the Agnatha (Fujii, Nakagawa & Murakawa, 1979).

Mitogen responsiveness in teleosts has similarly suggested two different subpopulations of lymphocytes,

although some species show less clear-cut responses than others (Rijkers, 1980). In the bluegill, the population of lymphocytes that responded to PHA and Con A, but not to LPS, contained surface antigens in common with bluegill brain, whereas the population that responded only to LPS did not (Cuchens & Clem, 1977). This situation is very similar to that in mice, where the Thy-1 antigen is present on the surface of T cells and nervous tissue, and is suggestive of a T and B cell population in fish. However, both subpopulations were found in the bluegill thymus and possessed readily demonstrable surface immunoglobulin.

In fact nearly all fish lymphocytes carry surface immunoglobulin, although there are some quantitative and qualitative differences in different lymphoid organs, in contrast to mammals where only B cells have readily demonstrable surface immunoglobulin. Surface immunoglobulins have also been detected on the thymus cells of urodele amphibians (Charlemagne & Tournefier, 1975) and larval anuran amphibians (Du Pasquier, Weiss & Loor, 1972), and a correlation between the presence of readily detectable immunoglobulin of amphibian thymocytes and the lack of a low molecular weight antibody class has been noted (Du Pasquier & Haimovich, 1976). Some of this earlier work may need re-evaluation in view of the recent finding of cross-reactive anti-carbohydrate activity in anti-trout and anti-frog immunoglobulin antisera (Mattes & Steiner, 1978; Yamaga, Kubo & Etlinger, 1978). However, in fish a positive reaction can still be obtained using antisera directed against immunoglobulin light chains

which lack a carbohydrate moiety (Fiebig & Ambrosius, 1976; Clem, McLean, Shankey & Cuchens, 1977). That two populations exist in the thymus should not be too surprising, in view of the fact that antibody synthesizing cells have been reported here in fish after immunization. This has also been noticed in several species of amphibians (Moticka, Brown & Cooper, 1973; Minagawa, Ohnishi & Murakawa, 1975) and the work of Horton, Edwards, Ruben and Mette (1979) suggests that the thymus may actually spawn some B cell subsets.

Nevertheless, in the few experiments in which thymectomy has been attempted in fish it has been suggested that distinct lymphocyte populations do exist. Thus in Tilapia adult thymectomy results in abrogated responses to T-dependent but not to T-independent antigens (Jayaraman, Mohan & Muthukkaruppan, 1979) and thymectomy in young fish, if performed early enough, can completely suppress the response to SRBC's (Sailendri, 1973). So, even though the segregation of T and B cells and reconstitution experiments have not yet been achieved, it is evident that functional T- and B-like fish lymphocytes do exist.

One can see how T cell functions may have evolved from the pre-existing defences in deuterostome invertebrates. Cells resembling lymphocytes are present in the echinoderms and the urochordates, and are involved with graft rejection, and echinoderm coelomocytes contain a substance having similar functions to mammalian migration inhibition factor.

However, the origin of vertebrate immunoglobulins is more obscure. Immunoglobulins first appear in the Agnatha,

where it has been proposed they exist primarily in a membrane bound state, and that their presence in serum may be the result of shedding of receptors rather than active secretion of immunoglobulins, but these animals lack a defined thymus, plasma cells and cell to cell co-operation, so a separate humoral immune response system equivalent to the B lymphocyte-plasmacyte system of higher vertebrates may be lacking. The first integrated cell- and humoral antibody immunity appears to have evolved at the level of advanced bony fish, coincident with the appearance of almost every tumour type (Tam, Reddy, Karp & Hildemann, 1976). It may be that the appearance of malignant cells carrying surface determinants resembling self determinants was paralleled by the appearance of a new class of highly variable and specific antigen receptors. With the evolution of immunoglobulins complex control mechanisms have arisen to prevent unrestrained synthesis, while maximising the production of specific antibodies on a subsequent challenge. These mechanisms are associated in homiotherms with antigen-trapping at germinal centres in the spleen and lymph nodes. In fish it seems (from the work of Ellis (1980) and the studies described here) that a primitive form of antigen-trapping does occur, in the spleen and pronephros, and may also be associated with such functions.

Fish probably produce only one class of immunoglobulin, and their immunoregulatory mechanisms appear to be less sophisticated than in higher vertebrates; also there may be fewer B cell and T cell subsets. Both primary and secondary lymphoid organs are rather simple in structure and both

T and B cells may arise from within the thymus. Why then, has the greater diversity of immunoglobulin classes, the greater complexity of lymphoid organ architecture and the progressive separation of T cells and B cells arisen in the higher vertebrates and not in fish which are as diverse and highly specialized in their own right as the higher tetrapods on land?

To answer such a question it must be realised that the immune system is as much related to the animal's anatomy, physiology and way of life as are the other major body systems. During evolution three major changes have occurred with the emergence of vertebrates from water to land, all of which may relate to changes in the immune system (Manning, 1981). Changes in the blood vascular system have occurred, increasing the efficiency of circulation and inevitably affecting the distribution of antigen, antibody and lymphoid cells. At the reptilian stage of evolution a change from free-living larvae to the amniote condition occurs. When larvae are free-living foreign antigens may be encountered when the lymphoid system is still very immature and this may necessitate a rapid maturation of the pathways which lead to immunocompetence, perhaps at the expense of further differentiation. In the amniote embryo the need to produce immunocompetent cells is less urgent and may allow a further build-up of cellular populations before functional commitments need be made. Thirdly, in the two advanced vertebrate classes, mammals and aves, homoiothermy has been perfected. In warm-blooded animals potential pathogens can multiply quickly and antibody production must be equally rapid.

The thymus, the first lymphoid organ to appear during ontogeny and phylogeny, has remained virtually unchanged through vertebrate evolution. However, secondary lymphoid organs, particularly the spleen, have undergone radical changes (Pitchappan, 1980; Chapter 4 in this thesis). The spleen is present in all jawed vertebrates, whereas other secondary organs appear to be peculiar to the group. These include the lymph nodes of mammals, the bursa of Fabricius and Harder's gland in birds, the jugular bodies of anuran amphibians and the important role of the pronephros in teleost fish (see Chapters 3 and 4).

So both the spleen and kidney of teleosts have an important proliferative role in the response to antigens (Chapter 3) leading to antibody production. They also trap antigen which may have regulatory effects. These are organs with sinusoidal blood flow, where lymphoid cells can cluster and respond to the presence of antigen. It has been suggested by Manning and Turner (1976) that as the circulation becomes more efficient in response to the demands of terrestrial life, the regions where blood flow is sinusoidal are restricted. Further, fish do not need hollow bones for locomotion and lack extensive bone marrow. The kidney is important for haemopoiesis, as well as lymphoid cell functions. The kidney of amniotes no longer has a sinusoidal blood flow and its functions as a lymphoid and haemopoietic organ are lost. With the evolution of hollow bones for locomotion in tetrapods, a new site is available with a slow blood flow to rehouse the lymphocytic and haemopoietic tissue. This new site has an additional advantage in that

the bone surrounding the stem cells protects them from irradiation, now that water is no longer acting as a radio-opaque shield (Cooper, Klempau, Ramirez & Zapata, 1980). The lymphoid tissue in the gut of fish is restricted to only scattered cells between the epithelial cells and in the lamina propria. There are no organs equivalent to Peyer's patches or the bursa of Fabricius. The bursa in birds may represent a unique case where a special micro-environment is needed to hasten and amplify B cell differentiation, in order to protect the warm-blooded, non-suckling, newly-hatched chick.

In relation to increased rates of circulation more complex antigen-trapping arrangements may be necessary and correlated with an increase in sophistication of lymphoid organ architecture. In fish the spleen and kidney are the major lymphoid organs that trap antigen, and both lack a permanently present white pulp. Further evolutionary advances are first seen in the anuran amphibians, which show a mixture of fish-like and more advanced features. The more advanced species possess jugular bodies, which like the kidney of fish and amphibians has a sinusoidal blood flow. They have an important role in antigen-trapping (Manning, 1981) and so are functionally as well as structurally similar to the pronephros of teleosts. In more primitive species lacking jugular bodies, such as in Xenopus laevis, structural advances appear in the spleen which have similarities to the complex organisation in homiotherm secondary lymphoid organs (Chapter 4). Distinct areas of antigen-trapping are seen in the permanently present white pulp,

associated with reticulin fibres. These advances suggest certain evolutionary trends that have occurred in the spleen from the situation in fish, where white pulp areas become conspicuous only after immunization, through the situation in amphibians discussed above, to the germinal centres seen in homiotherms. Mammals have further elaborated this system by perfecting the lymph nodes, allowing discrete and well controlled local responses while at the same time being part of the highly integrated total immune system of the animal.

Changes in the rate of circulation will also affect the movement of immunoglobulins, making it less easy for high molecular weight antibody to reach invading micro-organisms (Manning, 1978). In more advanced vertebrates, which possess a double circulation, blood is under high pressure and high molecular weight antibody tends to remain intra-vascular (except where permeability is increased locally as in inflammation). Under such conditions low molecular weight antibodies, which are able to distribute readily into extra-vascular compartments, become increasingly important. Nevertheless, IgM has been shown to be an extremely efficient agglutinating antibody, and when bound to cell membrane antigens is particularly effective in activating complement, thereby generating immune lysis of cells. It has been estimated that IgM antibodies are one thousand times as efficient at lysing red cells as IgG antibodies (Turner, 1977). Thus the IgM molecule may be regarded as functionally more flexible than IgG. Further, in rabbits both haemagglutinating and toxin-neutralizing

activity of IgM have been found to be more active in the cold (Bauer & Stavitsky, 1961). So IgM antibodies seem to have a lower temperature optimum than IgG antibodies and would be suitable for a poikilothermic animal, particularly one in water, where temperatures will probably always remain quite low. This coupled with the low-pressure single circulatory system of fish, allowing ample time for the migration of substances from the blood vessels, may mean that these animals are more than capable of mounting adequate immune responses without the assistance of low molecular weight antibody. It is interesting that IgM monomer is found in some species of fish, and that in Dipnoi, a new type of short heavy chain typifies a second class of monomeric immunoglobulin, i.e. the class IgN (Litman, 1976). Thus the regular appearance of a low molecular weight immunoglobulin class, with distinct heavy chains, may not have been such a large evolutionary step. In anurans their role in antibody responses is less conspicuous and they are rather slow to appear compared to mammals, and once they appear they do not cause inhibition of the IgM response. A further immunoglobulin class, IgA, appears to be confined to homiotherms (Jurd, 1977), whilst IgE is found only in mammals. The more evolutionary advanced the group the greater the diversity of immunoglobulin classes.

It is generally accepted that poikilotherms lack germinal centres, even though melano-macrophage centres seen in teleosts and the pyroninophilic cell clusters observed in the carp pronephros in the present study may be functionally analogous. The appearance of germinal centres

in secondary lymphoid organs, and the occurrence in the red pulp of large numbers of mature plasma cells directed towards synthesizing considerable amounts of antibody may be associated with homiothermy (Manning, 1981). Warm-blooded animals may need to bring immunological defences into play very rapidly after an infection. It has also been proposed that high avidity IgG and typical anamnoses are the outcome of germinal centre function (Pitchappan, 1980). Although both occur in poikilotherms, typical anamnesis with a copious amount of 2ME-resistant, high avidity IgG antibody in quick succession following immunization and the feedback control of IgM production by IgG, seems to occur only in homiotherms.

In conclusion it can be said that whilst fish lack many of the refinements of the immune system seen in higher vertebrates, their immune capabilities appear to be adequate for aquatic animals with a single-circuit circulatory system. High molecular weight antibody, with its multiple binding sites, is perhaps the antibody class best suited to the needs of lower groups where much of the response to antigens occurs on a local basis. Trapped antigen may induce the formation of memory cells and regulate a feedback control of the antibody response, possibly in addition to the regulatory role of suppressor cells. Therefore it may well be that, at this level of organisation, there was no evolutionary incentive to diversify further and specialize existing immunological apparatus.

SUMMARY

(1) Mirror carp immunized with human gamma globulin (HGG) in Freund's complete adjuvant (FCA) show pyroninophilia and a proliferative response, especially in the haemopoietic parenchyma of the pronephros and mesonephros. This response peaked at week 3, with the formation of clusters of pyroninophilic cells in the pronephros. Immunization with Aeromonas salmonicida gave rise to fewer pyroninophilic cells, but to a larger increase in pigment-containing cells which formed centres containing more than one pigment type.

(2) After a booster immunization with HGG in FCA a distinct response was observed in the spleen: pyroninophilic cells collected within the ellipsoid sheaths in large numbers and formed nodules. The reticulum of such nodules acquired spherical proportions and resembled the white pulp reticulum of the tetrapod spleen.

The roles of such pyroninophilic cells and pigment-containing cells, and the possibility that aggregations of either cell type may be functionally analogous to homiotherm germinal centres, are discussed.

(3) Antigen localization studies were carried out using immunofluorescent techniques, after immunization with either HGG or A. salmonicida. Fluorescence was found to occur in the ellipsoids of the spleen and in the haemopoietic parenchyma of the pronephros and mesonephros, and reached its greatest intensity at three to four weeks after antigen administration. In the spleen the fluorescence was dendritic in nature and appeared to be associated with

reticulin fibres in the ellipsoid walls, suggesting the trapping to be extracellular. The delay in trapping, the correlation with antibody titres and the fact that homologous antibody could be detected in the same areas, suggested that the trapping of antigen was in the form of immune complexes.

(4) Antigen could be detected in the splenic ellipsoids the day following a third injection of HGG. Furthermore, both immune complexes and heat aggregated HGG were trapped more quickly in the pronephros than was antigen alone.

(5) In the pronephros and mesonephros fluorescence due to the presence of HGG was associated with pyroninophilic cell clusters, whilst that due to A. salmonicida was associated only with scattered pyroninophilic cells. A direct comparison with antigen localization in the clawed toad Xenopus laevis, enabled possible evolutionary trends towards the complex dendritic retention of antigen seen in the higher vertebrates to be analysed.

(6) Mirror carp aged 1 year were able to produce agglutinating antibodies to both soluble and cellular antigens. Adjuvant and booster injections gave enhanced agglutinating and precipitating antibody responses to HGG, and the intramuscular route appeared to elicit better antibody responses than the intraperitoneal route. Agglutinating and precipitating antibodies to HGG were confined to the first, high molecular weight, protein peak on column chromatography traces, even though 2ME-resistant antibodies were present.

(7) During histogenesis the kidney develops before the spleen and histological findings in 8 week carp showed that whereas the pronephros was responding in a similar way to year old carp the spleen appeared immature in that it lacked pyroninophilic cells after a booster injection of HGG in FCA and did not trap HGG in the splenic ellipsoids. Nevertheless, carp aged 8 weeks were able to respond to HGG and A. salmonicida and no progressive increase in the magnitude of the agglutinating antibody response was seen as the fish matured from 8 weeks to 1 year.

(8) High doses of HGG (1 mg/g fish) administered intraperitoneally in saline or FCA, failed to elicit the usual primary antibody response and antigen was not detected in the splenic ellipsoids the day following a booster injection of a normal immunogenic dose in FCA into these animals.

(9) Cell-mediated responses, as exhibited by migration inhibition of peripheral blood leucocytes or pronephros leucocytes, were also demonstrated in year old carp. The largest inhibition was seen when using FCA stimulated leucocytes migrating in medium containing PPD, or when using mitogens such as PHA or Con A to non-specifically stimulate leucocytes. Pilot experiments suggested that such mitogens affect cell migration by causing the release of soluble factors from leucocytes.

(10) Adult thymectomized rainbow trout showed enhanced agglutinating and precipitating antibody responses to HGG in adjuvant, but normal responses to A. salmonicida. Conversely, adult thymectomized carp showed enhanced

antibody responses to A. salmonicida, at early timings (day 7) after immunization but normal responses thereafter. This suggests that the thymus in adult life has a role in suppressing the immune response in fish, possibly mediated through short lived T cells.

Rainbow trout thymectomized 14 days after hatching, however, showed decreased levels of proliferating cells after immunization with HGG in FCA, in autoradiographical studies.

(11) Rainbow trout fry were able to produce agglutinating antibodies to A. salmonicida before they were able to respond to HGG in FCA. This, and the fact that the spleen of 8 week carp can trap A. salmonicida in a similar way to year old carp, whereas the ability to trap HGG in the spleen is lacking, support the suggestion of previous workers that these fish are able to fully respond to T-independent antigens before they can fully respond to T-dependent antigens.

(12) Rainbow trout fry immunized with HGG on days 1, 7 or 14 after hatching were unable to respond to a subsequent injection of HGG in FCA, whereas fry initially immunized with BSA were able to produce agglutinating antibodies to a subsequent injection of HGG in FCA. The possibility that tolerance to HGG had been induced is discussed.

ACKNOWLEDGEMENTS

I should like to thank my supervisor, Dr. Margaret J. Manning, for her guidance and encouragement throughout this study, and for the valuable discussions we have shared.

I should like to thank the Department of Agriculture and Fisheries for Scotland for supplying the A. salmonicida and rabbit anti-A. salmonicida antiserum. Also, Dr. W. Van Muiswinkel of the Agricultural University, Wageningen, for supplying the rabbit anti-carp immunoglobulin antiserum.

I should also like to thank R. Crossley for valuable technical assistance with chromatography, Dr. I. G. Jones for operating the analytical ultracentrifuge, and R. Wheeler-Osman for photographic help.

I am indebted to Professor J. G. Phillips and Professor I. M. L. Donaldson for providing the facilities in their Department which enabled me to carry out this study.

Lastly, I should like to acknowledge the financial support of the Science Research Council in providing a CASE research studentship, awarded in conjunction with Dr. A. E. Ellis of DAFS Marine Laboratory, Aberdeen, Scotland. The help and encouragement given by Dr. Ellis is gratefully acknowledged.

BIBLIOGRAPHY

ACTON, R. T., WEINHEIMER, P.F., HALL, S. J., NIEDERMEIER, W.,
SHELTON, E. and BENNETT, J. C. (1971)

Tetrameric immune macroglobulins in three orders of
bony fishes.

Proc. Nat. Acad. Sci. 68: 107-111.

ADA, G. L., NOSSAL, G. J. V. and AUSTIN, C. M. (1964)

Antigens in immunity. V. The ability of cells in lymphoid
follicles to recognize foreignness.

Aust. J. Exp. Biol. Med. Sci. 42: 331-346.

ADLER, F. L. (1965a)

Studies on mouse antibodies. I. The response to sheep
red cells.

J. Imm. 95: 26-38.

ADLER, F. L. (1965b)

Studies on mouse antibodies. II. Mercaptoethanol-sensitive
7S antibodies in mouse antisera to protein antigens.

J. Imm. 95: 39-47.

AGIUS, C. (1979)

Aspects of the melano-macrophage centres in fish.

Ph.D. Thesis, University of Stirling, Stirling, UK.

AL-ASKARI, S. and LAWRENCE, H.S. (1972)

In vitro studies on transplantation immunity. I. MIF
production by sensitive lymphocytes in mice.

Cell Imm. 5: 402-409.

AL-ASKARI, S. and LAWRENCE, H. S. (1973)

In vitro studies on transplantation immunity. II. The
migration inhibition in homograft reactions in guinea pigs.

Cell Imm. 6: 292-299.

ALDO-BENSON, M. and BOREL, Y. (1974)

The tolerant cell: direct evidence for receptor blockade by tolerogen.

J. IMM. 112: 1793-1803.

ALEXANDER, J. B., WILSON, J. G. M. and KERSHAW, W. E. (1970)

Immune response in salmon.

J. Fish Biol. 2: 384.

ALLISON, A. C. and DAVIES, A. J. S. (1971)

Requirement of thymus-dependent lymphocytes for potentiation by adjuvants of antibody formation.

Nature 233: 330-332.

ALM, G. V. and PETERSON, R. D. A. (1969)

Antibody and immunoglobulin production at the cellular level in bursectomized-irradiated chickens.

J. Exp. Med. 129: 1247-1259.

AMBROSIUS, H. and DROSSLER, K. (1972)

Specific cell-mediated immunity in batrachians.

I. Quantitative demonstration by macrophage migration inhibition test for peritoneal exudate cells and spleen fragment cells.

Acta biol. med. germ. 29: 437-440.

AMBROSIUS, H. and LEHMANN, R. (1965)

Contributions to the immune biology of poikilothermic vertebrates. III. The influence of adjuvants on the antibody production of teleostei.

Acta biol. med. germ. 14: 830-844.

AMBROSIUS, H., RICHTER, R. AND KÖNIG, I. (1967)

Über die Immunglobuline von Knochenfischen.

Acta biol. med. germ. 19: 599-601.

AMEND, D. F. and FENDER, D. C. (1976)

Uptake of bovine serum albumin by rainbow trout from hyperosmotic solutions: a model for vaccinating fish. *Science* 192: 793-794.

AMIRANTE, G. A. (1968)

Immuno-cyto-adherence tests in Xenopus laevis Daud. *Experientia* 24: 1059-1060.

AMOS, D. B. (1962)

The use of simplified systems as an aid to the interpretation of mechanisms of graft rejection. *Progr. Allergy* 6: 468-538.

ANDERSON, D. P. and DIXON, O. W. (1980)

Immunological memory in rainbow trout to a fish disease bacterin administered by flush exposure. In: *Phylogeny of Immunological Memory* (Ed. by M.J. Manning), pp. 103-111.

Elsevier/North-Holland Biomedical Press, Amsterdam.

ANDERSON, D. P., DIXON, O. W. and ROBERSON, B. S. (1979)

Kinetics of the primary immune response in rainbow trout after flush exposure to Yersinia ruckeri O-antigen. *Dev. Comp. Imm.* 3: 739-744.

ANDERSON, D. P., ROBERSON, B. S. and DIXON, O. W. (1979a)

Induction of antibody-producing cells in rainbow trout, Salmo gairdneri Richardson; by flush exposure. *J. Fish Biol.* 15: 317-322.

ANDERSON, D. P., ROBERSON, B. S. and DIXON, O. W. (1979b)

Plaque-forming cells and humoral antibody in rainbow trout (Salmo gairdneri) induced by immersion in a Yersinia ruckeri O-antigen preparation.

J. Fish. Res. Bd. Can. 36: 636-639.

ANDERSON, J. I. W. and CONROY, D. A. (1970)

Vibrio disease in marine fishes.

Am. Fish. Soc. Spec. Publ. 5: 266-272.

ANTIPA, R. and AMEND, D. F. (1977)

Immunization of Pacific Salmon: Comparison of Intraperitoneal Injection and Hyperosmotic Infiltration of Vibrio anguillarum and Aeromonas salmonicida Bacterins.

J. Fish. Res. Bd. Can. 34: 203-208.

ANTIPA, R. G. and CROY, T. R. (1979)

Fish vaccine development and testing.

Prog. Fish-Culturist 41: 46-48.

ATWELL, J. L. and MARCHALONIS, J. J. (1976)

Immunoglobulin classes of lower vertebrates distinct from IgM immunoglobulin.

In: Comparative Immunology (Ed. By J. J. Marchalonis), pp. 276-297.

Blackwell.

AUERBACH, R. and RUBEN, L. N. (1970)

Studies of antibody formation in Xenopus laevis.

J. Imm. 104: 1242-1246.

AVTALION, R. R. (1969a)

Temperature effect on antibody production and immunological memory, in carp (Cyprinus carpio) immunized against bovine serum albumin (BSA).

Immunology 17: 927-931.

AVTALION, R. R. (1969b)

Secondary response and immunological memory in carp (Cyprinus carpio) immunized against bovine serum albumin.

Israel J. Med. Sci. 5: 441-442.

AVTALION, R. R., MALIK, Z., LEFLER, E. and KATZ, E. (1970)

Temperature effect on immune resistance of fish to pathogens.

Bamidgeh 22: 33-38.

AVTALION, R. R., WEISS, E., MOALEM, T. and MILGROM, L. (1975)

Evidence for co-operation of T and B cells in fish.

Israel J. Med. Sci. 11: 1385.

AVTALION, R. R., WOJDANI, A., MALIK, Z., SHAHRABANI, R. and

DUCZYMINER, M. (1973)

Influence of environmental temperature on the immune response in fish.

Current topics in Microbiol. and Imm. 61: 1-35.

BABES, V. and RIEGLER, P. (1903)

Ueber eine Fischepidemie bei Bukarest.

Centralbl. f. Bakt. 33: 438-449.

BACH, R., CHEN, P. K. and CHAPMAN, G. B. (1978)

Changes in the spleen of the channel catfish Ictalurus punctatus Rafinesque induced by infection with Aeromonas hydrophila.

J. Fish Diseases 1: 205-217.

BALDWIN, W. M. and COHEN, N. (1981)

A primitive dendritic splenocyte in Xenopus laevis with morphological similarities to Reed-Sternberg cells.

In: Proceedings of the 1st Congress of the International Society for Developmental and Comparative Immunology (Ed. by J. B. Solomon).

Pergamon, Oxford (in press).

BALFOUR, B. M. and HUMPHREY, J. H. (1967)

Localization of γ -globulin and labeled antigen in germinal centers in relation to the immune response.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 80-85.
Springer-Verlag, Berlin.

BALINSKY, B. I. (1948)

On the development of specific characters in cyprinid fishes.
Proc. Zool. Soc. Lond. 118: 335-344.

BANCROFT, J. D. and STEVENS, A. (Eds.) (1977)

Theory and practice of histological techniques.
Churchill-Livingstone, Edinburgh, London and New York.

BASKAR, S. and MUTHUKKARUPPAN, VR. (1980)

Induction of low zone tolerance to sheep erythrocytes in
the lizard Calotes versicolor.
Dev. Comp. Imm. 4: 295-308.

BASTEN, A., MILLER, J. F. A. P. and JOHNSON, P. (1975)

T cell-dependent suppression of an anti-hapten antibody
response.
Transplant. Rev. 26: 130-169.

BASTEN, A., MILLER, J. F. A. P., SPRENT, J. and CHEERS, C. (1974)

Cell-to-cell interaction in the immune response.
X. T-cell-dependent suppression in tolerant mice.
J. Exp. Med. 140: 199-217.

BAUER, D. C. and STAVITSKY, A. B. (1961)

On the different molecular forms of antibody synthesized
by rabbits during the early response to a single injection
of protein and cellular antigens.
Proc, Nat. Acad. Sci. 47: 1667-1680.

BEARD, J. (1894)

The development and probable function of the thymus.
Anat. Anz. 9: 476-486.

BEHRING, E. and KITASATO, S. (1890)

Ueber das zustandekommen der diphtherie-immunitat und der tetanus-immunitat bei thieren.

Dt. med. Wschr. 16: 1113-1114.

BENJAMIN, D. C. (1975)

Evidence for specific suppression in the maintenance of immunologic tolerance.

J. Exp. Med. 141: 635-646.

BENNER, R., MEIMA, F., VAN DER MEULEN, G. M. and

VAN EWIJK, W. (1974)

Antibody formation in mouse bone marrow. III. Effects of route of priming and antigen dose.

Immunology 27: 747-760.

BERENBAUM, M. C. and STRINGER, I. M. (1970)

The localization of haemolytic antibody in sections of lymphoid organs: an improved method.

Immunology 18: 85-90.

BERMAN, M. A. and WEIGLE, W. O. (1977)

B-lymphocyte activation by the Fc region of IgG.

J. Exp. Med. 146: 241-256.

BIANCO, C., DUKOR, P. and NUSSENZWEIG, V. (1971)

Follicular localization of antigen: possible role of lymphocytes bearing a receptor for antigen-antibody-complement complexes.

In: Morphological and Functional Aspects of Immunity (Ed. by K. Lindahl-Kiesling, G. Alm and M. G. Hanna).

Adv. Exp. Med. Biol. 12: 251-256.

Plenum Press, New York.

BISSET, K. A. (1948)

The effect of temperature upon antibody production in cold-blooded vertebrates.

J. Path. Bact. 60: 87-92.

BLOOM, B. R. (1971)

In vitro approaches to the mechanism of cell-mediated immune reactions.

Advan. Imm. 13: 101-208.

BLOOM, B. R. and BENNETT, B. (1966)

Mechanism of a reaction in vitro associated with delayed-type hypersensitivity.

Science 153: 80-82.

BLYTHMAN, H. E. and WHITE, R. G. (1977)

Effect of early bursectomy on germinal centre and immunoglobulin production in chickens.

Immunology 33: 671-677.

BOREL, Y., KILHAM, L., KURTZ, S. E. and REINISCH, C. L. (1980)

Dichotomy between the induction of suppressor cells and immunologic tolerance by adult thymectomy.

J. Exp. Med. 151: 743-748.

BORYSENKO, M. (1976)

Phylogeny of immunity: an overview.

Immunogenetics 3: 305-326.

BOTHAM, J. W., GRACE, M. F. and MANNING, M. J. (1980)

Ontogeny of first set and second set alloimmune reactivity in fishes.

In: Phylogeny of Immunological Memory (Ed. by M.J. Manning), pp. 83-92.

Elsevier/North-Holland Biomedical Press, Amsterdam.

BOWERS, A. (1979)

Hyperosmotic immunisation of teleosts: the portal of entry of the bacterium.

M.Sc. Thesis, University of Salford, Salford, UK.

BROWN, A. L. (1978)

Immunity and practical vaccine development.

Mar. Fish. Rev. 40: 2-5.

BROWN, J. C., DEJESUS, D. G., HOLBOROW, E. J. and

HARRIS, G. (1970)

Lymphocyte mediated transport of aggregated human γ -globulin into germinal centre areas of normal mouse spleen.

Nature 228: 367-369.

BROWN, J. C., HARRIS, G., PAPAMICHAIL, M., SLJIVIC, V. S. and

HOLBOROW, E. J. (1973)

The localization of aggregated human γ -globulin in the spleens of normal mice.

Immunology 24: 955-968.

BROWN, J. C., SCHWAB, J. H. and HOLBOROW, E. J. (1970)

The localization of immunoglobulin and immune complexes in lymphoid tissue.

Immunology 19: 401-415.

BURNET, F. M. (1957)

A modification of Jerne's theory of antibody production using the concept of clonal selection.

Aust. J. Sci. 20: 67-69.

BURTIN, P. and BUFFE, D. (1967)

Synthesis of human immunoglobulins in germinal centers.

In: Germinal Centers in Immune Responses (Ed. by

H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon),

pp. 120-125.

Springer-Verlag, Berlin.

CHAN, E. L., MISHELL, R. I. and MITCHELL, G. F. (1970)

Cell interaction in an immune response in vitro: requirement for theta-carrying cells.

Science 170: 1215-1217.

CHAN, P. L. and SINCLAIR, N. R. ST.C. (1971)

Regulation of the immune response. V. An analysis of the function of the Fc portion of antibody in suppression of an immune response with respect to interaction with components of the lymphoid system.

Immunology 21: 967-981.

CHARLEMAGNE, J. and TOURNEFIER, A. (1975)

Cell surface immunoglobulins of thymus and spleen lymphocytes in Urodele amphibian Pleurodeles waltlii (Salamandridae).

In: Immunologic Phylogeny (Ed. by W. H. Hildemann and A. A. Benedict). Adv. Exp. Med. Biol. 64: 251-255.

CHARLEMAGNE, J. and TOURNEFIER, A. (1977)

Anti-horse red blood cells antibody synthesis in the mexican Axolotl (Ambystoma mexicanum). Effect of thymectomy.

In: Developmental Immunobiology (Ed. by J. B. Solomon and J. D. Horton), pp. 267-275.

Elsevier/North-Holland Biomedical Press, Amsterdam.

CHILLER, J. M., HABICHT, G. S. and WEIGLE, W. O. (1970)

Cellular sites of immunologic unresponsiveness.

Proc. Nat. Acad. Sci. 65: 551-556.

CHILLER, J. M., HODGINS, H. O., CHAMBERS, V. C. and WEISER, R. S. (1969)

Antibody response in rainbow trout (Salmo gairdneri).

I. Immunocompetent cells in the spleen and anterior kidney.

J. Imm. 102: 1193-1201.

CHILLER, J. M., HODGINS, H. O. and WEISER, R. S. (1969)

Antibody response in rainbow trout (Salmo gairdneri).

II. Studies on the kinetics of development of antibody-producing cells and on complement and natural hemolysin.

J. Imm. 102: 1202-1207.

CHILLER, J. M., LOUIS, J. A., SKIDMORE, B. J. and WEIGLE, W.O. (1974)

Cellular parameters of the tolerant state induced to human γ -globulin in mice and its modulation by bacterial lipopolysaccharides.

In: Immunological tolerance: mechanisms and potential therapeutic applications (Ed. by D. H. Katz and B. Benaceraf), pp. 373-395.

Academic Press, New York.

CISAR, J. O. and FRYER, J. L. (1974)

Characterization of anti-Aeromonas salmonicida antibodies from coho salmon.

Inf. Imm. 9: 236-243.

CLAMAN, H. N., CHAPERON, E. A. and SELNER, J. C. (1968)

Thymus-marrow immunocompetence. III. The requirement for living thymus cells.

Proc. Soc. Exp. Biol. Med. 127: 462-466.

CLAWSON, C. C., FINSTAD, J. and GOOD, R. A. (1966)

Evolution of the immune response. V. Electron microscopy of plasma cells and lymphoid tissue of the paddlefish.

Lab. Invest. 15: 1830-1847.

CLEM, L. W. and MCLEAN, W. E. (1975)

Phylogeny of immunoglobulin structure and function.

VII. Monomeric and tetrameric immunoglobulins of the

margate, a marine teleost fish.

Immunology 29: 791-799.

CLEM, L. W., MCLEAN, W. E., SHANKEY, V. T. and CUCHENS, M.A.
(1977)

Phylogeny of lymphocyte heterogeneity. I. Membrane immunoglobulins of teleost lymphocytes.

Dev. Comp. Imm. 1: 105-118.

CLEM, L. W. and SIGEL, M. M. (1963)

Comparative immunochemical and immunological reactions in marine fishes with soluble, viral, and bacterial antigens.

Fed. Proc. 22: 1138-1144.

CLEM, L. W. and SMALL, P. A. (1967)

Phylogeny of immunoglobulin structure and function.

I. Immunoglobulins of the lemon shark.

J. Exp. Med. 125: 893-920.

COE, J. E. and PEEL, L. F. (1970)

Antibody production in the bull frog (Rana catesbeiana).

Immunology 19: 539-550.

COHEN, J. J. and CLAMAN, H.N. (1971)

Thymus-marrow immunocompetence. V. Hydrocortisone-resistant cells and processes in the hemolytic antibody response of mice.

J. Exp. Med. 133: 1026-1034.

COHEN, M. W. and THORBECKE, G. J. (1963)

Interference by newborn lymphoid cells with establishment of immunologic unresponsiveness to a protein antigen.

Proc. Soc. Exp. Bio. Med. 112: 10-15.

COLLIE, M. H. (1974)

The location of soluble antigen in the spleen of

Xenopus laevis.

Experientia 30: 1205-1206.

COLLIE, M. H. (1976)

Studies on the role of the thymus and the spleen in the immune system of Xenopus laevis (Daudin).

Ph.D. Thesis, University of Hull, Hull, UK.

COLLIE, M. H. and TURNER, R. J. (1975)

Influence of antigen dose on antibody production of intact and splenectomised Xenopus laevis.

J. Exp. Zoo. 192: 173-180.

CONGDON, C. C. and MAKINODAN, T. (1961)

Splenic white pulp alteration after antigen injection: relation to time of serum antibody production.

Amer. J. Path. 39: 697-709.

CONROY, D. A. and WITHNELL, G. C. (1974)

The use of a slide agglutination test as an aid in the diagnosis of *Vibrio* disease in fish.

Riv. It. Piscic. Ittiop. 3: 69-74.

COONS, A. H. LEDUC, E. H. and CONNOLLY, 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.

J. Exp. Med. 102: 49-60.

COOPER, E. L. (1969)

Specific tissue graft rejection in earthworms.

Science 166: 1414-1415.

COOPER, E. L. (1971)

Phylogeny of transplantation immunity: graft rejection in earthworms.

Trans. Proc. 3: 214-216.

COOPER, E. L., KLEMPAU, A. E., RAMIREZ, J. A. and ZAPATA, A. G. (1980)

Source of stem cells in evolution.

In: Development and Differentiation of Vertebrate Lymphocytes (Ed. by J. D. Horton), pp. 3-14.

Elsevier/North-Holland Biomedical Press, Amsterdam.

COOPER, M. D., PETERSON, R. D. A., SOUTH, M. A. and GOOD, R. A. (1966)

The functions of the thymus system and the bursa system in the chicken.

J. Exp. Med. 123: 75-102.

CORBEL, M. J. (1975)

The immune response in fish: a review.

J. Fish Biol. 7: 539-563.

COUTINHO, A. and MOLLER, G. (1973)

B cell mitogenic properties of thymus-independent antigens.

Nature New Biol. 245: 12-14.

COWDEN, R. R., DYER, R. F., GEBHARDT, B. M. and VOLPE, E. P. (1968)

Amphibian plasma cells.

J. Imm. 100: 1293-1295.

CROY, T. R. and AMEND, D. F. (1977)

Immunization of sockeye salmon (Oncorhynchus nerka) against vibriosis using the hyperosmotic infiltration technique.

Aquaculture 12: 317-325.

CUCHENS, M. A. and CLEM, L. W. (1977)

Phylogeny of lymphocyte heterogeneity. II. Differential effects of temperature on fish T-like and B-like cells.

Cell. Imm. 34: 219-230.

CUCHENS, M., MCLEAN, E. and CLFM, L. W. (1976)

Lymphocyte heterogeneity in fish and reptiles.

In: Phylogeny of Thymus and Bone Marrow-Bursa Cells
(Ed. by R. K. Wright and E. L. Cooper), pp. 205-213.

Elsevier/North-Holland Biomedical Press, Amsterdam.

CUSHING, J. E. JR. (1942)

An effect of temperature upon antibody-production in fish.

J. Imm. 45: 123-126.

CUSHING, J. E. JR. (1945)

A comparative study of complement. I. The specific
inactivation of the components.

J. Imm. 50: 61-74.

CUSHING, J. E. (1970)

Immunology of fish.

In: Fish Physiology. Vol. IV. The nervous system,
circulation and respiration (Ed. by W. S. Hoar and
D. J. Randall), pp. 465-500.

Academic Press, New York and London.

DAVID, J. R. (1966)

Delayed hypersensitivity in vitro: its mediation by cell-
free substances formed by lymphoid cell-antigen interaction.

Proc. Nat. Acad. Sci. 56: 72-77.

DAVID, J. R., AL-ASKARI, S., LAWRENCE, H. S. and THOMAS, L.

(1964)

Delayed hypersensitivity in vitro. I. The specificity of
inhibition of cell mediated migration by antigens.

J. Imm. 93: 264-273.

DAVID, J. R. and DAVID, R. R. (1972)

Cellular hypersensitivity and immunity. Inhibition of
macrophage migration and the lymphocyte mediators.

Progr. Allergy 16: 300-449.

DAVIES, H. G. and HAYNES, M. E. (1975)

Light- and electron-microscope observations on certain leukocytes in a teleost fish and a comparison of the envelope-limited monolayers of chromatin structural units in different species.

J. Cell Sci. 17: 263-285.

DAVINA, J. H. M., RIJKERS, G. T., ROMBOUT, J. H. W. M.,

TIMMERMANS, L. P. M. and VAN MUISWINKEL, W. B. (1980)

Lymphoid and non-lymphoid cells in the intestine of cyprinid fish.

In: Development and Differentiation of Vertebrate Lymphocytes (Ed. J. D. Horton), pp.129-140.

Elsevier/North-Holland Biomedical Press, Amsterdam.

DEANESLY, R. (1927-28)

The structure and development of the thymus in fish, with special reference to Salmo fario.

Quart. J. Micro. Sci. 71: 113-145.

DIENER, E. and MARCHALONIS, J. (1970)

Cellular and humoral aspects of the primary immune response of the toad, Bufo marinus.

Immunology 18: 279-293.

DIENER, E. and NOSSAL, G. J. V. (1966)

Phylogenetic studies on the immune response. I. Localization of antigens and immune response in the toad, Bufo marinus.

Immunology 10: 535-542.

DIXON, F. J., MAURER, P. H. and DEICHMILLER, M. P. (1954)

Primary and specific anamnestic antibody responses of rabbits to heterologous serum protein antigens.

J. Imm. 72: 179-186.

- DORIA, G., MARTINOZZI, M., AGAROSI, G. and DIPIETRO, S. (1970)
In vitro primary immune response resulting from the interaction between bone marrow-derived and thymus cells.
 Experientia 26: 410-411.
- DORSON, M. (1972)
 Some characteristics of antibodies in the primary immune response of rainbow trout Salmo gairdneri.
 In: Diseases of Fish (Ed. by L. E. Mawdesley-Thomas).
 Symp. Zool. Soc. Lond. 30: 129-140.
 Academic Press, London and New York.
- DORSON, M. M. (1974)
 Immunologie-Production d'anticorps précipitants anti-dinitrophénol chez les alevins de truit Arc-en-Ciel (Salmo gairdneri) immunisés à L'âge d'un mois.
 C. R. Acad. Sc. Paris 278: 3151-3152.
- DRESSER, D. W. (1962)
 Specific inhibition of antibody production - I. Protein-overloading paralysis.
 Immunology 5: 161-168.
- DRESSER, D. W. (1968)
 An assay for adjuvanticity.
 Clin. exp. Imm. 3: 877-888.
- DRESSER, D. W. and MITCHISON, N. A. (1968)
 The mechanism of immunological paralysis.
 Advan. Imm. 8: 129-181.
- DROSSLER, K. and AMBROSIUS, H. (1972)
 Specific cell-mediated immunity in batrachians.
 II. Delayed hypersensitivity to BCG in toad (Bufo bufo L.)
 Acta biol. med. germ. 29: 441-445.

DUFF, D. C. B. (1942)

The oral immunization of trout against Bacterium salmonicida.
J. Imm. 44: 87-94.

DUPASQUIER, L. (1973)

Ontogeny of the immune response in cold-blooded vertebrates.
Current Topics in Microbiol. and Imm. 61: 37-88.

DUPASQUIER, L. and HAIMOVICH, J. (1976)

The antibody response during amphibian ontogeny.
Immunogenetics 3: 381-391.

DUPASQUIER, L., WEISS, N. and LOOR, F. (1972)

Direct evidence for immunoglobulins on the surface of
thymus lymphocytes of amphibian larvae.
Eur. J. Imm. 2: 366-370.

EDELSTEIN, L. M. (1971)

Melanin: a unique bio polymer.

In: Pathobiology Annual. Vol. I (Ed. by H. L. Ioachim),
pp. 309-324.

Butterworth, London.

EHLINGER, N. F. (1977)

Selective breeding of trout for resistance to furunculosis.
N.Y. Fish and Game J. 24: 25-36.

ELLIS, A. E. (1974)

Aspects of the lymphoid and reticuloendothelial systems
in the plaice, Pleuronectes platessa L.

Ph.D. Thesis, University of Aberdeen, Aberdeen, UK.

ELLIS, A. E. (1976)

Leucocytes and related cells in the plaice Pleuronectes
platessa.

J. Fish. Biol. 8: 143-156.

ELLIS, A. E. (1977)

Ontogeny of the immune response in Salmo salar. Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leucocyte reactivity.

In: Developmental Immunobiology (Ed. by J. B. Solomon and J. D. Horton), pp. 225-231.

Elsevier/North-Holland Biomedical Press, Amsterdam.

ELLIS, A. E. (1978)

The immunology of teleosts.

In: Fish Pathology (Ed. by R. J. Roberts), pp. 92-104.

Bailliere Tindall, London.

ELLIS, A. E. (1980)

Antigen-trapping in the spleen and kidney of the plaice, Pleuronectes platessa L.

J. Fish Diseases 3: 413-426.

ELLIS, A. E. and DESOUSA, M. (1974)

Phylogeny of the lymphoid system. I. A study of the fate of circulating lymphocytes in plaice.

Eur. J. Imm. 4: 338-343.

ELLIS, A. E., MUNROE, A. L. S. and ROBERTS, R. J. (1976)

Defence mechanisms in fish. I. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (Pleuronectes platessa L.).

J. Fish Biol. 8: 67-78.

ELLIS, A. E. and PARKHOUSE, R. M. E. (1975)

Surface immunoglobulins on the lymphocytes of the skate Raja naevus.

Eur. J. Imm. 5: 726-728.

ELLIS, A. E. and WOOTTEN, R. (1978)

Costiasis of Atlantic salmon, Salmo salar L. smolts in seawater.

J. Fish Diseases 1: 389-393.

EMBLING, P. H., EVANS, H., GUTTIEREZ, C., HOLBOROW, E. J.,
JOHNS, P., JOHNSON, P. M., PAPAMICHAIL, M. and STANWORTH,
D. R. (1978)

The structural requirements for immunoglobulin aggregates to localize in germinal centres.

Immunology 34: 781-786.

EMMRICH, F., RICHTER, R. F. and AMBROSIUS, H. (1975)

Immunoglobulin determinants on the surface of lymphoid cells of carps.

Eur. J. Imm. 5: 76-78,

ETLINGER, H. M. and CHILLER, J. M. (1979)

Maturation of the lymphoid system. I. Induction of tolerance in neonates with a T-dependent antigen that is an obligate immunogen in adults.

J. Imm. 122: 2558-2563.

ETLINGER, H. M., CHILLER, J. M. and HODGINS, H. O. (1979)

Evolution of the lymphoid system - IV. Murine T-independent but not T-dependent antigens are very immunogenic in rainbow trout (Salmo gairdneri).

Cell. Imm. 47: 400-406.

ETLINGER, H. M., HODGINS, H. O. and CHILLER, J. M. (1976a)

Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity in rainbow trout revealed by the organ distribution of mitogenic responses.

J. Imm. 116: 1547-1553.

- ETLINGER, H. M., HODGINS, H. O. and CHILLER, J. M. (1976b)
Rainbow trout leukocyte culture: a simplified method.
In Vitro 12: 599-601.
- ETLINGER, H. M., HODGINS, H. O. and CHILLER, J. M. (1977)
Evolution of the lymphoid system. II. Evidence for
immunoglobulin determinants on all rainbow trout lympho-
cytes and demonstration of mixed leukocyte reaction.
Eur. J. Imm. 7: 881-887.
- ETLINGER, H. M., HODGINS, H. O. and CHILLER, J. M. (1978)
Evolution of the lymphoid system. III. Morphological and
functional consequences of mitogenic stimulation of rainbow
trout lymphocytes.
Dev. Comp. Imm. 2: 263-276.
- EVANS, E. A. (1966)
Tritium and its compounds.
Butterworths, London.
- EVANS, E. E., KENT, S. P., BRYANT, R. E. and MOYER, M. (1966)
Antibody formation and immunological memory in the marine
toad.
In: *Phylogeny of Immunity* (Ed. by R. T. Smith, P. A.
Miescher and R. A. Good), pp. 218-226.
University of Florida Press, Gainesville, USA.
- EVELYN, T. P. T. (1971)
The agglutinin response in sockeye salmon vaccinated
intraperitoneally with a heat-killed preparation of the
bacterium responsible for salmonid kidney disease.
J. Wildlife Dis. 7: 328-335.
- EVERHART, D. L. (1972)
Antibody response of goldfish to a protein immunogen
and to haptenic determinants.
Immunology 22: 503-507.

EVERHART, D. L. and SHEFNER, A. M. (1966)

Specificity of fish antibody.

J. Imm. 97: 231-234.

FAGRAEUS, A. (1948)

Antibody production in relation to the development of plasma cells. In vivo and in vitro experiments.

Acta Med. Scand. 204: 3-122.

FALK, R. F., COLLSTE, L. and MOLLER, G. (1969)

In vitro correlates of transplantation immunity: the release of substances by immune lymphocytes confronted with specific antigens.

Surgery 66: 51-57,

FELDMAN, J. D. (1964)

Ultrastructure of immunologic processes.

Advan. Imm. 4: 175-248.

FENDER, D. C. and AMEND, D. F. (1978)

Hyperosmotic infiltration: factors influencing uptake of bovine serum albumin by rainbow trout (Salmo gairdneri).

J. Fish. Res. Bd. Can. 35: 871-874.

FERGUSON, H. W. (1976)

The relationship between ellipsoids and melano-macrophage centres in the spleen of turbot (Scophthalmus maximus).

J. Comp. Path. 86: 377-380.

FERREN, F. A. JR. (1967)

Role of the spleen in the immune response of teleosts and elasmobranchs.

J. Florida Med. Assoc. 54: 434-437.

FFY, F. (1967)

Vergleichende Hamozytologie niederer Vertebraten. IV.
Monozyten-Plasmozyten-Lymphozyten.

Folia Haemat. 86: 133-147.

FIEBIG, H. and AMBROSIUS, H. (1976)

Cell surface immunoglobulin of lymphocytes in lower
vertebrates.

In: Phylogeny of Thymus and Bone Marrow-Bursa Cells
(Ed. by R. K. Wright and E. L. Cooper), pp. 195-203.

Elsevier/North-Holland Biomedical Press, Amsterdam.

FIEBIG, H. and AMBROSIUS, H. (1977)

Studies on the regulation of the IgM immune response.

I. Changes of the affinity of anti-dinitrophenyl anti-
bodies of carp during immunization in dependence on the
antigen dose.

Acta biol. med. germ. 36: 79-86.

FINSTAD, J. and GOOD, R. A. (1966)

Phylogenetic studies of adaptive immune responses in the
lower vertebrates.

In: Phylogeny of immunity (Ed. by R. T. Smith, P. A.
Miescher and R. A. Good), pp. 173-189.

University of Florida Press, Gainesville, USA

FINSTAD, J., PAPERMASTER, B. W. and GOOD, R. A. (1964)

Evolution of the immune response. II. Morphologic
studies on the origin of the thymus and organized lymphoid
tissue.

Lab. Invest. 13: 490-512.

FISHMAN, M. (1961)

Antibody formation in vitro.

J. Exp. Med. 114: 837-856.

FISHMAN, M. and ADLER, F. L. (1963)

Antibody formation initiated in vitro. II. Antibody synthesis in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen.

J. Exp. Med. 117: 595-602.

FLEMMING, W. (1885)

Studien uber regeneration der gewebe.

Arch. Mikr. Anat. 24: 50-97.

FLETCHER, T. C. and GRANT, P. T. (1969)

Immunoglobulins in the serum and mucus of the plaice (Pleuronectes platessa).

Biochem. J. 115: 65P.

FLETCHER, T. C. and WHITE, A. (1973)

Antibody production in the plaice (Pleuronectes platessa L.) after oral and parenteral immunization with Vibrio anguillarum antigens.

Aquaculture 1: 417-428.

FLIEDNER, T. M., KESSE, M., CRONKITE, E. P. and ROBERTSON, J. S. (1964)

Cell proliferation in germinal centers of the rat spleen.

Ann. New York Acad. Sci. 113: 578-594.

FORD, W. L., GOWANS, J. L. and MCCULLAGH, P. J. (1966)

The origin and function of lymphocytes.

In: The Thymus: Experimental and Clinical Studies

(Ed. by G. E. W. Wolstenholme and R. Porter), pp. 58-85.

Churchill, London.

FRENZEL, E.-M. and AMBROSIUS, H. (1971)

Antihapten antibodies in lower vertebrates.

Acta biol. med. germ. 26: 165-171.

FRIEDMAN, H. (1965)

Prevention of immunologic tolerance by lymphoid cell transplants. Effect of time, cell concentration, injection route and type of cells transferred on establishment of unresponsiveness to shigella antigens in neonatal mice.

J. Imm. 94: 205-213.

FROMMEL, D., LITMAN, G. W., FINSTAD, J. and GOOD, R. A. (1971)

The evolution of the immune response. XI. The immunoglobulins of the horned shark, Heterodontus francisci: purification, characterization and structural requirement for antibody activity.

J. Imm. 106: 1234-1243.

FROST, P. and LANCE, E. M. (1978)

On the mechanism of action of adjuvants.

Immunology 35: 63-68.

FUJIHARA, M. P., OLSON, P. A. and NAKATANI, R. E. (1964)

Antibody production and immune response of fish to C. columnaris.

Pacific Northwest Laboratories Annual Report 122: 194-196.

FUJII, T., NAKAGAWA, H. AND MURAKAWA, S. (1979)

Immunity in lamprey. II. Antigen-binding responses to sheep erythrocytes and hapten in the ammocoete.

Dev. Comp. Imm. 3: 609-620.

GALLILY, R. and FELDMAN, M. (1967)

The cellular components in the induction of antibody by X-irradiated animals.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 333-342.

Springer-Verlag, Berlin.

GEORGE, M. and VAUGHAN, J. H. (1962)

In vitro cell migration as a model for delayed hypersensitivity.

Proc. Soc. Exp. Biol. Med. 111: 514-521.

GLICK, B., CHANG, T. S. and JAAP, R. G. (1956)

The bursa of Fabricius and antibody production.

Poultry Sci. 35: 224-225.

GOMORI, G. (1937)

Silver impregnation of reticulum in paraffin sections.

Am. J. Path. 13: 993-1001.

GONCHAROV, G. D. (1971)

Dynamics of the accumulation of antibodies in carp in relation to the intervals of injection.

Biologiya vnutrennikh vod informatsionnyi byulleten No. 9, AS USSR "Nauka", Leningrad. 44-47.

GOOD, R. A., DALMASSO, A. P., MARTINEZ, C., ARCHER, O. K.,

PIERCE, J. C. and PAPERMASTER, B. W. (1962)

The role of the thymus in development of immunologic capacity in rabbits and mice.

J. Exp. Med. 116: 773-795.

GOOD, R. A. and FINSTAD, J. (1967)

The phylogenetic development of immune responses and the germinal center system.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 4-27.

Springer-Verlag, Berlin.

GOOD, R. A., FINSTAD, J., POLLARA, B. and GABRIELSEN, A. E. (1966)

Morphologic studies on the evolution of the lymphoid tissues among the lower vertebrates.

In: Phylogeny of Immunity (Ed. by R. T. Smith, P. A. Miescher and R. A. Good), pp. 149-170.

University of Florida Press, Gainesville, USA.

GOOD, R. A. and PAPERMASTER, B. W. (1964)

Ontogeny and phylogeny of adaptive immunity.

Advan. Imm. 4: 1-115.

GORDON, J. and YU, H. (1973)

Relationship of T cells involved in cell-mediated immunity and antibody synthesis.

Nature New Biol. 244: 21-22.

GOULD, R. W., ANTIPA, R. and AMEND, D. F. (1979)

Immersion vaccination of sockeye salmon (Oncorhynchus nerka) with two pathogenic strains of Vibrio anguillarum.

J. Fish. Res. Bd. Can. 36: 222-225.

GOULD, R. W., O'LEARY, P. J., GARRISON, R. L., ROHOVEC, J. S. and FRYER, J. L. (1978)

Spray vaccination: a method for the immunization of fish.

Fish Pathology 13: 63-68.

GOWANS, J. L. (1962)

The fate of parental strain small lymphocytes in F₁ hybrid rats.

Ann. New York Acad. Sci. 99: 432-455.

GOWANS, J. L., MCGREGOR, D. D., COWEN, D. M. and FORD, C. E. (1962)

Initiation of immune responses by small lymphocytes.

Nature 196: 651-655.

GRACE, M. F., BOTHAM, J. W. and MANNING, M. J. (1981)

- Ontogeny of lymphoid organ function in fish.
 In: Proceedings of the 1st Congress of the International Society for Developmental and Comparative Immunology.
 (Ed. by J. B. Solomon).
 Pergamon, Oxford (in press).
- GRACE, M. F. and MANNING, M. J. (1980)
 Histogenesis of the lymphoid organs in rainbow trout, Salmo gairdneri Rich. 1836.
 Dev. Comp. Imm. 4: 255-264.
- GRANGER, G. A. and WILLIAMS, T. W. (1968)
 Lymphocyte cytotoxicity in vitro: activation and release of a cytotoxic factor.
 Nature 218: 1253-1254.
- GRANTHAM, W. G. (1972)
 The secondary response to high and low dose priming in mice.
 J. Imm. 108: 562-565.
- GRANTHAM, W. G. and FITCH, F. W. (1975)
 The role of antibody feedback inhibition in the regulation of the secondary antibody response after high and low dose priming.
 J. Imm. 114: 394-398.
- GREY, H. M. (1963a)
 Production of mercaptoethanol sensitive, slowly sedimenting antibody in the duck.
 Proc. Soc. Exp. Bio. Med: 113: 963-967.
- GREY, H. M. (1963b)
 Phylogeny of the immune response. Studies on some physical, chemical and serologic characteristics of antibody produced in the turtle.
 J. Imm. 91: 819-825.

GREY, H. M. (1966)

Structure and kinetics of formation of antibody in the turtle.

In: Phylogeny of Immunity (Ed. by R. T. Smith, P. A. Miescher and R. A. Good), pp. 227-235.

University of Florida Press, Gainesville, USA.

GREY, H. M. (1969)

Phylogeny of immunoglobulins.

Advan. Imm. 10: 51-104.

GROBLER, P., BUERKI, H., COTTIER, H., HESS, M. W. and

STONER, R. D. (1974)

Cellular bases for relative radioresistance of the antibody-forming system at advanced stages of the secondary response to tetanus toxoid in mice.

J. Imm. 112: 2154-2165.

HADJI-AZIMI, I. (1971)

Studies on Xenopus laevis immunoglobulins.

Immunology 21: 463-474.

HAFTER, E. (1952)

Histological age changes in the thymus of the teleost, Astyanax.

J. Morph. 90: 555-582.

HALPERN, B. N., PREVOT, A.-R., BIOZZI, G., STIFFEL, C.,

MOUTON, D., MORARD, J. C., BOUTHILLIER, Y. and DECREUSE-

FOND, C. (1963)

Stimulation de l'activité phagocytaire du système réticuloendothélial provoquée par Corynebacterium parvum.

J. Retic. Soc. 1: 77-96.

HANNA, M. G. JR. (1965)

Germinal center changes and plasma cell reaction during

the primary immune response.

Int. Arch. Allergy 26: 230-251.

HANNA, M. G. JR., SWARTZENDRUBER, D. C. and CONGDON, C. C. (1967)

Morphologic and autoradiographic studies of spleen white pulp germinal centers after antigen injection.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 189-198.

Springer-Verlag, Berlin.

HARRELL, L. W., ETLINGER, H. M. and HODGINS, H. O. (1975)

Humoral factors important in resistance of salmonid fish to bacterial disease. I. Serum antibody protection of rainbow trout (Salmo gairdneri) against vibriosis.

Aquaculture 6: 211-219.

HARRIS, J. E. (1973)

The immune response of dace Leuciscus leuciscus (L.) to injected antigenic materials.

J. Fish Biol. 5: 261-276.

HARTMAN, K.-U. (1970)

Induction of a hemolysin response in vitro. Interaction of cells of bone marrow origin and thymic origin.

J. Exp. Med. 132: 1267-1278.

HEARTWELL III, C. M. (1975)

Immune response and antibody characterization of the channel catfish (Ictalurus punctatus) to a naturally pathogenic bacterium and virus.

U.S. Department of the Interior Fish and Wildlife Service.

Technical Papers of the U.S. Fish and Wildlife Service

85: 1-34.

HERD, Z. L. and ADA, G. L. (1969)

Distribution of ¹²⁵I-Immunoglobulins, IgG subunits and

antigen-antibody complexes in rat lymph nodes.

Aust. J. exp. Biol. med. Sci. 47: 73-80.

HILDEMANN, W. H. (1958)

Tissue transplantation immunity in goldfish.

Immunology 1: 46-53.

HILDEMANN, W. H. and COOPER, E. L. (1963)

Immunogenesis of homograft reactions in fishes and amphibians.

Fed. Proc. 22: 1145-1151.

HILDEMANN, W. H. and HAAS, R. (1960)

Comparative studies on homotransplantation in fishes.

J. Cell. Comp. Phys. 55: 227-233.

HILDEMANN, W. H., RAISON, R. L., CHEUNG, G., HULL, C. J.,

AKAKA, L. and OKAMOTO, J. (1977)

Immunological specificity and memory in a scleractinian coral.

Nature 270: 219-223.

HINES, R. and YASHOUV, A. (1970)

Differential leukocyte counts and total leukocyte and erythrocyte counts for some normal Israeli mirror carp.

Bamidgeh 22: 106-113.

HODGINS, H. O., WEISER, R. S. and RIDGWAY, G. J. (1967)

The nature of antibodies and the immune response in rainbow trout (Salmo gairdneri)

J. Imm. 99: 534-544.

HOLMGREN, N. (1950)

On the pronephros and the blood in Myxine glutinosa.

Acta Zool. - Stock. 31: 233-348.

HORTON, J. D., EDWARDS, B. F., RUBEN, L. N. and METTE, S. (1979)

Use of different carriers to demonstrate thymic-dependent and thymic-independent anti-trinitrophenyl reactivity in the amphibian Xenopus laevis.

Dev. Comp. Imm. 3: 621-633.

HORTON, J. D. and MANNING, M. J. (1974)

Effect of early thymectomy on the cellular changes occurring in the spleen of the clawed toad following administration of soluble antigen.

Immunology 26: 797-807.

HOSKINS, E. R. and HOSKINS, M. M. (1918)

The reaction of selachii to injections of various non-toxic solutions and suspensions (including vital dyes) and to excretory toxins.

J. Ex. Zool. 27: 101-155.

HUMASON, G. L. (1979)

Animal Tissue Techniques 4 th Edition.

Freeman and Company, San Francisco.

HUMPHREY, J. H. and FRANK, M. M. (1967)

The localization of non-microbiol antigens in the draining lymph nodes of tolerant, normal and primed rabbits.

Immunology 13: 87-100.

INGRAM, G. A. and ALEXANDER, J. B. (1976)

The immune response of brown trout (Salmo trutta L.) to injection with soluble antigens.

Acta biol. med. germ. 35: 1561-1570.

INGRAM, G. A. and ALEXANDER, J. B. (1979)

The immunoglobulin of the brown trout, Salmo trutta and its concentration in the serum of antigen-stimulated and non-stimulated fish.

J. Fish Biol. 14: 249-260.

INGRAM, G. A. and ALEXANDER, J. B. (1980)

The immune response of the brown trout Salmo trutta to lipopolysaccharide.

- J. Fish Biol. 16: 181-197.
- JAYARAMAN, S., MOHAN, R. and MUTHUKKARUPPAN, VR. (1979)
 Relationship between migration inhibition and plaque-forming cell responses to sheep erythrocytes in the teleost, Tilapia mossambica.
 Dev. Comp. Imm. 3: 67-75.
- JAYARAMAN, S. and MUTHUKKARUPPAN, VR. (1977)
 In vitro correlate of transplantation immunity: spleen cell migration inhibition in the lizard, Calotes versicolor.
 Dev. Comp. Imm. 1: 133-144.
- JAYARAMAN, S. and MUTHUKKARUPPAN, VR. (1978a)
 Detection of cell-mediated immunity to sheep erythrocytes by the capillary migration inhibition technique in the lizard, Calotes versicolor.
 Immunology 34: 231-240.
- JAYARAMAN, S. and MUTHUKKARUPPAN, VR. (1978b).
 Influence of route and dose of antigen on the migration inhibition and plaque-forming cell responses to sheep erythrocytes in the lizard, Calotes versicolor.
 Immunology 34: 241-246.
- JOHNSON, A.G., JACOBS, A., ABRAMS, G. and MERRITT, K. (1967)
 Comparative changes in the mouse spleen during immunostimulation or immunosuppression.
 In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 234-239.
 Springer-Verlag, Berlin.
- JOHNSTON, W. H. JR., ACTON, R. T., WEINHEIMER, P. F., NIEDERMEIER, W., EVANS, E. E., SHELTON, E. and BENNETT, J. C. (1971)

Isolation and physico-chemical characterization of the "IgM-like" immunoglobulin from the stingray Dasyatis americana.

J. Imm. 107: 782-793.

JURD, R. D. (1977)

Secretory immunoglobulins and gut-associated lymphoid tissue in Xenopus laevis.

In: Developmental Immunobiology (Ed. by J. B. Solomon and J. D. Horton), pp. 307-314.

Elsevier/North-Holland Biomedical Press, Amsterdam.

KALLMAN, K. D. and GORDON, M. (1958)

Genetics of fin transplantation in xiphophorin fishes.

Ann. New York Acad. Sci. 73: 599-610.

KALTREIDER, H. B., SOGHOR, D., TAYLOR, J. B. and DECKER, J. L. (1969)

Capillary tube migration for detection of human delayed hypersensitivity: difficulties encountered with "buffy coat" cells and tuberculin antigen.

J. Imm. 103: 179-184.

KAPPLER, J. W., HUNTER, P. C., JACOBS, D. and LORD, E. (1974)

Functional heterogeneity among the T-derived lymphocytes of the mouse. I. Analysis by adult thymectomy.

J. Imm. 113: 27-38.

KARP, R. D. and HILDEMANN, W. H. (1976)

Specific allograft reactivity in the sea star Dermasterias imbricata.

Transplantation 22: 434-439.

KATZ, D. H. (1977)

Lymphocyte Differentiation, Recognition and Regulation.

Academic Press, San Francisco, New York and London.

KATZ, D. H. and BENACERRAF, B. (1972)

The regulatory influence of activated T cells on B cell responses to antigen.

Advan. Imm. 15: 1-94.

KERBEL, R. S. and EIDINGER, D. (1972)

Enhanced immune responsiveness to a thymus-independent antigen early after adult thymectomy: evidence for short-lived inhibitory thymus-derived cells.

Eur. J. Imm. 2: 114-118.

KHALIFA, K. A. and POST, G. (1976)

Immune response of advanced rainbow trout fry to Aeromonas liquefaciens.

Prog. Fish-Culturist 38: 66-68.

KLAUS, G. G. B. (1978)

The generation of memory cells. II. Generation of B memory cells with preformed antigen-antibody complexes.

Immunology 34: 643-652.

KLONTZ, G. W. (1967)

Immunopathology. Oral immunization of coho salmon against furunculosis.

U.S. Dept. Int. Fish Wildl. Ser. Bur. Sport Fish.

Wildl. Res. Pub. 39: 81-82.

KLONTZ, G. W. and ANDERSON, D. P. (1970)

Oral immunization of salmonids: a review.

Am. Fish Soc. Special Publication 5: 16-20.

KRANTZ, G. E., REDDECLIFF, J. M. and HEIST, C. E. (1963)

Development of antibodies against Aeromonas salmonicida in trout.

J. Imm. 91: 757-760.

KURAMOCHI, T. (1974)

In vitro detection of transplantation immunity: leukocyte migration inhibition in the dog.

Japan. J. Exp. Med. 44: 223-226.

LAISSUE, J., COTTIER, H., HESS, M. W. and STONER, R. D. (1971)

Early and enhanced germinal center formation and antibody responses in mice after primary stimulation with antigen-isologous antibody complexes as compared with antigen alone.

J. Imm. 107: 822-831.

LAMELIN, J. P. and VASSALLI, P. (1971)

Inhibition of macrophage migration by a soluble factor from lymphocytes stimulated with PHA or ALS.

Nature 229: 426-428.

LANG, P. G. and ADA, G. L. (1967)

Antigen in tissues. IV. The effect of antibody on the retention and localization of antigen in rat lymph nodes.

Immunology 13: 523-534.

LANGEVOORT, H. L. (1963)

The histophysiology of the antibody response. I. Histogenesis of the plasma cell reaction in rabbit spleen.

Lab. Invest. 12: 106-118.

LANGEVOORT, H. L., ASOFSKY, R. M., JACOBSON, E. B., DEVRIES, T. and THORBECKE, G. J. (1963)

γ -Globulin and antibody formation in vitro. II. Parallel observations on histologic changes and on antibody formation in the white and red pulp of the rabbit spleen during the primary response, with special reference to the effect of endotoxin.

J. Imm. 90: 60-71.

LANNAN, J. E. (1978)

Vibriosis vaccination of chum salmon by hyperosmotic infiltration.

Prog. Fish-Culturist 40: 43-45.

LELE, S. H. (1933a)

On the phasical history of the thymus gland in plaice of various ages with note on the involution of the organ, including also notes on the other ductless glands in this species.

J. Univ. Bombay 1: 37-53.

LELE, S. H. (1933b)

On the phasical morphology of the thymus gland in some common European fishes and in two cyclostomes.

J. Univ. Bombay 2: 33-42.

LIEW, F. Y. and PARISH, C. R. (1974)

Lack of a correlation between cell-mediated immunity to the carrier and the carrier-hapten helper effect.

J. Exp. Med. 139: 779-784.

LIN, H. H., CAYWOOD, B. E. and ROWLANDS, D. T. JR. (1971)

Primary and secondary immune responses of the marine toad (Bufo marinus) to bacteriophage f2.

Immunology 20: 373-380.

LITMAN, G. W. (1976)

Physical properties of immunoglobulins of lower species: a comparison with immunoglobulins of mammals.

In: Comparative Immunology (Ed. by J. J. Marchalonis), pp. 239-275.

Blackwell Scientific Publications, Oxford.

LYKAKIS, J. J. (1969)

The production of two molecular classes of antibody in the toad, Xenopus laevis, homologous with mammalian γ M (19S) and γ G (7S) immunoglobulins.

Immunology 16: 91-98.

LYKAKIS, J. J. and COX, F. E. G. (1968)

Immunological responses of the toad, Xenopus laevis, to the antigens of the ciliate, Tetrahymena pyriformis.

Immunology 15: 429-437.

MACKMULL, G. and MICHELS, N. A. (1932)

Absorption of colloidal carbon from the peritoneal cavity in the teleost Tautoglabrus adspersus.

Am. J. Anat. 51: 3-47.

MANNING, M. J. (1975)

The phylogeny of thymic dependence.

Amer. Zool. 115: 63-71.

MANNING, M. J. (1978)

The amphibian immune system and emerging adaptations to life on land.

In: Proceedings of the Zodiac Symposium on Adaptation.

Wageningen, The Netherlands, pp. 88-91.

Centre for Agricultural Publishing and Documentation, Wageningen.

MANNING, M. J. (1981)

The evolution of vertebrate lymphoid organs.

In: Proceedings of the 1st Congress of the International Society for Developmental and Comparative Immunology

(Ed. by J. B. Solomon).

Pergamon, Oxford (in press).

MANNING, M. J., DONNELLY, N. and COHEN, N. (1976)

Thymus-dependent and thymus-independent components of the amphibian immune system.

In: Phylogeny of Thymus and Bone Marrow-Bursa Cells

(Ed. by R. K. Wright and E. L. Cooper), pp. 123-132.

Elsevier/North-Holland Biomedical Press, Amsterdam.

MANNING, M. J. and JURD, R. D. (1981)

Antibody production in thymectomized Xenopus

In: Proceedings of the 1st Congress of the International Society for Developmental and Comparative Immunology.

(Ed. by J. B. Solomon).

Pergamon, Oxford (in press).

MANNING, M. J. and TURNER, R. J. (1976)

Comparative Immunobiology.

Blackie and Son Ltd., Glasgow.

MARCHALONIS, J. J. (1969)

Isolation and characterization of immunoglobulin-like proteins of the Australian lungfish (Neoceratodus forsteri).

Aust. J. Exp. Bio. Med. Sci. 47: 405-419.

MARCHALONIS, J. J. (1971a)

Immunoglobulins and antibody-production in amphibians.

Amer. Zool. 11: 171-181.

MARCHALONIS, J. J. (1971b)

Isolation and partial characterization of immunoglobulins of goldfish (Carassius auratus) and carp (Cyprinus carpio).

Immunology 20: 161-172.

MARCHALONIS, J. J., ALLEN, R. B., and SAARNI, E. S. (1970)

Immunoglobulin classes of the clawed toad, Xenopus laevis.

Comp. Biochem. Physiol. 35: 49-56.

MARCHALONIS, J. J. and COHEN, N. (1973)

Isolation and partial characterization of immunoglobulin from a urodele amphibian (Necturus masculosus).

Immunology 24: 395-407.

MARCHALONIS, J. J. and EDELMAN, G. M. (1966)

Phylogenetic origins of antibody structure. II. Immunoglobulins in the primary immune response of the bullfrog, Rana catesbiana.

J. Exp. Med. 124: 901-913.

MARCHALONIS, J. J. and GERMAIN, R. N. (1971)

Tolerance to a protein antigen in a poikilotherm, the marine toad Bufo marinus.

Nature 231: 321-322.

MATTES, M. J. and STEINER, L. A. (1978)

Antisera to frog immunoglobulins cross-react with a periodate-sensitive cell surface determinant.

Nature 273: 761-763.

MCGREGOR, D. D. and GOWANS, J. L. (1963)

The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct.

J. Exp. Med. 117: 303-320.

MCKINNEY, E. C., ORTIZ, G., LEE, J. C. SIGEL, M. M., LOPEZ,

D. M., EPSTEIN, R. S. and MCLEOD, T. F. (1976)

Lymphocytes of fish: multipotential or specialized?

In: Phylogeny of Thymus and Bone Marrow-Bursa Cells

(Ed. by R. K. Wright and E. L. Cooper), pp. 73-82.

Elsevier/North-Holland Biomedical Press, Amsterdam.

MCKINNEY, E. C., PETTEY, C. L., LOPEZ, D. M. and SIGEL,

M. M. (1977)

Cytotoxicity of nurse shark (Ginglymostoma cirratum)
leukocytes.

In: Developmental Immunobiology (Ed. by J. B. Solomon and
J. D. Horton), pp. 217-224.

Elsevier/North-Holland Biomedical Press, Amsterdam.

MCQUEEN, A., MACKENZIE, K., ROBERTS, R. J. and YOUNG, H. (1973)

The effect of temperature on the inflammatory response to
the metacercariae of Cryptocotyle lingua (Creplin, 1825)
(Digenea: Heterophyidae).

J. Fish Biol. 5: 241-247.

MELLORS, R. C. and KORNGOLD, L. (1963)

The cellular origin of human immunoglobulins.

J. Exp. Med. 118: 387-395.

METCALF, D. (1965)

Delayed effect of thymectomy in adult life on immunological
competence.

Nature 208: 1336-1337.

METCHNIKOFF, E. (1905)

Immunity in Infective Diseases.

Cambridge University Press, London.

MICHEL, C. (1979)

Furunculosis of salmonids: vaccination attempts in rain-
bow trout (Salmo gairdneri) by formalin-killed germs.

Ann. Rech. Vet. 10: 33-40.

MILLER, J. F. A. P. (1965)

Effect of thymectomy in adult mice on immunological
responsiveness.

Nature 208: 1337-1338.

MILLER, J. F. A. P., DUKOR, P., GRANT, G., SINCLAIR, N. R. SIC.
and SACQUET, E. (1967)

The immunological responsiveness of germ-free mice thymec-
tomized at birth. I. Antibody production and skin
homograft rejection.

Clin. Exp. Imm. 2: 531-542.

MILLER, J. F. A. P. and MITCHELL, G. F. (1968)

Cell to cell interaction in the immune response.

I. Hemolysin-forming cells in neonatally thymectomized
mice reconstituted with thymus or thoracic duct lymphocytes.

J. Exp. Med. 128: 801-820.

MILLER, J. F. A. P. and MITCHELL, G. F. (1970)

Cell to cell interaction in the immune response.

V. Target cells for tolerance induction.

J. Exp. Med. 131: 675-699.

MILLER, J. F. A. P., MITCHELL, G. F. and WEISS, N. S. (1967)

Cellular basis of the immunological defects in thymec-
tomized mice.

Nature 214: 992-997.

MINAGAWA, Y., OHNISHI, K. and MURAKAWA, S. (1975)

Structure and immunological function of lymphomyeloid
organs in the bullfrog, Rana catesbeiana.

In: Immunologic Phylogeny (Ed. by W. H. Hildemann and
A. A. Benedict). Adv. Exp. Med. Biol. 64; 257-266.

MITCHELL, G. F. and MILLER, J. F. A. P. (1968)

Cell to cell interaction in the immune response. II. The
source of hemolysin-forming cells in irradiated mice
given bone marrow and thymus or thoracic duct lymphocytes.

J. Exp. Med. 128: 821-837.

MITCHELL, J. (1972)

Antigens in immunity. XVII. The migration of antigen-binding, bone-marrow-derived and thymus-derived spleen cells in mice.

Immunology 22: 231-245.

MITCHELL, J. (1973)

Lymphocyte circulation in the spleen. Marginal zone bridging channels and their possible role in cell traffic.

Immunology 24: 93-107.

MITCHISON, N. A. (1965)

Induction of immunological paralysis in two zones of dosage.

Proc. Roy. Soc. Lond. B 161: 275-292.

MITCHISON, N. A. (1969)

The immunogenic capacity of antigen taken up by peritoneal exudate cells.

Immunology 16: 1-14.

MITSUHASHI, S., KURASHIGE, S., MISHIMA, S., YAMAGUSHI, N. and FUKAI, K. (1971)

Antibody production without reactive proliferation of pyroninophilic cells in rainbow trout and African clawed toad.

Tohoku J. Exp. Med. 103: 7-10.

MOLLER, G. and WIGZELL, H. (1965)

Antibody synthesis at the cellular level. Antibody-induced suppression of 19S and 7S antibody response.

J. Exp. Med. 121: 969-989.

MORROW, W. J. W. (1978)

The immune response of the dogfish Scyliorhinus canicula L.

Ph.D. Thesis, School of Environmental Sciences, Plymouth Polytechnic, Plymouth, UK.

MOSIER, D. E. (1967)

A requirement for two cell types for antibody formation
in vitro.

Science 158: 1573-1575.

MOTICKA, E. J., BROWN, B. A. and COOPER, E. L. (1973)

Immunoglobulin synthesis in bullfrog larvae.

J. Imm. 110: 855-861.

MUROGA, K. and EGUSA, S. (1969)

Immune response of the Japanese eel to Vibrio anguillarum.

I. Effects of temperature on agglutinating antibody
production in starved eels.

Bull. Jap. Soc. Sci. Fish. 35: 868-874.

MUTHUKKARUPPAN, VR., PITCHAPPAN, RM. and RAMILA, G. (1976)

Thymic dependence and regulation of the immune response
to sheep erythrocytes in the lizard.

In: Phylogeny of Thymus and Bone Marrow-Bursa Cells
(Ed. by R. K. Wright and E. L. Cooper), pp. 185-193.

Elsevier/North-Holland Biomedical Press, Amsterdam.

NEALE, N. L. and CHAVIN, W. (1971)

Lymphocytic tissue alterations during the primary immune
response of the goldfish (Carassius auratus L.).

Michigan Academician 3: 23-30.

NICOL, T., QUANTOCK, D. C. and VERNON-ROBERTS, B. (1966)

Stimulation of phagocytosis in relation to the mechanism
of action of adjuvants.

Nature 209: 1142-1143.

NORDQVIST, B. and RORSMAN, H. (1970)

Capillary tube migration of human peripheral blood cells.

Acta Dermatovener (Stockholm) 50: 435-439.

NOSSAL, G. J. V. and ADA, G. L. (1971)

Antigens, Lymphoid Cells and the Immune Response.

Academic Press, London and New York.

NOSSAL, G. J. V., ADA, G. L. and AUSTIN, C. M. (1964)

Antigens in immunity. IV. Cellular localization of ^{125}I - and ^{131}I -labelled flagella in lymph nodes.

Aust. J. Exp. Bio. Med. Sci. 42: 311-330.

NOSSAL, G. J. V., AUSTIN, C. M., PYE, J. and MITCHELL, J. (1966)

Antigens in immunity. XII. Antigen trapping in the spleen.

Int. Arch. All. App. Imm. 29: 368-383.

NOSSAL, G. J. V., CUNNINGHAM, A., MITCHELL, G. F. and MILLER, J. F. A. P. (1968)

Cell to cell interaction in the immune response. III.

Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated, or thymectomized mice.

J. Exp. Med. 128: 839-853.

OHARA, T., SHIMIZU, M., KAKINUMA, M., KIMURA, T. and

OKADA, M. (1979)

Induction of immunological tolerance by intrathymic injection of antigens.

Bull. Inst. Imm. Sci. Hokkaido Univ. 39: 48-51.

O'NEILL, J. G. (1978)

The immune response in teleosts: the effects of temperature and heavy metals.

Ph.D. Thesis, Department of Life Sciences, Trent Polytechnic, Nottingham, UK.

O'NEILL, J. G. (1979)

The immune response of the brown trout, Salmo trutta, L. to MS2-bacteriophage: immunogen concentration and adjuvants.

J. Fish Biol. 15: 237-248.

OPSTAD, A. M. (1959)

A methyl green-pyronin stain for plasma cells in tissues.
Stain. Techn. 34: 293.

ORTEGA, L. G. and MELLORS, R. C. (1957)

Cellular sites of formation of gamma globulin.
J. Exp. Med. 106: 627-640.

ORTIZ-MUNIZ, G. and SIGEL, M. M. (1971)

Antibody synthesis in lymphoid organs of two marine teleosts.
J. Retic. Soc. 9: 42-52.

PACHA, R. E. and KINGSBURY, D. T. (1962)

Preparation of chromosomes for light microscopy utilizing an agar fixation procedure.
Proc. Soc. Exp. Bio. Med. 111: 710-712.

PAPAMICHAIL, M., GUTIERREZ, C., EMBLING, P., JOHNSON, P.,

HOLBOROW, E. J. and PEPYS, M. B. (1975)

Complement dependence of localisation of aggregated IgG in germinal centres.
Scand. J. Imm. 4: 343-347.

PAPERMASTER, B. W., CONDIE, R. M., FINSTAD, J. and

GOOD, R. A. (1964)

Evolution of the immune response. I. The phylogenetic development of adaptive immunologic responsiveness in vertebrates.
J. Exp. Med. 119: 105-130.

PARISH, C. R. and ADA, G. L. (1971)

The mechanism of antigen localization in lymphoid follicles and the patterns of localization of tolerance-inducing

flagellar proteins.

In: Morphological and Functional Aspects of Immunity

(Ed. by K. Lindahl-Kiesling, G. Alm and M. G. Hanna),

pp. 221-225.

Plenum Press, New York.

PARKER, R. E. (1973)

Introductory Statistics for Biology. The Institute of

Biology's Studies in Biology No. 43.

Edward Arnold, Southampton, UK.

PARKS, D. E., DOYLE, M. V. and WEIGLE, W. O. (1978)

Induction and mode of action of suppressor cells generated against human gamma globulin. I. An immunologic unresponsive state devoid of demonstrable suppressor cells.

J. Exp. Med. 148: 625-638.

PATERSON, W. D. and FRYER, J. L. (1974)

Immune response of juvenile coho salmon (Oncorhynchus kisutch) to Aeromonas salmonicida cells administered intraperitoneally in Freund's Complete Adjuvant.

J. Fish. Res. Bd. Can. 31: 1751-1755.

PERNIS, B. (1967)

The immunoglobulins present in the germinal centers.

In: Germinal Centers in Immune Responses (Ed. by

H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon),

pp. 112-119.

Springer-Verlag, Berlin.

PICK, E., BROSTOFF, J., KREJCI, J. and TURK, J. L. (1970)

"Interaction between sensitized lymphocytes" and antigen in vitro. II. Mitogen-induced release of skin reactive and macrophage migration inhibitory factors.

Cell. Imm. 1: 92-109.

PICK, E., KREJCI, J. and TURK, J. L. (1970)

Release of skin reactive factor from guinea-pig lymphocytes by mitogens.

Nature 225: 236-238.

PICK, E. and TURK, J. L. (1972)

Review. The biological activities of soluble lymphocyte products.

Clin. Exp. Imm. 10: 1-23.

PITCHAPPAN, RM. (1980)

Review on the phylogeny of splenic structure and function.

Dev. Comp. Imm. 4: 395-416.

PITCHAPPAN, RM. and MUTHUKKARUPPAN, VR. (1977)

Role of the thymus in the immune response to sheep erythrocytes in the lizard Calotes versicolor.

Proc. Indian Acad. Sci. 85B: 25-33.

POLLARA, B., CAIN, W. A., FINSTAD, J. and GOOD, R. A. (1969)

The amphibian as a key step in the evolution of lymphoid tissue and diverse immunoglobulin classes.

In: Biology of Amphibian Tumors (Ed. by M. Mizell), pp. 177-183.

Springer-Verlag, Berlin.

POLLARA, B., FINSTAD, J. and GOOD, R. A. (1966)

The phylogenetic development of immunoglobulins.

In: Phylogeny of Immunity (Ed. by R. T. Smith, P. A. Miescher and R. A. Good), pp. 88-98.

University of Florida Press, Gainesville, USA.

POLLARA, B., FINSTAD, J. and GOOD, R. A. (1969)

Specific immunoglobulin synthesis in lower vertebrates lacking germinal centres.

- In: Lymphatic Tissue and Germinal Centers in Immune Response (Ed. by L. Fiore-Donati and M. G. Hanna, Jr.), Adv. Exp. Med. Bio. 5: 1-8.
Plenum Press, New York.
- POST, G. (1966)
Response of rainbow trout (Salmo gairdneri) to antigens of Aeromonas hydrophila.
J. Fish. Res. Bd. Can. 23: 1487-1494.
- PRENDERGAST, R. A. and SUZUKI, M. (1970)
Invertebrate protein simulating mediators of delayed hypersensitivity.
Nature 227: 277-279.
- RABINOWITZ, S. G. (1976)
Measurement and comparison of the proliferative and antibody responses of neonatal, immature and adult murine spleen cells to T-dependent and T-independent antigens.
Cell. Imm. 21: 201-216.
- REDDY, A. L. and BRYAN B. (1974)
Progr. Imm. 2: 295.
- REMOLD, H. G., KATZ, A. B., HABER, E. and DAVID, J. R. (1970)
Studies on migration inhibitory factor (MIF): recovery of MIF activity after purification by gel filtration and disc electrophoresis.
Cell. Imm. 1: 133-145.
- RICH, A. R. and LEWIS, M. R. (1932)
The nature of allergy in tuberculosis as revealed by tissue culture studies.
Bull. Johns Hopkins Hosp. 50: 115-131.

RICHTER, M. and HAUROWITZ, F. (1960)

Continuous synthesis of antibody after primary immunization with protein antigens.

J. Imm. 84: 420-425.

RICHTER, R. and AMBROSIUS, H. (1972)

Studies on "competition of antigens" in carp (Cyprinus carpio L.)

Acta bio. med. germ. 29: 897-906.

RICHTER, R., FRENZEL, E.-M., HADGE, D., KOPPERSCHLAGER, G. and AMBROSIUS, H. (1973)

Structural and immunochemical studies on the immunoglobulin of carp (Cyprinus carpio L.). I. Analysis at the total molecule.

Acta biol. med. germ. 30: 735-749.

RIDGWAY, G. J., HODGINS, H. O. and KLONTZ, G. W. (1966)

The immune response in teleosts.

In: Phylogeny of Immunity (Ed. by R. T. Smith, P. A. Miescher and R. A. Good), pp. 199-208.

University of Florida Press, Gainesville, USA.

RIJKERS, G. T. (1980)

The immune system of cyprinid fish.

Ph.D. Thesis, Landbouwhogeschool, Wageningen,

The Netherlands.

RIJKERS, G. T., FREDERIX-WOLTERS, L. M. H. and VAN

MUISWINKEL, W. B. (1980a)

The immune system of cyprinid fish. The effect of antigen dose and route of administration on the development of immunological memory in carp (Cyprinus carpio).

In: Phylogeny of Immunological Memory (Ed. by M. J. Manning), pp. 93-102.

Elsevier/North-Holland Biomedical Press, Amsterdam.

RIJKERS, G. T., FREDERIX-WOLTERS, E. M. H. and VAN
MUISWINKEL, W. B. (1980b)

The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (Cyprinus carpio).

Immunology 41: 91-97.

RIJKERS, G. T. and VAN MUISWINKEL, W. B. (1977)

The immune system of cyprinid fish. The development of cellular and humoral responsiveness in the rosy barb (Barbus conchonus).

In: Developmental Immunobiology (Ed. by J. B. Solomon and J. D. Horton), pp. 233-240.

Elsevier/North-Holland Biomedical Press, Amsterdam.

RIMMER, J. J. and GEARING, A. J. H. (1980)

Antigen specific migration inhibition of peritoneal exudate cells in an anuran (Rana temporaria).

In: Development and Differentiation of Vertebrate Lymphocytes (Ed. by J. D. Horton), pp. 195-200.

Elsevier/North-Holland Biomedical Press, Amsterdam.

RINGERTZ, N. and ADAMSON, C. A. (1950)

The lymph-node response to various antigens.

Acta Path. Microbiol. Scand. 86: 1-69.

RIO, G. J. and RECCO, R. A. (1971)

Effects of cold storage on serum antibodies of the goldfish.

Progr. Fish-Cult. 33: 37-41.

ROBERTS, R. J. (1975)

Melanin-containing cells of teleost fish and their relation to disease.

In: The Pathology of Fishes (Ed. by W. E. Ribelin and G. Migaki), pp. 399-428.

The University of Wisconsin Press, Madison, Wisconsin, USA.

ROGERS, A. W. (1967)

Techniques of Autoradiography.

Elsevier Publishing Company, Amsterdam.

ROSENBERG, S. A. and DAVID, J. R. (1970)

Inhibition of leukocyte migration: an evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen.

J. Imm. 105: 1447-1452.

ROWLEY, D. (1962)

Phagocytosis.

Adv. Imm. 2: 241-264.

ROWLEY, D. (1966)

Phagocytosis and immunity.

Experientia 22: 1-13.

RUBEN, L. N. (1975)

Ontogeny, phylogeny and cellular cooperation.

Amer. Zool. 15: 93-106.

RUBEN, L. N., VAN DER HOVEN, A. and DUTTON, R. W. (1973)

Cellular cooperation in hapten-carrier responses in the newt, Triturus viridescens.

Cell. Imm. 6: 300-314.

RUBEN, L. N., WARR, G. W., DECKER, J. M. and MARCHALONIS, J. J. (1977)

Phylogenetic origins of immune recognition: lymphoid heterogeneity and the hapten carrier effect in the goldfish, Carassius auratus.

Cell. Imm. 31: 266-283.

RUBIN, B. (1971)

The influence of amount and avidity of persisting primary antibodies on the secondary response to human serum albumin in rabbits.

Immunology 21: 475-481.

RUSSELL, P. J. and DIENER, E. (1970)

The early antibody-forming response to Salmonella antigens. A study of morphology and kinetics in vivo and in vitro.

Immunology 19: 651-667.

SAILENDRI, K. (1973)

Studies on the development of lymphoid organs and immune responses in the teleost Tilapia mossambica (Peters)

Ph.D. Thesis, Madurai University, India.

SAILENDRI, K. and MUTHUKKARUPPAN, VR. (1975a)

The immune response of the teleost, Tilapia mossambica to soluble and cellular antigens.

J. Exp. Zoo. 191: 371-382.

SAILENDRI, K. and MUTHUKKARUPPAN, VR. (1975b)

Morphology of lymphoid organs in a cichlid teleost,

Tilapia mossambica (Peters)

J. Morph. 147: 109-122.

SALVIN, S.B. and SMITH, R. F. (1960)

The specificity of allergic reactions. I..Delayed versus arthus hypersensitivity.

J. Exp. Med. 111: 465-483.

SCHAPERCLAUS, W. (1972)

Orale und parenterale aktive immunisierung von Karfen gegen Aeromonas punctata.

Archiv. Exp. Veterinaermed. 26: 863-874.

SCHIMPL, A. and WECKER, E. (1971)

Reconstitution of a thymus cell-deprived immune system by syngeneic and allogeneic thymocytes in vitro.

Eur. J. Imm. 1: 304-306.

SCHIRRMACHER, V. and RAJEWSKY, K. (1970)

Determination of antibody class in a system of co-operating antigenic determinants.

J. Exp. Med. 132: 1019-1034.

SCHWARTZ, H.J., LEON, M.A. and PELLELY, R.P. (1970)

Concanavalin A-induced release of skin-reactive factor from lymphoid cells.

J. Imm. 104: 265-268.

SECOMBES, C.J. and MANNING, M.J. (1980)

Comparative studies on the immune system of fishes and amphibians: antigen localization in the carp

Cyprinus carpio L.

J. Fish Diseases 3: 399-412.

SELL, S. and GELL, P.G.H. (1965)

Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with an antiallotype serum.

J. Exp. Med. 122: 423-440.

SERERO, M. and AVTALION, R. R. (1978)

Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. III. Establishment of immunological suppression in fish.

Dev. Comp. Imm. 2: 87-94.

SHELTON, E. and SMITH, M. (1970)

The ultrastructure of carp (Cyprinus carpio) immunoglobulin: a tetrameric macroglobulin.

J. Mol. Biol. 54: 615-617.

SHULMAN, S., HUBLER, L. and WITEBSKY, E. (1964)

Antibody response to immunization by different routes.

Science 145: 815-817.

SIGEL, M. M. and CLEM, L. W. (1965)

Antibody response of fish to viral antigens.

Ann. New York Acad. Sci. 126: 662-677.

SIGEL, M. M. and CLEM, L. W. (1966)

Immunologic anamnesis in elasmobranchs.

In: Phylogeny of Immunity (Ed. by R.T. Smith, P. A. Miescher and R.A. Good), pp. 190-198.

University of Florida Press, Gainesville, USA.

SIGEL, M. M., RUSSELL, W. J., WILLIAMS, J. A., GAINES, J. F. and LUCAS, A. H. (1968)

Studies on immunosuppression in teleosts.

Fed. Proc. 27: 685 (2636).

SILVERSTEIN, A. M. and LUKES, R. J. (1962)

Fetal response to antigenic stimulus. I. Plasma cellular and lymphoid reactions in the human fetus to intra-uterine infection.

Lab. Invest. 11: 918-932.

SIMAR, L., BETZ, E. H. and LEJEUNE, G. (1967)

Ultrastructural modifications of the lymph nodes after homologous skin grafting in the mouse.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 60-70.

Springer-Verlag, Berlin.

SIMIC, M. M. and PETROVIC, M. Z. (1967)

Effects of local irradiation of the spleen on the primary response in rats.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp, 240-249.

Springer-Verlag, Berlin.

SIMPSON, E. and CANTOR, H. (1975)

Regulation of the immune response by subclasses of T lymphocytes. II. The effect of adult thymectomy upon humoral and cellular responses in mice.

Eur. J. Imm. 5: 337-343.

SINCLAIR, N. R. STC., LEES, R. K., ABRAHAMS, S., CHAN, P. L.,

FAGAN, G. and STILLER, C. R. (1974)

Regulation of the immune response. X. Antigen-antibody complex inactivation of cells involved in adoptive transfer.

J. Imm. 113: 1493-1500.

SMITH, A. M., POTTER, M. and MERCHANT, E. B. (1967)

Antibody-forming cells in the pronephros of the teleost Lepomis macrochirus.

J. Imm. 99: 876-882.

SMITH, A. M., WIVEL, N. A., and POTTER, M. (1970)

Plasmacytopoiesis in the pronephros of the carp (Cyprinus carpio).

Anat. Rec. 167: 351-370.

SMITH, R. T. (1961)

Immunological tolerance of non-living antigens.

Advan. Imm. 1: 67-129.

SMITH, R. T., MIESCHER, P. A. and GOOD, R. A. (Eds.) (1966)

Phylogeny of Immunity.

University of Florida Press, Gainesville, USA.

SMITH, W. W. (1940)

Production of anti-bacterial agglutinins by carp and trout at 10°C.

Proc. Soc. Exp. Bio. Med. 45: 726-729.

SNIESZKO, S. F. (1970)

Immunization of fishes: a review.

J. Wildlife Dis. 6: 24-30.

SØBORG, M. (1968)

In vitro migration of peripheral human leucocytes in cellular hypersensitivity.

Acta Med. Scand. 184: 135-139.

SØBORG, M. and BENDIXEN, G. (1967)

Human lymphocyte migration as a parameter of hypersensitivity.

Acta Med. Scand. 181: 247-256.

SPENCE, K. D., FRYER, J. L. and PILCHER, K. S. (1965)

Active and passive immunization of certain salmonid fishes against Aeromonas salmonicida.

Can. J. Microbiol. 11: 397-405.

STAVITSKY, A. B. (1954)

Micromethods for the study of proteins and antibodies.

I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells.

J. Imm. 72: 360-375.

STERBA, G. (1951)

Untersuchungen an der Milz des Krallenfrosches (Xenopus laevis Daudin).

Morphol. Jahr. 90: 221-248.

STERZL, J. (1966)

Immunological tolerance as the result of terminal differentiation of immunologically competent cells.

Nature 209: 416-417.

STOCKINGER, B., BOTZENHARDT, U. and LEMMEL, E.-M. (1979)

On the feedback regulation of humoral immune response.

I. Evidence for 'B suppressor cells'.

Immunology 36: 87-94.

STOLEN, J. S. and MAKELA, O. (1975)

Carrier preimmunisation in the anti-hapten response of a marine fish.

Nature 254: 718-719.

STOLEN, J. S. and MAKELA, O. (1980)

Cell collaboration in a marine teleost. A demonstration of specificity in the carrier effect.

Immunology Letters 1: 341-345.

SVEJCAR, J., JOHANOVSKY, J. and PEKAREK, J. (1966)

Studies on the mechanism of delayed type hypersensitivity.

V. The influence of the specific antigen concentration upon the spleen cell migration in normal and sensitized guinea-pigs.

Z. Imm. Forsch. 131: 301-316.

TAHAN, A. M. and JURD, R. D. (1979)

Delayed hypersensitivity in Ambystoma mexicanum.

Dev. Comp. Imm. 3: 299-306.

TAM, M. R., REDDY, A. L., KARP, R. D. and HILDEMANN, W. H. (1976)

Phylogeny of cellular immunity among vertebrates.

In: Comparative Immunology (Ed. by J. J. Marchalonis), pp. 98-119.

Blackwell Scientific Publications, Oxford.

TAUB, R. N., KRANTZ, A. R. and DRESSER, D. W. (1970)

The effect of localized injection of adjuvant material on the draining lymph node.

Immunology 18: 171-186.

TAUSSIG, M. J. (1974)

The effect of Freund's complete adjuvant on the specificity of an immune response, and its relationship to antigenic competition.

Cell. Imm. 11: 484-487.

TAYLOR, R. B. (1965)

Decay of immunological responsiveness after thymectomy in adult life.

Nature 208: 1334-1335.

TAYLOR, R. B. (1968)

Immune paralysis of thymus cells by bovine serum albumin.

Nature 220: 611.

TAYLOR, R. B. (1969)

Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells.

Transplant. Rev. 1: 114-149.

TERRES, G., HABICHT, G. S. and STONER, R. D. (1974)

Carrier-specific enhancement of the immune response using antigen-antibody complexes.

J. Imm. 112: 804-811.

TERRES, G., MORRISON, S. L., HABICHT, G. S. and STONER, R. D. (1972)

Appearance of an early "primed state" in mice following the concomitant injections of antigen and specific antiserum.

J. Imm. 108: 1473-1481.

THORBECKE, G. J. (1969)

Germinal centers and immunological memory.

In: Lymphatic Tissue and Germinal Centers in Immune Response (Ed. by L. Fiore-Donati and M. G. Hanna, Jr.), Adv. Exp. Med. Bio. 5: 83-92.

Plenum Press, New York.

THORBECKE, G. J. and BENACERRAF, B. (1962)

The reticulo-endothelial system and immunological phenomena.

Progr. Allergy 6: 559-598.

THORBECKE, G. J., GORDON, H. A., WOSTMAN, B., WAGNER, M. and REYNIERS, J. A. (1957)

Lymphoid tissue and serum gamma globulin in young germ-free chickens.

J. Infect. Dis. 101: 237-251.

THORBECKE, G. J. and HURLIMANN, J. (1965)

Les nodules lymphoïdes secondaires.

Nouv. Rev. Franc. Hematol. 5: 385-392.

THORBECKE, G. J., JACOBSON, E. B. and ASOFSKY, R. (1964)

γ -Globulin and antibody formation in vitro. IV. The effect on the secondary response of X-irradiation given at varying intervals after a primary injection of bovine γ -globulin.

J. Imm. 92: 734-746.

THORPE, J. E. and ROBERTS, R. J. (1972)

An aeromonad epidemic in the brown trout, (Salmo trutta L.).

J. Fish Biol. 4: 441-451.

TIMUR, G., ROBERTS, R. J. and MCQUEEN, A. (1977)

The experimental pathogenesis of focal tuberculosis in the plaice (Pleuronectes platessa L.).

J. Comp. Path. 87: 83-87.

TIMUR, M. (1975)

A study of the Carrageenin Granuloma in the plaice
(Pleuronectes platessa L.).

Ph.D. Thesis, Unit of Aquatic Pathology, University of
Stirling, Stirling, UK.

TRUMP, G. N. (1970)

Goldfish immunoglobulins and antibodies to bovine serum
albumin.

J. Imm. 104: 1267-1275.

TRUMP, G. N. and HILDEMANN, W. H. (1970)

Antibody responses of goldfish to bovine serum albumin.
Primary and secondary responses.

Immunology 19: 621-627.

TURNER, C. (1966)

General Endocrinology, 4th edition.

W. B. Saunders Co., Philadelphia and London.

TURNER, M. W. (1977)

The immunoglobulins.

In: Immunology in Medicine. A comprehensive guide to
clinical immunology (Ed. by E. J. Holborow and W. G.
Reeves), pp. 83-129.

Academic Press, London.

TURNER, R. J. (1969)

The functional development of the reticuloendothelial
system in the toad, Xenopus laevis (Daudin).

J. Exp. Zoo. 170: 467-480.

TURNER, R. J. (1970)

Lymphoreticular organization and immune responses in the
toad, Xenopus laevis (Daudin).

Ph.D. Thesis, University of Hull, Hull, UK.

TURNER, R. J. (1973)

Response of the toad, Xenopus laevis, to circulating antigens. II. Responses after splenectomy.

J. Exp. Zoo. 183: 35-46.

TURNER, R. J. and MANNING, M. J. (1973)

Response of the toad, Xenopus laevis, to circulating antigens. I. Cellular changes in the spleen.

J. Exp. Zoo. 183: 21-34.

TURNER, R. J. and MANNING, M. J. (1974)

Thymic dependence of amphibian antibody responses.

Eur. J. Imm. 4: 346-349.

UEKI, A., FUKUSHIMA, Y., HYODOH, F. and KIMOTO, T. (1978)

Distribution of C3 receptor sites on cell surfaces of vertebrate fibroblasts.

Dev. Comp. Imm. 2: 547-553.

UHR, J. W., FINKELSTEIN, M. S. and FRANKLIN, E. C. (1962)

Antibody response to bacteriophage ϕ X174 in non-mammalian vertebrates.

Proc. Soc. Exp. Bio. Med. 111: 13-15.

UHR, J. W. and MOLLER, G. (1968)

Regulatory effect of antibody on the immune response.

Advan. Imm. 8: 81-127.

UNANUE, E. R. (1970)

Thymus dependency of the immune response to hemocyanin: an evaluation of the role of macrophages in thymectomized mice.

J. Imm. 105: 1339-1343.

UNANUE, E. R. (1978)

The regulation of lymphocyte functions by the macrophage.

Imm. Rev. 40: 227-255.

UNANUE, E. R. and FELDMAN, J. D. (1971)

Role of macrophages in delayed hypersensitivity.

I. Induction with macrophage-bound antigen.

Cell. Imm. 2: 269-274.

VAN DUIJN, C. (1967)

Diseases of Fishes. 2nd Edition.

Iliffe Books, London.

VAN LOON, J. J. A., VAN OOSTEROM, R. and VAN MUISWINKEL,
W. B. (1981)

Development of the immune system in carp.

In: Proceedings of the 1st Congress of the International
Society for Developmental and Comparative Immunology
(Ed. by J. B. Solomon).

Pergamon, Oxford, (in press).

VAN MUISWINKEL, W. B., RIJKERS, G. T. and VAN OOSTEROM, R.
(1978)

The origin of vertebrate immunity. The lymphoid system
in fish.

In: Proceedings of the Zodiac Symposium on Adaptation.
Wageningen, The Netherlands, pp. 84-87.

Centre for Agricultural Publishing and Documentation,
Wageningen, The Netherlands.

VAN ROOIJEN, N. (1972a)

The vascular pathways in the white pulp of the rabbit
spleen.

Acta Morphol. Neerl.-Scand. 10: 351-357.

VAN ROOIJEN, N. (1972b)

Antigens in the spleen. The non-specificity of the
follicles in the process of antigen trapping and the

role of antibody.

Immunology 22: 757-765.

VAN ROOIJEN, N. (1973a)

Mechanism of follicular antigen trapping. Migration of antigen-antibody complexes from marginal zone towards follicle centres.

Immunology 25: 847-852.

VAN ROOIJEN, N. (1973b)

Nonspecific nature of antigen trapping in lymphoid follicles.

A comparative study on the localization of the antigens ^3H -DNP-BGG and ^{125}I -HGG in the spleen of rabbits, using an autoradiographic technique for distinction between two isotopes in the same tissue section.

Immunology 25: 853-867.

VAN ROOIJEN, N. (1978)

Binding of labelled antigens and immune complexes to macrophages and dendritic cells in cryostat sections of normal mouse spleen.

Acta Morphol. Neerl.-Scand. 16: 121-127.

VAN ROOIJEN, N. (1980)

Immune complex trapping in lymphoid follicles: a discussion on possible functional implications.

In: Phylogeny of Immunological Memory (Ed. by M. J. Manning), pp. 281-290.

Elsevier/North-Holland Biomedical Press, Amsterdam.

VERNIER, J.-M. (1969)

Table chronologique du développement embryonnaire de la truite arc-en-ciel, Salmo gairdneri Rich 1836.

Ann. Embryol. Morphogenese 2: 495-520.

VESTERGARD JØRGENSEN, P. E. (1973)

The nature and biological activity of IPN virus neutralizing antibodies in normal and immunized rainbow trout (Salmo gairdneri).

Arch. gesamte Virusforsch. 42: 9-20.

WARD, P. A., JOHNSON, A. G. and ABELL, M. R. (1959)

Studies on the adjuvant action of bacterial endotoxins on antibody formation. III. Histologic response of the rabbit spleen to a single injection of a purified protein antigen.

J. Exp. Med. 109: 463-474.

WARR, G. W., DELUCA, D., DECKER, J. M., MARCHALONIS, J. J. and RUBEN, L. N. (1977)

Lymphoid heterogeneity in teleost fish: studies on the genus Carassius.

In: Developmental Immunobiology (Ed. by J. B. Solomon and J. D. Horton), pp. 241-248.

Elsevier/North-Holland Biomedical Press, Amsterdam.

WARR, G. W., DELUCA, D. and MARCHALONIS, J. J. (1980)

Phylogeny and ontogeny of antigen-specific T cell receptors.

In: Development and Differentiation of Vertebrate Lymphocytes (Ed. by J. D. Horton), pp. 99-110.

Elsevier/North-Holland Biomedical Press, Amsterdam.

WEIGLE, W. O. (1973)

Immunological unresponsiveness.

Advan. Imm. 16: 61-122.

WEIGLE, W. O., SIECKMANN, D. G., DOYLE, M. V. and

CHILLER, J. M. (1975)

Possible roles of suppressor cells in immunological tolerance.

Transplant. Rev. 26: 186-205.

WEINBERG, S. (1975)

Occurrence of lymphoid cells in the intestine of the goldfish.

Bijdr. Dierk. 45: 195-204.

WEIR, D. M. (1973)

Immunology for undergraduates. 3rd Edition.

Churchill-Livingstone, Edinburgh.

WEISS, E. and AVTALION, R. R. (1977)

Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. II. Primary enhancement of anti-hapten antibody response at high and low temperatures.

Dev. Comp. Imm. 1: 93-104.

WHITE, R.G. (1958)

Antibody production by single cells.

Nature 182: 1383-1384.

WHITE, R.G. (1963)

Functional recognition of immunologically competent cells by means of the fluorescent antibody technique.

In: The Immunologically Competent Cell. Ciba Foundation Study Group No. 16 (Ed. by G. E. W. Wolstenholme and J. Knight), pp. 6-19.

Churchill, London.

WHITE, R. G. (1972)

Concepts of the mechanism of action of adjuvants.

In: Immunogenicity (Ed. by F. Borek).

Frontiers of Biology 25: 112-130.

North-Holland Publishing Company, Amsterdam.

WHITE, R. G. (1973)

Immunopotentialiation by mycobacteria in complete Freund-type adjuvant as the failure of normal immunological

homeostasis.

In: Immunopotential (Ed. by G. E. W. Wolstenholme and J. Knight). Ciba Foundation Symp. 18: 47-72.

Elsevier, Amsterdam.

WHITE, R. G., FRENCH, V. I. and STARK, J. M. (1967)

Germinal center formation and antigen localization in malpighian bodies of the chicken spleen.

In: Germinal Centers in Immune Responses (Ed. by H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 131-144.

Springer-Verlag, Berlin.

WHITE, R. G., FRENCH, V. I. and STARK, J. M. (1970)

A study of the localisation of a protein antigen in the chicken spleen and its relation to the formation of germinal centres.

J. Med. Microbiol. 3: 65-83.

WHITE, R. G., HENDERSON, D. C., ESLAMI, M. B. and NIELSEN, K. H. (1975)

Localization of a protein antigen in the chicken spleen. Effect of various manipulative procedures on the morphogenesis of the germinal centre.

Immunology 28: 1-21.

WHITE, R. G. and NIELSEN, K. H. (1975)

Interactions between the immunological responses of a thymus-independent antigen (Salmonella adelaide O antigen) with a thymus-dependent antigen (sheep erythrocytes) in the adult bird.

Immunology 28: 959-972.

WIGZELL, H. (1970)

Specific fractionation of immunocompetent cells.

- Transplant. Rev. 5: 76-104.
- WISLOCKI, G. B. (1917)
- The action of vital dyes in teleosts.
- Anat. Rec. 12: 415-427.
- WRIGHT, A. E. and DOUGLAS, S. R. (1903)
- An experimental investigation of the role of the blood fluids in connection with phagocytosis.
- Proc. Roy. Soc. B 72: 357-370.
- YAMAGA, K., KUBO, R. T. and ETLINGER, H. M. (1978)
- Studies on the question of conventional immunoglobulin on thymocytes from primitive vertebrates. II. Delineation between Ig-specific and cross-reactive membrane components.
- J. Imm. 120: 2074-2079.
- YAMAGUCHI, N., KURASHIGE, S. and MITSUHASHI, S. (1973)
- Immune response in Xenopus laevis and immunochemical properties of the serum antibodies.
- Immunology 24: 109-118.
- YOCUM, D., CUCHENS, M. and CLEM, L. W. (1975)
- The hapten-carrier effect in teleost fish.
- J. Imm. 114: 925-927.
- YOFFEY, J. M. (1929)
- A contribution to the study of the comparative histology and physiology of the spleen, with reference chiefly to its cellular constituents. I. In fishes.
- J. Anat. 63: 314-344.
- YOUNG, I. and FRIEDMAN, H. (1967)
- The morphologic demonstration of antibody formation in follicles of lymphoid tissue.
- In: Germinal Centers in Immune Responses (Ed. by

H. Cottier, N. Odartchenko, R. Schindler and C. C. Congdon), pp. 102-111.
Springer-Verlag, Berlin.

YU, M.-L., SAROT, D. A., FILAZZOLA, R. J. and PERLMUTTER, A.
(1970)

Effects of splenectomy on the immune response of the blue gourami Trichogaster trichopterus to infectious pancreatic necrosis (IPN) virus.

Life Sci. 9: 749-755.

ZAPATA, A. (1979)

Ultrastructural study of the teleost fish kidney.

Dev. Comp. Imm. 3: 55-65.

PLATE 1

Cyprinus carpio: external appearance of fry 14 days after hatching at $22 \pm 1^{\circ}\text{C}$. Stage 41 of Balinsky's Normal Table for cyprinid fish.

PLATE 2

Cyprinus carpio: external appearance of fry 28 days after hatching at $22 \pm 1^{\circ}\text{C}$.

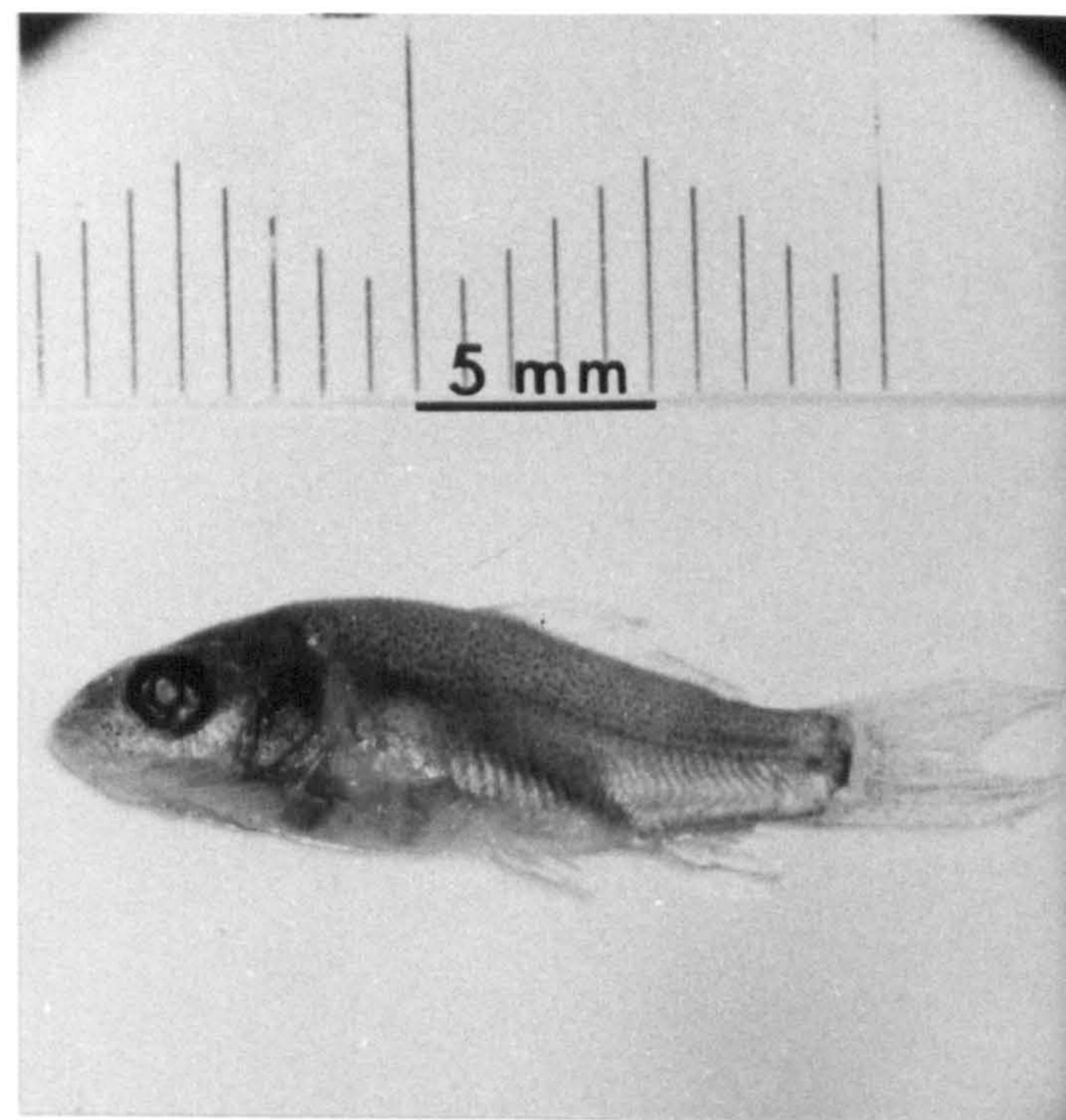


PLATE 3

Salmo gairdneri: external appearance of fry 1 day after hatching at 14°C. Stage 30 of Vernier's Normal Table for trout. Note the very large yolk sac (ys).

PLATE 4

Salmo gairdneri: external appearance of fry 14 days after hatching at 14°C. Stage 36 of Vernier's Normal Table for trout. The yolk sac is gradually being resorbed.

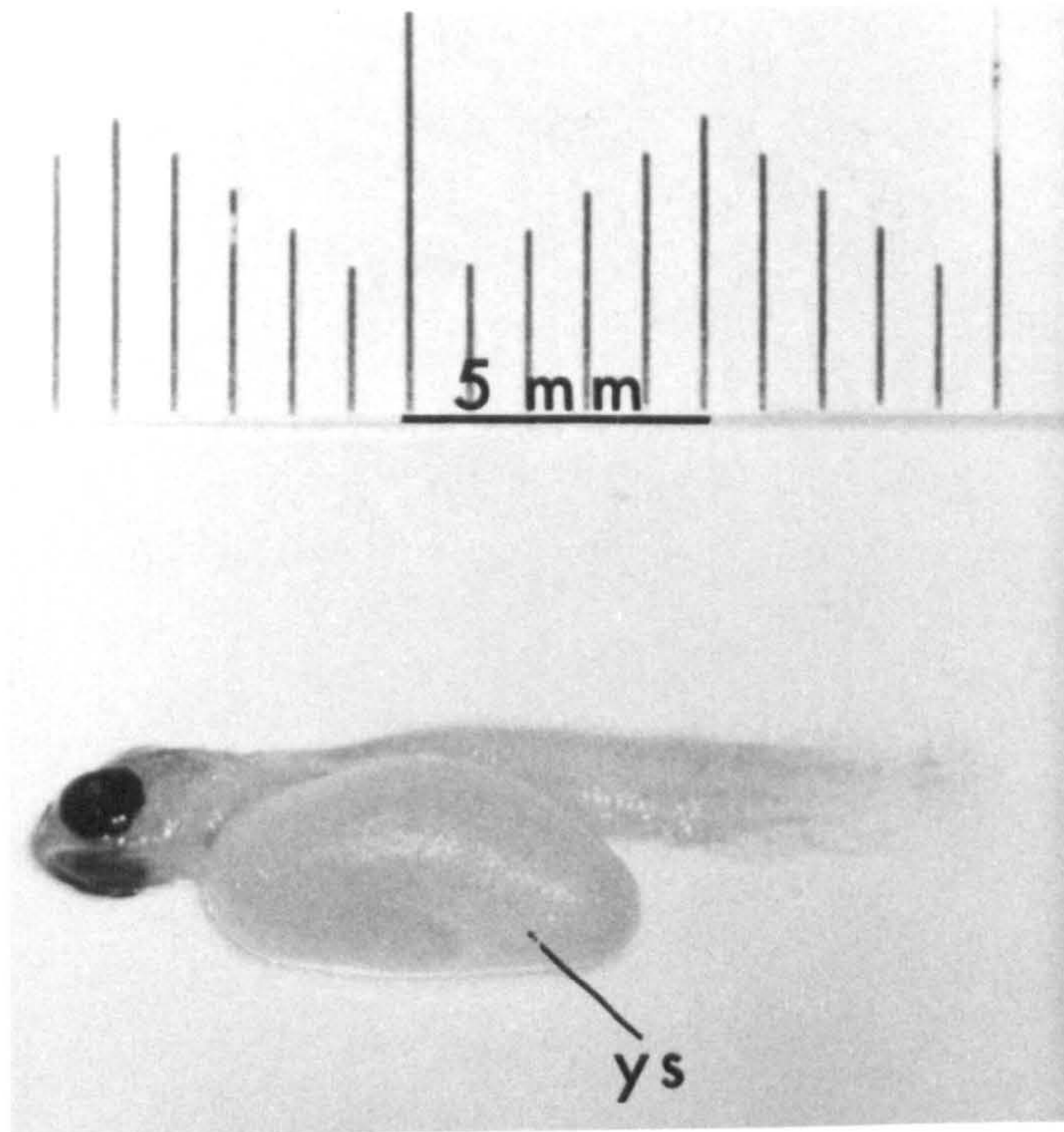


PLATE 5

A transverse section through a splenic ellipsoid stained with haematoxylin and eosin. Macrophages (M) are often present.

PLATE 6

Silver impregnated ellipsoids showing the reticulum.

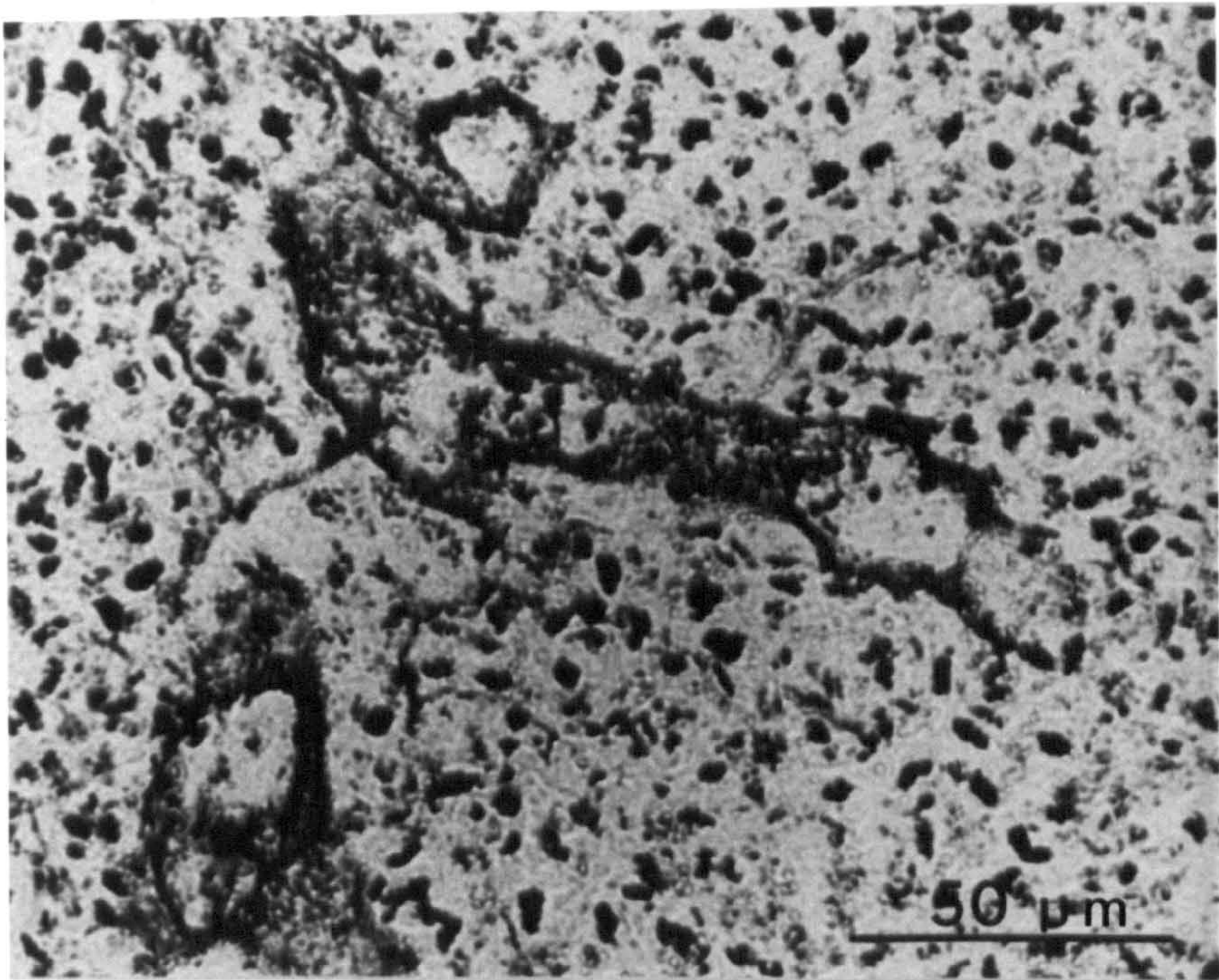
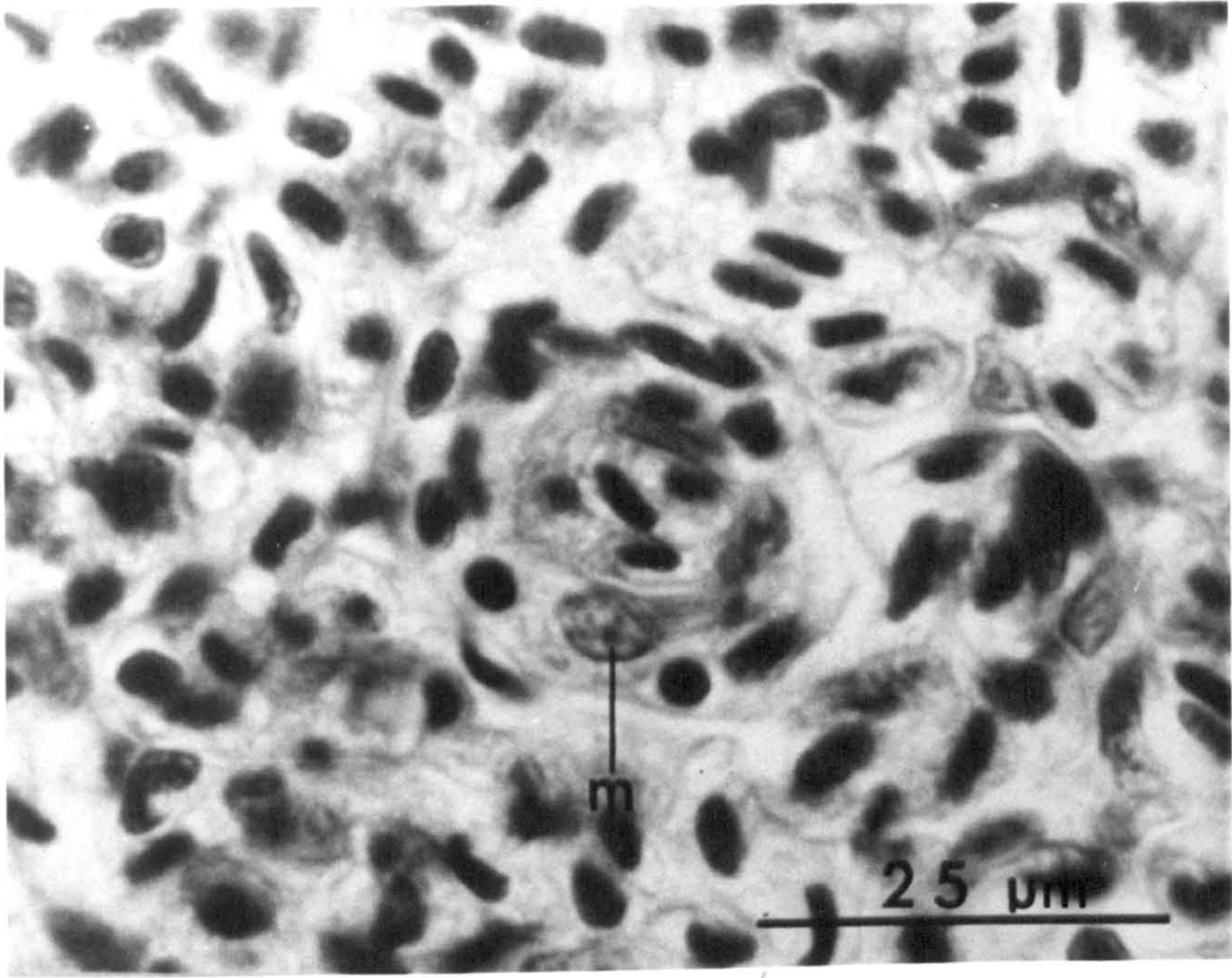


PLATE 7

A section of pronephros stained with methyl green-pyronin,
low power. Note the thick lining of steroidogenic cells (s).

PLATE 8

A section of pronephros stained with methyl green-pyronin,
high power.

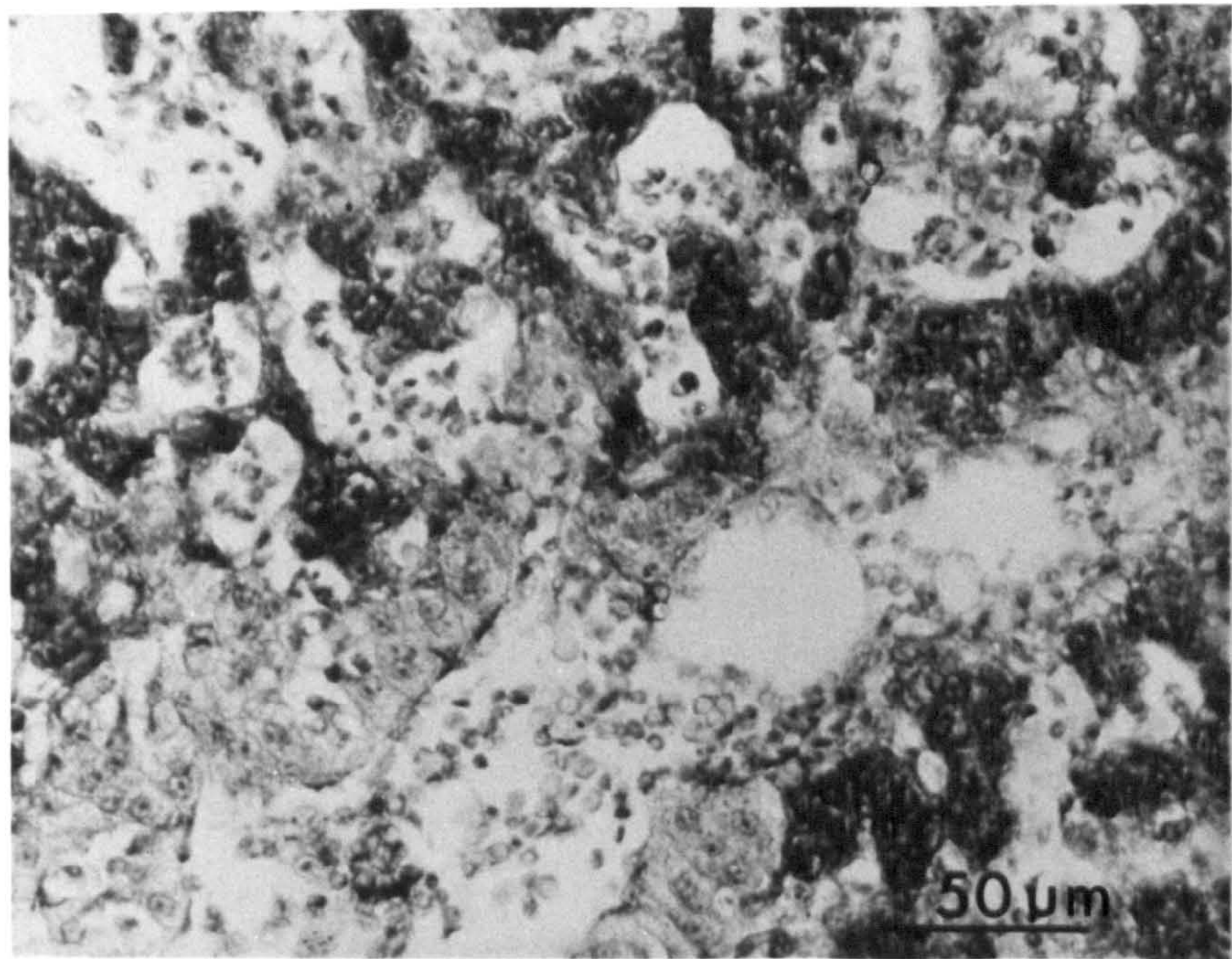
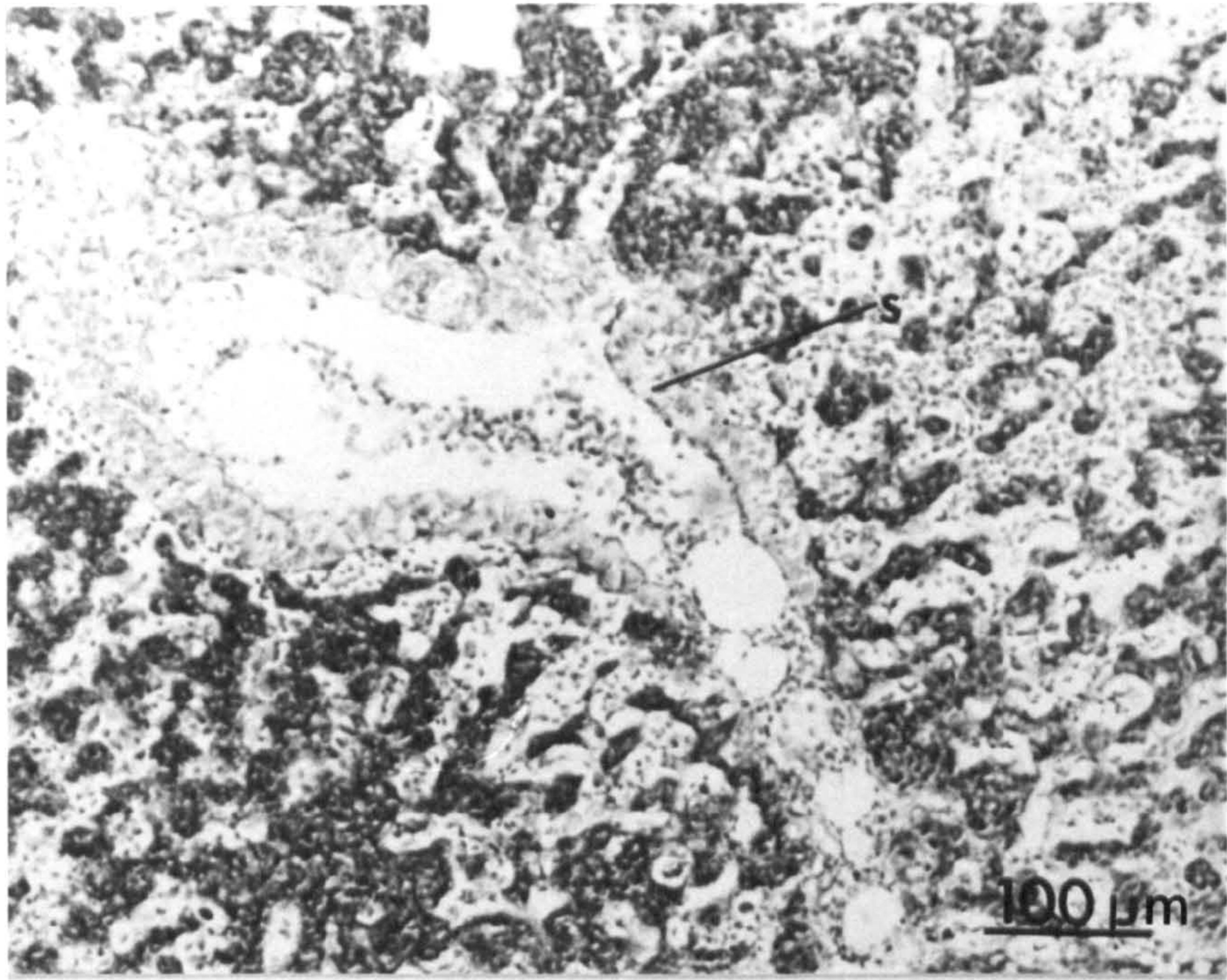


PLATE 9

Electron micrograph of a lymphocyte in the pronephros. The largest part of the cell is occupied by the nucleus, with dense chromatin (n). There are few mitochondria and the endoplasmic reticulum is absent. Free ribosomes (r) are scattered throughout the cytoplasm.

PLATE 10

Electron micrograph of a plasma cell in the pronephros. There is an eccentric nucleus (n) and a large amount of rough endoplasmic reticulum (rer). Mitochondria (m) occur between the cisternae.

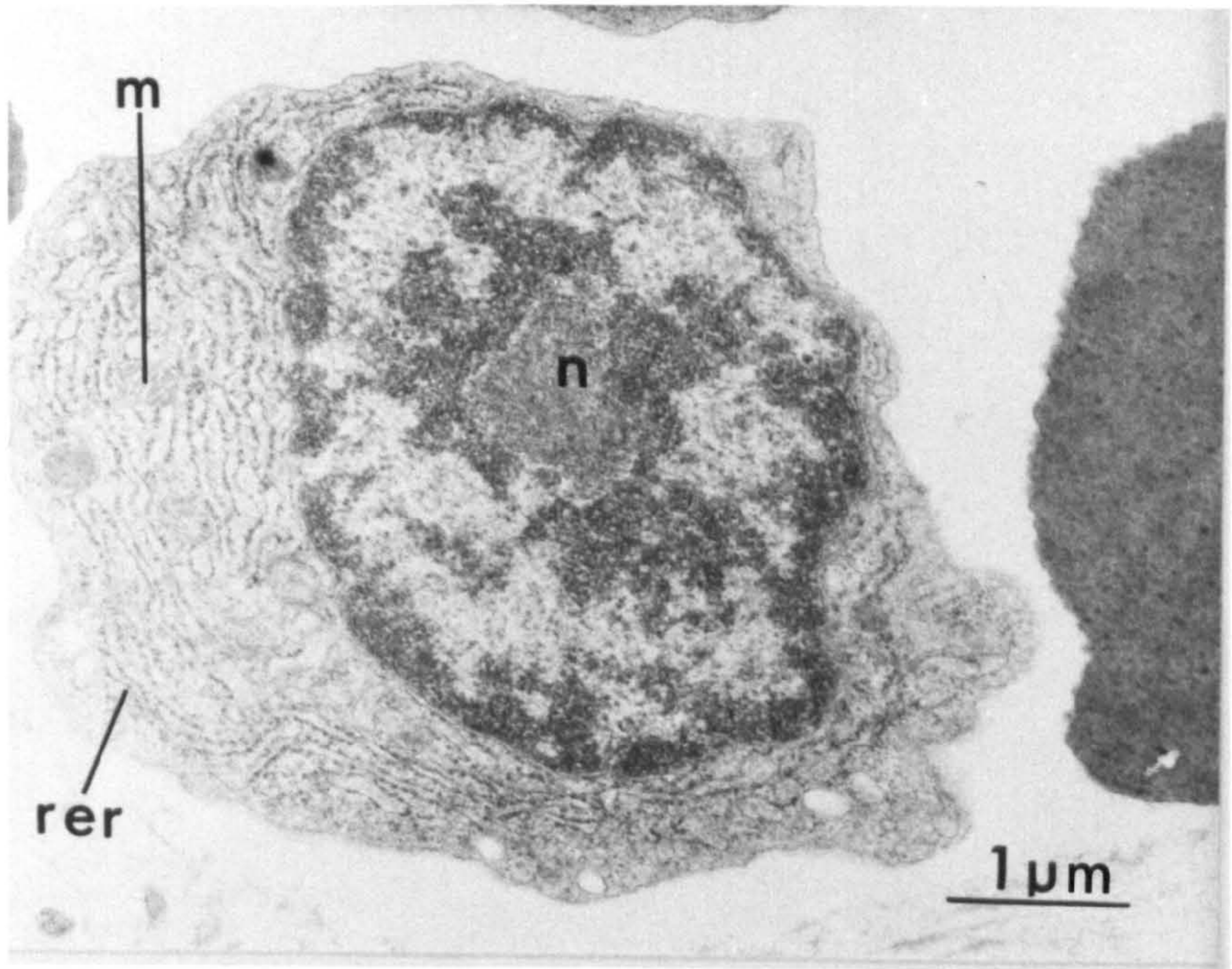


PLATE 11

Electron micrograph of a granulocyte in the pronephros resembling a mammalian eosinophil. The cytoplasmic granules have characteristic crystalline inclusions (ci), with an electron dense equatorial crystal. There is some rough endoplasmic reticulum (rer) present, and a few mitochondria (m) and multivesicular bodies (mb).

PLATE 12

Electron micrograph of a second type of granulocyte in the pronephros. There are many large vacuoles (v), but little endoplasmic reticulum.

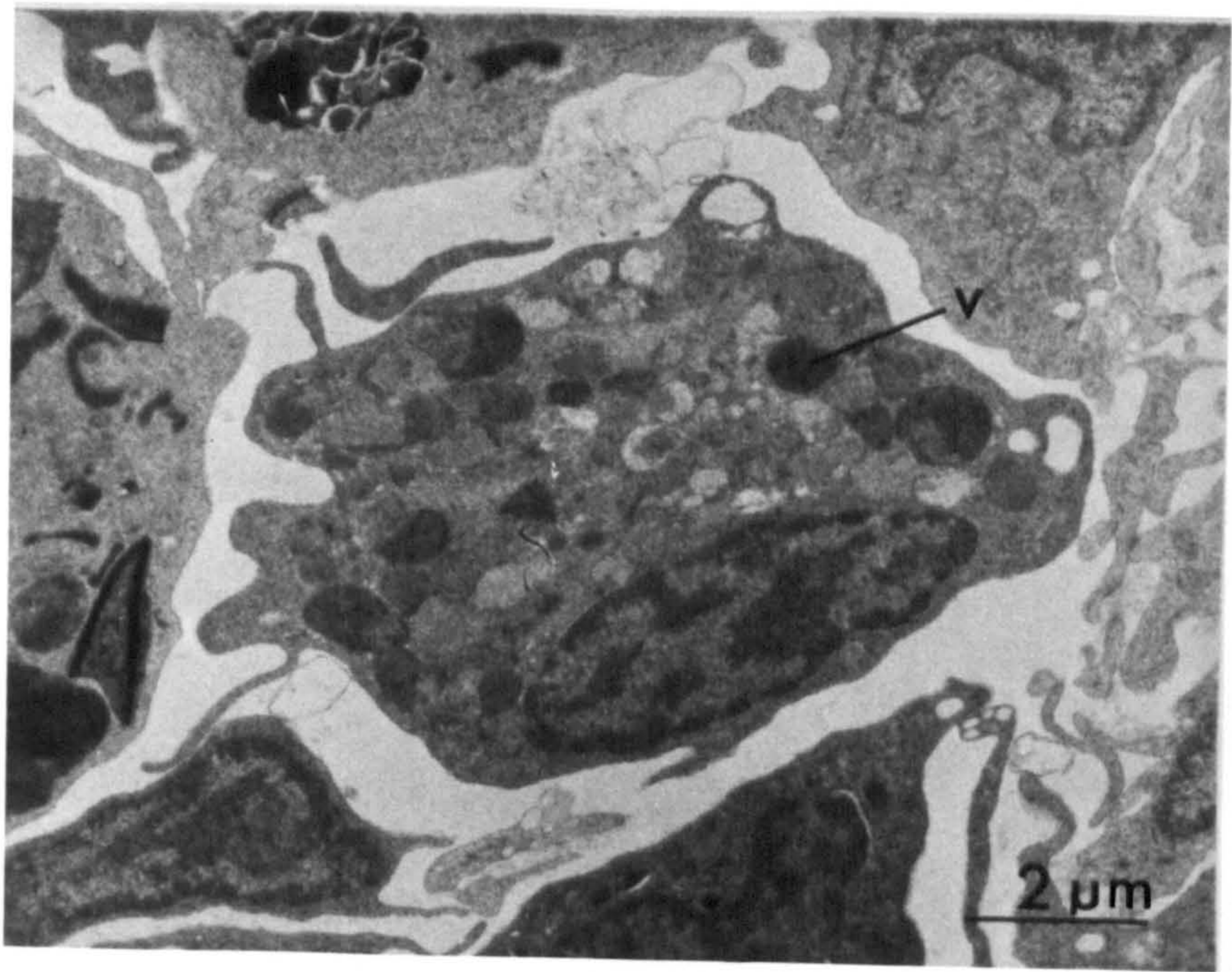
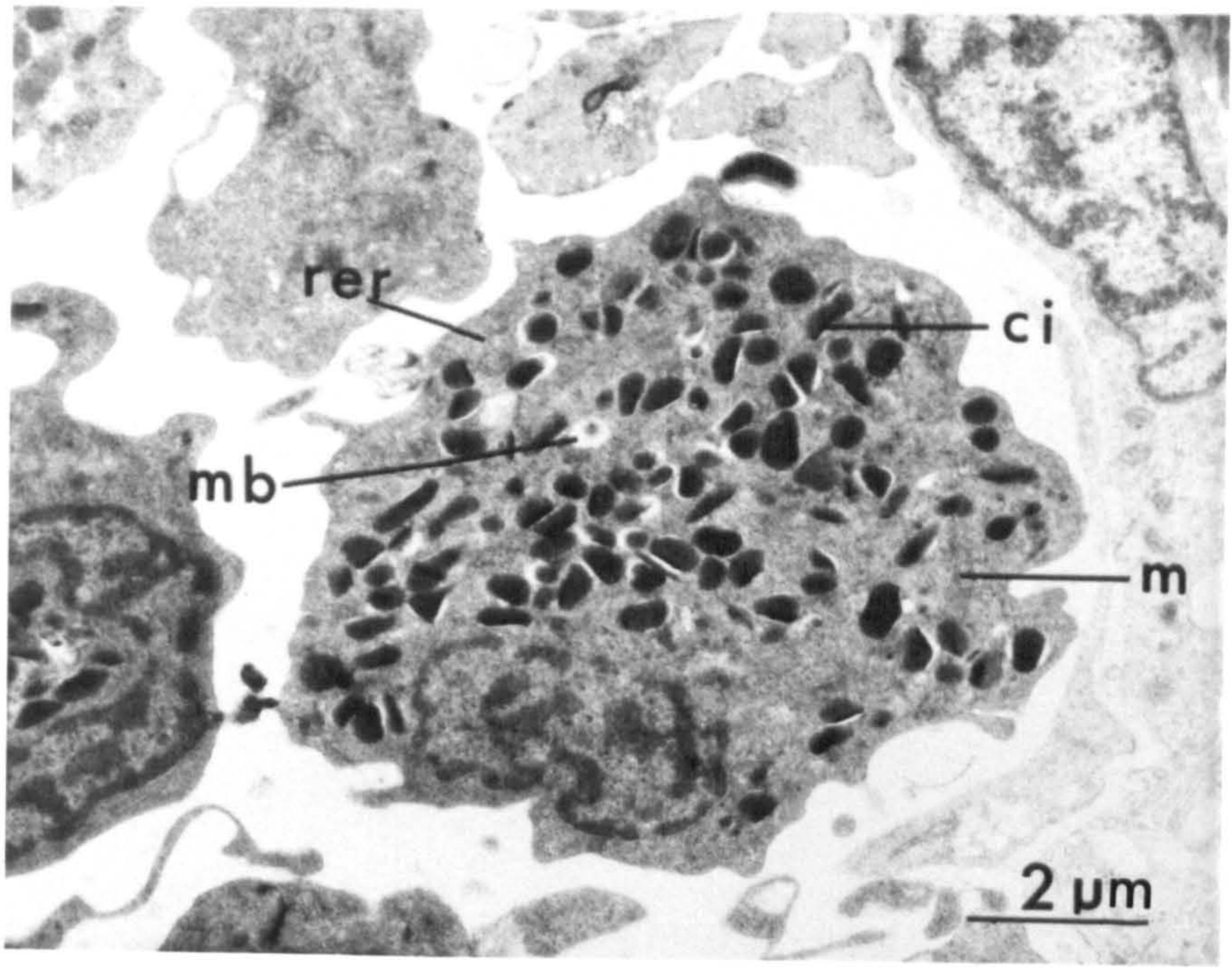


PLATE 13

A section of mesonephros stained with methyl green-pyronin, from a carp immunized with HGG in FCA. Note the cluster of pyroninophilic cells (pc) between the excretory tubules.

PLATE 14

A section of mesonephros stained with methyl green-pyronin, from a carp immunized with HGG in FCA, to show (a) a pyroninophilic tubule (pt) and (b) a normal tubule (nt).

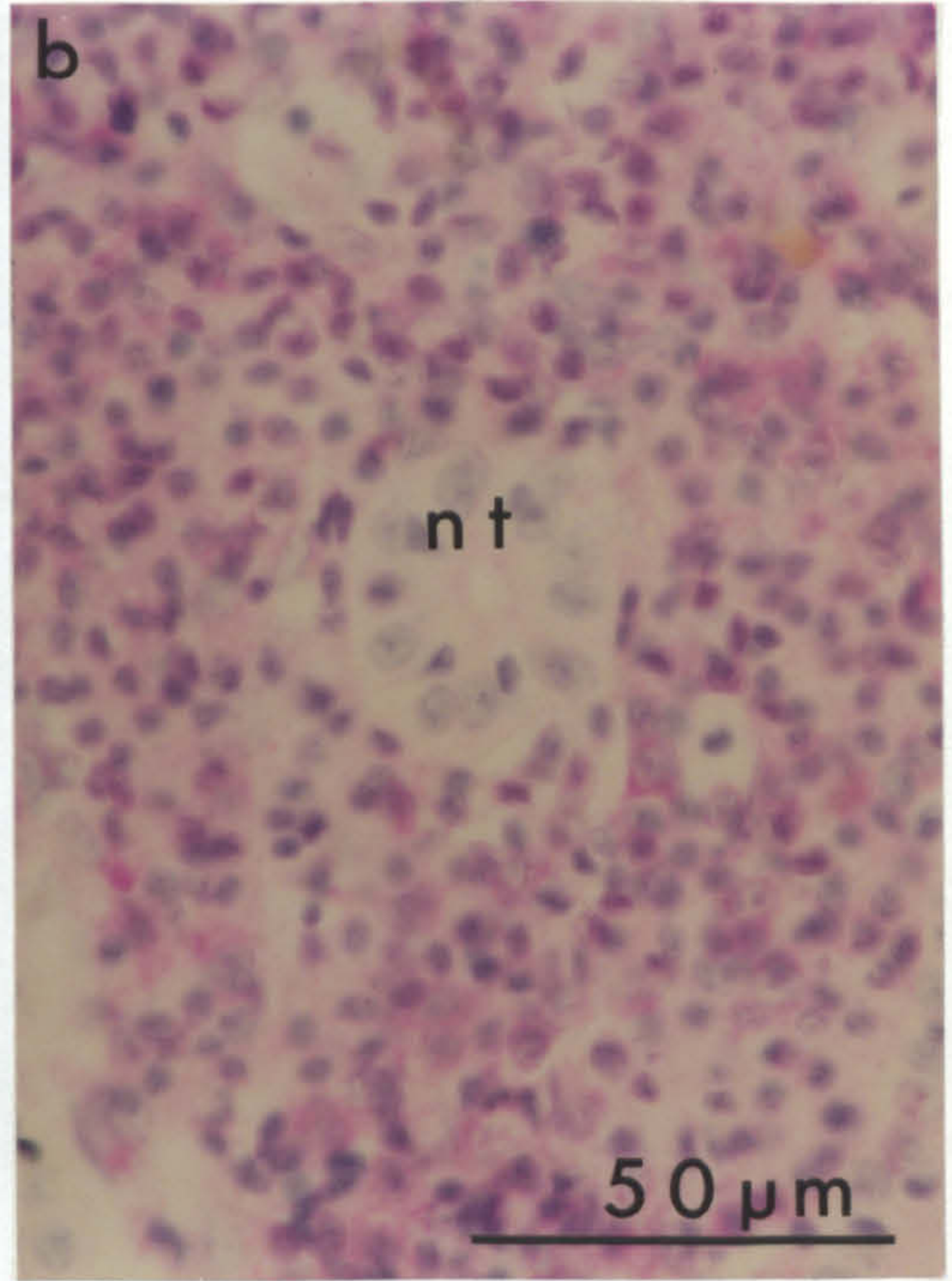
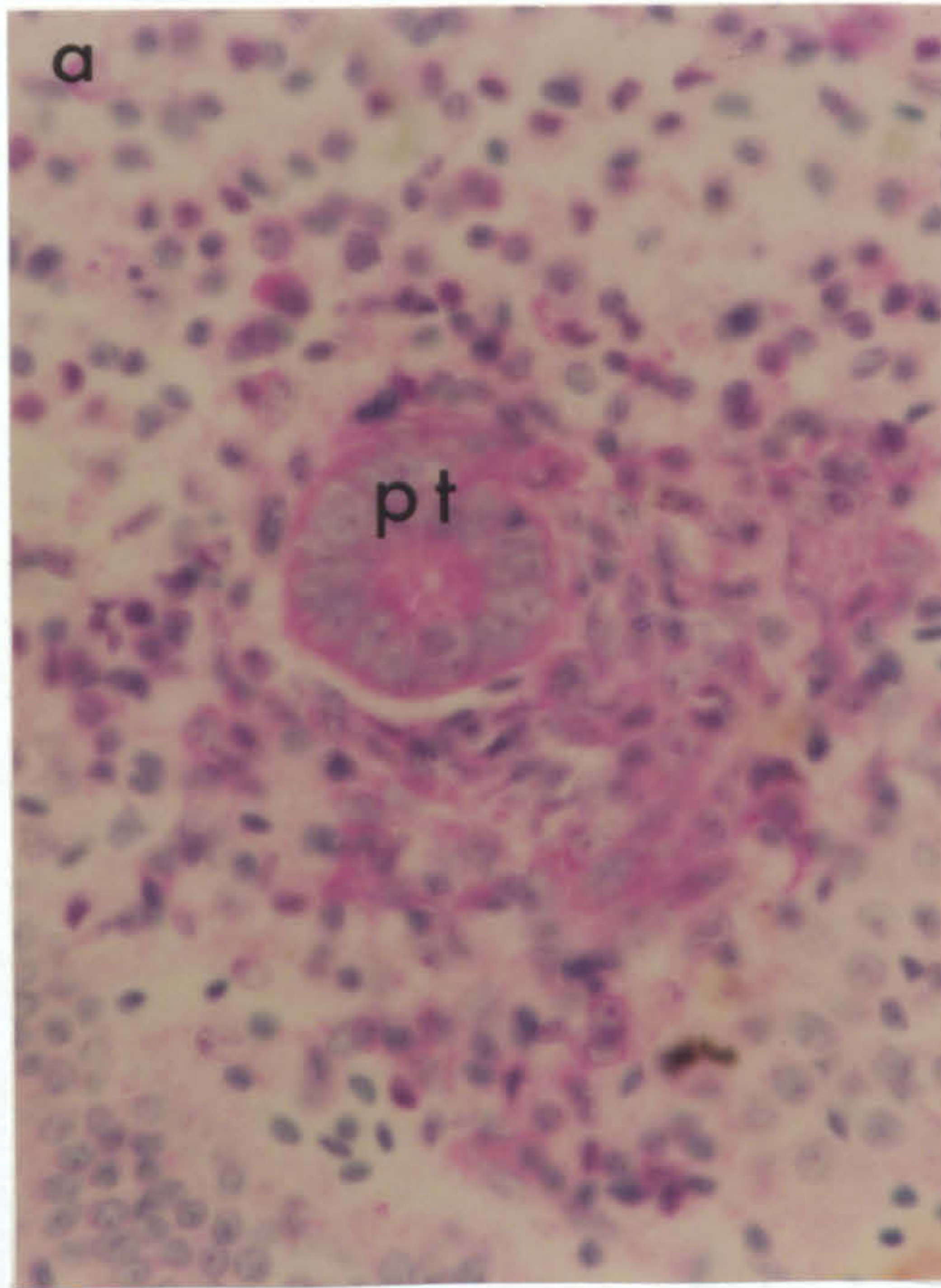
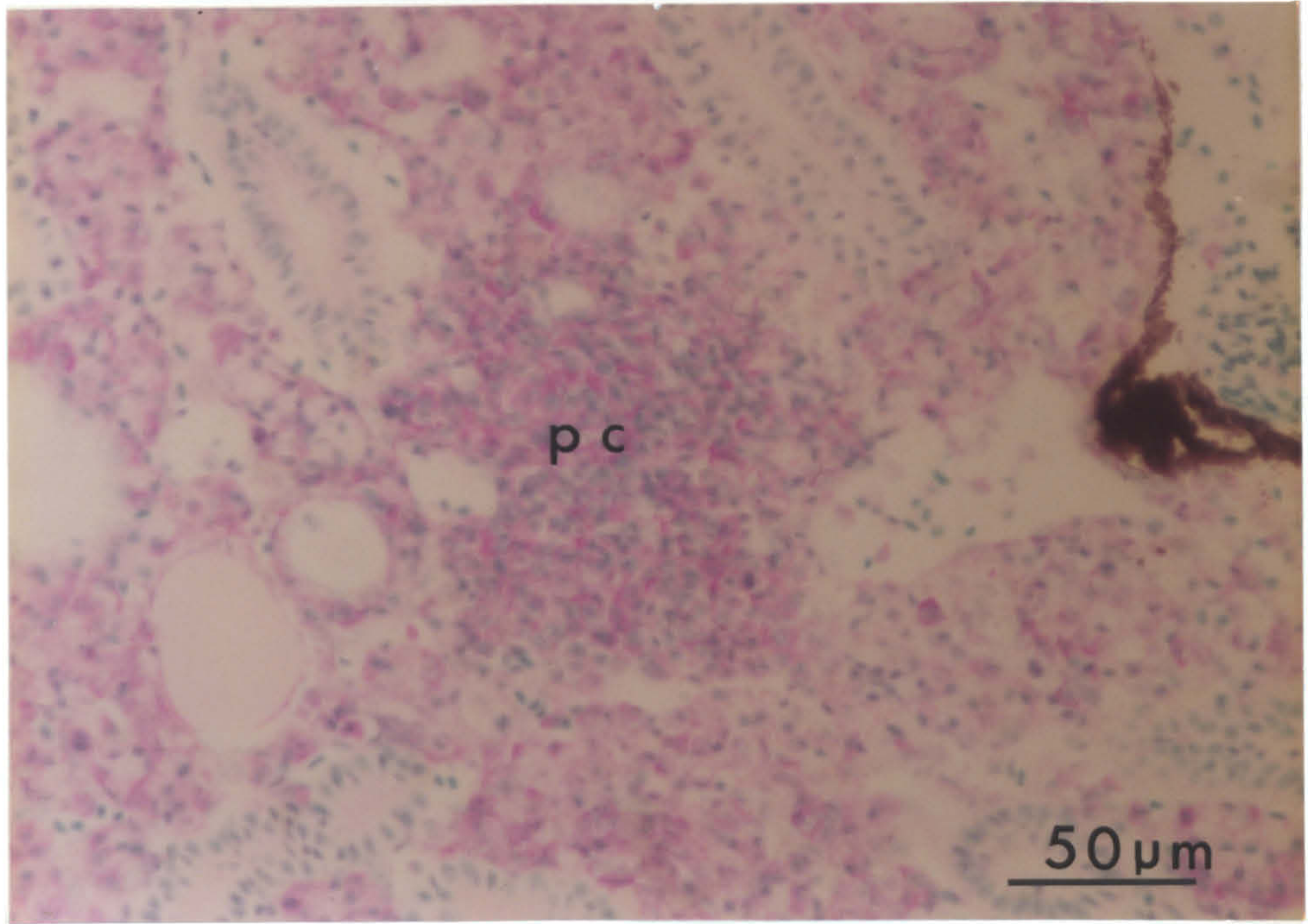


PLATE 15

Silver impregnated ellipsoids showing fine extensions (fe) of the reticulum of the ellipsoid sheath surrounding melano-macrophage aggregates (m).

PLATE 16

Electron micrograph of a large lymphocyte in the pronephros. There is a large nucleus (n) of loose chromatin structure, more abundant cytoplasm, more obvious endoplasmic reticulum (er) and many large mitochondria (m).

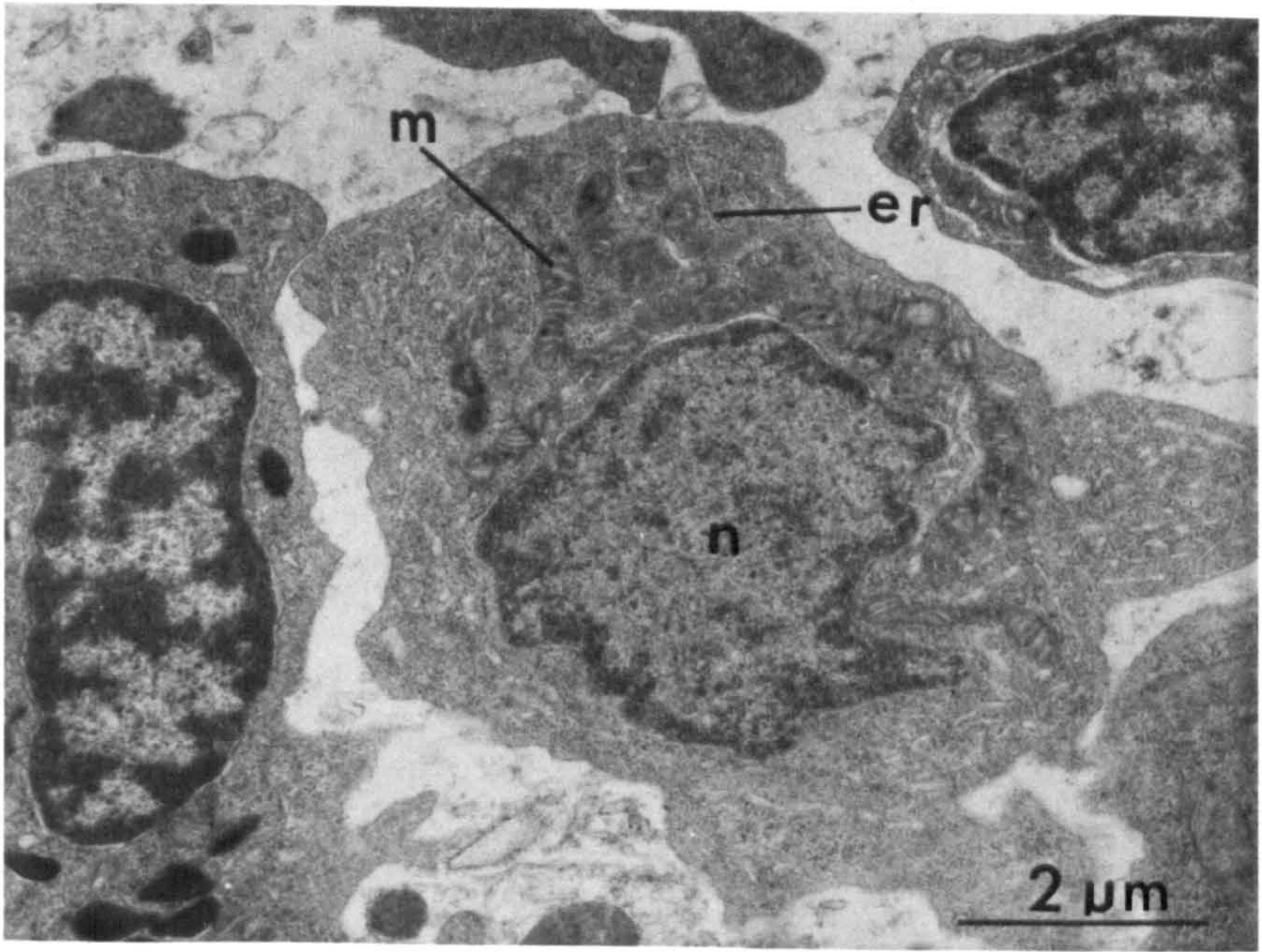
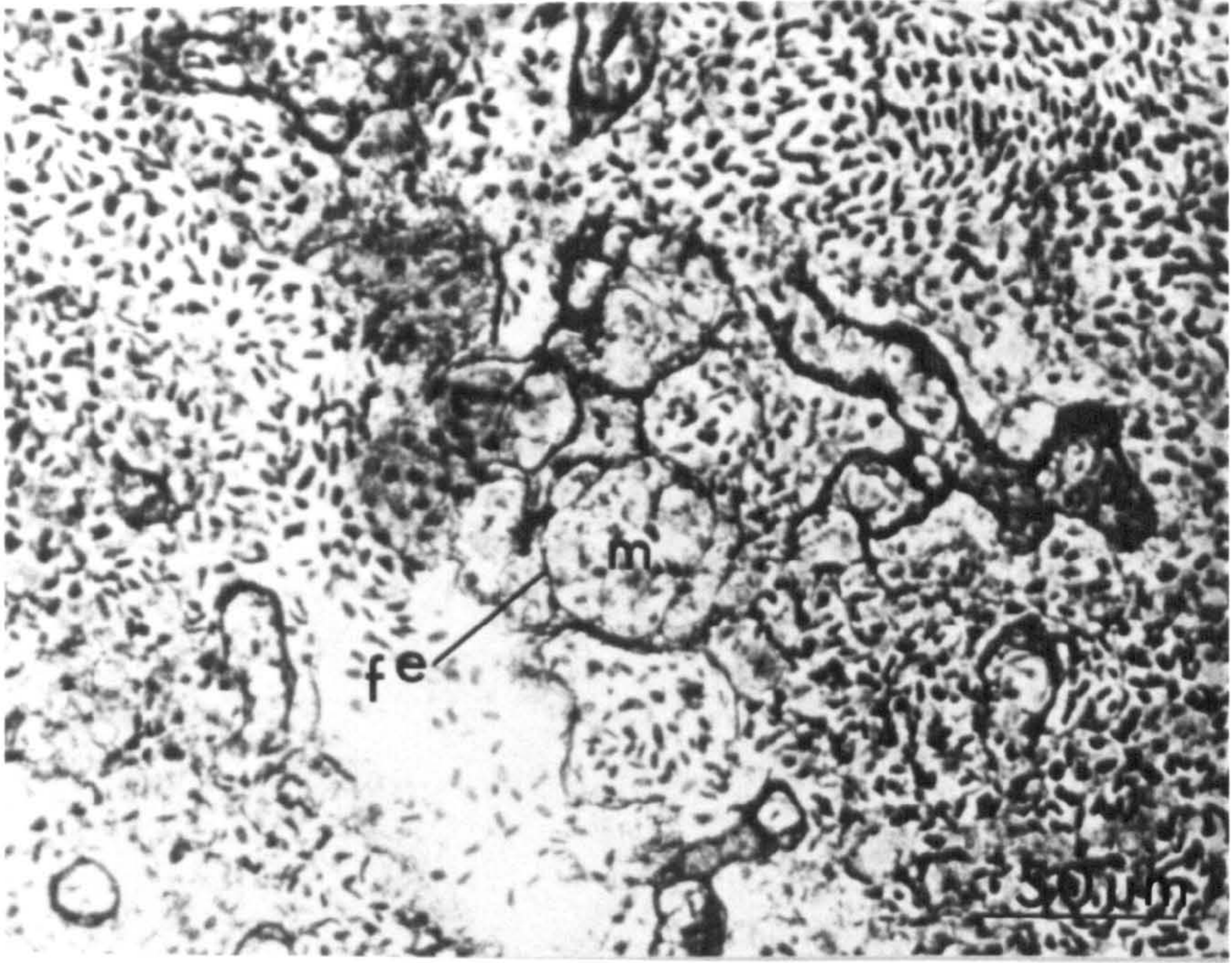


PLATE 17

A section of pronephros stained with methyl green-pyronin, from a carp immunized with HGG in FCA. At day 21 numerous clusters of pyroninophilic (pc) cells can be seen.

Low power.

PLATE 18

A section of pronephros stained with methyl green-pyronin, from a carp immunized with HGG in FCA, to show a high power view of a single pyroninophilic cell cluster (pc).

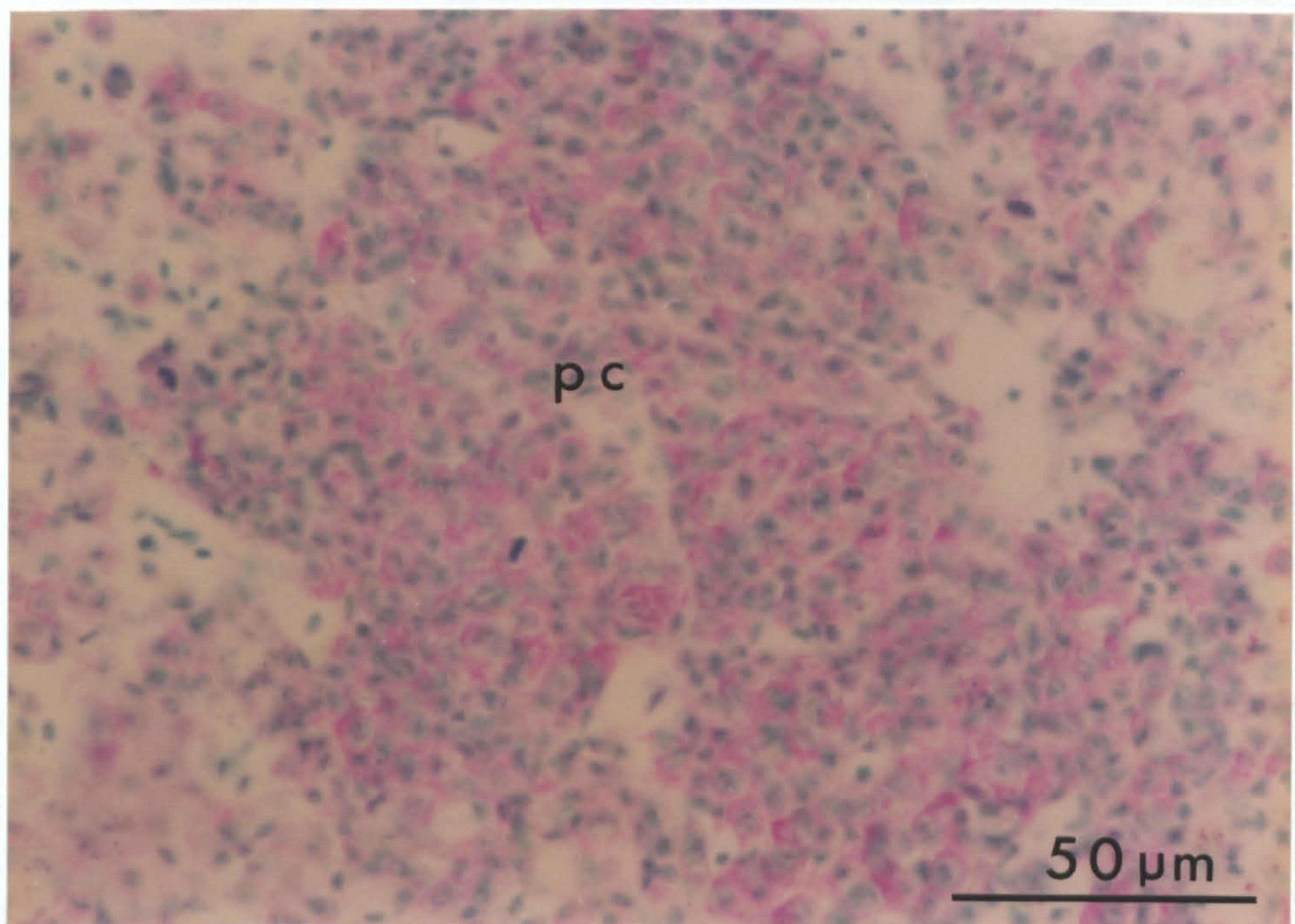
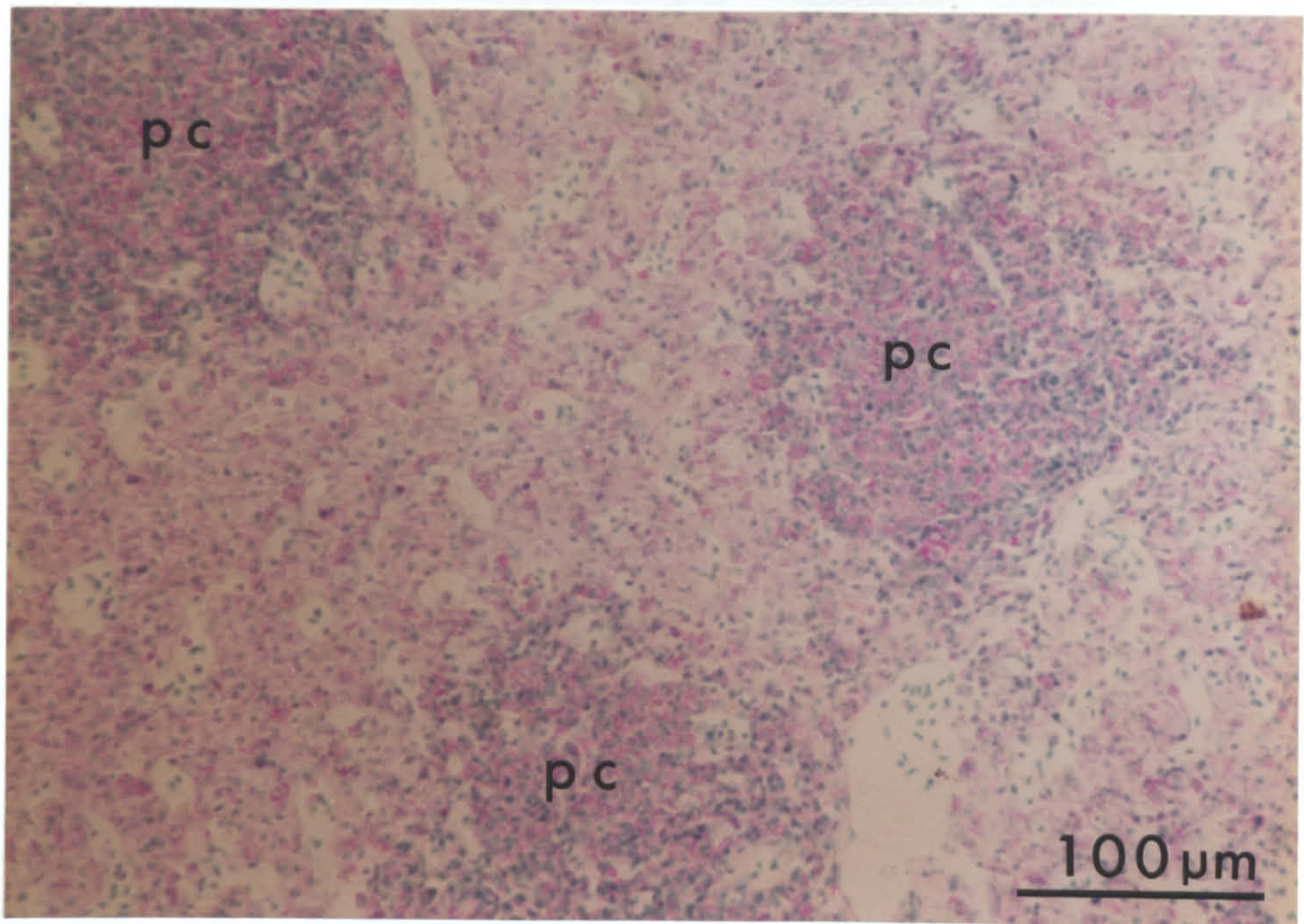


PLATE 19

A section of pronephros stained with methyl green-pyronin, from a carp immunized with HGG in FCA at ten-times the normal dosage. During the first week after immunization pyroninophilic cells (pc) can be seen in the blood spaces.

PLATE 20

A transverse section through a splenic ellipsoid stained with methyl green-pyronin, to show dark pigment (p) confined completely within it.

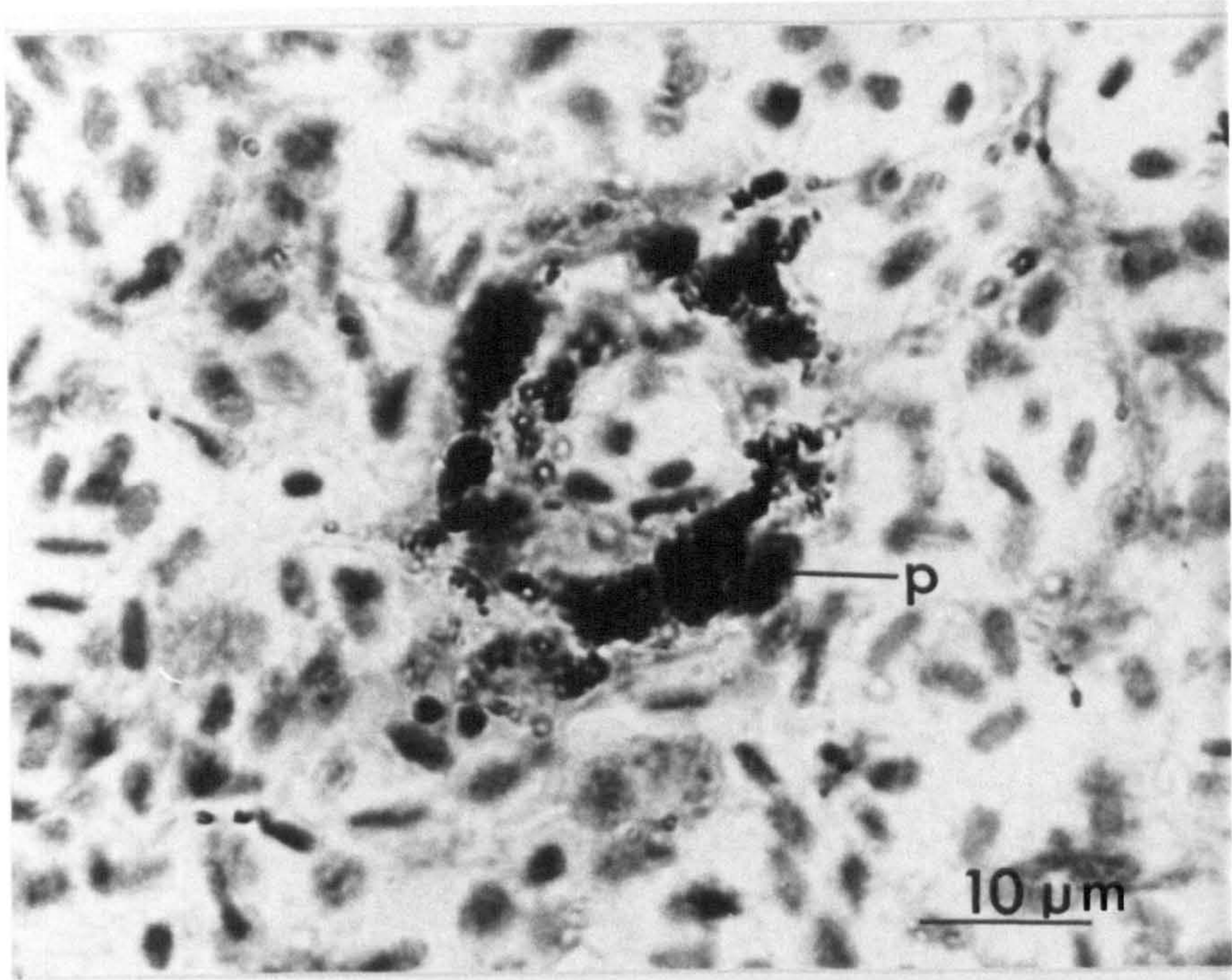
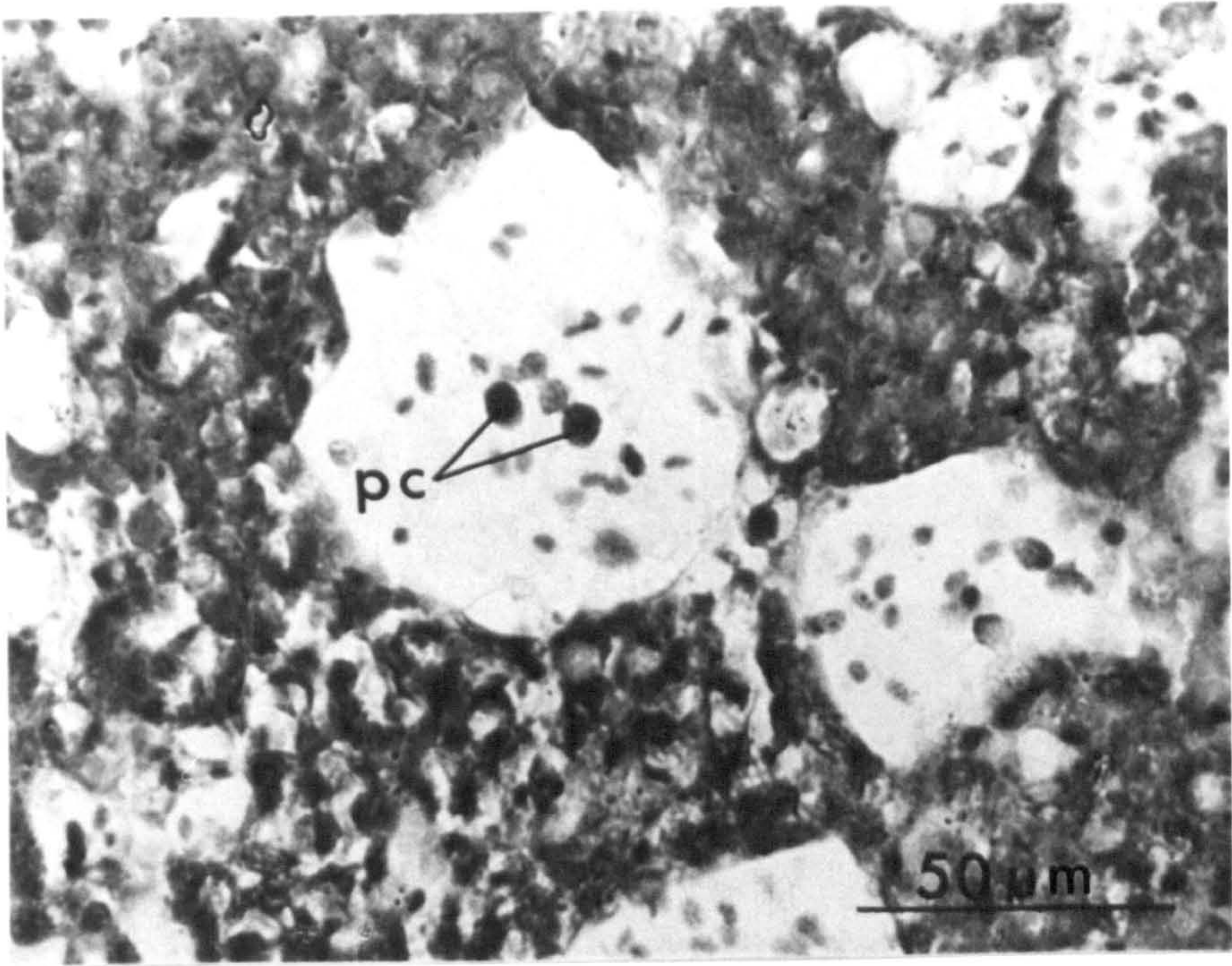


PLATE 21

Sections of spleen stained with methyl green-pyronin, from a carp immunized with HGG in FCA on days 0 and 56.

- (a) Pyroninophilic cells (pc) begin to collect along the length of the ellipsoid.
- (b) Pyroninophilic cells (pc) begin to cluster in certain areas of the ellipsoid.
- (c) A large collection of pyroninophilic cells (pc) swelling the ellipsoid sheath.

Note.

With reference to Tables III to X (Chapter 3) Plate 21a would be classified as +, relatively few pyroninophilic cells and Plate 21c would be classified as ++, large numbers of pyroninophilic cells. The category of + (many pyroninophilic cells) in Tables III to X would represent a level of pyroninophilia between a) and c).

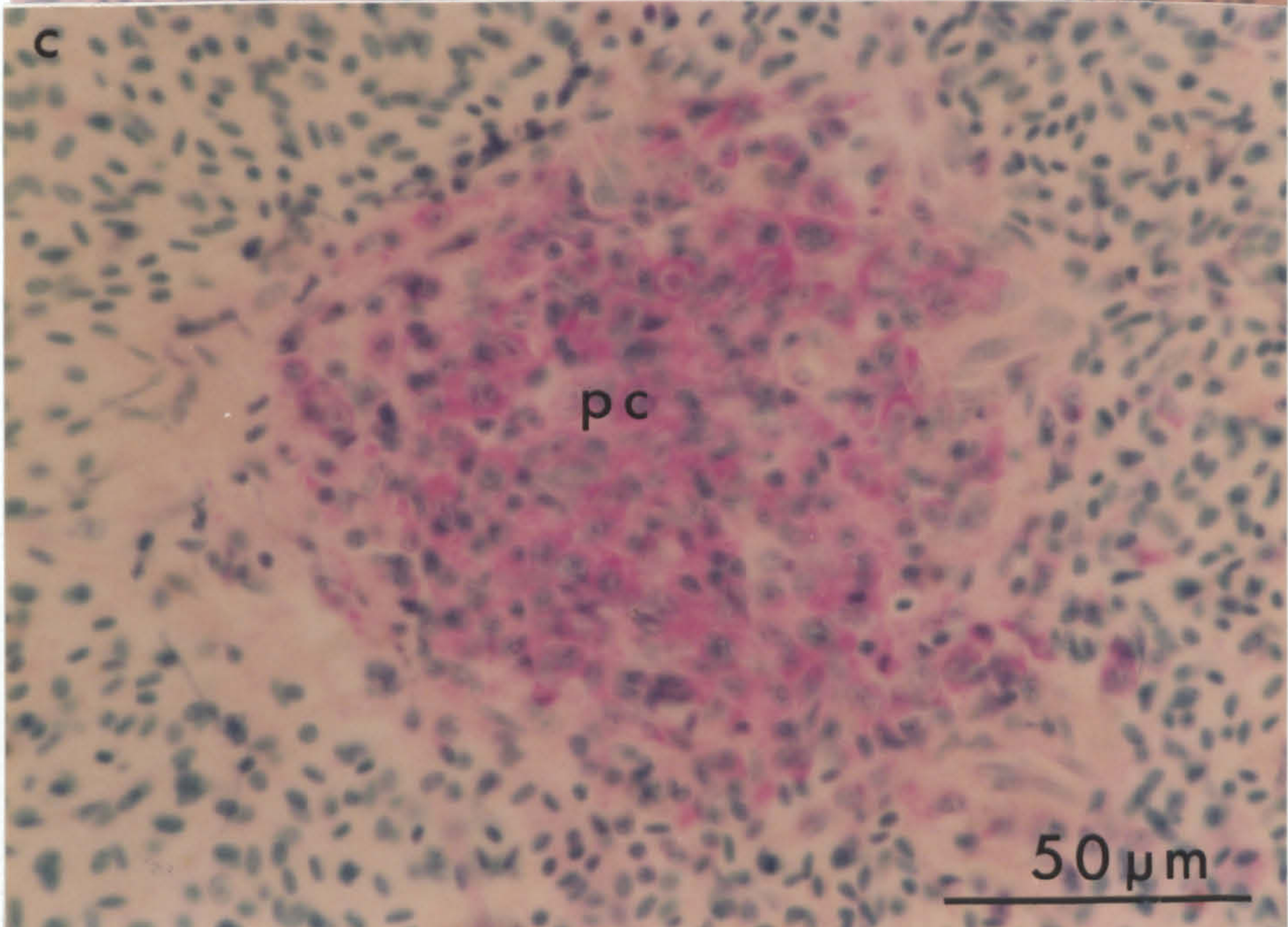
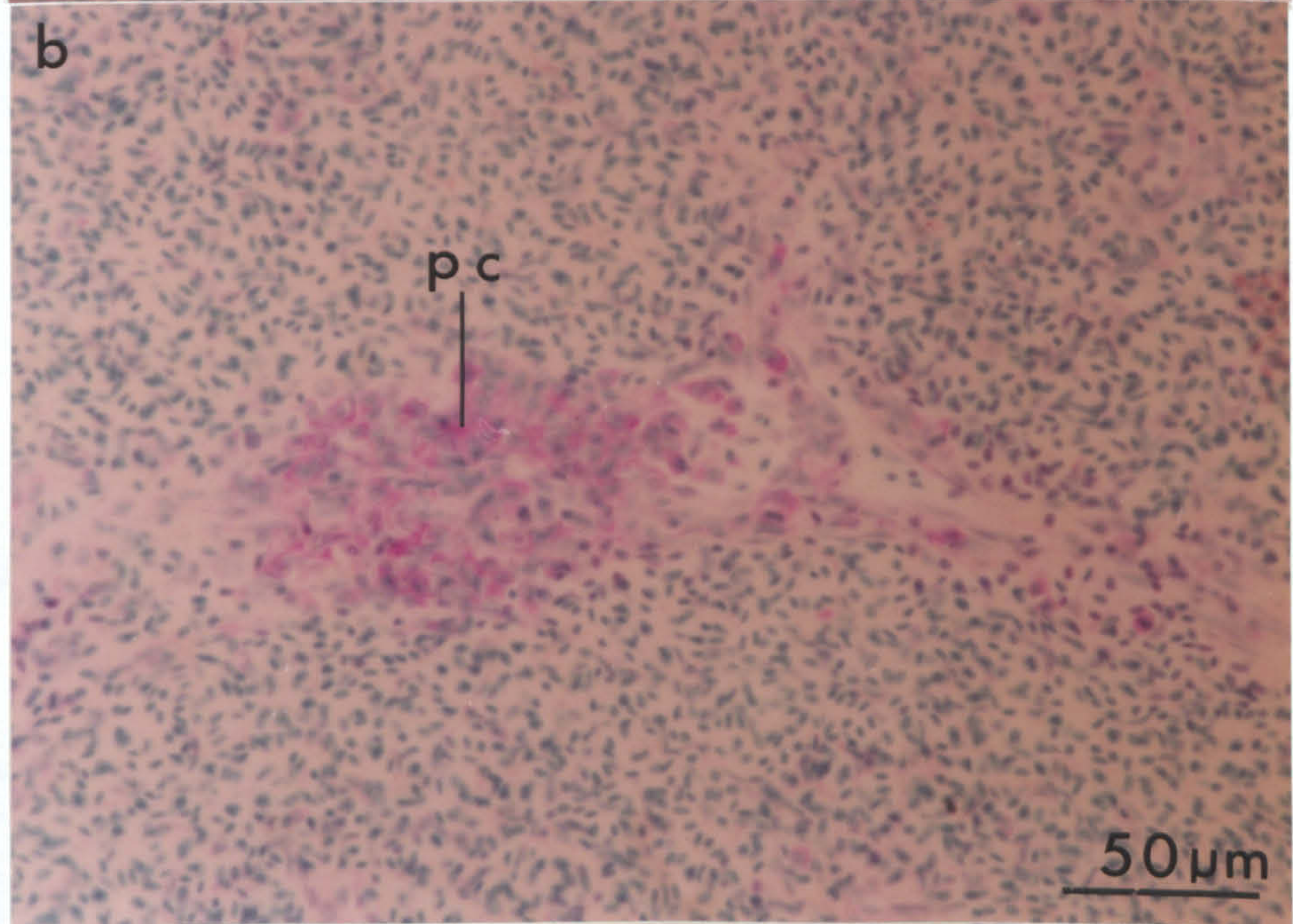
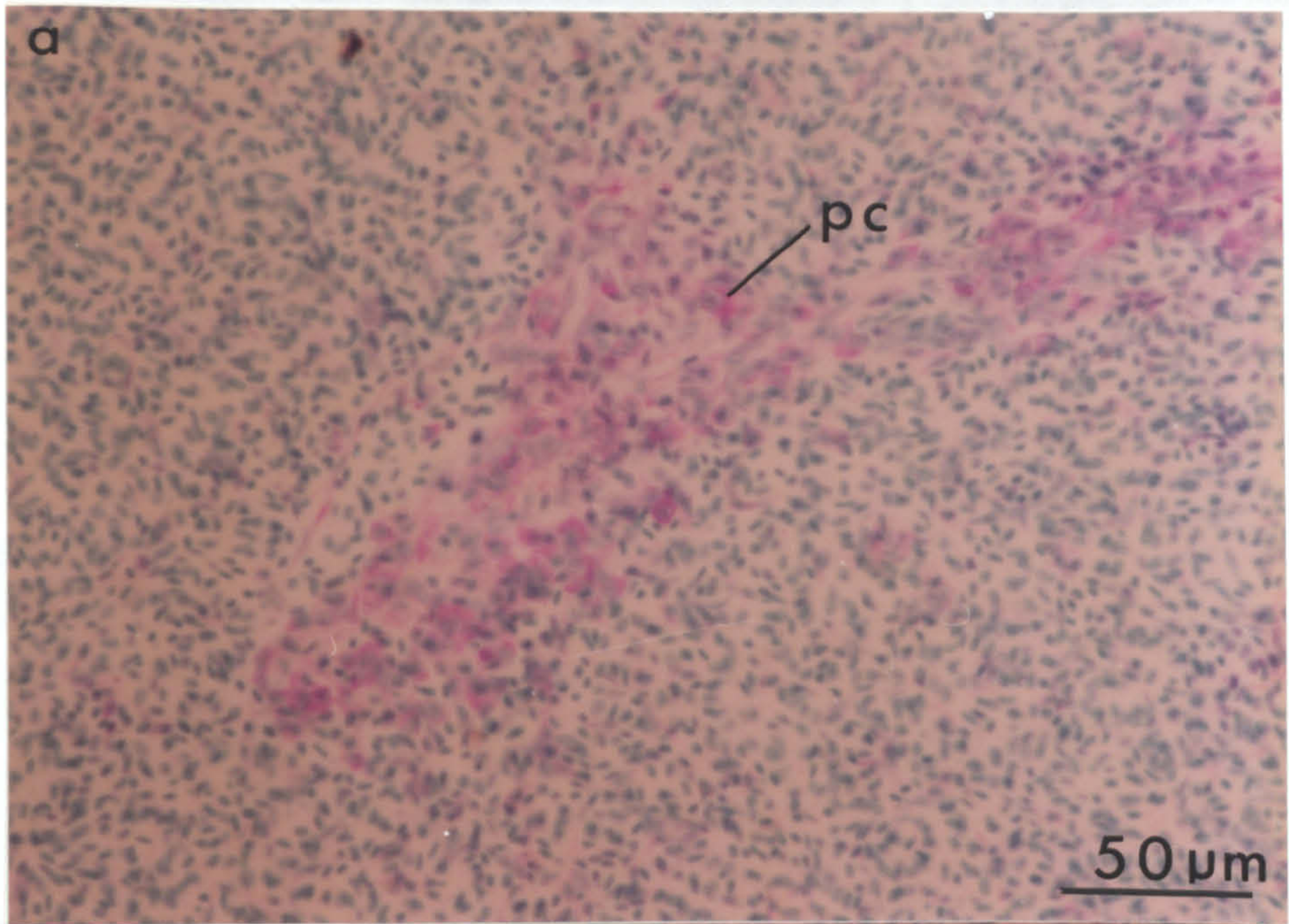


PLATE 22

Silver impregnated ellipsoids showing an expanded portion of the reticulum (er), resembling in a rudimentary form the white pulp of the tetrapod spleen.

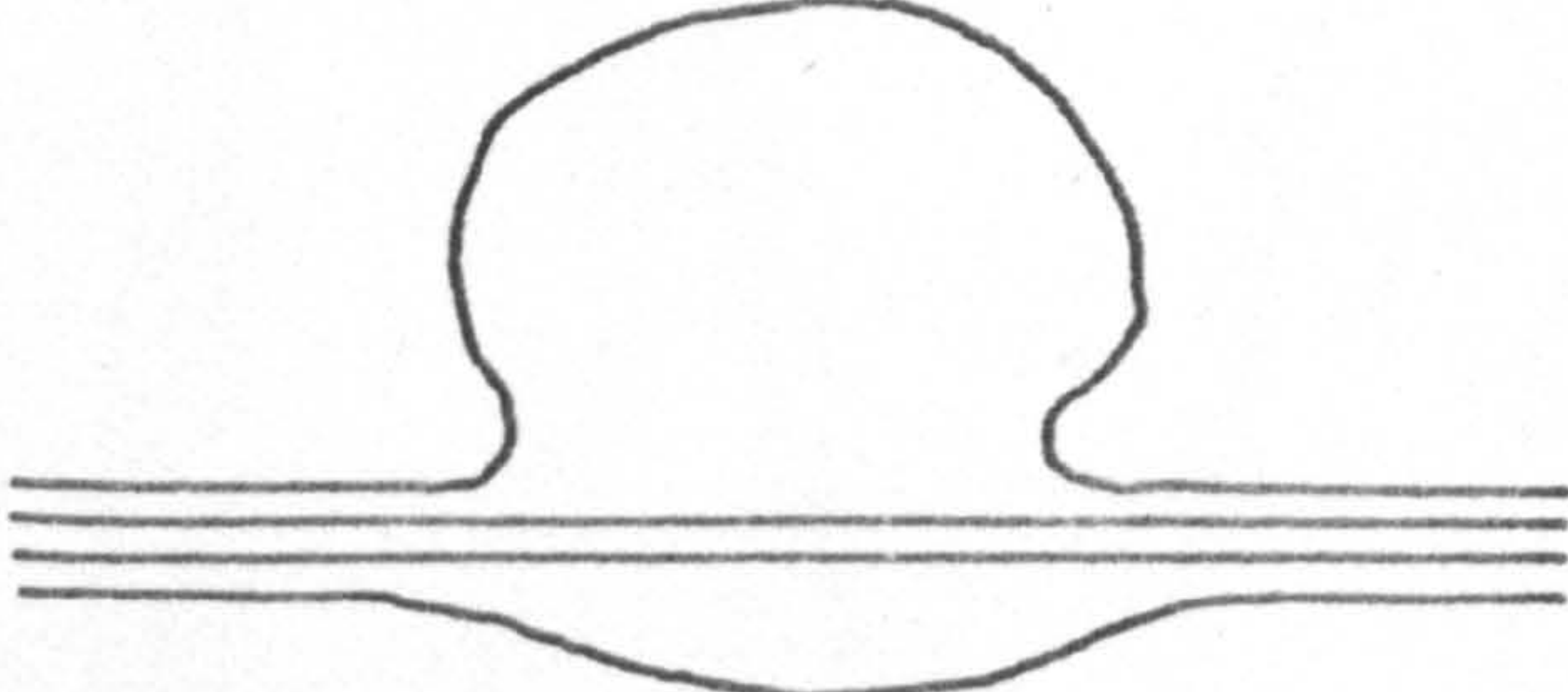
PLATE 23

A section of spleen, stained with methyl green-pyronin, from a carp immunized with heat aggregated HGG. Dark pigment-containing cells (p) are scattered throughout the pulp as well as in the ellipsoids.

Possible explanation of the enlarged ellipsoid shown in

Plate 22

(a)  Normal ellipsoid
(as seen in
Plate 6).

(b)  Ellipsoid as seen in
Plate 22. The swollen
sheath now accommodates
large numbers of pyro-
ninophilic cells
(Plate 21c).

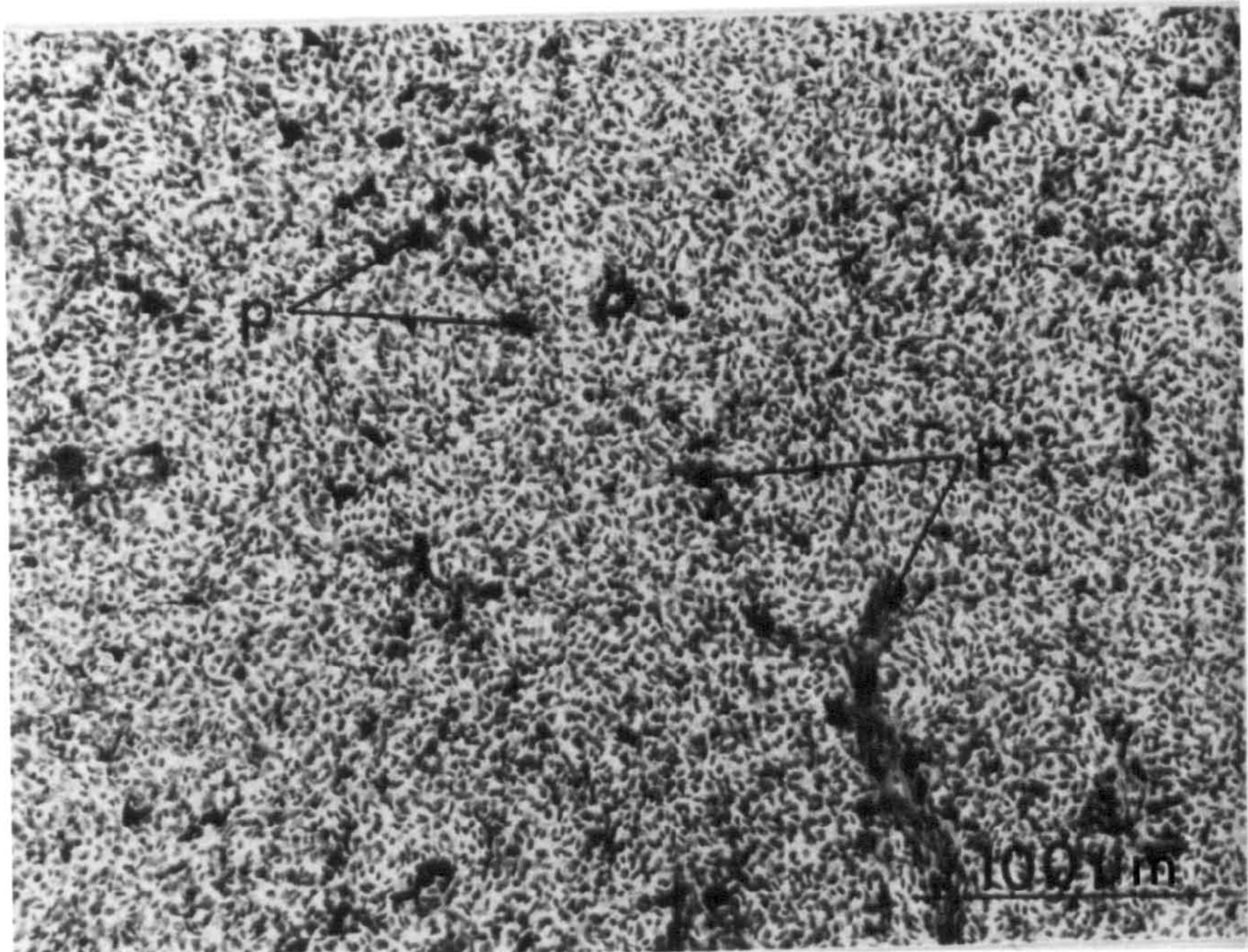
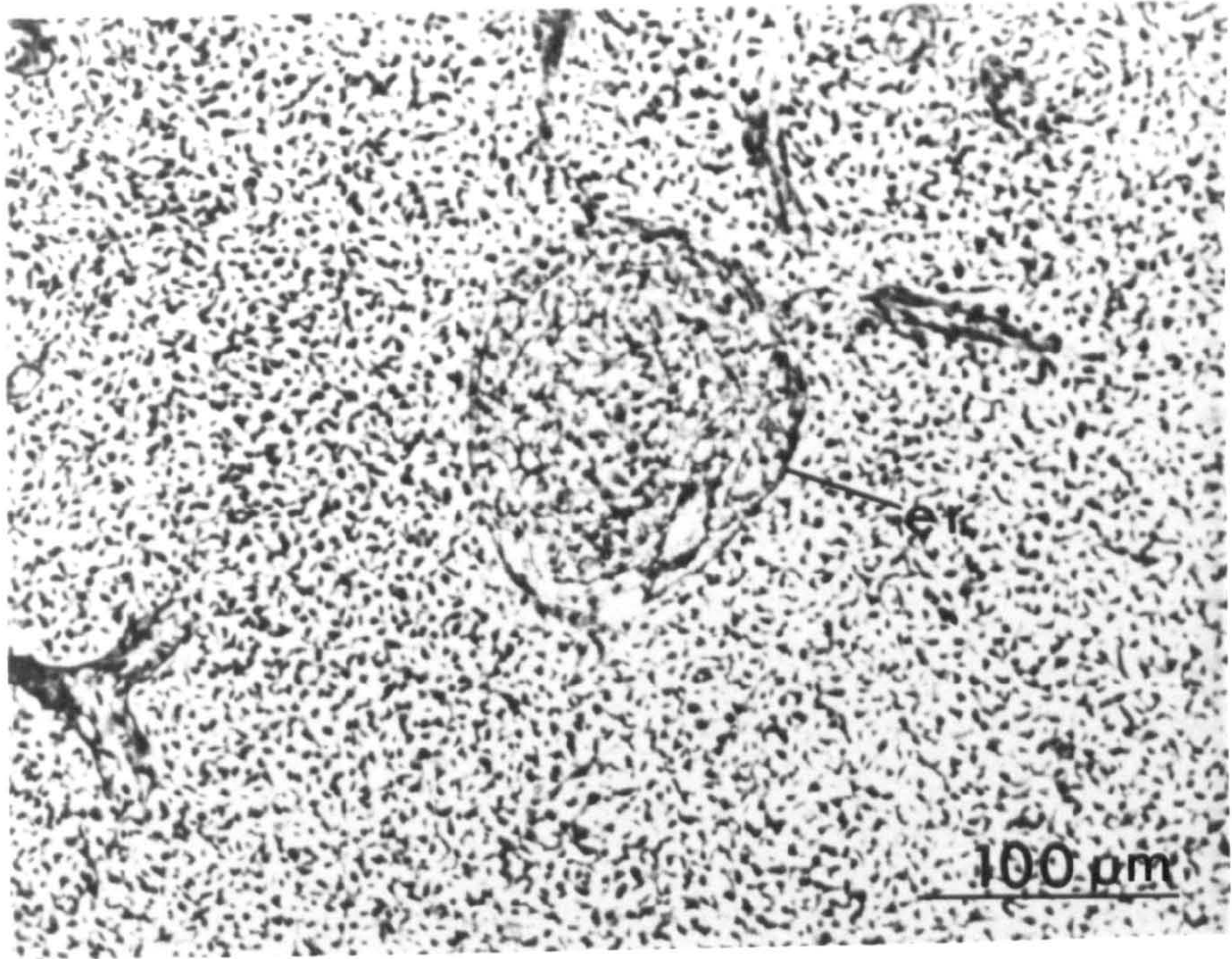


PLATE 24

Fluorescence seen in the splenic ellipsoids after injecting HGG in FCA and incubating with fluorescein-labelled sheep anti-HGG antiserum.

PLATE 25

Fluorescence seen in the splenic ellipsoids after injecting HGG in FCA and incubating with rabbit anti-carp immunoglobulin antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum.

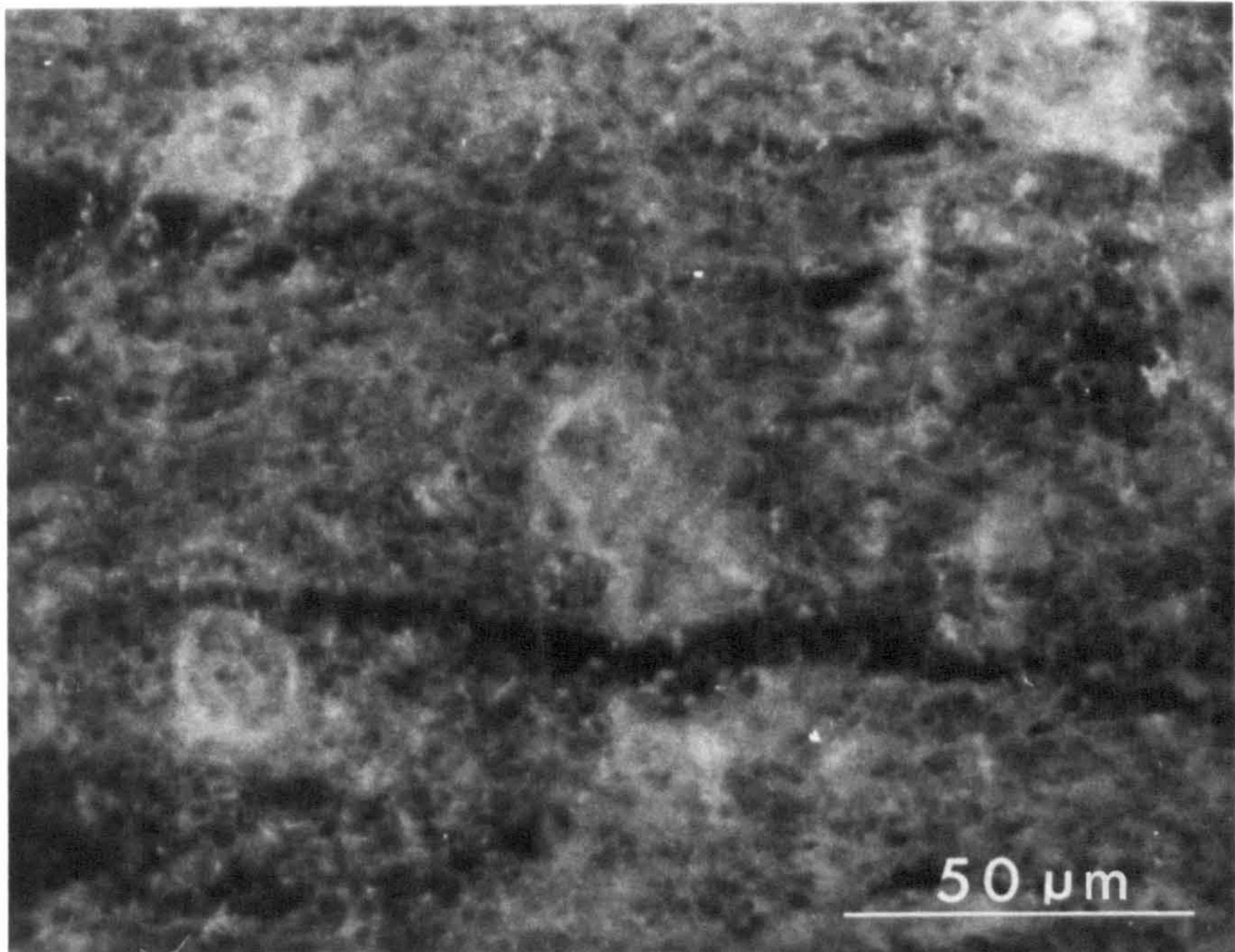
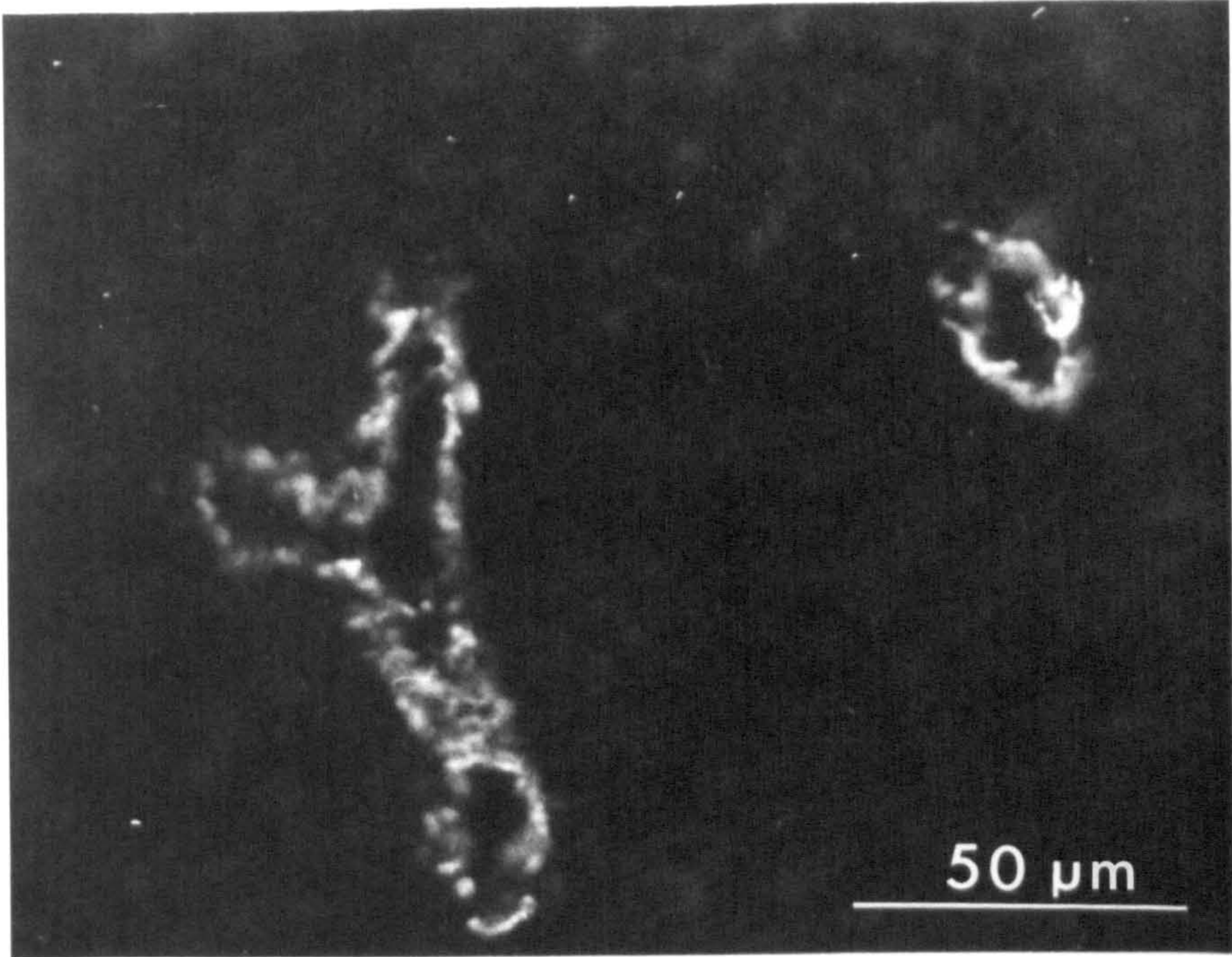


PLATE 26

Fluorescence seen in the pronephros after injecting HGG in FCA and incubating with fluorescein-labelled sheep anti-HGG antiserum.

PLATE 27

Fluorescence seen in the mesonephros between the excretory tubules (t) after injecting HGG in FCA and incubating with fluorescein-labelled sheep anti-HGG antiserum.

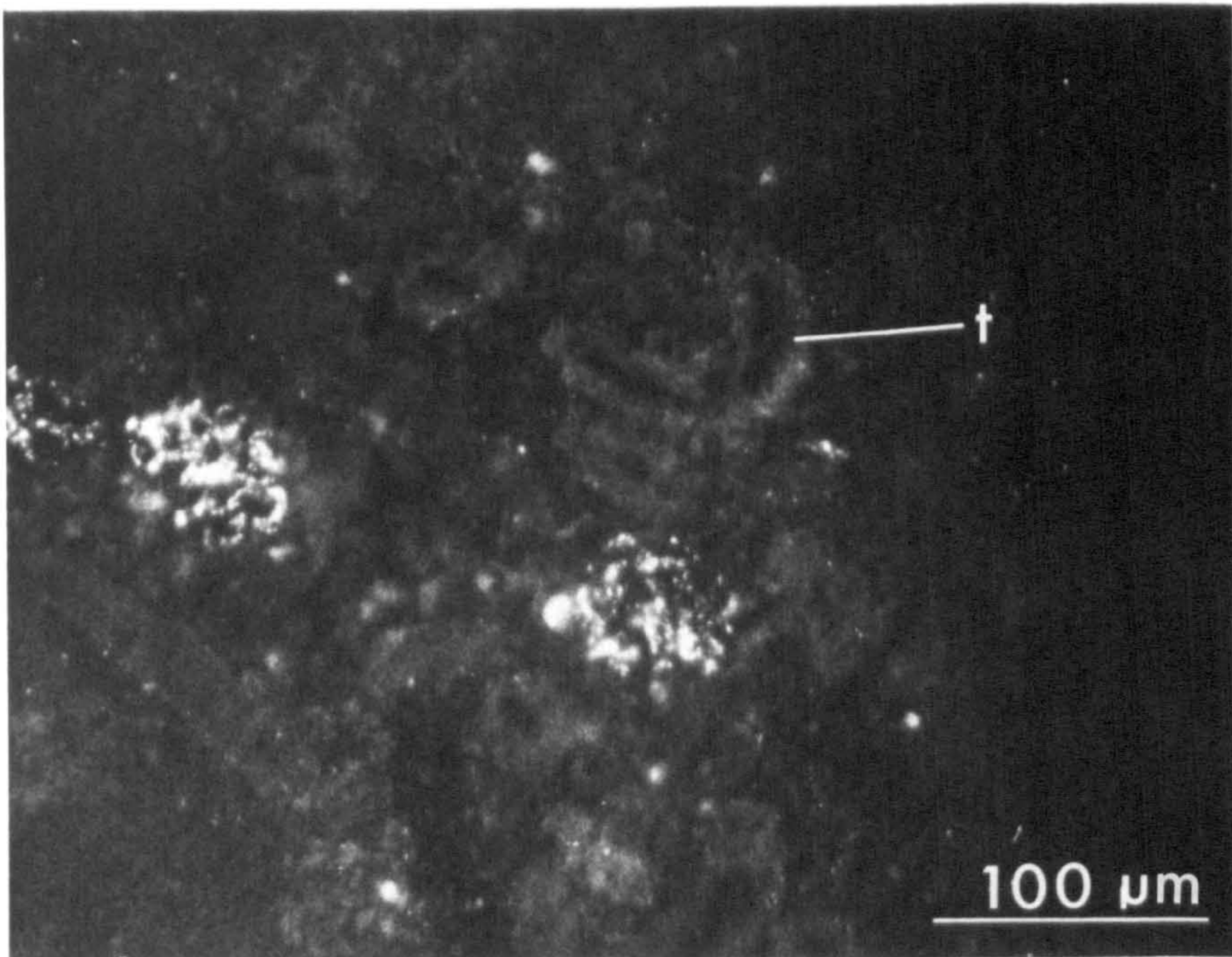
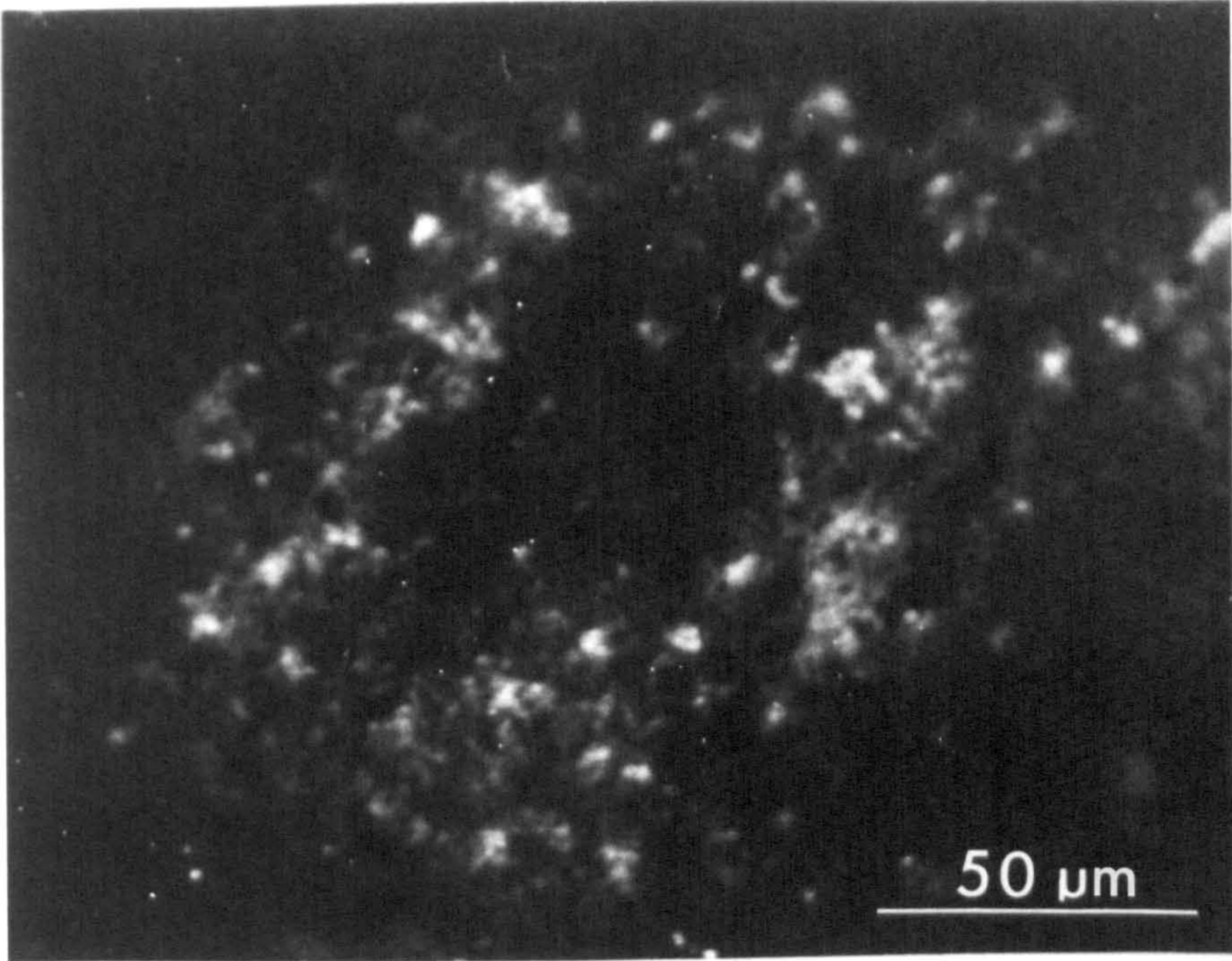


PLATE 28

Fluorescence seen in the spleen after injecting A. salmonicida and incubating with rabbit anti-A. salmonicida antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum.

PLATE 29

m:fluorescence seen in the spleen around a melano-macrophage centre, after injecting A. salmonicida and incubating with rabbit anti-A. salmonicida antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum.

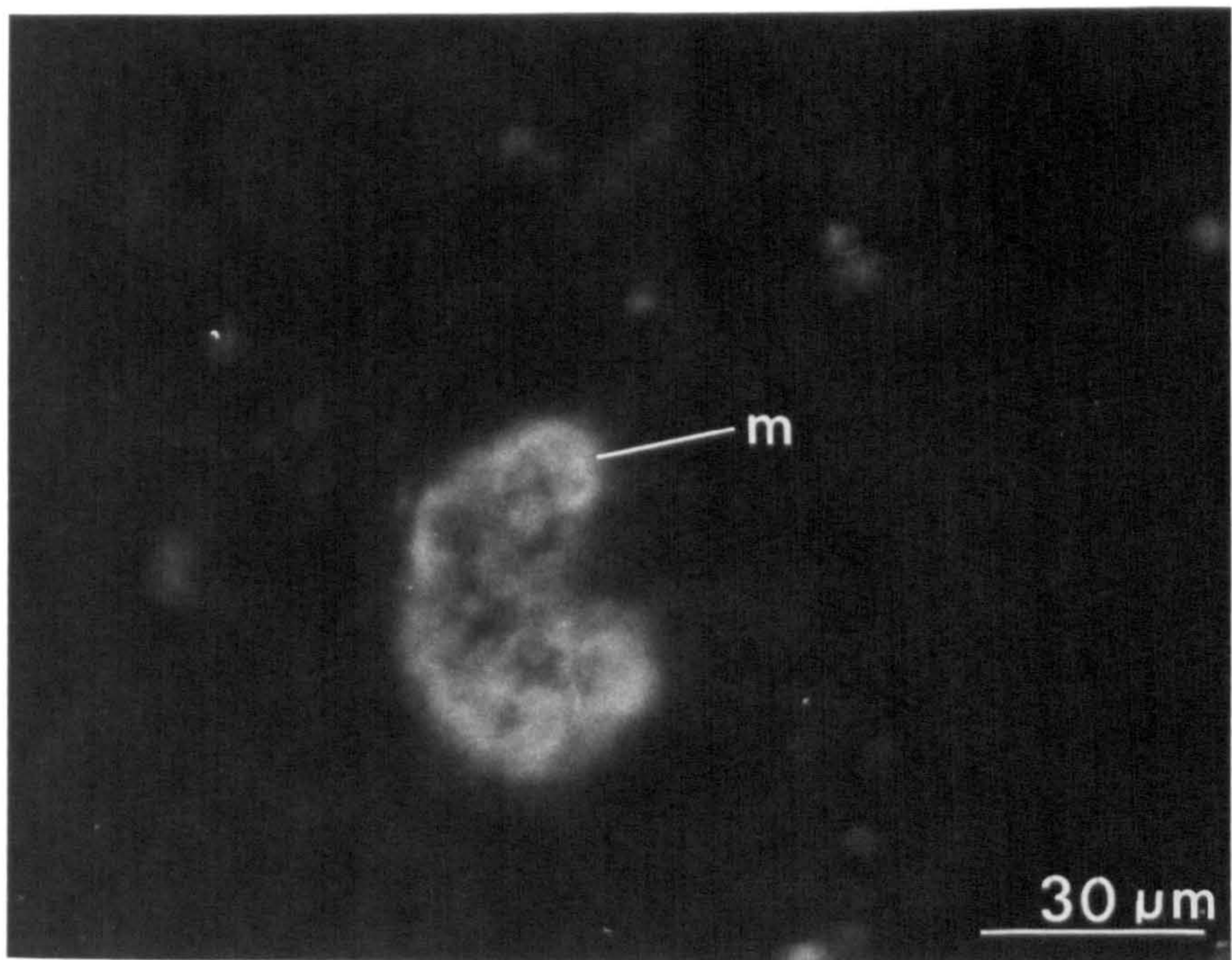
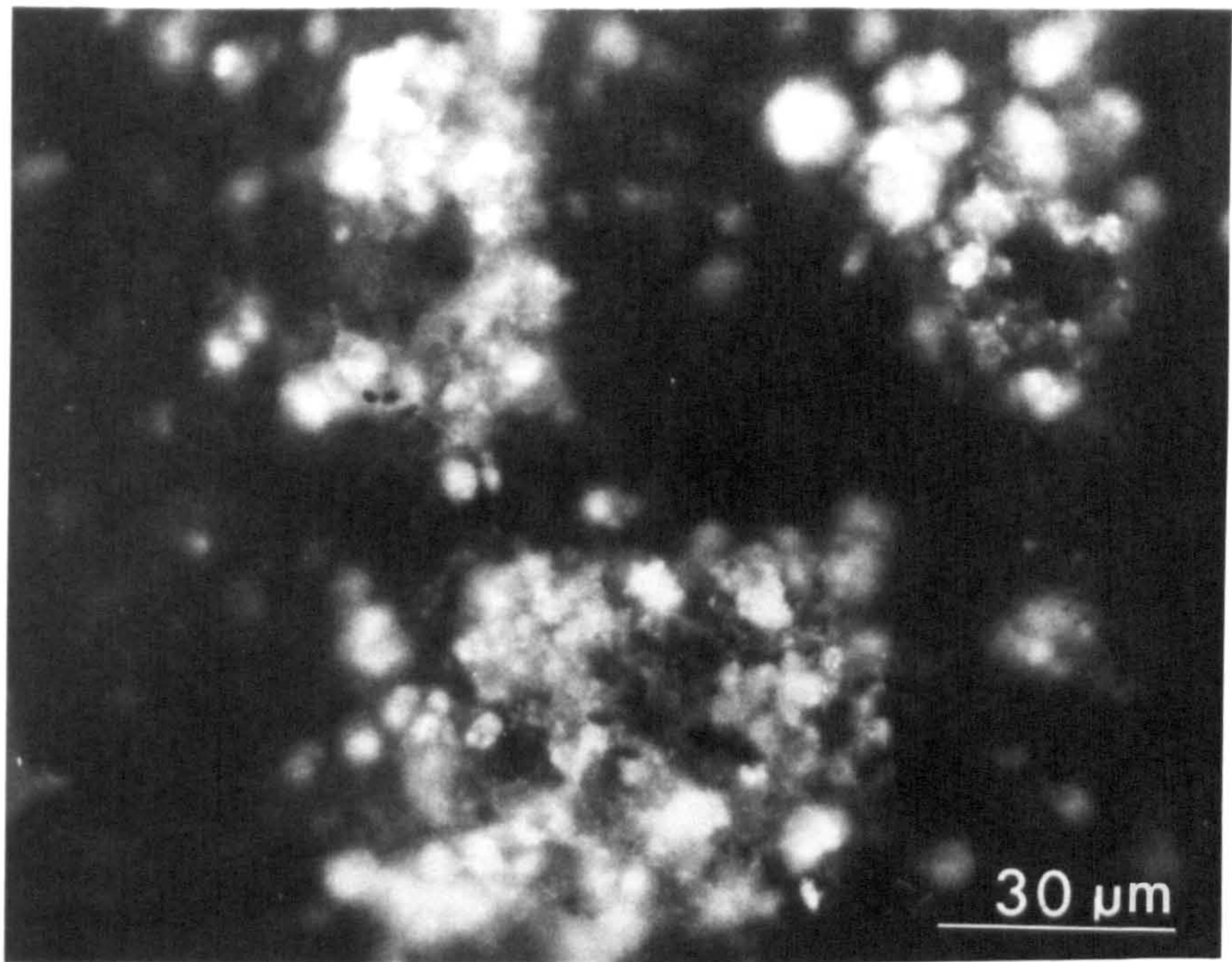


PLATE 30

Fluorescence seen in the pronephros after injecting A. salmonicida and incubating with rabbit anti-A. salmonicida antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum.

PLATE 31

Fluorescence seen in the mesonephros after injecting A. salmonicida and incubating with rabbit anti-A. salmonicida antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum.

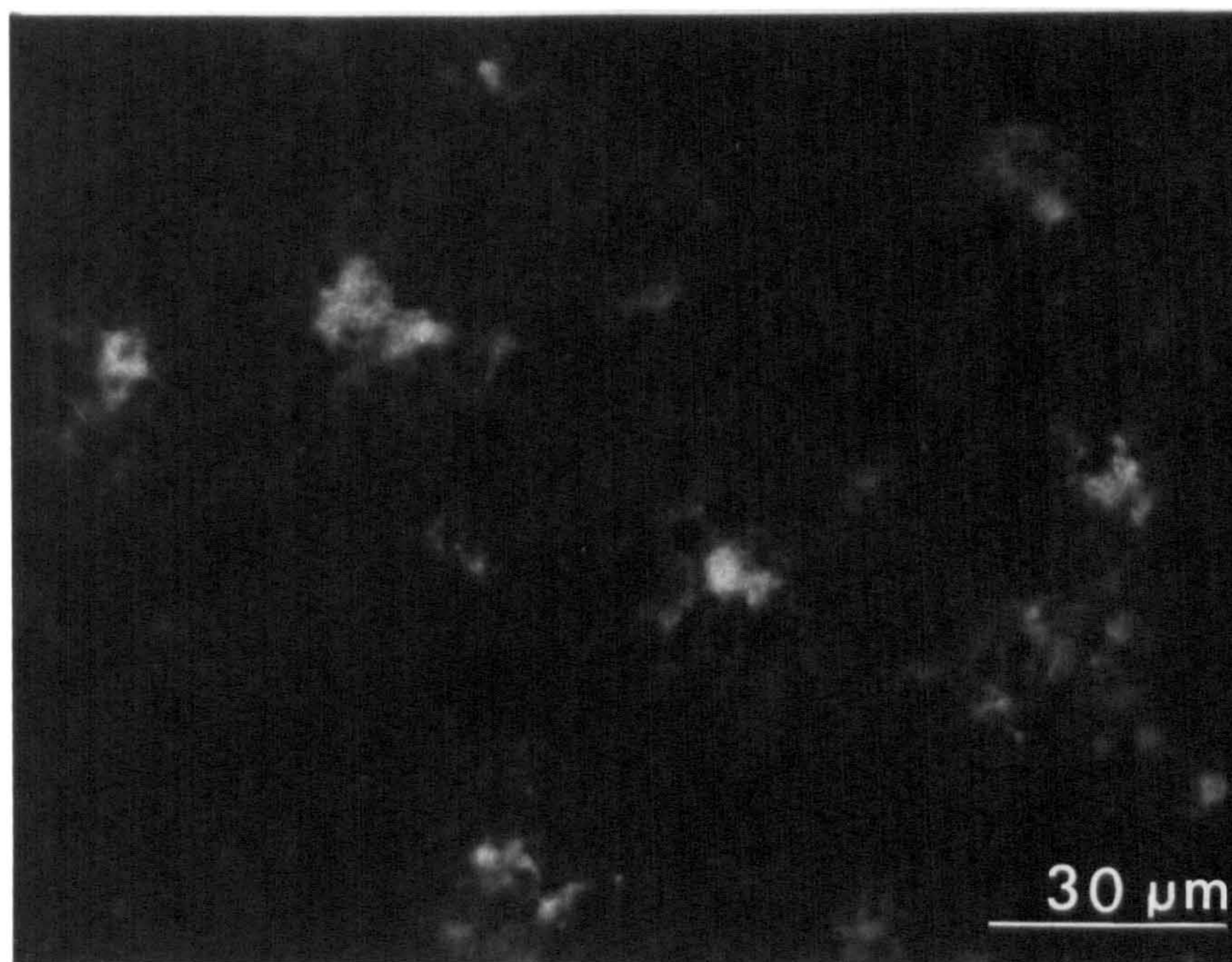
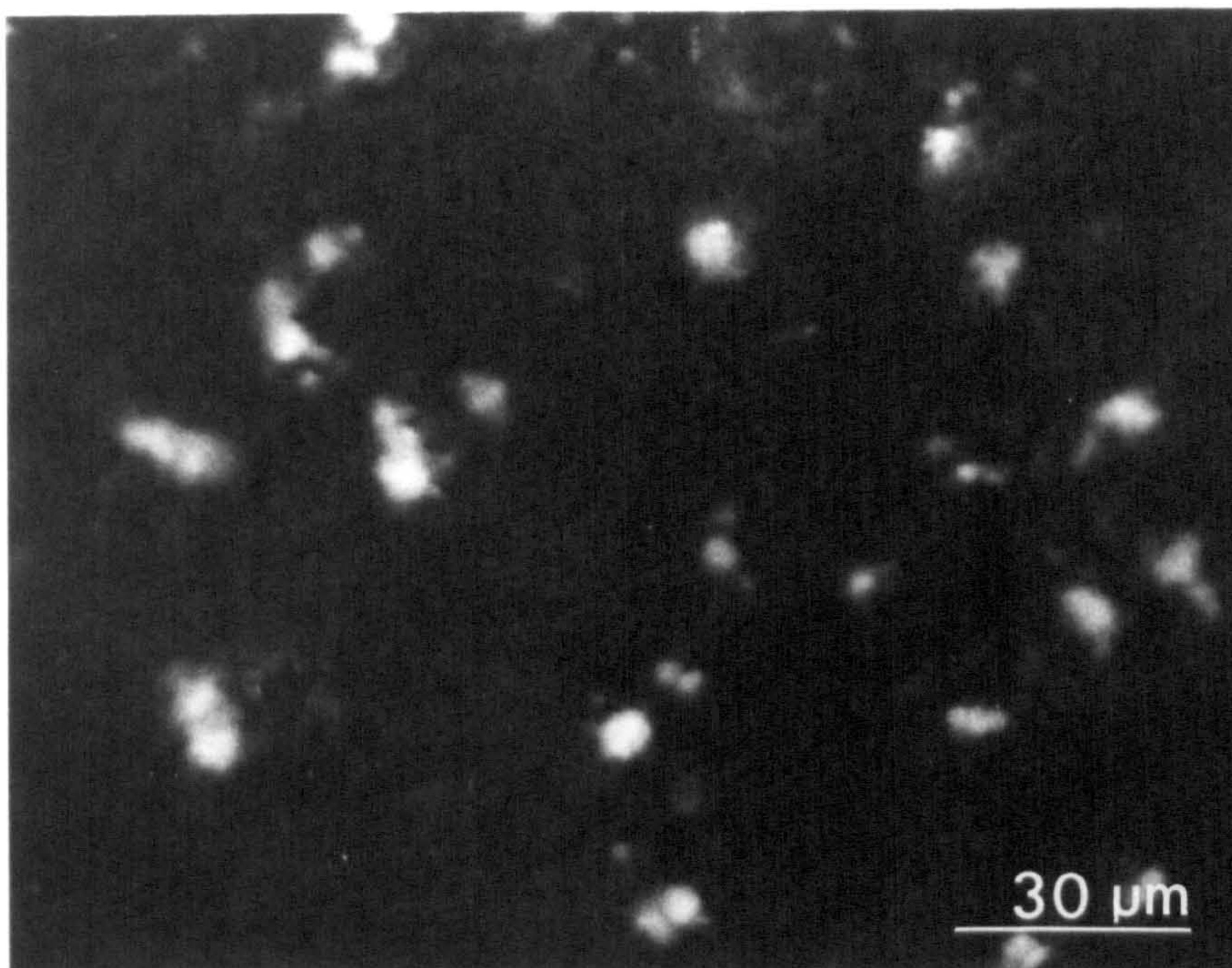


PLATE 32

Fluorescence seen in the pronephros after injecting immune complexes and incubating with fluorescein-labelled sheep anti-HGG antiserum.

PLATE 33

Fluorescence seen in the mesonephros between the tubules (t) after injecting immune complexes and incubating with fluorescein-labelled sheep anti-HGG antiserum.

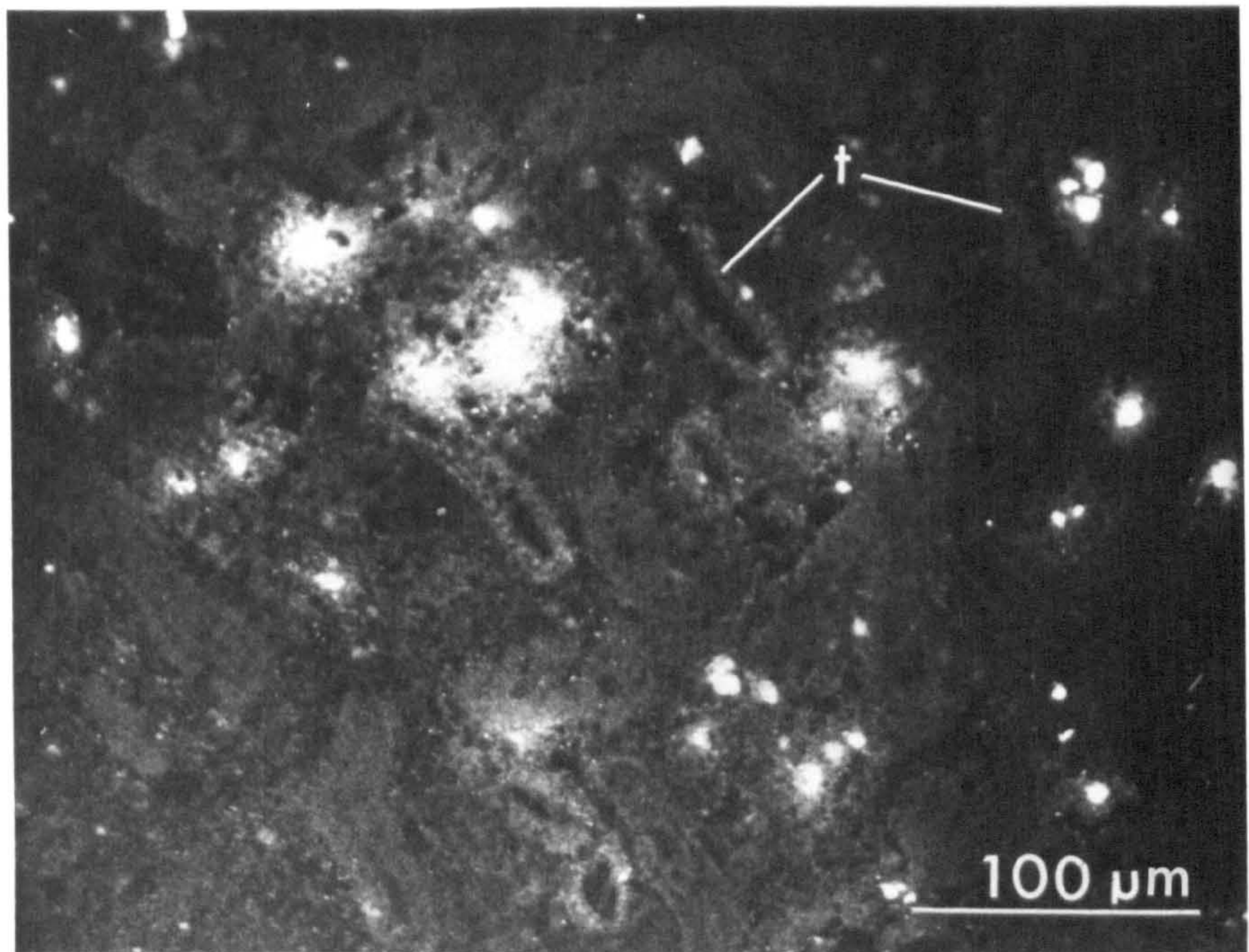
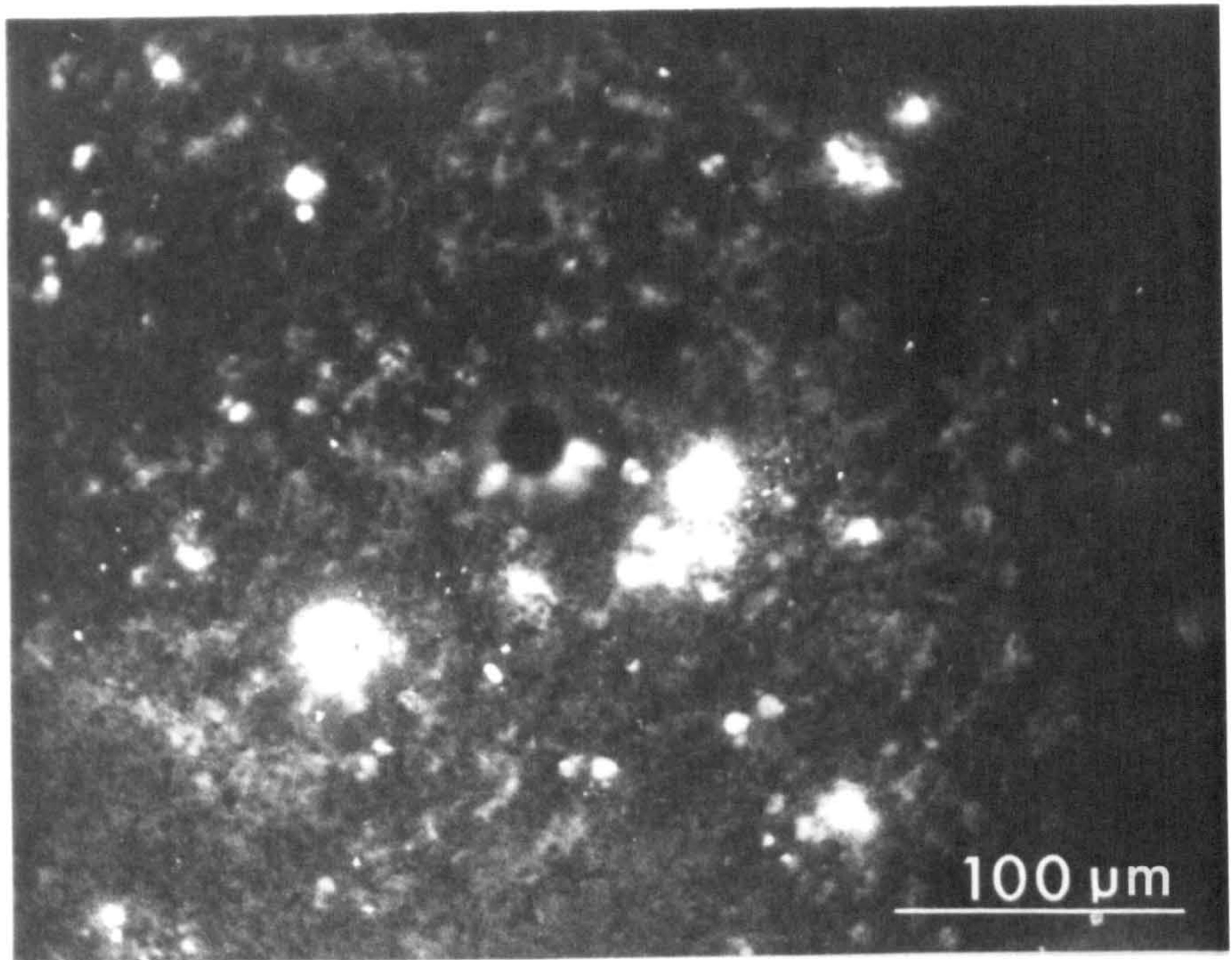


PLATE 34

A section of spleen stained with haematoxylin and eosin, from a carp injected with colloidal carbon. Carbon laden macrophages (m) can be seen within the ellipsoids.

PLATE 35

Fluorescence seen within the boundary layer surrounding the white pulp areas in the spleen of Xenopus laevis after injection with HGG in FCA and incubation with fluorescein-labelled sheep anti-HGG antiserum. Presented for comparison with the carp (Plate 24).

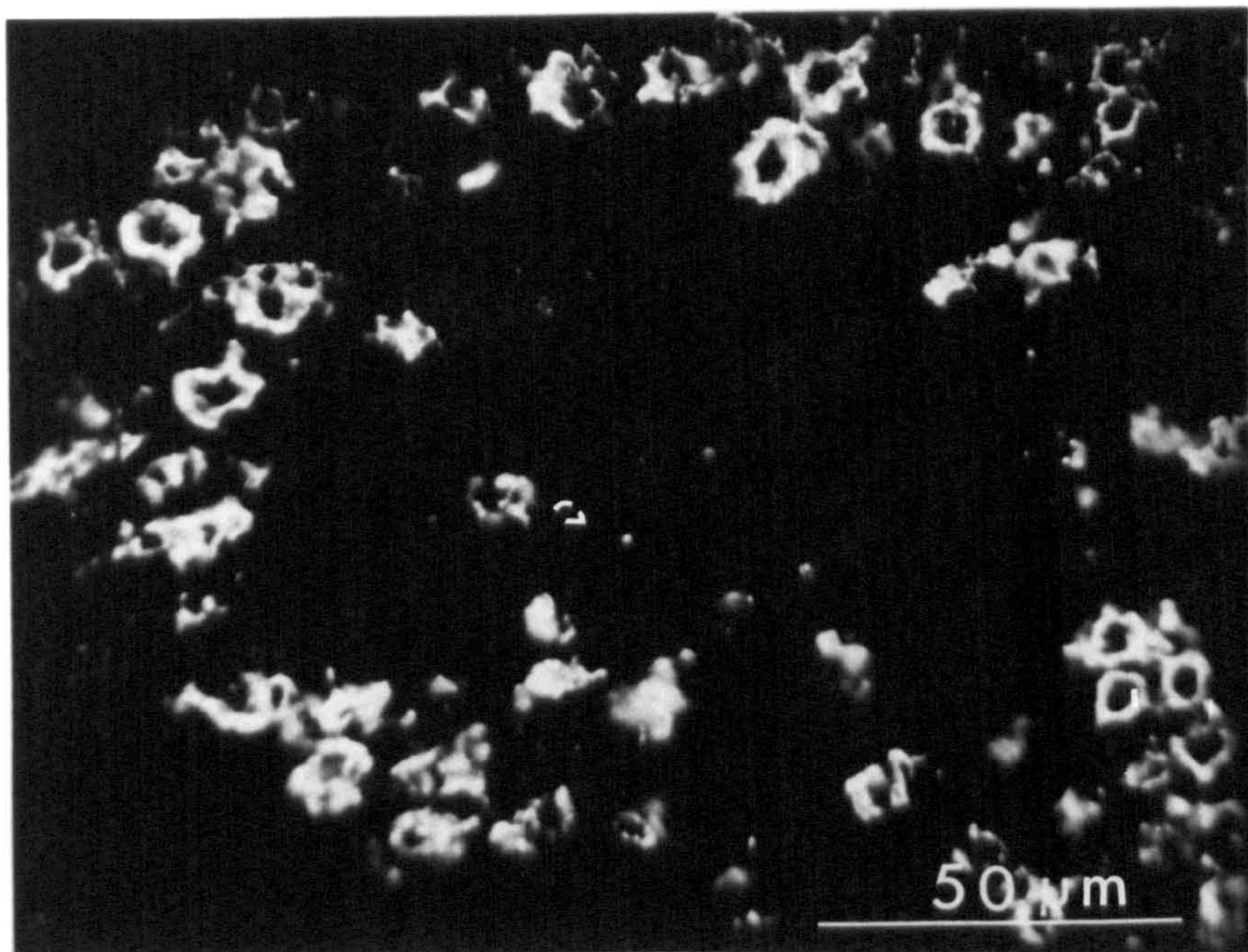
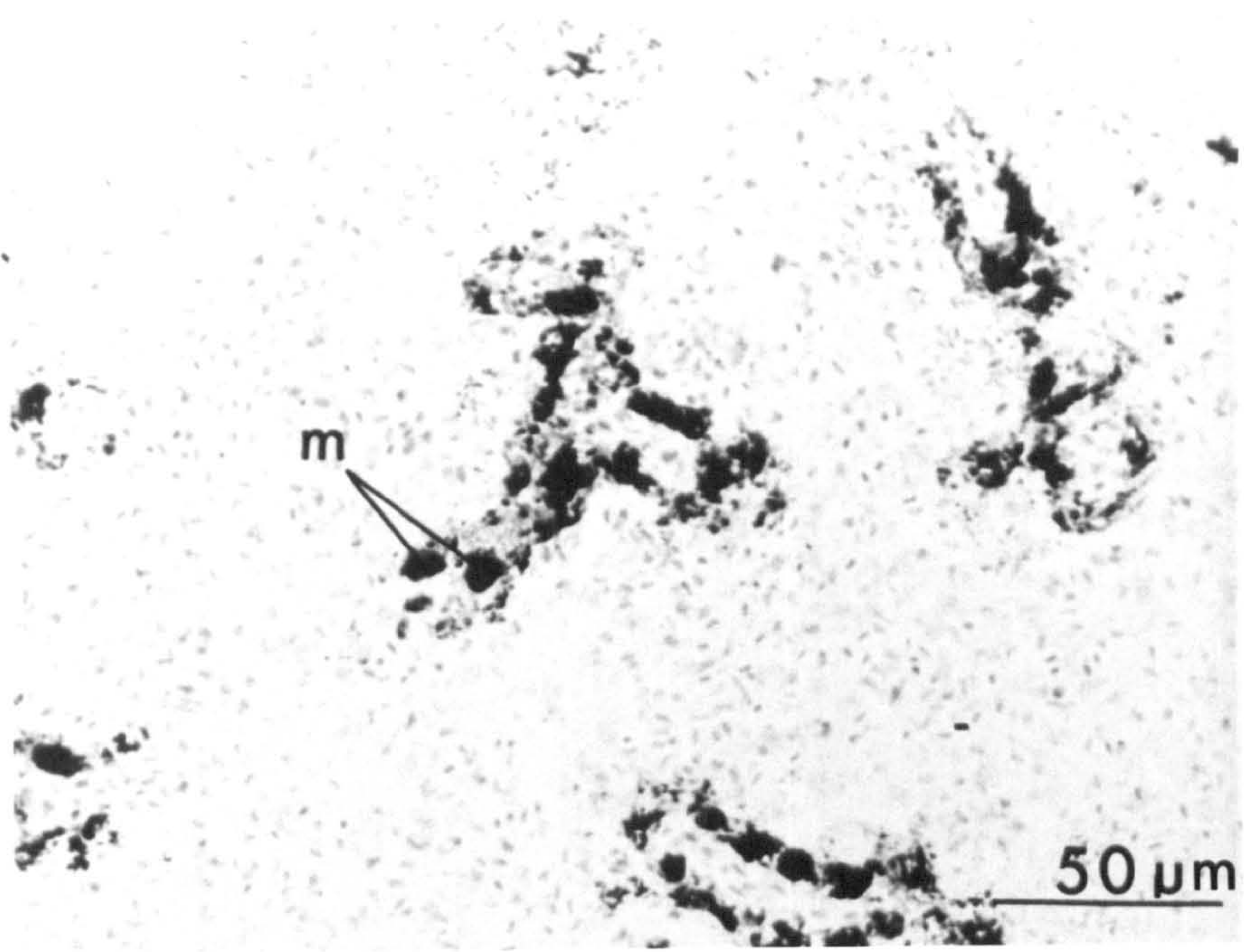


PLATE 36

Fluorescence seen in the white pulp and boundary layer in the spleen of X. laevis after injecting A. salmonicida and incubating with rabbit anti-A. salmonicida antiserum and fluorescein-labelled sheep anti-rabbit immunoglobulin antiserum. Presented for comparison with the carp (Plate 28).

PLATE 37

A section through a white pulp island in the spleen of X. laevis. Presented for comparison with the carp (Plate 5). Arrows show the boundary layer surrounding the white pulp.

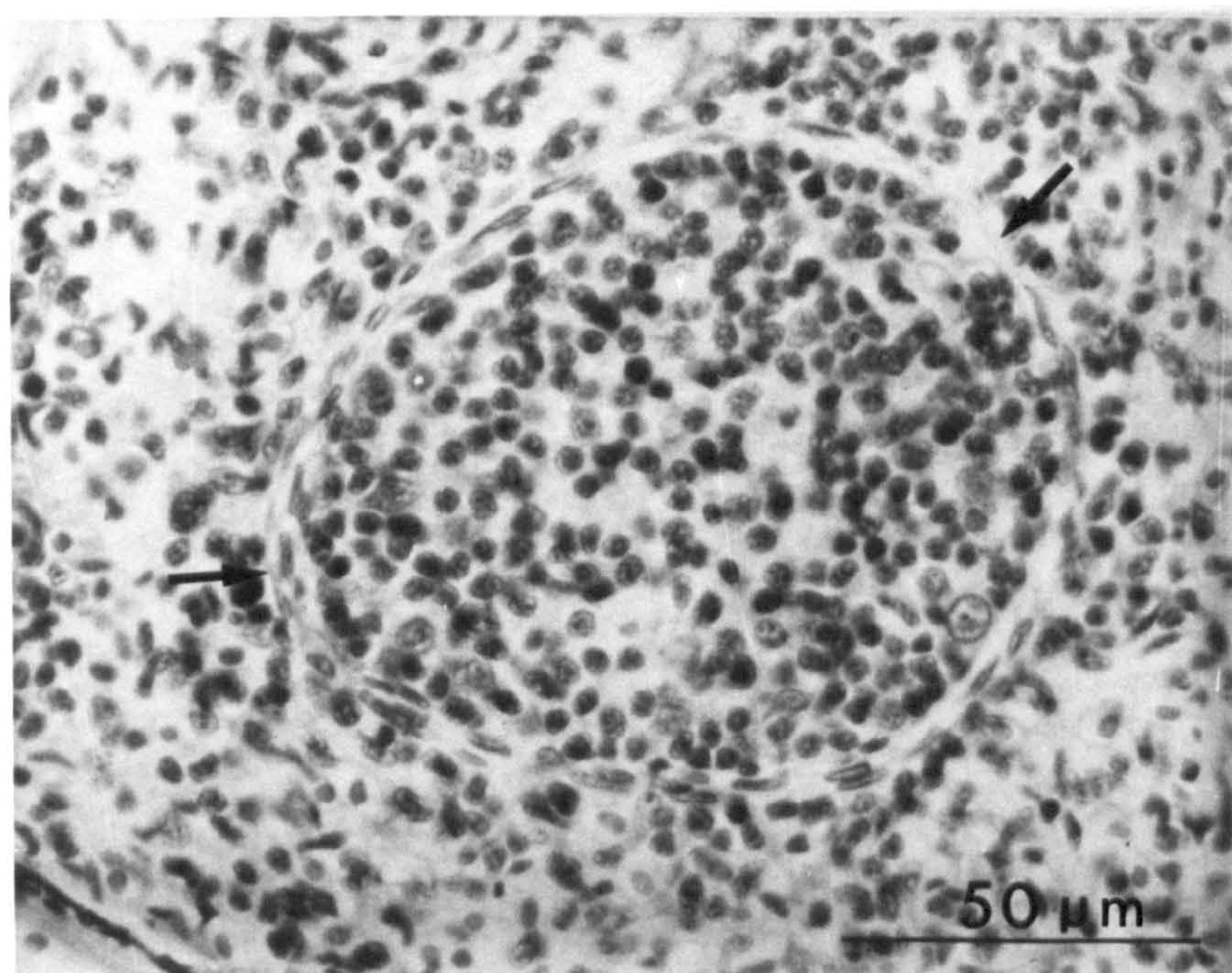
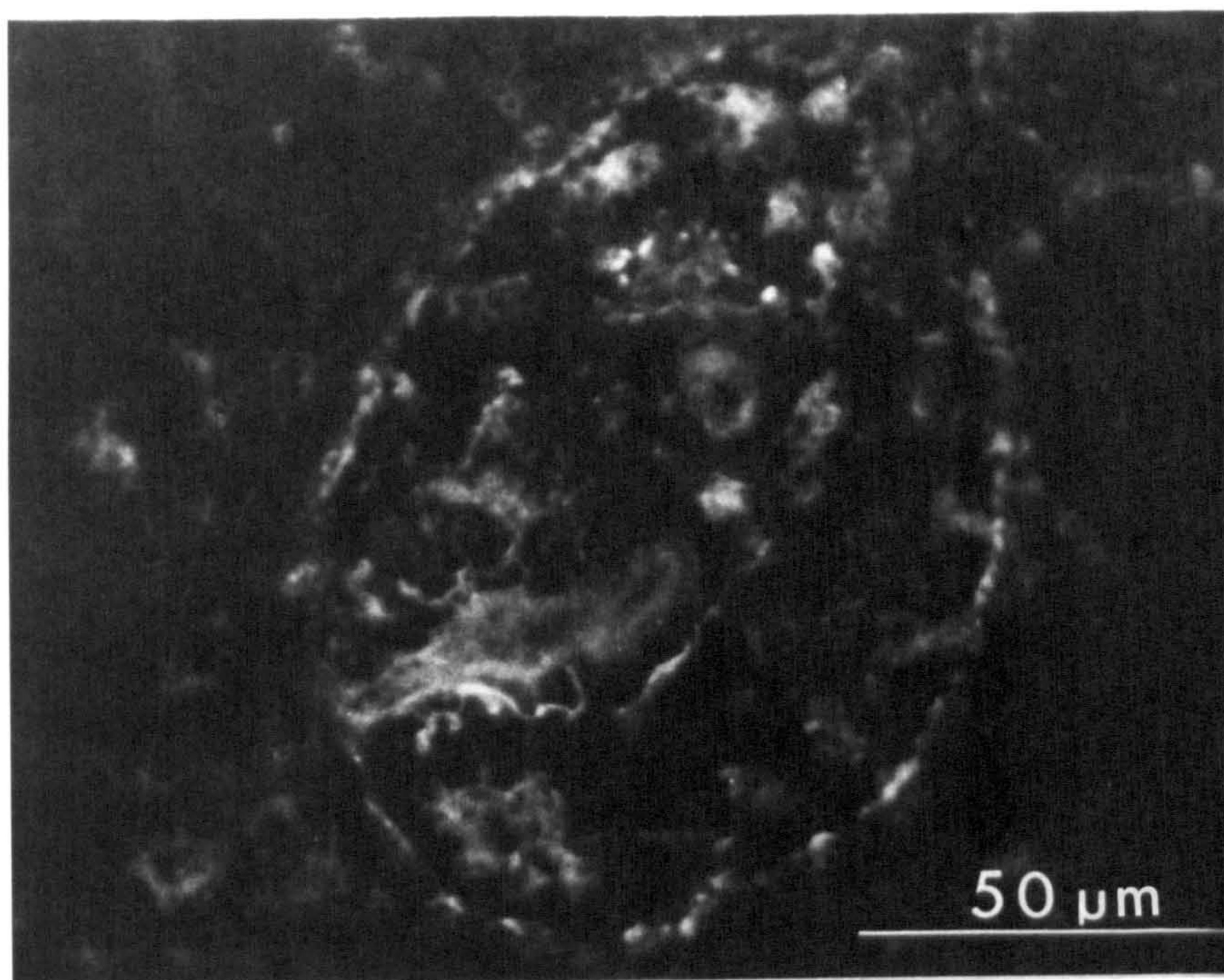


PLATE 38

Silver impregnation in the spleen of X. laevis showing the reticulum in the white pulp of an unimmunized animal, presented for comparison with carp (Plate 6). Arrows show the boundary layer which delineates the white pulp area.

PLATE 39

Silver impregnation in the spleen of X. laevis showing the reticulum in the white pulp after immunization with A. salmonicida, presented for comparison with carp (Plate 22).

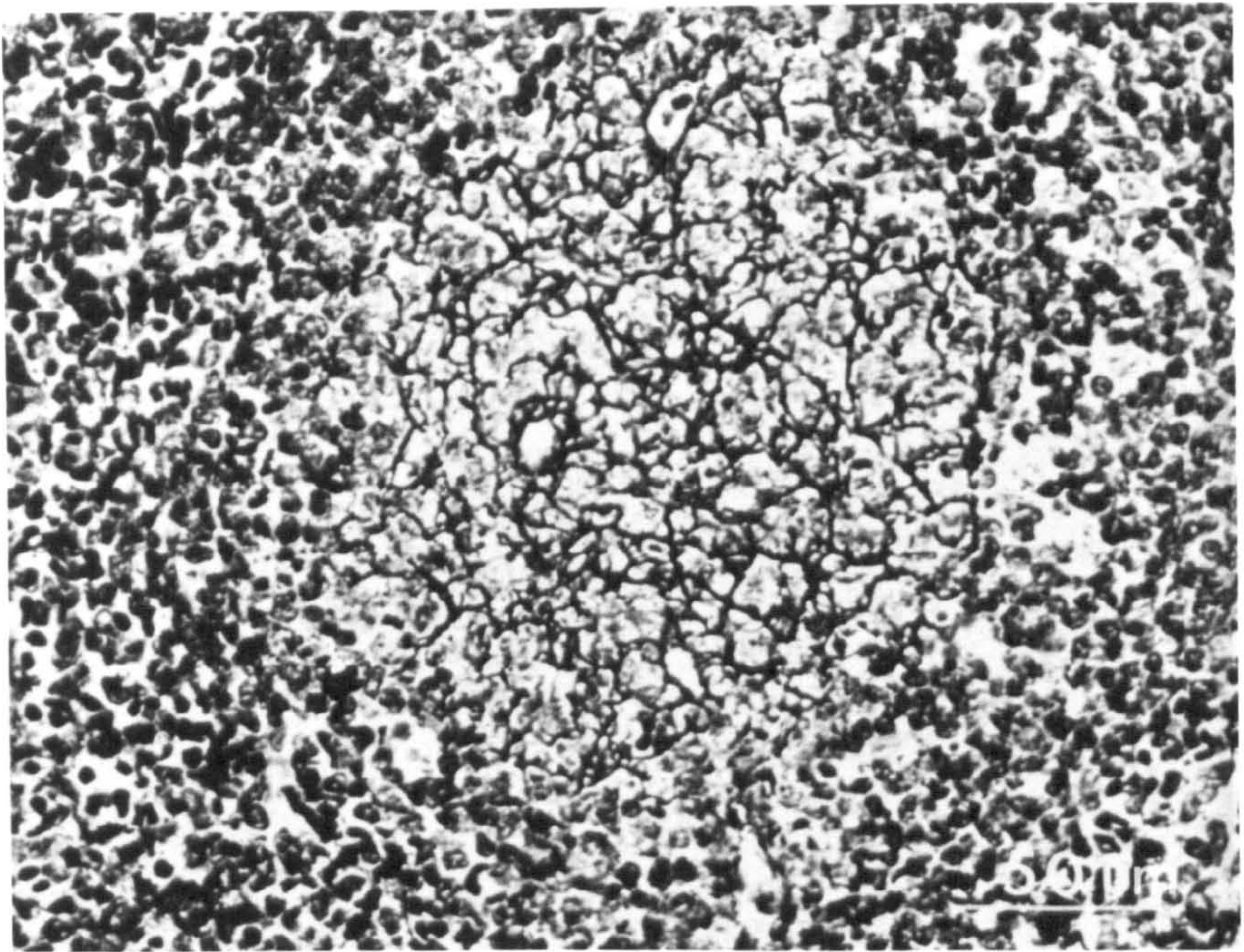
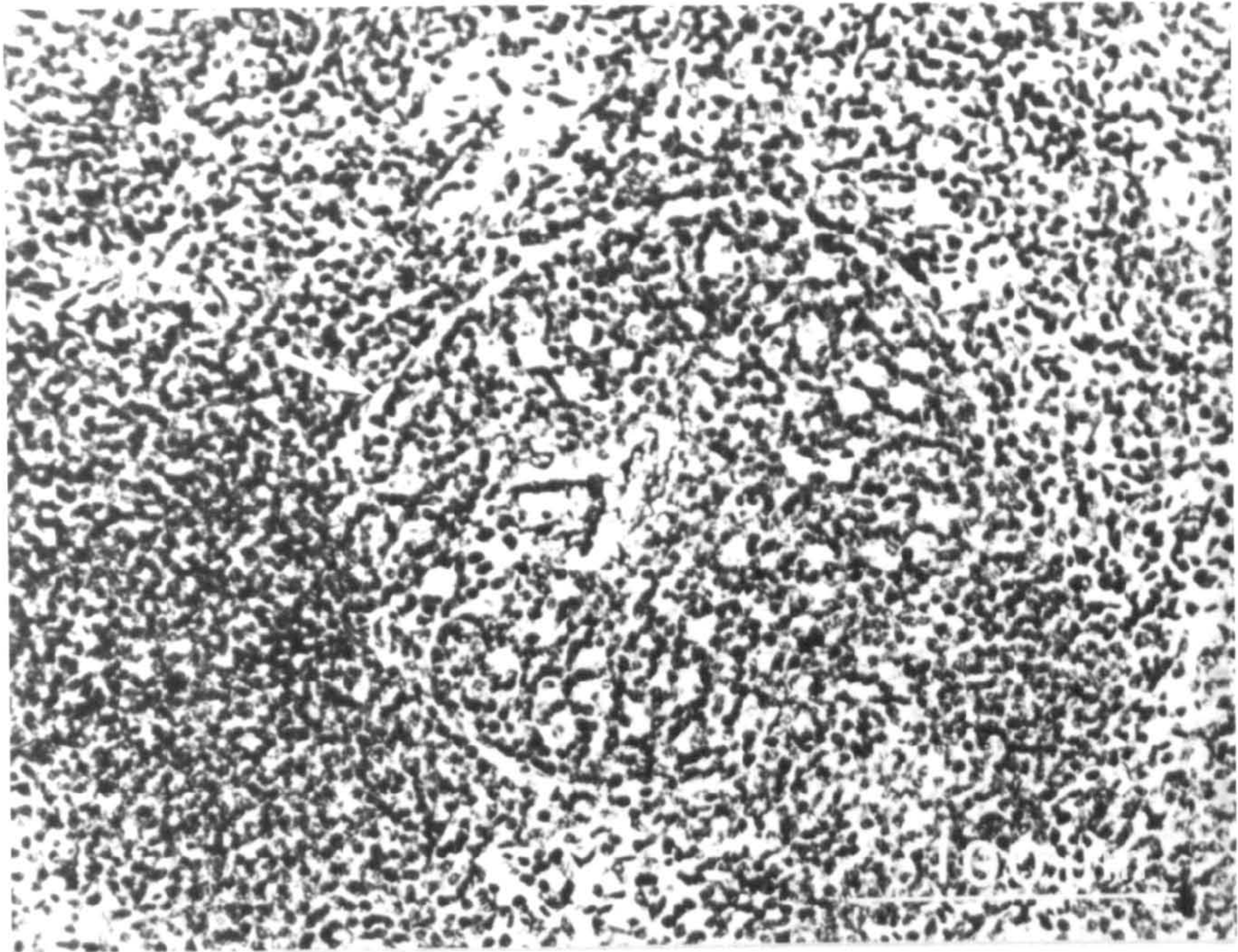


PLATE 40

Passive haemagglutination titres of sera from carp immunized with HGG in FCA. Each sample was diluted from well 1 to 20 and 21 to 24. End points are wells 10, 6, 14 and 15.

PLATE 41

Tube agglutination titres of sera from carp immunized with A. salmonicida. Each sample was diluted from well 1 to 18. End points are wells 13, 11, 11 and 10.

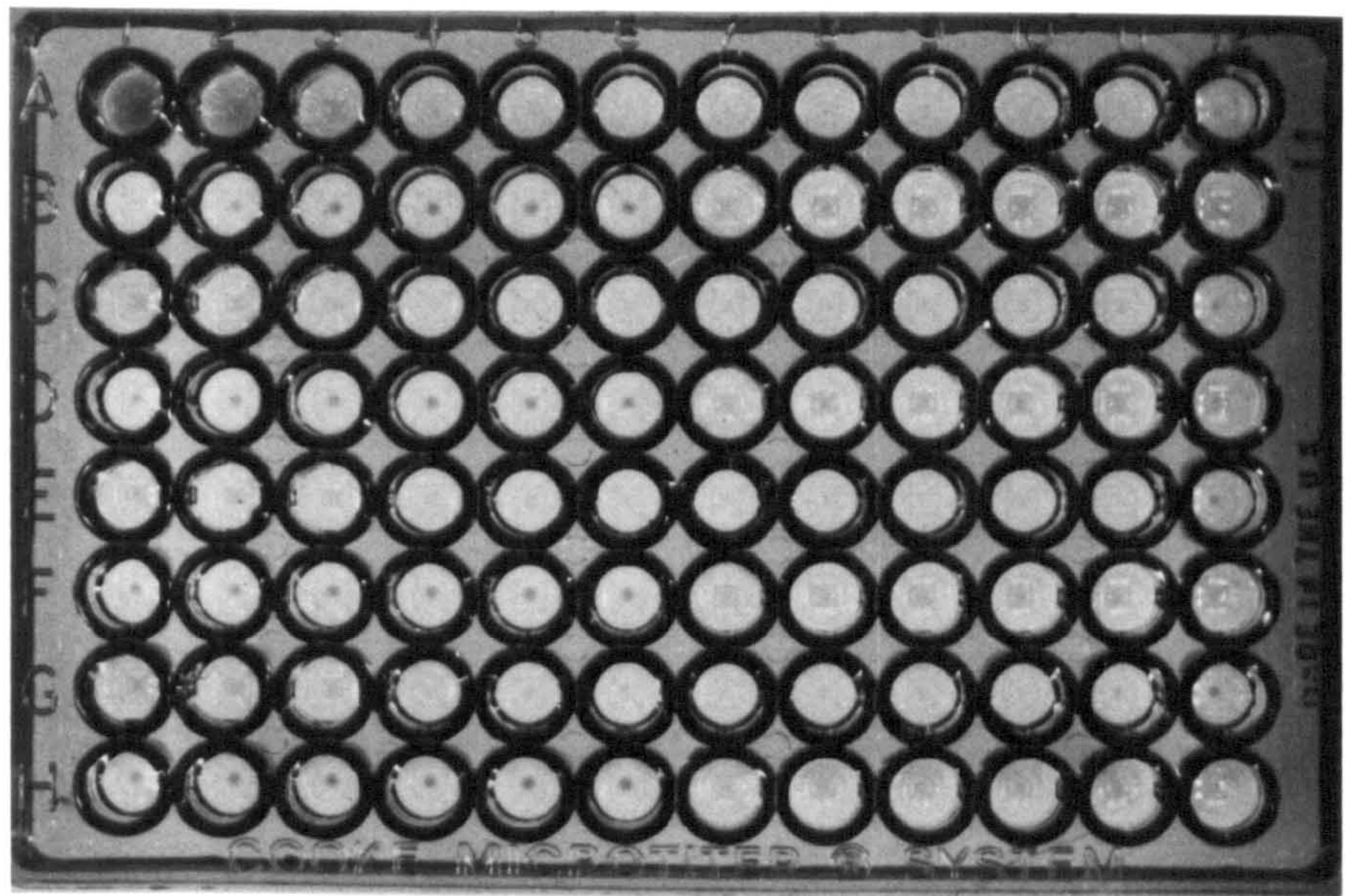
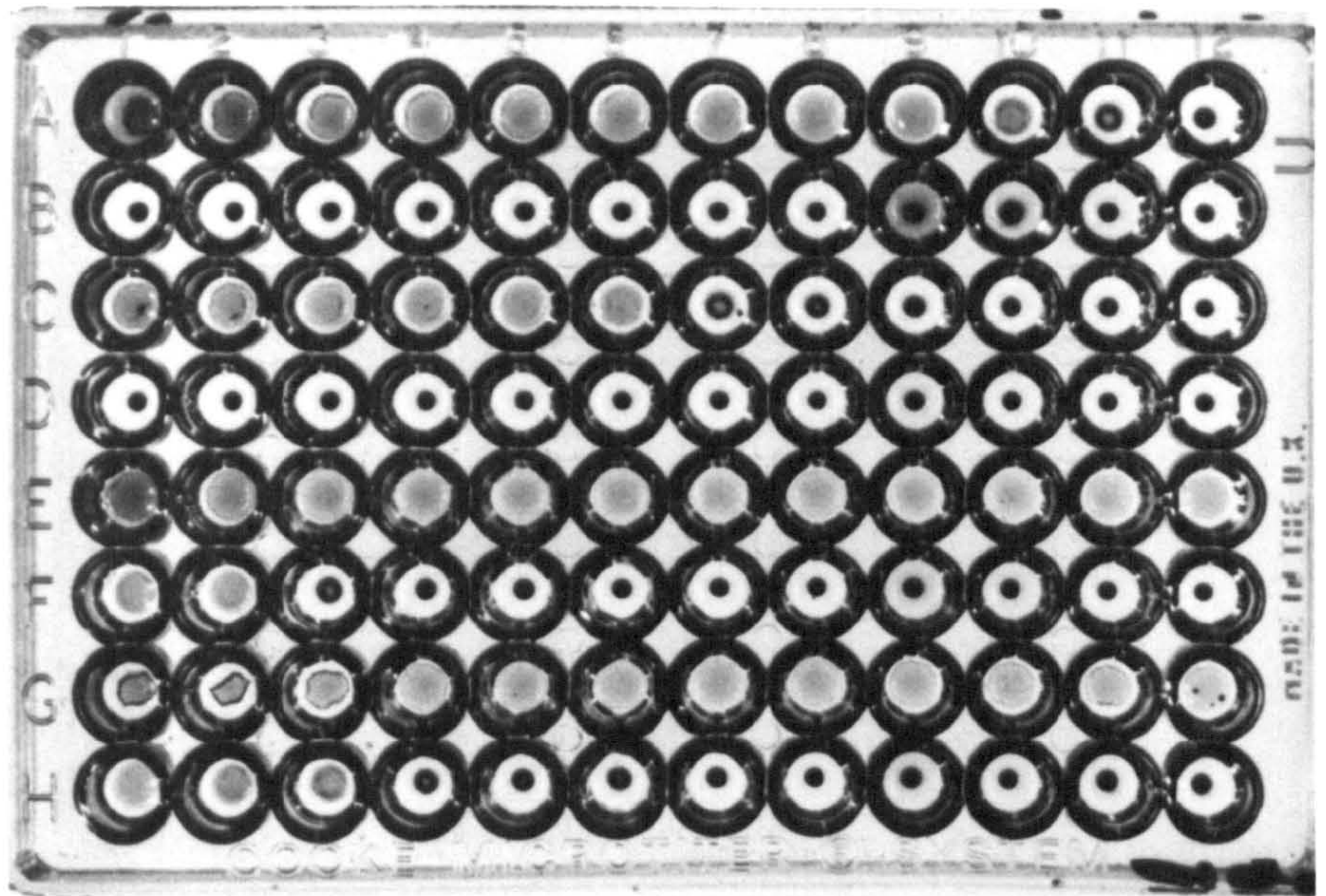


PLATE 42

Ouchterlony plate, demonstrating production of precipitating antibody by carp immunized intramuscularly with HGG in FCA. Neat serum was placed in the central well and dilutions of HGG antigen in the peripheral wells.

PLATE 43

Ouchterlony plate showing a lack of cross-reactivity of anti-HGG antiserum to BGG. Neat anti-HGG antiserum was placed in the central well, BGG in well 1 at 1 mg/ml and HGG in the other wells also at 1 mg/ml.

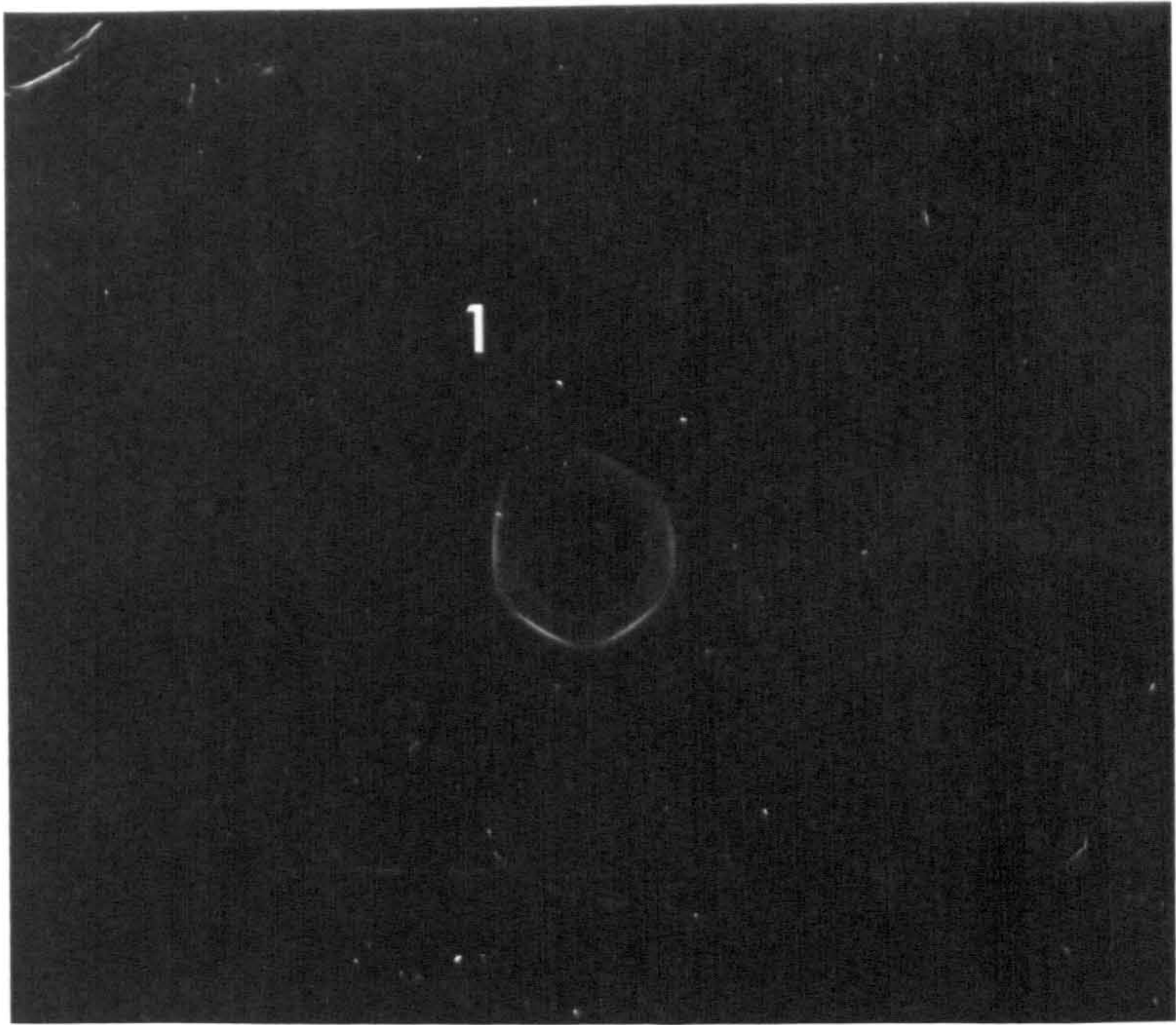
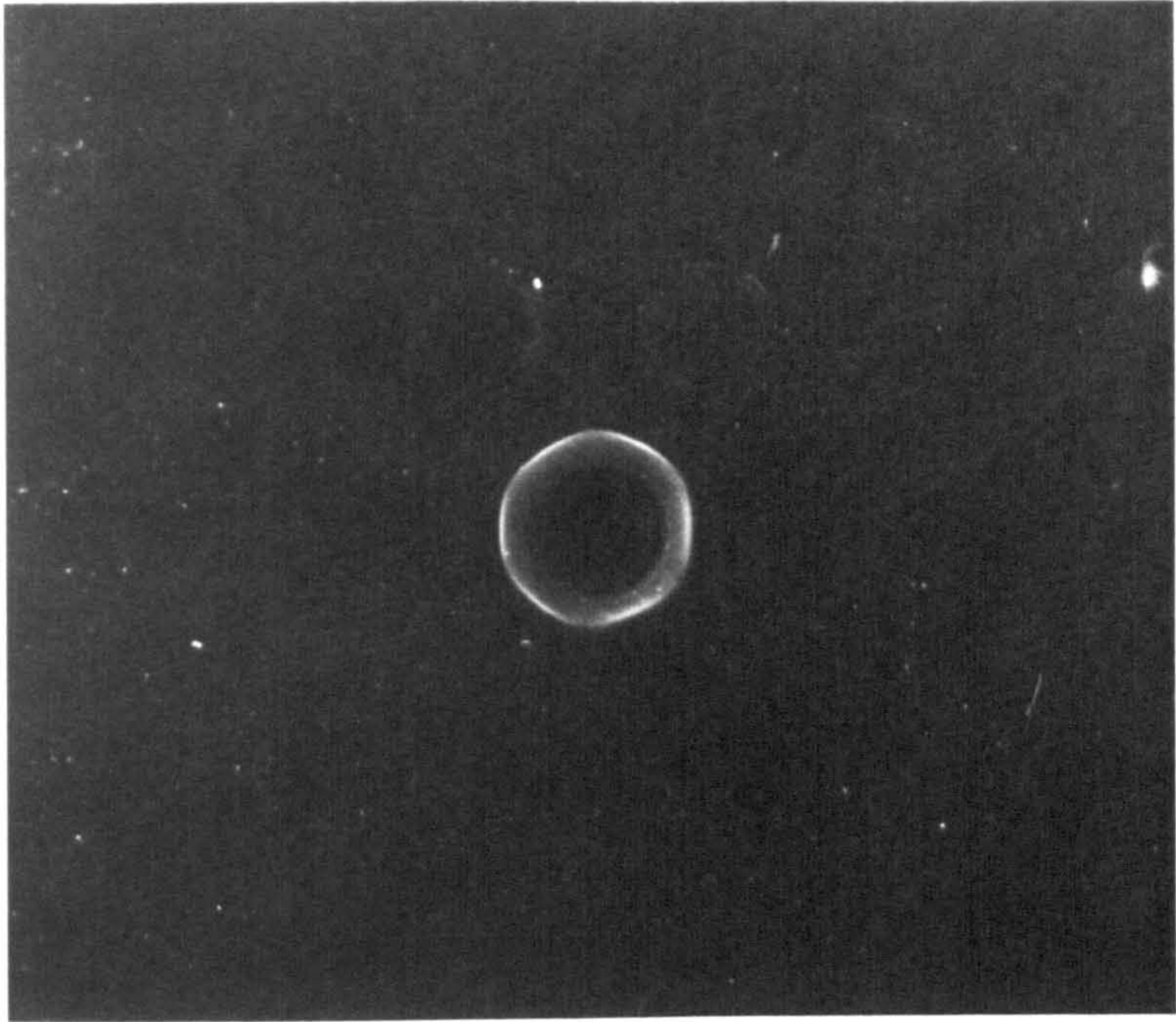


PLATE 44

Protein peak of carp anti-HGG antiserum undergoing ultracentrifugation in an MSE Analytical Ultracentrifuge. The temperature was 25°C and the rotor speed was 48,100 rpm. (160,000 g on average).

PLATE 45

Section through the thymic region of a 4 week old carp. The thymus (t) can be seen on both sides of the head, above the gills.

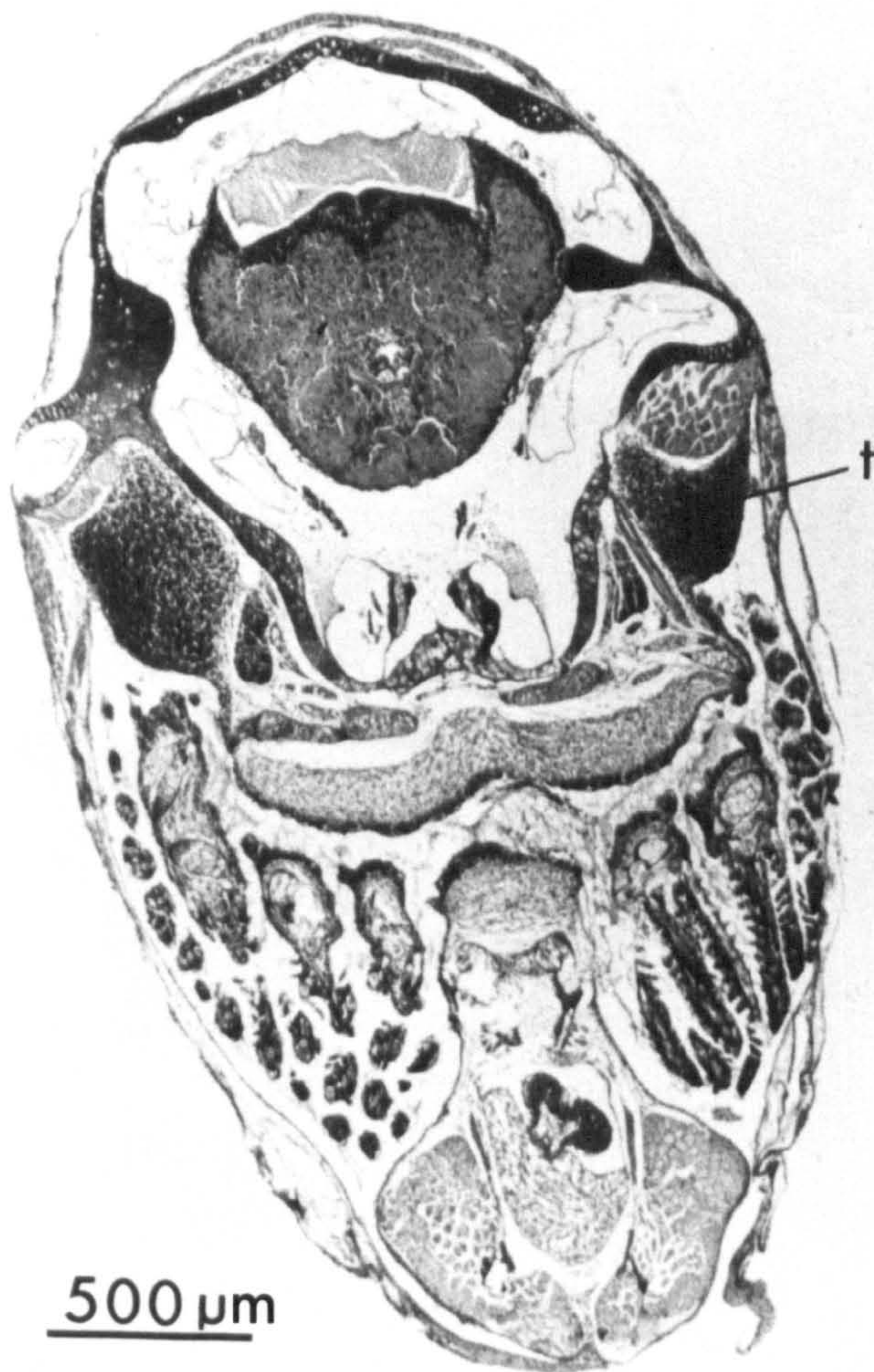
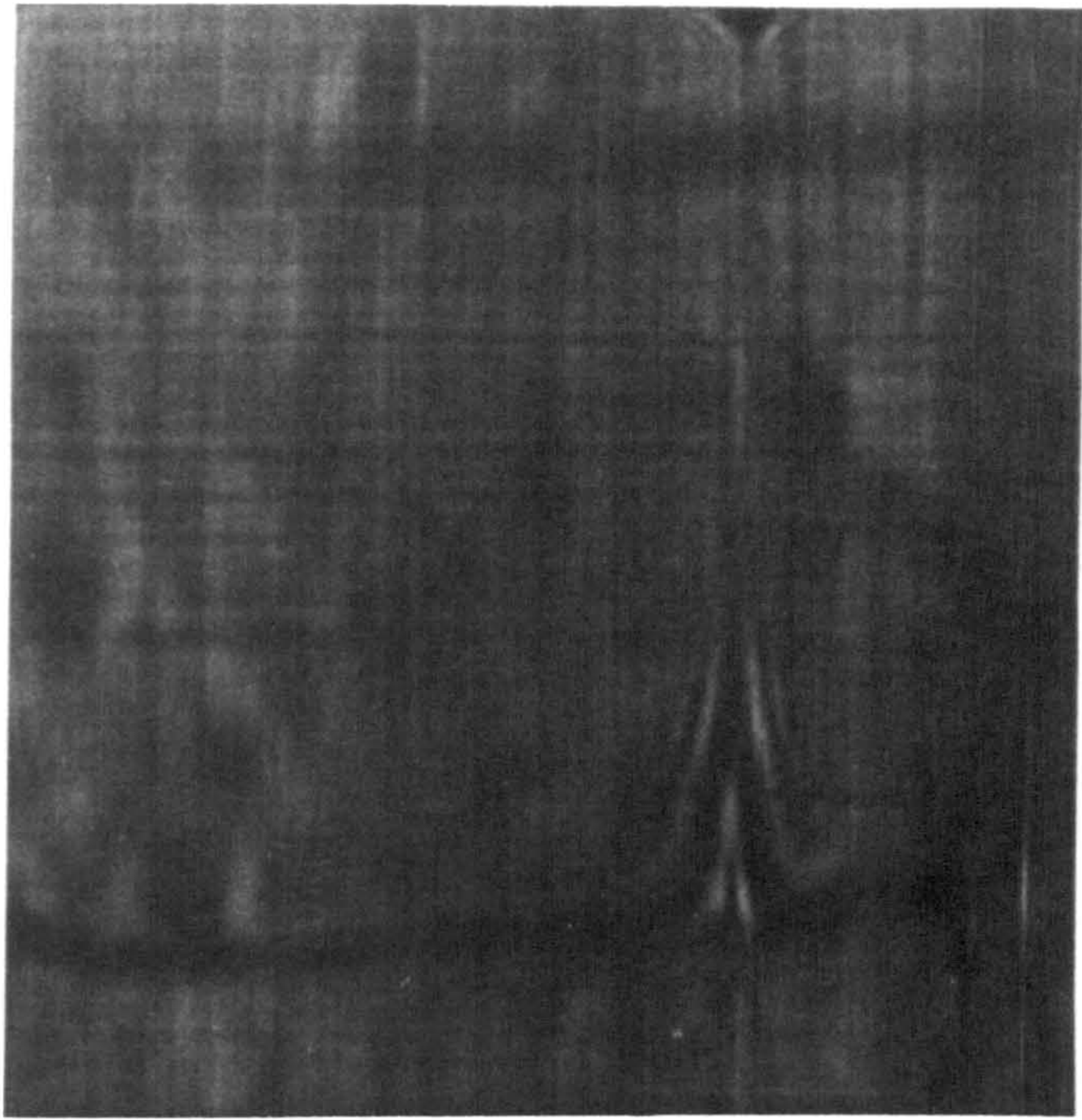


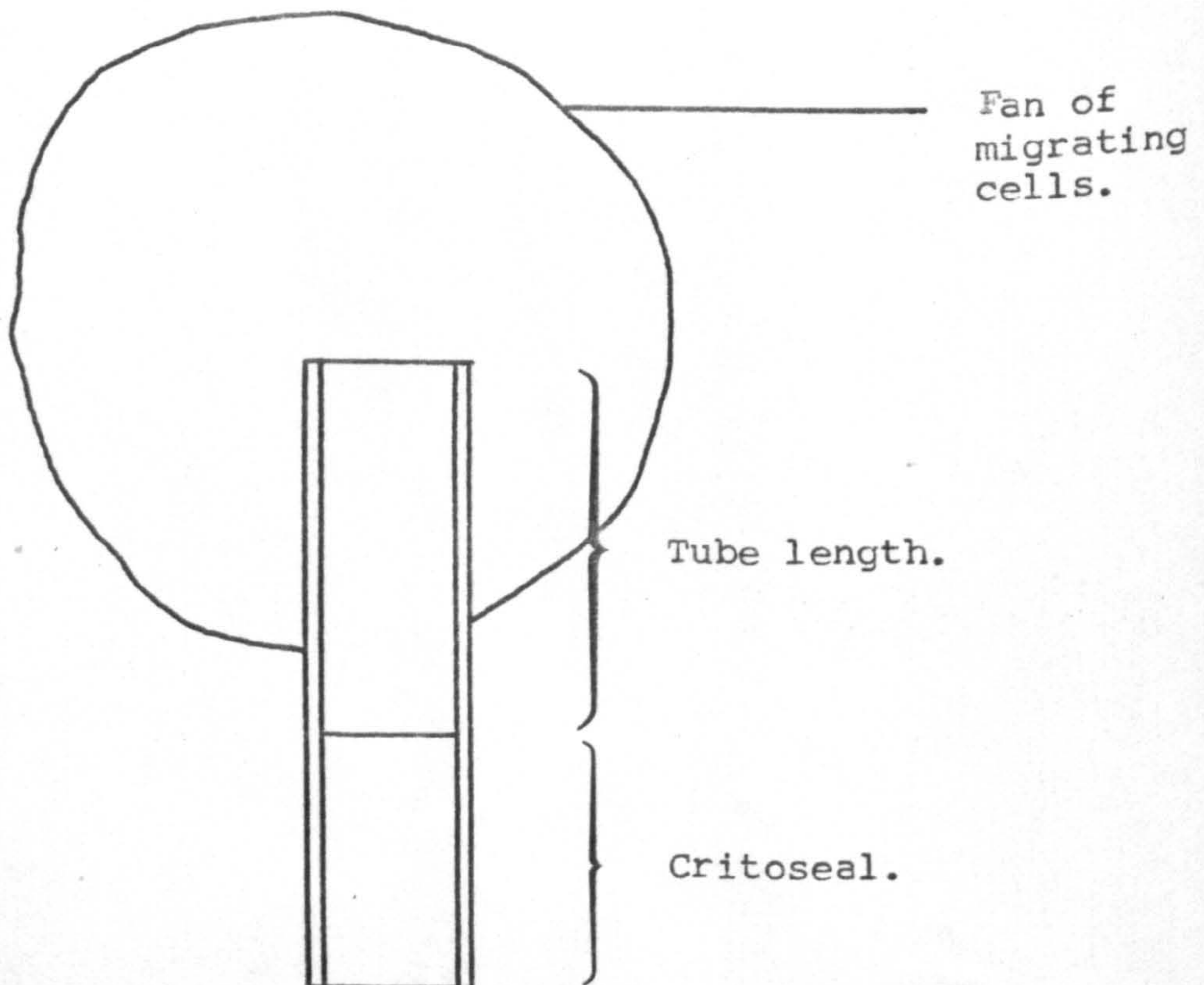
PLATE 46

Migration of FCA sensitized blood leucocytes in
(a) medium alone and (b) medium containing
3,000 i.u. PPD/ml.

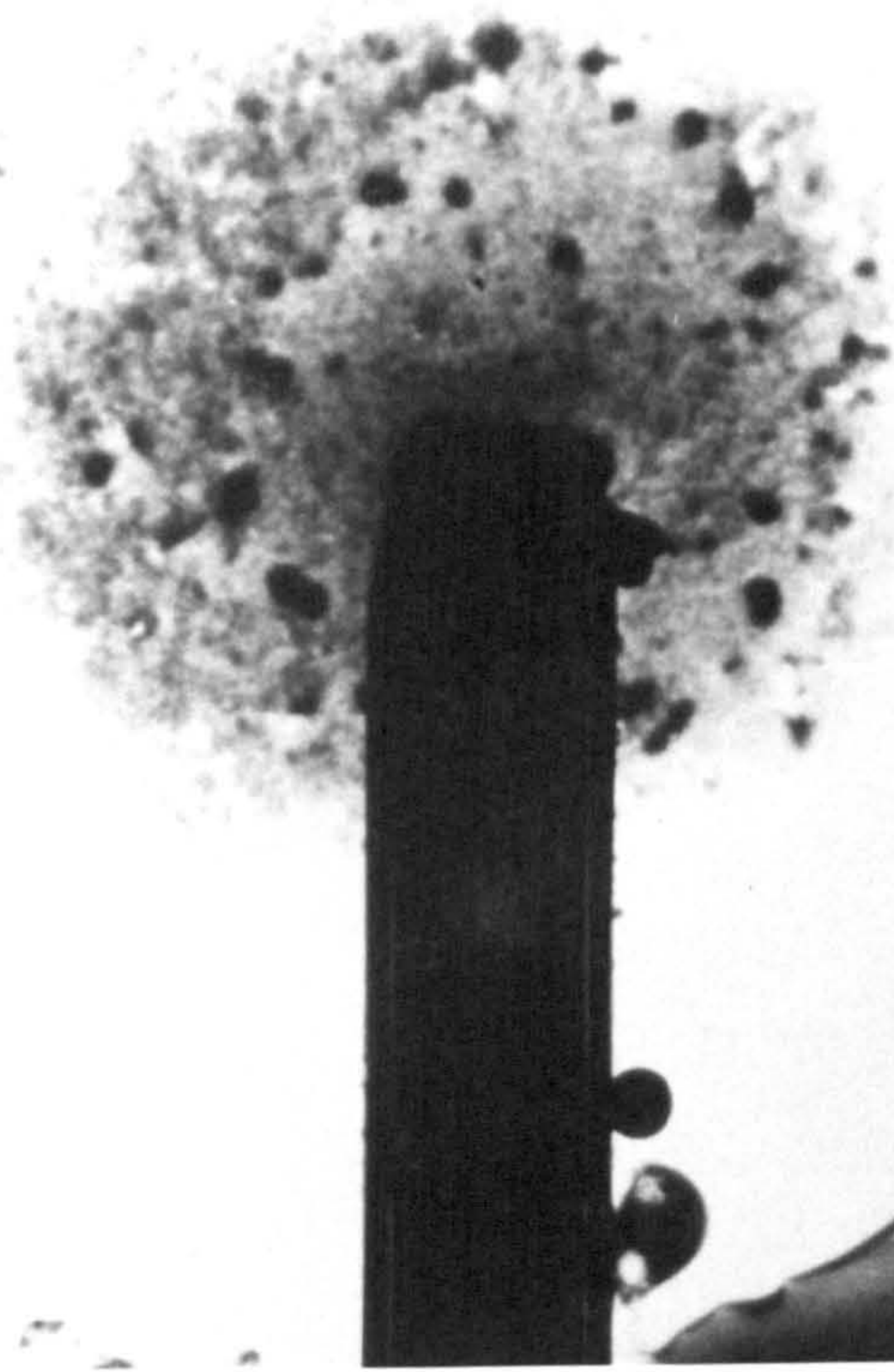
PLATE 47

Migration of FCA sensitized pronephros leucocytes in
(a) medium alone and (b) medium containing
3,000 i.u. PPD/ml.

Explanation of the areas used to calculate the 'fan area'
in Plate 46.



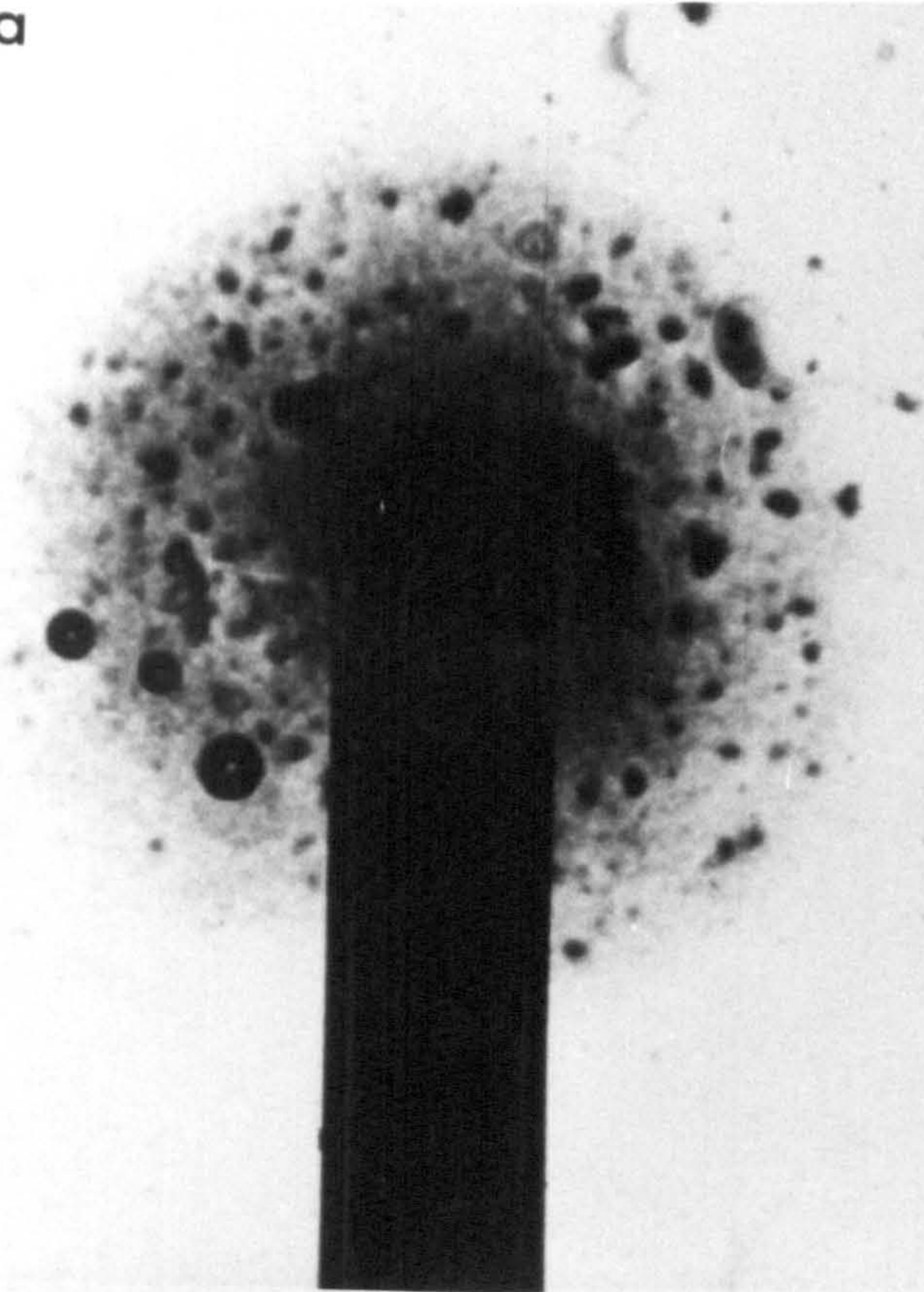
a



b



a



b

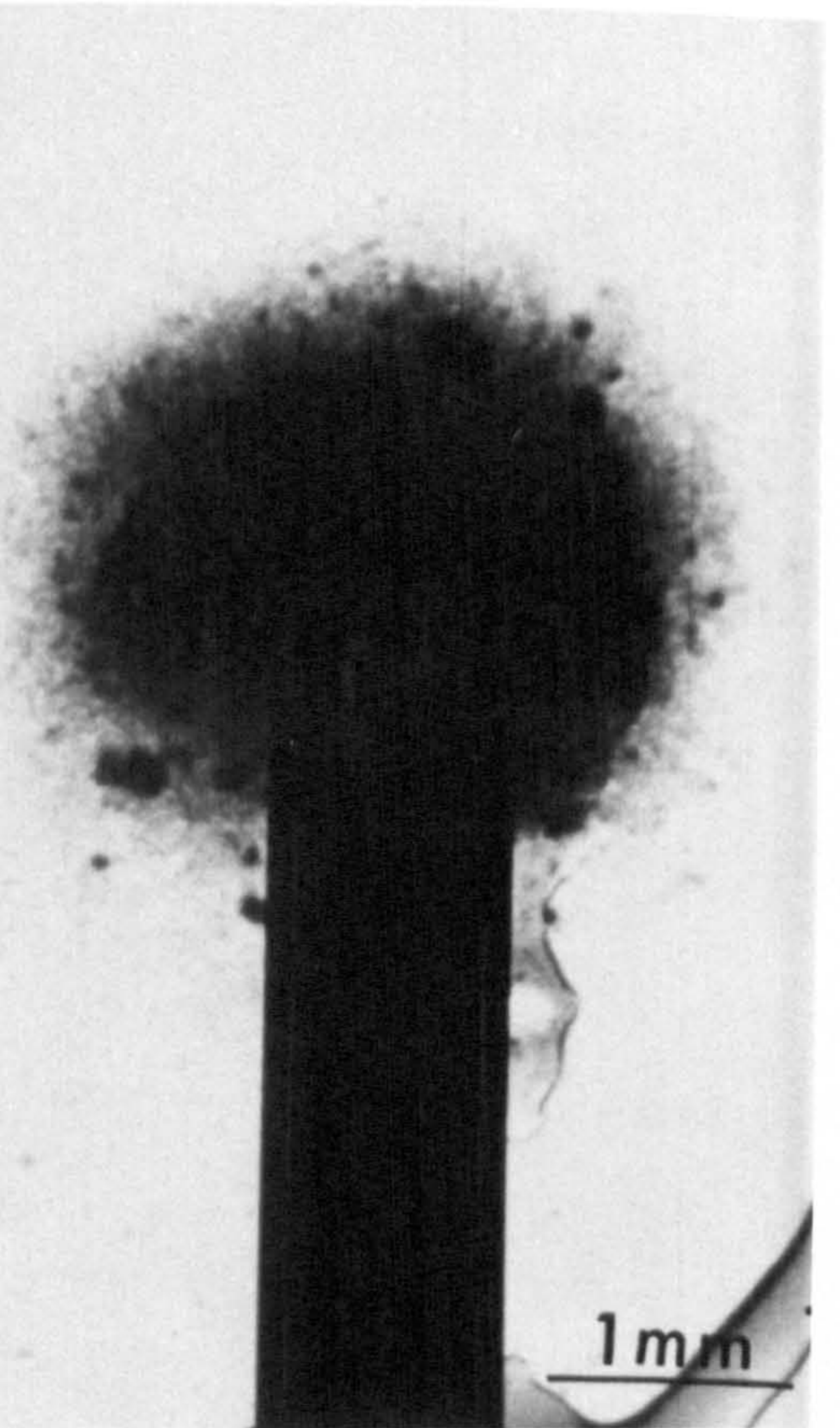


PLATE 48

Composite picture of cell types identified in carp peripheral blood and pronephros leucocyte cell suspensions. Two types of granulocyte (g_1 , g_2) can be seen, as can lymphocytes (l), thrombocytes (t) and a macrophage (m).

PLATE 49

Migration of pronephros leucocytes in (a) medium alone and (b) medium containing 200 μ g PHA/ml.

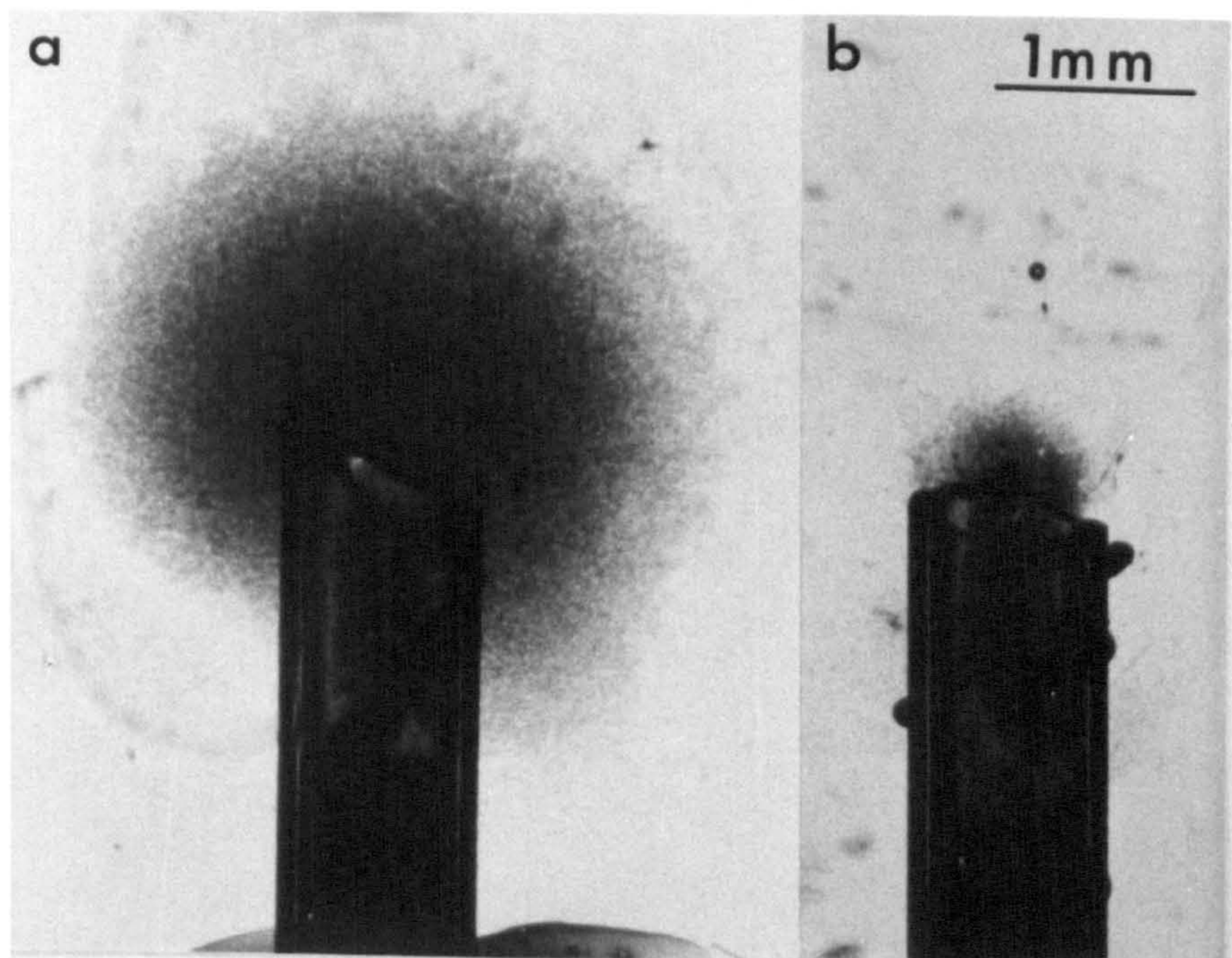
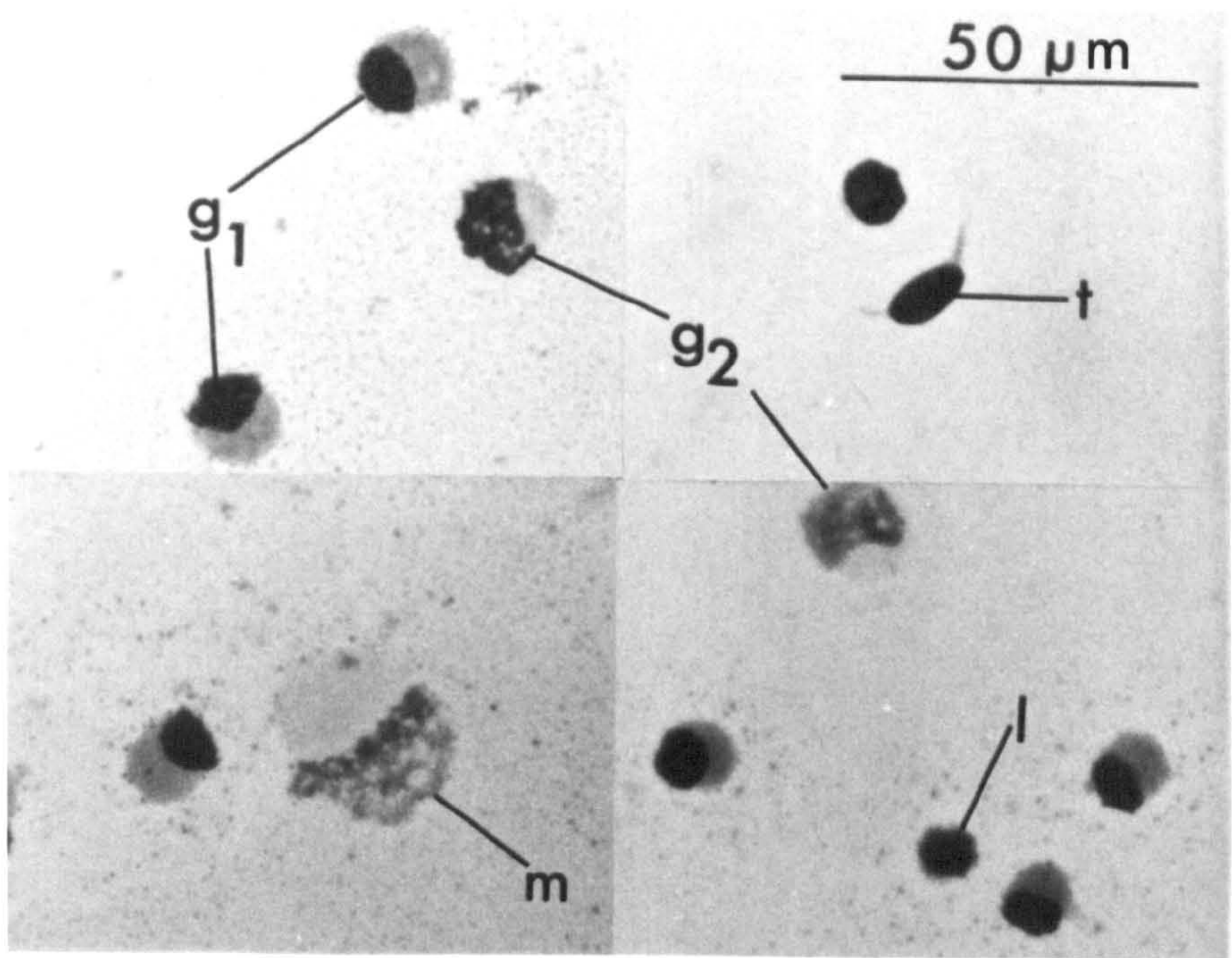


PLATE 50

Section through the thymic region of a unilaterally thymectomized adult rainbow trout. The thymus (t) can be seen on the left side of the head, but is completely absent from the right side.

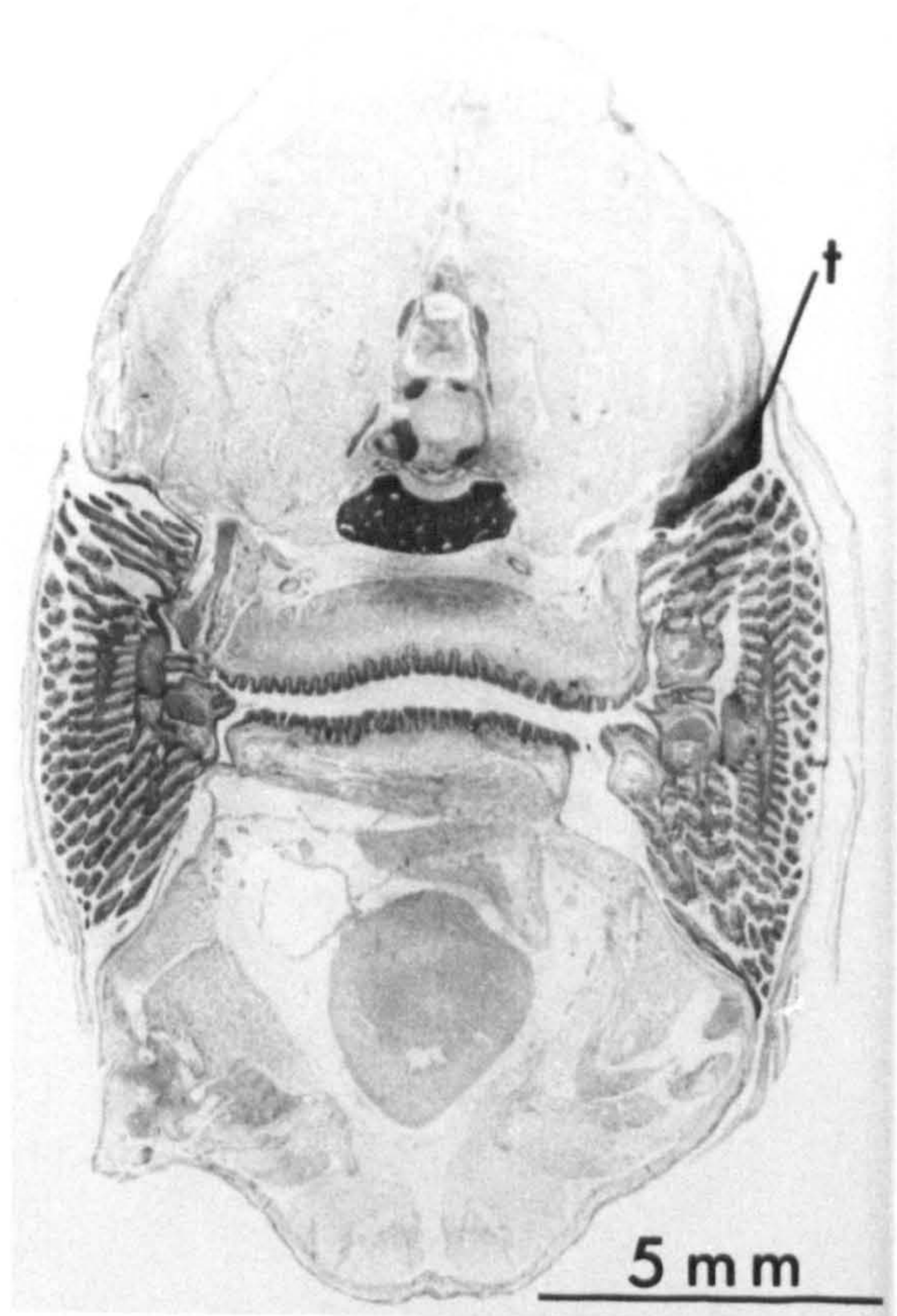


PLATE 51

Autoradiograph of the spleen from a sham-thymectomized trout immunized with HGG in FCA. The section is stained with methyl green-pyronin. The silver grains can be seen in great numbers over proliferating cells (pc) in contrast to the large dark spot of pigment (p) seen in other cells.

PLATE 52

Autoradiograph of the mesonephros from a sham-thymectomized trout immunized with HGG in FCA. Silver grains can be detected in proliferating cells (pc) between the excretory tubules which are also easily distinguished from pigment-containing cells (p).

