STUDIES OF PROLIFERATIVE KIDNEY DISEASE IN SALMONID FISHES

by

Richard Seymour Clifton-Hadley

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Ἐν δ' αὐτῇ πλωτοι χρυσωπιδες ἰχθυες ἑλλοι
νηχοντες παιζουσι δι' ὑδατος ἀμβροσιοιο.

Silent too, with glance of gold, fish dance,

playful in the paradisal deep.

Titanomachia

Preface

The experimental work described in this dissertation was performed mainly at the Ministry of Agriculture, Fisheries and Food Fish Diseases Laboratory, Weymouth, from January 1982 to January 1985, under the supervision of Dr R.H. Richards and Mr D. Bucke.

The dissertation describes original research carried out by the author. Work performed in co-operation with others has been acknowledged, where appropriate, in the text. This dissertation has not been submitted in full or in part to any other University.

August, 1986.

Richard Seymour Clifton-Hadley

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Summary

Sequential clinical and pathological changes were studied in 277 naturally-infected rainbow trout. Infection was histologically detectable at the beginning of June; gross pathology developed from the beginning of July; clinical signs, principally abdominal distension, were evident from mid-July. The main changes were associated with the kidney. Renal haemopoietic hyperplasia preceded the development of vascular lesions and intravascular haemoglobin crystallization. Diffuse inflammation disrupted nephrons and caused renal enlargement.

A kidney grading system was elaborated to assess disease severity.

Haematological changes were studied in 181 naturally-infected rainbow trout. A hypoplastic anaemia developed, exacerbated in some by hydraemia.

The infective period at the experimental site was shown to be between May and September when maximum water temperatures exceeded 10°C. Fish infected from the end of July did not become clinically affected. In subclinically-infected fish held in the laboratory at temperatures between 9°C and 18°C, PKD was delayed at the lower temperatures although the causative organism persisted in fish held at 9°C for several months. In clinically-affected fish held at 7°C to 21°C the rate of recovery was enhanced at the lower temperatures.

Resistance to PKD was assessed. Rainbow trout appeared more susceptible than Atlantic salmon and brown trout. Previously unexposed 0+ and 1+ rainbow trout were equally susceptible to the disease. Fish recovering from subclinical infection without renal swelling remained susceptible. Survivors of clinical disease could be re-infected but remained clinically unaffected when re-challenged. Fry from broodstock surviving clinical disease were susceptible.

PKD was transmitted using infected rainbow trout renal tissue by inoculation of rainbow trout via the peritoneal and subcutaneous routes, and by inoculation of brown trout via the peritoneal route. In rainbow trout, challenges via gill, skin and stomach were unsuccessful. No direct transmission was demonstrated from infected rainbow trout or infected Atlantic salmon to uninfected rainbow trout.

Publications

The following papers include work carried out by the author for this dissertation.

PUBLISHED

Clifton-Hadley, R., Richards, R.H. and Bucke, D. (1983) Method for the rapid diagnosis of proliferative kidney disease in salmonids. Veterinary Record, 112, 609.

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Clifton-Hadley, R.S., Bucke, D. and Richards, R.H. (1984) Proliferative kidney disease of salmonid fish: a review. Journal of Fish Diseases, 7, 363-377.

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Chapter 1

General introduction

Proliferative kidney disease (PKD) in salmonid fishes has assumed increasing importance as a cause of economic loss to the fish farming industry in the United Kingdom since the first recorded cases in Scotland in 1968 and Northern Ireland in 1976 (Roberts and Shepherd, 1974; Ferguson and Adair, 1977; Ferguson and Needham, 1978). With the rapid expansion of the industry and intensification of production during the past decade, PKD has become the primary limiting factor to profitability at several sites, especially in the south of England. No chemotherapeutic agent has been successful in treating affected fish, and control measures have been directed at ameliorating the effects of infection rather than preventing or curing it. The extent of the disease was highlighted by a survey of farms producing rainbow trout, Salmo gairdneri Richardson. Twenty of 45 sites were found to be infected (Seagrave, Bucke, Hudson and McGregor, 1981).

The work described in this dissertation was initiated in 1982 to investigate aspects of PKD which it was hoped would aid the development of a rational approach to the control of the disease.

Prior to this work, the aetiology of the disease was uncertain, although distinctive cells had been identified and postulated as the causative agent. Attempts to isolate and culture this agent had been unsuccessful. PKD had not been transmitted experimentally (Ferguson and Adair, 1977; Ferguson and Needham, 1978; Ferguson and Ball, 1979). The signs, gross pathology and histopathology of PKD in naturally-infected, clinically-affected rainbow trout had been recorded (de Kinkelin and Gérard, 1977a,b; Ghittino, Andruetto and Vigliani, 1977; Ferguson and Needham, 1978; Roberts, 1978), but the sequence of changes throughout the

course of infection had not been reported. There was little known of the epidemiology of the disease, although age of host and water temperature had been identified as factors affecting susceptibility (Ferguson and Ball, 1979; Ferguson, 1981). The work described in this dissertation was concerned with these aspects of the disease. The purposes of the work were:

1. To study the sequential clinical and pathological changes from the initial signs of infection until recovery or death from the disease.
2. To study the haematological changes during the course of the disease.
3. To study some epidemiological aspects of the disease including the time course and infective period of PKD at one river-fed site and the effects of water temperature, age, previous exposure and species of fish on the development of the disease.
4. To study the experimental transmission of PKD.

In this chapter, the following aspects of PKD are reviewed:

1. The history of PKD.
2. The worldwide occurrence of PKD.
3. Species affected.
4. Incidence of PKD.
5. Epidemiology of PKD.
6. Aetiology.
7. Experimental transmission.
8. Clinical signs.
9. Post-mortem findings.
10. Haematology.
11. Treatment and control.

The economic importance of PKD in England and Wales is assessed.

1. THE HISTORY OF PKD

The disease syndrome in fingerling rainbow trout in which gross kidney changes are seen macroscopically was first named "proliferative kidney disease" (PKD) by Roberts and Shepherd (1974). Other names are encountered in the literature, but this title has received widespread acceptance. Possible cases of PKD have been described as "Amöbeninfektion der Niere" by Plehn (1924), "Infektiöse Nierenschwellung und Leberdegeneration" by Schäperclaus (1954), "L'anémie infectieuse" by Besse (1954) (which Snieszko (1961) equated with the condition described by Schäperclaus in 1954), "L'anémie pernicieuse des truites" by Besse (1956), "Hépathonéphrétique", "Hépathonéphrite" and "Hépato-néphrite" by de Kinkelin and Gérard (1976, 1977a,b), "L'Amebiasi della trota iridea d'Allevamento" by Ghittino et al. (1977), "Iperplasia Emopoietica Parassitaria della Trota (IEPT)" by Ghittino, Andruetto and Vigliani (1980), and "lupus" (Hedrick, Kent, Foot, Rosemark and Manzer, 1985a,b).

Plehn (1924) was the first to describe in detail the clinical signs and post-mortem appearance of a condition in both rainbow and brook trout, Salvelinus fontinalis Mitchill, which resembled PKD.

Schäperclaus (1938) recorded an increasing incidence of a condition in hatchery rainbow trout, where the predominant sign was gross swelling of the kidney. He was unsuccessful in isolating the causative agent, but considered that he had transmitted the disease by injecting a cell-free, bacteria-free filtrate from the grossly affected organs of diseased fish (Schäperclaus, 1954). This led him to ascribe a viral aetiology to the condition. The similarities between this disease and chronic viral haemorrhagic septicaemia (VHS) were noted by Ghittino (1970).

Confusion over the distinct nature of PKD has continued since these

earlier reports, especially as PKD often occurs with intercurrent disease such as VHS, nephrocalcinosis, infectious pancreatic necrosis (IPN), furunculosis (Hoffmann and Dangschat, 1981) and Exophiala infection (Alderman and Feist, 1985). This confusion has been resolved with the detailed descriptions of the disease by Ghittino et al. (1977) and Ferguson and Needham (1978). However, an aetiological agent has yet to be isolated and shown to reproduce the disease.

2. THE WORLDWIDE OCCURRENCE OF PKD

PKD has been diagnosed by histological examination of infected tissues in Western Europe, Canada and the United States of America.

In Western Europe, it has been reported from Denmark (Olesen, 1985), France (de Kinkelin and Gérard, 1976, 1977a,b), Holland (Helder, 1985), Italy (Ghittino et al., 1977), Norway (Roberts, 1978), Republic of Ireland (O'Brien, McArdle and Doyle, 1977), Sweden (Ljüngberg, 1980) and West Germany (Hoffmannand Dangschat, 1981; Schlotfeldt, 1983a,b). In the United Kingdom it has been reported from England (Scott, 1979; Dalton, 1980; Seagrave et al., 1981), Northern Ireland (Ferguson and Adair, 1977; Ferguson and Needham, 1978), Scotland (Ferguson and Needham, 1978; Ellis, McVicar and Munro, 1982) and Wales (Seagrave et al., 1981). In Denmark, PKD was diagnosed for the first time in 1982 on three farms, then in 1983 on seven farms, and in 1984 on three farms (Olesen, 1985); in Italy, 38 cases of PKD were diagnosed in 1982, 28 cases in 1983 and 26 cases in 1984 (Giorgetti, 1985); in West Germany the disease has been observed in 33 trout farms and on 10 rivers (Hoffmann, Graaff, Braun, Körting, Dangschat and Manz, 1984).

PKD has been diagnosed in Canada at three salmon hatcheries in British Columbia (Hoskins, 1984) and in the United States of America in

Idaho (Smith, Morrison and Ramsey, 1982) and in California (Hedrick, Kent, Rosemark and Manzer, 1984).

3. SPECIES AFFECTED

Most reported cases of PKD have occurred in rainbow trout. However, infection has been diagnosed in other species of farmed and wild fish. Plehn (1924) described a disease similar to PKD in both rainbow and brook trout. Roberts (1978) reported a single case of PKD in brown trout, Salmo trutta Linnaeus, and Seagrave et al. (1981) found five of 100 brown trout sampled at one infected farm positive for PKD. Ellis et al. (1982, 1985) described serious outbreaks in farmed brown trout in their first summer (0+), while Wootten and McVicar (1982) found wild brown trout in both their first (0+) and second (1+) summers clinically infected with PKX cells and with pathological changes similar to those described by Ferguson and Needham (1978) in rainbow trout. Outbreaks in brown trout were recorded in Eire by McArdle (1982) and in West Germany by Hoffmann et al. (1984). Ellis et al. (1982, 1985) also recorded serious outbreaks of PKD in Atlantic salmon parr, Salmo salar L., on the Isle of Mull. A further outbreak of PKD in Atlantic salmon fry was observed on the Isle of Lewis (Clifton-Hadley, unpublished data). Hedrick et al. (1984) reported the first recorded cases of PKD in two Pacific salmon species, the chinook, Oncorhynchus tshawytscha Walbaum, and coho, O. kisutch Walbaum, both at the Mad River Hatchery in the state of California. Char, Salvelinus alpinus L., held in captivity, were found to be infected (Clifton-Hadley, unpublished data).

Seagrave et al. (1981) found renal changes histologically similar to those of PKD in pike, Esox lucius L., and grayling, Thymallus thymallus L.

Other wild fish examined during the survey from rivers supplying

infected farms showed no signs of PKD. These included specimens of bream, Abramis brama L., bullhead, Cottus gobio L., chub, Leuciscus (squalius) cephalus L., dace, Leuciscus (leuciscus) leuciscus L., eel, Anguilla anguilla L., gudgeon, Gobio gobio L., minnow, Phoxinus phoxinus L., roach, Rutilus rutilus L. and stone loach, Noemacheilus barbatulus L. Hoffmann and Dangschat (1981) also found histological evidence of PKD infection in grayling. Bucke (1984) found organisms with similarities to the possible causative organism of PKD in a liver specimen from a gudgeon, although no lesions similar to those seen in PKD were present.

4. INCIDENCE OF PKD

100% infection of 0+ rainbow trout fingerlings was recorded on a farm in Northern Ireland from 1972-1978 (Ferguson and Ball, 1979). During a survey of PKD (Seagrave et al., 1981) it was found that infection in 0+ rainbow trout was between 60% and 100% on 19 of 20 PKD-positive farms and 15% on the remaining site. In this survey ten rainbow trout were examined as a minimum batch. Where no parasite was found the sample was increased to 50 fish. A farm was considered uninfected if these were negative.

Recorded mortality rates in infected rainbow trout vary widely and appear to depend on the type of farm, the management procedures, the age of the fish, the water temperature and the presence of other diseases. Seagrave and Bucke (1979) recorded cumulative mortalities in PKD outbreaks in England of 0-100% of infected fish. During PKD outbreaks in rainbow trout on a farm on the west coast of Scotland in 1968 and 1969, Ferguson and Needham (1978) recorded that over 75% of fish died in 1968, and 72.4% died during a four-month period

from August to November 1969. Further mortalities occurred at a reduced rate in this stock until March 1970. Mortalities were thought to be exacerbated by salt water acclimatization commencing during the preclinical period of the disease. On one farm in Northern Ireland (Ferguson and Needham, 1978; Ferguson and Ball, 1979) mortalities in 0+ fingerling rainbow trout ranged from 10-15% of each year's production from 1972 to 1976. In 1977 and 1978 this figure increased to 40% in fingerlings moved to the farm site in May of each year, compared with 0% mortality in fingerlings moved in July, despite these fish becoming infected.

On a farm in Italy, losses of 100-120 mm rainbow trout corresponding to 6-7% of total stock were recorded over a 12-day period (Ghittino et al, 1977). In France, mortalities of up to 90% were described (de Kinkelin and Gérard, 1977b). In three separate occurrences of PKD in West Germany (Schlotfeldt, 1983a,b) mortality rates of 5-7%, 30% and 60% were recorded, and were related to the type of rearing facility, age of fish, water temperature and concommitant diseases. The effect of secondary infections on mortalities due to PKD has been noted by other authors. A mortality rate of 20-30% was reported in outbreaks of PKD in Germany in 1980 and 1981 (Hoffmann and Dangschat, 1981), and was thought to depend on additional infections, especially furunculosis and IPN. In the first reports of PKD in North America (Smith et al., 1982; Smith, Morrison, Ramsey and Ferguson, 1984) the overall mortality rate was approximately 20%. However, other infections at the Hagerman State Fish Hatchery during the outbreak in 1981 included IPN, infectious haemopoietic necrosis (IHN) and Sanguinicola infestation. These reduced mortalities directly attributable to PKD in one particular raceway

to approximately 5% during October and 7% during October and half of November. Farmers' reports from several intensive farms in the south of England suggest that annual mortalities from PKD now exceed 10%.

Less information is available for infection and mortality rates in brown trout, Atlantic salmon and Pacific salmon species. An incidence of 75% based on gross pathology was reported in 0+ farmed brown trout in Scotland (Ellis et al., 1982, 1985), with a cumulative mortality of 8% over a five-month period. In two outbreaks in the Republic of Ireland during 1981 and 1982, a morbidity rate of 60% and 76% respectively was recorded in August, reducing to 25% and 20% respectively during October (McArdle, 1982). In 0+ Atlantic salmon parr at one farm on the Isle of Mull in 1981, incidence (based on gross pathological findings) ranged from 60-70% in stocks of Scottish origin, and up to 90% in stock of Norwegian origin. Cumulative mortalities in these fish ranged from 7-32% (Ellis et al., 1982). In chinook salmon, average weight 2.5 g, and coho salmon, average weight 3 g, Hedrick et al. (1984) reported cumulative mortalities during a period from June to September of 95% and 13%, respectively.

5. EPIDEMIOLOGY OF PKD

As noted previously, most reports of PKD refer to fingerling rainbow trout (Roberts and Shepherd, 1974; Ghittino et al., 1977; Ferguson and Needham, 1978; Roberts, 1978; Seagrave and Bucke, 1979). Fish had usually been hatched and reared for about 4 months on spring or bore-hole water and then moved to rearing facilities supplied with river water. Ghittino et al. (1980) reported that PKD was seen in trout reared in earth and gravel ponds but not in those reared in concrete tanks. However, Ferguson and Ball (1979) found fish affected with PKD which were reared in circular concrete tanks, and transferred to earthen ponds in

July. In the United Kingdom PKD has been diagnosed at most types of rearing facility fed by river water, including cages, circular tanks of various sizes constructed of concrete, fibre glass, asbestos or plastic, raceways and earth ponds (Clifton-Hadley, unpublished data). No histological signs of PKD have been reported in rainbow or brown trout fry reared continuously in holding facilities supplied by spring or bore-hole water.

In previous reports, water quality is considered an important factor in the occurrence of PKD. Roberts and Shepherd (1974) described PKD as occurring "under soft water conditions in summer", and Roberts (1978) considered that low pH of the water in PKD outbreaks was significant. Ferguson and Needham (1978) analysed the water in two occurrences of PKD in Northern Ireland and on the west coast of Scotland. In respective outbreaks, the water supply was acid (pH 6.5 and 5.6), soft (total hardness 18 mg $CaCO_3$/l and 4.5 mg $CaCO_3$/l; calcium ion concentration 8.0 mg/l and 0.80 mg/l), and oligotrophic (nitrate ion concentration 40 µg/l and 14.2 µg/l). Ferguson and Needham (1978) considered these aspects of water quality to be important factors in the establishment of a PKD outbreak. However, Scott (1979) described PKD outbreaks where the water supply was from a chalk stream, being alkaline (pH 7.9-8.3), hard ($CaCO_3$ about 230 mg/l), and "highly eutrophic". He suggested that extremes of pH predisposed towards PKD development rather than the acidity or alkalinity *per se*. Hoffmann *et al*. (1984) also recorded PKD in waters which were hard and alkaline.

Hoffmann and Dangschat (1981) found that pollution, especially organic loading, was often present when PKD occurred, and concluded that poor water quality was a predisposing factor.

The temperature, as well as the quality, of the water supply has been recognised as affecting the occurrence, severity and regression of PKD.

Several authors have noted the correlations between rising water temperatures and the development of PKD, and between falling water temperatures and the decline of the disease (Ferguson and Ball, 1979; Ferguson, 1981; Ellis et al. , 1982). Ferguson and Ball (1979) recorded the mean monthly values for temperature computed from daily measurements at one infected farm. From April to October 1978 the values were respectively 8.7, 15.6, 17.2, 17.4, 15.7, 14.0 and 11.5°C. To investigate the effects of water temperature further, Ferguson (1981) studied three groups of fish removed from an infected farm and held at a constant water temperature under laboratory conditions. Group 1 comprised 45 fingerling rainbow trout moved to the infected farm in July 1978 and held there until mid-October, when they were transferred to the laboratory and kept in recirculating water at 16°C. Histological examination showed these fish to be infected at the time of transfer. In January 1979, 10 of the 14 surviving fish were found to be clinically affected, whereas corresponding fish still at the farm showed no evidence of PKD. Ferguson (1981) concluded that the falling autumn water temperatures prevented the disease developing on the farm in July-infected fish. He suggested that decreasing daylight length may have been involved. The second group of fish were 20 rainbow trout which had been exposed at the farm in May as fingerlings and had survived the summer's PKD outbreak. They were moved to the laboratory in mid-October 1978 and maintained at 16°C until January 1979. These fish recovered from PKD, although Ferguson (1981) considered that the recovery may have been delayed. He concluded that falling water temperature increased the recovery rate. The third group of fish comprised 20 rainbow trout fry moved to the infected farm in May, removed to the laboratory 25 days later, and maintained there in water at

5-7°C. These fish showed no evidence of PKD throughout the experimental period which ended in October. Ferguson (1981) concluded that low water temperature prevented the development of PKD. He did not know whether this temperature effect was on the causative organism or on the host fish.

Ghittino et al. (1977) considered that the time course of PKD ranged from four to five months in fingerling rainbow trout held in water at temperatures of 12-14°C.

In their description of the epidemiological aspects of PKD relating to a farm in Northern Ireland, Ferguson and Ball (1979) found that 0+ rainbow trout, moved in May from a hatchery supplied with spring water to a farm supplied with river water, showed histological signs of infection 40 days after transfer to the farm, and clinical signs three to four weeks later during July. However, 0+ fish moved from the hatchery to the farm in July only showed histological lesions after 58 days and gross signs did not develop. They concluded that infection due to PKD occurred most readily during May either because infective stages of the causative organism were most numerous at that time, or because of the changing water temperature over that period. Mortalities in the fish moved in May occurred from the second half of July until the middle of September.

Olesen (1985) considered that in Denmark fish were at most risk from infection in June. He described the incubation period from initial infection to clinical disease as varying from four to 12 weeks. The longer period was seen early in the year, while the shorter periods occurred from June to August.

Ferguson and Needham (1978) reported that at a site on the west coast of Scotland mortalities started in late July and continued until late October, with a peak in early September.

Ellis et al. (1982) reported a similar pattern in farmed brown trout

and Atlantic salmon in Scotland, with gross pathological lesions developing in July and mortalities continuing until September. However, in the first reported outbreak of PKD in rainbow trout in the U.S.A., although the disease was probably present during July, Smith et al. (1984) found that mortalities continued into December with peaks occurring during July through to November. Few changes were seen in samples taken in January. The authors considered that the constant water temperature of 15°C at the infected site was an important factor in the prolonged course of the disease. Furthermore, Schlotfeldt (1983b) reported that, in a closed recirculation system in West Germany where the water temperature was maintained between 15°C-18°C, mortalities occurred throughout the year.

The age of the fish at the time of exposure to infected water has been considered with regard to susceptibility to PKD. Although most reported cases in the United Kingdom have occurred in fingerling rainbow trout, there are several reports of older fish with histological signs of PKD. Ghittino et al. (1977) reported one case from rainbow trout brood stock which showed histological but no gross lesions of PKD. Seagrave et al. (1981) found grayling and pike of 1+ age infected. Wootten and McVicar (1982) examined several wild brown trout of 1+ or more with clinical infections and suggested that these fish had not been exposed to infection the previous year. Ferguson and Ball (1979) reported that rainbow trout put out in river water in May and surviving a clinical outbreak of PKD as 0+ fish showed no gross post-mortem lesions the following year as 1+ fish. No histological findings were recorded. However, they found that 10% of a batch of similar fish, not involved in the clinical disease as 0+ fish, did develop clinical disease with gross pathology and mortalities as 1+ fish. 90% of this batch had been put out into river water in July and 10% in October. Of the fish put out in July, 50% of those sampled as 0+

fish had histological lesions of PKD despite showing no clinical or gross pathological signs. They suggested that these fish may have developed a degree of resistance as 0+ fish and were those that did not succumb to disease the following year. They suggested that the fish in this batch that did become ill were the 10% that had been put out in river water the previous October and had possibly not been exposed to infection that year. They also exposed 60 1+ fish, which had been hatched and reared on PKD-free water, at the infected farm in May and sampled these fish every 14 days to follow the development of PKD. Although none of these fish died from PKD, by August 100% were infected, and 60% showed gross pathological changes. They concluded that development of resistance to PKD was related to previous exposure to the disease and not to the age of the fish at the time of exposure. It is interesting to note that Plehn (1924) and Hoffmann and Dangschat (1981) describe outbreaks of PKD in Germany as affecting 1+ rainbow trout rather than fingerlings.

Apart from the experimental and epidemiological findings of Ferguson and Ball (1979), there is little evidence of an immune response in rainbow trout following infection. Scott (1984) described an elevation of the F_4 fraction of serum proteins in fish which had recovered from the previous year's PKD infection. He considered that this could represent a defence response associated with resistance, although the protein involved was probably not an immunoglobulin. Ellis *et al.* (1982) considered that some Atlantic salmon stocks were possibly more susceptible to PKD than others. They found that in two outbreaks of PKD, parr from stock of Norwegian origin showed greater susceptibility to PKD than parr from Scottish stocks.

6. AETIOLOGY

As discussed previously, PKD was first named as such in 1974 by Roberts and Shepherd. Prior to this, descriptions of kidney disease often encompassed several conditions under a single name. It is, therefore, difficult to trace the aetiology of PKD through the early literature. Several authors have ascribed a single cause to a variety of conditions. For example, Besse (1956) considered "L'anémie pernicieuse des truites" had a nutritional basis; de Kinkelin and Gérard (1977a) considered "L'hépatonéphrite des Salmonidés" was possibly equivalent to "lipoid degeneration of the liver" described by Davis (1953) and caused by a dietary imbalance. As noted earlier, Schäperclaus (1954) ascribed a viral origin to a condition similar to PKD named "Infektiöse Nierenschwellung und Leberdegeneration". However, other attempts to isolate a virus from PKD-affected tissue in rainbow trout gonad (RTG) 2 cells failed (Ferguson and Adair, 1977; Ferguson and Needham, 1978), and only cytopathic effects similar to those caused by IPN virus were found by Ghittino *et al*. (1977). Bacterial culture also failed. Inoculation of Earp and Ordal's medium, Dorset egg medium and Loeffler's serum medium with infected kidney, liver and spleen produced no growth (Ferguson and Needham, 1978) and the only isolate obtained by Ghittino *et al*. (1977) was *Vibrio anguillarum*.

Several authors have described protozoa associated with kidney conditions in fish. Plehn (1924) considered an amoeba, perhaps first described by Hofer (1906), to be the causative agent of a condition similar to PKD. Ghittino *et al*. (1977) considered that she was describing PKD and agreed that an amoeba was involved. However, from the details of her description, Bykhovskaya-Pavlovskaya, Gusev, Dublina, Izyumova, Smirnova, Sokolovskaya, Shtein, Shul'man and Epshtein (1962)

could not assess the genus or family of amoeba that she had found.

Ghittino et al. (1977) isolated an amoeba, Vexillefera bacillipedes, from PKD-infected fish. Ferguson, Campbell , Ghittino and Andruetto (1978) described the isolate in detail. However, the organism failed to reproduce the disease and was subsequently shown to be a contaminant (Ghittino et al., 1980).

Ferguson and Adair (1977), from histological and ultra-structural studies of PKD-affected tissues, concluded that certain cells, seen in areas of renal cellular proliferation, were parasitic. These cells were often multi-nucleated, possessed small pseudopodia, an easily recognisable plasmalemma, and many small electron-dense bodies closely associated with the cell membrane. The pseudopodia suggested an amoebic or Myxosporidan classification. However, the inability to culture the organism and the failure to find spores precluded a precise taxonomic classification. Ferguson and Needham (1978) made further detailed descriptions of the parasitic cells without being able to classify them. They did not exclude the possibility that the electron-dense inclusion bodies were either viral particles or spores. However, Seagrave, Bucke and Alderman (1980a,b) conducted ultra-structural studies of the parasitic cells, which they termed "PKX cells", and established certain similarities between these cells and organisms of the protozoan species Marteilia refringens, a pathogen found in the European flat oyster, Ostrea edulis (described in detail by Grizel, Comps, Bonami, Cousserans, Duthoit and Le Pennec, 1974, and Perkins, 1976).

Stages in the internal division of the PKX cell resembled those seen in the sporulation sequence of M. refringens up to the development of spore primordia. Electron-dense bodies in the PKX cell were similar to haplosporosomes in the cytoplasm of the primary cells of Marteilia.

Furthermore, as in Marteilia, there were multivesicular bodies in the cell cytoplasm, and both Marteilia and the PKX cell had striated inclusions. The primary cell wall was structurally similar to that found in Marteilia species and also in the genera Minchinia and Urosporidium. Because of these similarities, Seagrave et al. (1980b) considered that the PKX cell might be a member of the Haplosporidia, now reclassified in the order Occlusosporida, of the phylum Ascetospora (Sprague, 1982). However, it should be noted that other members of the Haplosporidia are marine rather than freshwater protozoa. Also, structures similar to haplosporosomes have now been described in the phylum Myxozoa, in a species of Henneguya (Current, 1979). This may have taxonomic importance for members of the Occlusosporida. It is interesting in this context that a dropsical condition was described by Davis (1953) in fingerling salmon that was similar to PKD where the kidneys were infected with Myxosporidan protozoa. The aetiological significance of these organisms was not established. It is also interesting that cells similar to myxosporidan trophozoites, some containing what are believed to be developing spores, have now been described within renal tubules in tissue from steelhead trout, Salmo gairdneri Richardson, recovering from PKD (Kent and Hedrick, 1985a,b,c), suggesting a further link between PKX cells and the phylum, Myxozoa. This phylum, unlike the Ascetospora, contains many freshwater fish pathogens including Myxosoma cerebralis. However, although Myxosporidans have been recognised within renal tubules and between tubular epithelial cells (Plehn, 1924) as well as in the bladder (Davis, 1953), when involving the kidney, they generally cause damage not by tissue destruction but by mechanical obstruction.

7. EXPERIMENTAL TRANSMISSION

Several attempts have been made to transmit PKD. It was shown by Ferguson and Ball (1979) that rainbow trout, held at a river-fed site where PKD was known to occur, became infected and developed PKD if kept there during May for four weeks before being moved to laboratory holding facilities and held in recirculating dechlorinated tap water at ambient temperature (15-19°C). Fish from the same stock moved directly from the hatchery, supplied with spring water at 9-11°C, and held under similar laboratory conditions did not develop PKD. Also, fry kept for 10 weeks in water circulating through the tank containing the infected fish failed to develop clinical or histological signs of PKD. Infected river water passed through precision mesh with a pore size of 60 µm was shown to contain the infective agent of PKD (Alderman and Rodgers, 1984).

A series of transmission experiments was undertaken by D'Silva, Mulcahy and de Kinkelin (1984) in France and Ireland. 1+ rainbow trout held in cages within concrete ponds containing PKD-affected fish developed PKD whether the cages rested on or were suspended above the bottom of the pond. They were infected with PKX cells in the fourth week and signs of proliferation in the kidney were found from the fifth week onwards. The water temperature in these ponds ranged from 17-22°C. In the laboratory, a similar experiment to that performed by Ferguson and Ball (1979) was undertaken where the water at 14-17°C from an aquarium containing formalin-treated, PKD-infected fish flowed into another aquarium containing uninfected fish. During the experimental period of eight weeks none of the fish in the second tank showed signs of infection. 0+ rainbow trout held in aquaria failed to develop infection whether sediment from PKD-infected ponds was added for six weeks, or if cultured, infected kidney and homogenates of fresh infected kidney were added to the water, or if

sterilized and unsterilized faeces from PKD-infected fish were introduced to the aquaria over a 20 week period at water temperatures ranging between 9°C and 15°C. Laboratory transmission was successful by intraperitoneal injection of a homogenate of PKD-infected kidney material in phosphate-buffered saline (PBS). Test fish were held at 18°C ± 2°C for eight weeks. Of 11 out of 31 fish surviving injection, no fish sampled after two weeks showed signs of infection, but five of 11 fish sampled at four weeks were PKX-positive and contained kidneys with proliferative changes. From these experiments, it was concluded by D'Silva et al. (1984) that contact with pond sediment was not necessary for PKD transmission and that it was "difficult to see" the involvement of an intermediate host in PKD transmission because of the time course of the disease under laboratory and field conditions.

8. CLINICAL SIGNS

The most detailed descriptions of the clinical and post-mortem appearance of PKD in rainbow trout were by Plehn (1924), Schäperclaus (1954), Ghittino et al. (1977), and Ferguson and Needham (1978). Clinical signs were considered to be non-specific and to result from circulatory imbalance and anaemia, both secondary to renal malfunction (Ghittino et al., 1977).

Affected fish often showed abnormal swimming behaviour (de Kinkelin and Gérard, 1977a,b; Ghittino et al., 1977), remaining at the water surface and tending to collect at tank outlets. They also showed signs of oxygen starvation, gasping spasmodically and unable to withstand stressful procedures such as grading and transportation (Plehn, 1924; Ghittino et al., 1977; Ferguson and Needham, 1978).

Abdominal distension was a frequent presenting sign (Plehn, 1924;

Schäperclaus, 1954; Roberts and Shepherd, 1974; Ghittino et al., 1977). Longitudinal swellings were sometimes visible at the level of the lateral line (Ferguson and Needham, 1978). Some fish exhibited melanosis (Schäperclaus, 1954; Roberts and Shepherd, 1974; de Kinkelin and Gérard, 1977a,b; Ferguson and Needham, 1978), although Plehn (1924) found affected fish lighter in colour. Exophthalmos sometimes occurred (Schäperclaus, 1954; Ghittino et al., 1977) with the eyes having a particularly dark appearance according to Plehn (1924). It was considered an infrequent finding by de Kinkelin and Gérard (1977a,b), and reported as monolateral by Ferguson and Needham (1978). Cloudiness of cornea and lens was described by Plehn (1924).

Anaemia was a more constant finding, noted clinically as gill pallor (Plehn, 1924; de Kinkelin and Gérard, 1977b; Ghittino et al., 1977; Ferguson and Needham, 1978). Affected fish stopped feeding (Plehn, 1924; Ghittino et al., 1977), although it was found by Ferguson and Needham (1978) that appetite was not reduced until fish were in extremis.

9. POST-MORTEM FINDINGS

Gross pathology

The abdominal distension in severely affected fish resulted from the enlargement of one or more internal organs, combined sometimes with the collection of ascitic fluid and oedema (Plehn, 1924; Schäperclaus, 1954; de Kinkelin and Gérard, 1977b; Ghittino et al., 1977), which could be stained with blood (Ferguson and Needham, 1978).

The kidney was most obviously enlarged, especially the mesonephros, although its whole length could be involved in severe cases (Plehn, 1924; Ferguson and Needham, 1978). This enlargement sometimes displaced the swim bladder laterally and caused its distortion, accounting for the

longitudinal swelling of the abdominal wall at the level of the lateral line (Ferguson and Needham, 1978). The kidney was lighter in colour than normal, being grey, and ridged (Ghittino et al., 1977; Ferguson and Needham, 1978). The consistency of the tissue was considered firmer than normal, with the cut surface having a marbled appearance (Plehn, 1924; Heuschmann-Brunner, 1966). Pale, raspberry-coloured nodules protruding from the surface and edges of the kidney were described by Ghittino et al. (1977). Similar, discrete nodules were described in the peritoneum, musculature and liver with, rarely, a massive localized enlargement occurring which was greater than the size of the liver. Plehn (1924) found no pathological changes in the other internal organs. However, de Kinkelin and Gérard (1977b), Ghittino et al. (1977) and Hoffmann et al. (1984) found spleens occasionally enlarged with rounded edges, while Ferguson and Needham (1978) found the majority of spleens smaller than normal. The liver and spleen were reported as occasionally grey and mottled in appearance like the kidney with the rest of the body and internal organs generally pale. Localized haemorrhages, confined to the internal organs, were described by de Kinkelin and Gérard (1977a). The intestines were reported as being empty by Plehn (1924), but as usually containing food by Ferguson and Needham (1978).

Histopathology

The principle description of the histopathology of PKD in rainbow trout was by Ferguson and Needham (1978). Less detailed accounts were given by Plehn (1924), Ferguson and Adair (1977), de Kinkelin and Gérard (1977a,b), Ghittino et al. (1977), Roberts (1978) and Smith et al. (1984). Findings in brown trout and Atlantic salmon were described by Ellis et al. (1985). These accounts describe the changes in grossly affected organs

during the clinical period of the disease.

In rainbow trout, renal pathology was related to a proliferation of haemopoietic and lymphoid cells, especially cells resembling macrophages and lymphocytes, both in the pro- and meso-nephros. There was little collagen initially within this reaction, but with time it was found diffusely throughout affected areas. Within these areas of interstitial inflammation, described by de Kinkelin and Gérard (1977b) as a chronic granulomatous inflammation, melanin was decreased. The excretory elements of the kidney appeared to be fewer in number and displaced by the increased interstitial tissue, such that, in severely affected kidneys, the tubules and glomeruli were found in small islands amongst the abnormal tissue. Glomeruli in these kidneys showed degrees of sclerosis and calcium deposition (Ferguson and Needham, 1978). Necrosis of tubules was described by Schäperclaus (1954), and distension of tubules and glomeruli by Hoffmann and Lommel (1984). The cellular reaction was often arranged into "whorls", and centrally placed within these were one or more PKX cells. PKX cells were only seen in the area of the mononuclear cell reaction according to Ferguson and Adair (1977). However, PKX cells were found within tubule lumina by Hedrick et al. (1984) and between tubule epithelial cells according to Ferguson and Needham (1978). Intraluminal myxosporidan trophozoites and sporoblasts were described by Kent and Hedrick (1985a,b,c) in PKD-affected fish. The sporoblasts contained two polar bodies and refractile granules.

An inflammatory reaction similar to that in the kidney was described in the spleen, liver, Islets of Langerhans and occasionally in the muscles dorsal to the kidney. Spleens contained collagen deposits in severe cases. PKX cells were found close to splenic ellipsoid sheaths, and it was suggested that reticular cells were hyperplastic in these areas

(Ferguson and Needham, 1978). Iron deposits were found in splenic macrophages by Hoffmann and Lommel (1984).

The ectopic nodules in liver, peritoneum and muscle described by Ghittino et al. (1977) consisted of bundles of connective tissue arranged into whorls within a loose connective tissue matrix similar to the more advanced renal lesions recorded by Ferguson and Needham (1978).

Vascular lesions were described by Smith et al. (1984) with PKX cells attached to vessel walls causing a necrotizing vasculitis with occlusion of renal and hepatic vessels. PKX cells were also found in the capillaries of the submucosa and lamina propria of the pyloric caeca and in other areas of the intestine, in capillaries of secondary lamellae of the gills, with apparent epithelial hypoplasia in some cases (Ferguson and Needham, 1978), and in the spinal canal (Smith et al., 1984).

The PKX cells stained orange with haematoxylin and eosin (H & E) and were periodic acid Schiff (PAS) positive. Their size was variously described as 5 to 30 μm in diameter and their shape usually rounded, although ovoid examples were described by Smith et al. (1984). Often they were surrounded by a clear area, producing a halo effect. PKX cell nuclei had an obvious nucleolus and chromatin in radiating strands attached to a prominent nuclear membrane. Their cytoplasm could be divided into a darkly staining endoplasmic layer and a lighter ectoplasmic layer. Within the cytoplasm round inclusions, described as "inclusion bodies" by Ferguson and Needham (1978), were found, with a diameter of approximately 4 μm and numbering up to six.

A similar inflammatory reaction to that in rainbow trout was described in both Atlantic salmon and brown trout by Ellis et al. (1985). In four of five salmon parr in one group, possibly suffering from a fungal disease as well as PKD, multinucleated giant cells were found in the

proliferative granulomatous reaction. In brown trout, in addition to the inflammatory reaction cells, considered to be activated reticulo-endothelial cells, were described in the renal interstitial tissue. Intravenous hepatic granulomata were seen in two of six brown trout, associated with liquefactive necrosis of hepatocytes.

Ultra-structural studies

Two studies briefly mention the host tissue response to the organism (Ferguson and Adair, 1977; Ferguson and Needham, 1978). In inflammatory lesions the main cell types were described by Ferguson and Adair (1977) as macrophages and transforming lymphocytes, with similarities to plasma cells. Where lesions contained little connective tissue, these cell types and lymphocytes, as well as some neutrophils and thrombocytes, were found by Ferguson and Needham (1978). No details of early tissue response or recovery were described.

Detailed ultra-structural studies of the PKX cell were undertaken by Ferguson and Needham (1978), Seagrave *et al*. (1980a,b), and Smith *et al*. (1984). They related mainly to the PKX cell as seen in well developed lesions, although one reference was made to organisms in recovering fish (Seagrave *et al*., 1980a). No differentiation was made between immature and mature forms.

The PKX cells were distinguished from host cells by the presence of intra-cytoplasmic inclusion cells and a granular cytoplasm. This granularity was caused mainly by the presence of electron-dense bodies, termed "haplosporosomes" by Seagrave *et al*. (1980a) by analogy with similar structures in members of the Haplosporidia. The PKX cells were often seen with host cells "attached" by Seagrave *et al*. (1980a). They were always found apparently within macrophages by Smith *et al*. (1982),

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but were encountered both within macrophages and extracellularly by Hedrick et al. (1984). The cells were described as having an amorphous cell wall 40-70 nm thick (Seagrave et al., 1980a), a plicated trilamellar delimiting plasma membrane and small pseudopodia (Ferguson and Adair, 1977; Ferguson and Needham, 1978). The cytoplasm contained, in addition to inclusion cells and haplosporosomes, mitochondria, rough endoplasmic reticulum (RER), and phagocytic vacuoles (Ferguson and Needham, 1978). It also contained occasional multilaminate structures and vacuoles containing membrane-bound material, these occurring especially in organisms in fish recovering from PKD (Seagrave et al., 1980a). Lipid droplets, lysosomes, vesicular bodies (the possible sites of haplosporosome production), and structures similar to Golgi apparatus (possibly packaging haplosporosomes), were also described within the cytoplasm of the PKX cell by Smith et al. (1984). Some parasites possessed a layer of ectoplasm free from structures except haplosporosomes (Ferguson and Needham, 1978; Smith et al., 1984).

The numerous haplosporosomes ranged in diameter between 0.1-0.2 μm. They were found throughout the cytoplasm of the PKX cell,sometimes concentrated at one end or beneath the plasma membrane. They were not found in the inclusion cells. The electron-dense material of the haplosporosome was surrounded by a membrane and contained a cleft, described as an electron-lucent bar by Ferguson and Needham (1978), which extended up to two-thirds across its width. Some haplosporosomes appeared to have an extension in the form of a tail (Ferguson and Needham, 1978).

The inclusion cells, designated secondary cells by analogy with the sporulation sequence in Marteilia refringens (Seagrave et al., 1980b), were more electron-dense than the primary cells, but contained similar structures except for the haplosporosomes and their production and pack-

aging sites. Some secondary cells contained further, more electron-dense cells with their own delimiting membrane, nucleus, mitochondria and RER.

10. HAEMATOLOGY

Anaemia, clinically noted as gill pallor, has been described as a presenting sign in PKD (Plehn, 1924; Ghittino *et al.*, 1977; de Kinkelin and Gérard, 1977b; Ferguson and Needham, 1978; Roberts, 1978; Smith *et al.*, 1984). Ferguson and Needham (1978) recorded haematocrit values and total erythrocyte counts from an unspecified number of rainbow trout and found all fish "very anaemic" with an average haematocrit value of 0.112 and an average erythrocyte count of 0.38×10^{12} cells/l. They compared these figures with those of Barnhart (1969), who studied various blood parameters under standardized conditions in four groups of 70 rainbow trout. He considered that the range of mean values of 0.280-0.308 for the haematocrit and $1.03\text{-}1.143 \times 10^{12}$ cells/l for total blood cell counts were within the normal ranges for these parameters. Although other haematological values were not recorded, Ferguson and Needham (1978) stated that blood from affected fish contained many immature and small cells.

Ghittino *et al.* (1977) compared two blood smears, one from a healthy rainbow trout, the other from a fish with clinical PKD. Although they did not perform total red or white cell counts, they did compare the number of white blood cells seen while counting 10,000 erythrocytes. These numbered 198 in the "normal" slide, and 491 in the "pathological" slide. They also observed anisochromasia and anisocytosis of mature erythrocytes and increased numbers of neutrophils, and large basophilic and monocytic cells in the "pathological" slide. They concluded that in PKD-affected fish the leucocyte count had increased by 250% above the

normal value, that the anisocytosis and anisochromasia of erythrocytes indicated anaemia and that the increase in leucocytes, especially of neutrophils, indicated a leucocytosis due to inflammation. However, without total cell counts, these conclusions could not be substantiated.

Hoffmann and Lommel (1984), in a more detailed report, studied haematological parameters in fish with clinical signs of PKD and compared the values obtained with those, which they considered to be normal, from fish held and sampled under similar conditions. Histological changes in diseased kidneys and spleens were apparently typical of those previously described in the active, inflammatory stage of PKD, although renal tubules and glomeruli in the posterior kidney were reported as distended and iron deposits were found in most splenic macrophages. In 24 affected fish they found anaemia, with decreased haematocrit (0.28 ± 0.13 compared with a normal value of 0.40 ± 0.07), haemoglobin (3.9 ± 2.1 g/100 ml compared with a normal value of 9.4 ± 1.0 g/100 ml) and erythrocyte count values (1.0 ± 0.4 x 10^{12} cells/l compared with a normal value of 1.6 ± 0.4 x 10^{12} cells/l). They found increased numbers of immature erythrocytes, decreased mean corpuscular haemoglobin concentration (MCHC), and poikilocytosis, hypochromasia and polychromasia of mature erythrocytes. The nuclear area of erythrocytes from affected fish (17.65 ± 4.37 μm^2) was significantly smaller than that of cells from unaffected fish (18.78 ± 3.44 μm^2), while cytoplasmic areas were similar. Unlike Ghittino et al. (1977), Hoffmann and Lommel (1984) found only insignificant changes in leucocyte numbers and composition in affected fish. In 12 affected fish, blood urea levels were higher than in unaffected fish (12.3 ± 5.5 mg % compared with 6.4 ± 1.2 mg %), while total serum protein concentration was lower (3.16 ± 1.43 g % compared with 4.795 ± 0.95 g %). This last finding disagrees with that of Scott (1984), who recorded total serum protein

levels in 37 normal fish as having a mean value of 5.83 g %, whereas that in 71 affected fish was 6.13 g %. These fish were from various farms and held under varying conditions. Scott (1984) also recorded the mean value in 12 recovered fish (tested in December) as 7.5 g %, and in eight fish re-challenged with PKD infection in their second summer (tested in June) as 6.8 g %.

Neither Ghittino et al. (1977) nor Ferguson and Needham (1978) categorised the type of anaemia in PKD. Roberts (1978) considered it to be one of hypoplasia, although he did not offer evidence to support this. However, Hoffmann and Lommel (1984) concluded that the anaemia was not one of aplasia, but one of chronic haemolysis.

11. TREATMENT AND CONTROL

As mentioned previously, most forms of chemotherapy have so far been unsuccessful in treating PKD, despite extensive field and laboratory trials of antibacterial, anti-inflammatory and antiprotozoal compounds (Ghittino et al. 1977; Ferguson and Ball, 1979; Bucke, McGregor, Hudson and Scott, 1981). However, some evidence suggests that salination at 8-12 ppt was successful in relieving the symptoms of PKD in 0+ Atlantic salmon (O'Hara, 1985).

Once an outbreak of PKD has occurred, control measures have been directed towards minimizing the mortalities. It has been suggested that stressful procedures should be avoided as far as possible (Ferguson and Ball, 1979) and handling, for example at grading and transporting, should be kept to a minimum, since affected fish are debilitated and subject to respiratory distress secondary to anaemia (Seagrave and Bucke, 1979). It has been suggested that chemotherapy of secondary infections may itself cause additional mortalities (Dalton, 1980). Furthermore, it was considered

that feeding ad lib increased mortalities in PKD-affected fish (Ferguson and Ball, 1979). Therefore, by feeding a maintenance diet, losses were reduced, although morbidity was not changed.

It was found by Ferguson and Ball (1979) that, by delaying the transfer of rainbow trout fingerlings to river-fed, on-growing sites until July, substantial reductions in mortalities caused by PKD resulted, although morbidity was unchanged. This reduction was considered by Ferguson (1981) to be a water temperature effect, with decreasing temperatures in late summer and autumn inhibiting full development of the disease process. Unfortunately, the same control measure was ineffective on farms in southern England (Bucke et al., 1981).

Other general measures for minimizing losses have been advocated such as careful hygiene precautions at the hatchery and rearing sites; careful handling of fish; no overstocking; avoidance of other debilitating infections; attention to quality of the water supply; and maintenance of a generally high standard of husbandry (Ghittino et al., 1977; Seagrave et al., 1981).

12. ECONOMIC IMPORTANCE OF PKD

The economic importance of PKD in England and Wales has been assessed from data collected between 1982 and 1985 from published information, records at FDL Weymouth and the Institute of Aquaculture, University of Stirling, and figures supplied by individual farmers. The figures relate to the situation at the beginning of 1986.

Seagrave et al. (1981) reported that 20 out of 45 sites tested in England and Wales were positive for PKD by histological examination of renal specimens. Since this survey PKD has been diagnosed at a further 25 farms in England. Field evidence suggests that a further 20 farms are

infected, but this has not been confirmed by histological examination. Of the 65 infected sites, 24 have been shown to be infected in two or more years, implying that the disease is well established on the rivers supplying these farms. On sites where the disease has been recorded only once, it is possible that tested fish were derived from stock infected prior to movement. The distribution of infected farms is shown in Fig.1.1.

There are at least a further 77 farms situated on rivers and their tributaries supplying positive sites, and these may also be infected. It appears, therefore, that at least 142 salmonid-producing farms in England and Wales are directly at risk from losses due to PKD, out of an approximate total of 400 farms which are supplied with river water. Table 1.1 summarizes the total numbers of salmonid farms in each Water Authority in England and Wales supplied with river water, the number of positive sites in each area, the number of untested salmonid farms on rivers supplying positive sites and the approximate production of fish in tonnes per annum at the positive sites.

Annual production at histologically-confirmed positive sites is about 3,240 tonnes per annum, equivalent to more then 30% of total trout production in England and Wales. If mortalities at these farms average 5-10% of production, this represents an annual loss of 160-320 tonnes, equivalent to £240,000-480,000 at existing wholesale prices. If the food conversion ratio deteriorates from an average 1.5:1 to 2.0:1 on 3,240 tonnes of fish produced, this represents a further loss of about £640,000 per annum, assuming an average cost of £400/tonne of fish food.

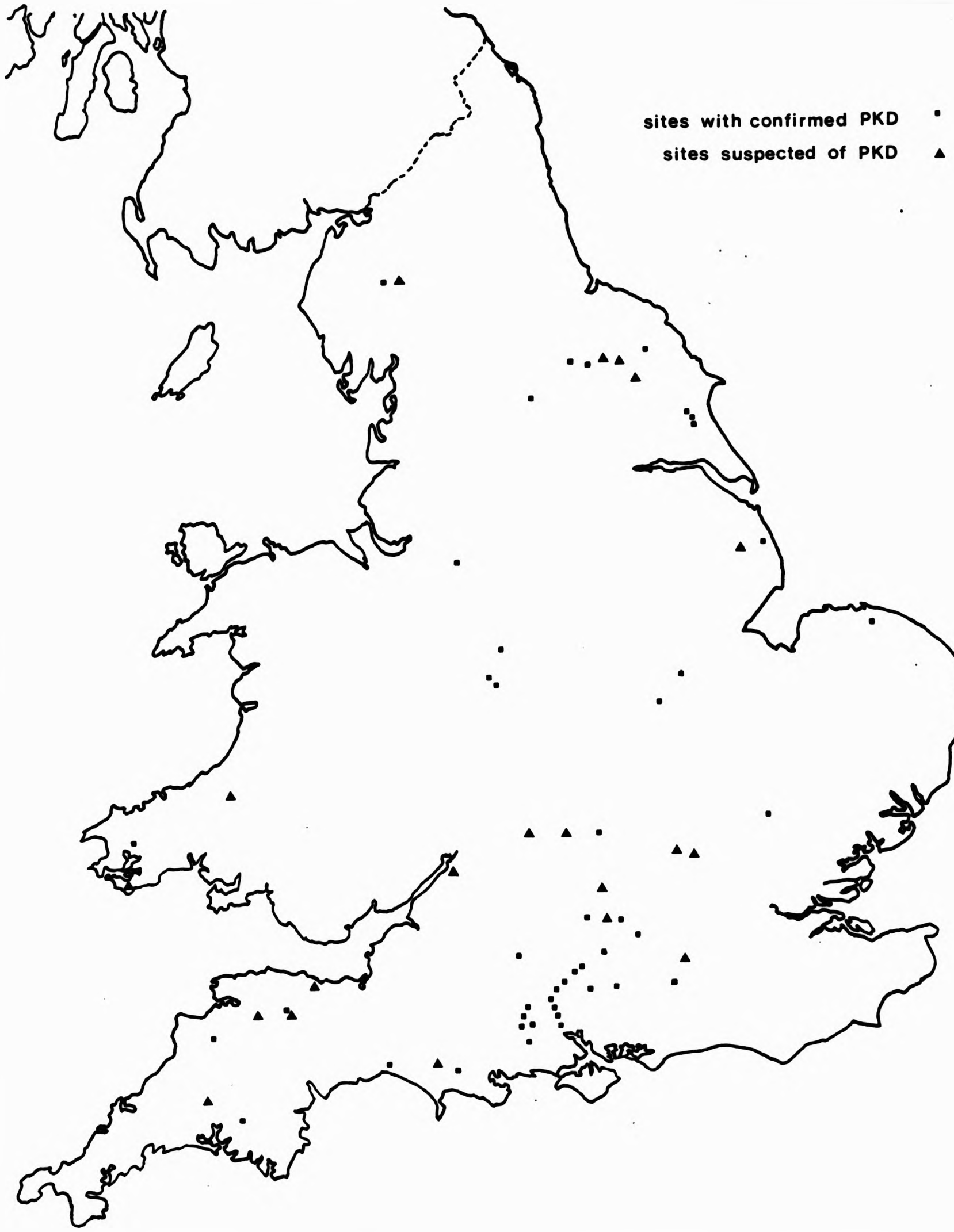
As these figures only account for average losses on histologically-positive farms, it would appear that a conservative figure for annual losses caused by PKD in England and Wales is £1 million.

Table 1.1. Distribution and production of PKD-positive farms in England and Wales.

Water Authority Area	*Salmonid farms supplied by river water	Number of PKD-positive farms (1) Confirmed histologically +ve	(2) Suspected +ve	*Number of untested farms on rivers supplying PKD-positive farms	*Production in tonnes per annum of PKD-positive farms (1) Confirmed histologically +ve	(2) Suspected +ve
Anglian	58	4	1	6	75	100
Northumbrian	4	0	0	0	0	0
North-West	25	2	1	1	25	75
Severn-Trent	40	3	0	1	60	0
Southern	60	11	0	11	990	0
South-West	35	3	3	6	85	90
Thames	40	6	7	20	420	165
Welsh	35	1	2	0	30	Not known
Wessex	55	8	3	12	1065	30
Yorkshire	40	7	3	20	490	70
Totals	392	45	20	77	3240	530

* Figures approximate

Fig.1.1. Distribution of PKD in England and Wales : sites with confirmed PKD or suspected of PKD.



Chapter 2

General Materials and Methods

SECTION 1. THE EXPERIMENTAL FIELD SITE

(a) Situation and water supply

All experimental field studies were conducted at a site situated on the River Avon in Wiltshire, approximately 4 miles south east of Salisbury and 1.5 miles north of Downton, grid reference SU181236 (Fig. 2.1). The River Avon has a reputation for being one of the best game and coarse fishing rivers in the country, although runs of Atlantic salmon have been variable over the past few years. In its course from the headwaters north east of Pewsey to Downton the Avon supplies water to several fish farms, one approximately 4 miles from the spring source, another approximately 0.5 miles north of Downton. Both farms rear rainbow trout, either for the table or for selling on, and both suffer annual losses from PKD. The River Ebble, joining the Avon approximately 2 miles north of the experimental site, supplies a fish farm where PKD occurs but seldom causes mortalities.

The River Avon at the level of the experimental site is eutrophic, with a rich invertebrate fauna, including various species of molluscs, oligochaetes, the amphipod crustacean, Gammarus pulex, swimming nymphs including genera of Baetis and Ephemerella, Hemiptera (water bugs), and various stages of Diptera, especially of the midges (Chironomidae) and the black-flies (Simulidae), The water is alkaline and has a varying dissolved oxygen content, with low values especially during the summer months (Fig. 2.2). Various water quality parameters were monitored at the level of the experimental site by the Wessex Water Authority over a two-year period, and the measurements are summarized in Table 2.1.

Maximum and minimum diurnal water temperatures at the site

during 1983 were recorded on a Foster Cambridge seven-day thermograph. Seven day rolling average maximum and minimum temperatures are recorded in Fig. 2.3.

The experimental site was originally designed for research into salmon hatchery procedures and salmon rearing. However, since 1981 the facilities have been used by the Ministry of Agriculture, Fisheries and Food, Fish Diseases Laboratory (FDL), Weymouth, for a range of field experiments. PKD has occurred on the site annually since 1981.

(b) Holding facilities

The site consisted originally of 18 circular concrete tanks, six having an internal diameter of approximately 6 m, and 12 an internal diameter of 2 m. The smaller tanks were designed for rearing salmon fry, the larger for holding older salmon. Apart from these tanks, an additional row of circular polythene tanks, capacity 115 l or 230 l, was plumbed into the water supply to the site during 1982 and 1983. During the field studies in the present dissertation only the 6 m concrete tanks (Fig. 2.4) were used unless otherwise stated. These tanks, with an edge depth of 0.92 m and centre depth of 1.06 m, had an adjustable valve at the side of each tank controlling water inflow directly from the River Avon. A central drain discharging into the Avon was covered by an upright, circular, aluminium monk. Depth of water was controlled by inflow rate and hole size of the aluminium mesh of the monk (in this case the holes were 3 mm diameter). The water level was generally maintained at an edge depth of about 0.35 m, although this increased if the river water level rose or the holes of the monk were obstructed with debris. Adequate flow rates could be maintained throughout the year by altering water flow through weirs above and below the water inlet to the

site.

Experimental fish were either held without restriction in the 6 m tanks or, if the numbers and size of fish permitted, they were kept in single or double 1 m^3 mesh cages placed on the bottom of the 6 m tank. These cages were constructed from a framework of 12 mm internal diameter Durapipe (Durapipe Ltd., Norton Canes, Staffordshire) initially covered with 6 mm mesh size Netlon (Aquatic Services [International] Ltd., East Boldre, Hampshire). However, this covering often became clogged with debris, reducing water flow, and was difficult to clean. Therefore, 1 m^3 net cages were made by a local manufacturer from number 2 knotless netting, which was subsequently treated with Ren Not (Monopol, Bergen, Norway) antifouling paint and then attached with nylon ties to the Durapipe frames. These nets remained relatively free from fouling and were easily cleaned.

(c) Management of experimental fish

Tanks and cages were cleaned weekly during the summer, and as necessary at other times.

The "Omega" range of trout and salmon foods, manufactured by Edward Baker Ltd (subsequently Ewos-Baker), Sudbury, Suffolk, was fed throughout the period of research both at the experimental site and at the laboratory. The size of pellet fed was determined by the manufacturer's recommendations (Table 2.2). Pellet composition is detailed in Table 2.2, although water-soluble vitamin values were not available.

Fish, free in the 6 m tank, were fed from a 100 l capacity automatic feeder (Tess Aquaculture Ltd., Newport, Shropshire), set to distribute food every 40 minutes for five seconds during the hours of 05.30 to 11.00 and 15.00 to 21.00. Rate of feeding was adjusted throughout the year to

supply approximately 4% of total body weight of the fish each day. However, this rate was reduced throughout July and August to approximately 1% of total body weight daily when PKD pathology was most marked. Fish confined within the 1 m^3 net cages were fed by hand to appetite twice daily, although the amount fed was reduced if mortalities due to PKD were occurring.

SECTION 2. THE LABORATORY TANK ROOMS AND HOLDING FACILITIES

(a) General description

The fish-holding facilities used during the period of laboratory experimentation comprised two main areas, namely, an open holding area for disease-free fish, and a closed tank room for experimental purposes.

The open holding area contained seven 1000 l circular glass fibre tanks, each with a lid which was partially raised at feeding times. Fish from disease-free sources were sometimes held in these tanks prior to being taken to the experimental field site or prior to use at the laboratory. In this way stocks of fish, free from PKD, were readily available throughout the year for experimental purposes.

The closed tank room contained sixteen 55 l (small) rectangular tanks, six 385 l (medium) rectangular tanks, and four 1000 l (large) circular tanks (Fig. 2.5). The rectangular tanks had solid lids which did not admit light. The circular tanks had lids with a perspex square inset so that some light was admitted. Lids were only raised when feeding fish or when cleaning out the tanks.

(b) Management of experimental fish

Prior to use, experimental tanks were drained and scrubbed with a 10% solution of Chloros (Boots, Nottingham, Notts.). This was left in the

tanks for two hours and then hosed out before the water supply was resumed. Fish were not added to tanks for at least 12 hours after the water supply was restarted.

Different-sized nets were available for handling fish. These were used once then disinfected with a 10% solution of Chloros (Boots, Nottingham, Notts.).

Apart from fry, experimental fish were fed by hand once a day at a rate of 1-2% body weight/day with the appropriate grade of Omega trout food. Fry were fed to appetite 2-3 times/day.

Tanks were routinely cleaned once a week, but were cleaned more often if skin or gill infections were suspected.

A recurrent problem in rainbow trout brought from the field site to the laboratory and held in the experimental tanks was ichthyophthiriasis. When this was suspected, confirmatory skin scrapings were made and affected fish were subsequently treated either with salt water at 5-10 ppt for 7-10 days, or with a mixture of 10% formalin at 20 ppm and malachite green at 1 ppm. Treatment with formalin and malachite green was administered by reducing the water level in the affected tank by 30%, adding the required volume of formalin and malachite, and then increasing both the water flow and aeration until the dye had disappeared. Usually three treatments on alternate days were given. During the period of research, it became apparent that the formalin/malachite treatment was interfering with the development of clinical PKD and was, therefore, discontinued in favour of saline treatment.

(c) <u>The water supply</u>

Water supplying the holding and experimental tanks was dechlorinated tap water. Dechlorination was achieved by sucking water through activated

charcoal filters which needed replacing every 18-24 months. Dechlorinated water was then pumped into glass fibre storage tanks, each with a capacity of 135,000 l. The water, therefore, was not sterile. Analysis of the water after dechlorination is recorded in Table 2.3.

Between the storage and experimental tanks was a buffer tank, holding the water at ambient temperature. Three supplies led from this tank; one supply was at ambient temperature, one passed through an immersion heating unit capable of heating the water to +30°C, the remaining supply passed through a refrigeration unit which could chill water down to +2°C. The water passing through the immersion heater was sterilized with ultra-violet light prior to entering the experimental tank rooms.

All experimental tanks had a direct water supply at ambient temperature. The large tanks also had direct chilled and heated supplies, whereas the small and medium tanks were fed heated and chilled supplies via header tanks. The proportions of heated, ambient and chilled water entering a particular tank could be controlled by individual valves and so a required water temperature could be maintained to within ± 1°C within a possible range of +2°C to +30°C, although during the summer the minimum stable temperature was +9°C.

Having passed through the experimental tanks, the water was collected and sterilized by ozonization prior to being pumped into the main sewer.

Flow rates to the holding area tanks were adjusted to give approximately two changes of water every 24 h. Water level was controlled by side outlets. In the experimental tanks, water levels were controlled by internal stand pipes for the small tanks and side outlets for the medium and large tanks. Water levels were maintained at 66-75% of total capacity. Flow rates to the small tanks allowed four to five changes of water per day, for the medium tanks about three changes per day, and for

the large tanks about two changes a day.

SECTION 3. SOURCES OF EXPERIMENTAL FISH

(a) Rainbow trout

Rainbow trout were derived from three farms designated A, B and C.

1. FARM A

Farm A is situated at the head of the River Wey in Dorset (Fig. 2.1) (grid reference SY662851). It has been established for 10 years, deriving its water supply entirely from spring sources. Certain water quality parameters of these spring sources are summarized in Table 2.4. The water temperature remains stable at 8-10°C. The farm produces eggs from its own broodstock (derived originally from the U.S.A. and Denmark) each year for fry and fingerling production. These fish are sold to other farms throughout the United Kingdom for on-growing. The farm participated in a research project evaluating the feasibility of brood-stock testing in developing a supply of health accredited salmonid stock (Hudson and McGregor, 1984). Despite stringent regulation of stock importation and regular monitoring for notifiable diseases, the farm was found positive for bacterial kidney disease in 1983 necessitating complete eradication of existing stocks, and disinfection of the site. Restocking has since been completed. The site also has a history of nephrocalcinosis. However, PKD has not been found at the site.

2. FARM B

This small farm is situated at the head of Watergates stream, a tributary of the River Frome (Fig. 2.1) (grid reference SY740871). It was formerly a salmon hatchery but at the time of this investigation functioned as a fish holding facility for restocking fisheries. The

water supply is from pumped spring water. Rainbow trout from this site are derived from its own broodstock. No history of PKD is associated with the farm.

3. FARM C

Farm C is situated at the head of a tributary of the Upper Avon near Pewsey in Wiltshire (Fig, 2.1) (grid reference SU176616). It is a spring and borehole water fed site and, like farm A, participated in a broodstock testing research project (Hudson and McGregor, 1984). The farm produces rainbow trout eggs and fry from its own broodstock for supply to other farms. The site has been regularly monitored for diseases listed as "notifiable" under the Diseases of Fish Act, 1937, for several years and, like farm A, was found positive for BKD. Other diseases, including bacterial gill disease and nephrocalcinosis have been identified on several occasions. This farm has no history of PKD, although fry moved to the farm's nearby on-growing facilities on the River Avon do develop PKD. Fry were only obtained from the hatchery during the present project.

(b) Brown trout

Brown trout were derived from eggs, stripped from broodstock held at farm D, and hatched at a bore-hole water fed hatchery site, farm E, close to the experimental site near Downton (grid reference SU177242), Wiltshire (Fig. 2.1). This hatchery supplies both brown and rainbow trout fry to farms on the Rivers Ebble and Avon owned by the Longford Estate. The sites have no history of PKD.

(c) Atlantic salmon

Atlantic salmon parr, derived from eggs stripped from fish on the Isle

of Uist, Scotland, were obtained from a spring-fed farm, farm F, at the head of a tributary of the River Frome near Shaftesbury, Dorset (grid reference ST924214) (Fig. 2.1). The site has no history of PKD.

SECTION 4. TRANSPORTATION OF EXPERIMENTAL FISH

Experimental fish were transported either in 225 l capacity glass fibre tanks filled with 150 l of aerated water, or in double thickness plastic bags containing 30 l of aerated water. Up to 500 x 80 mm rainbow trout could be transported in the glass fibre tanks, and up to 100 in the plastic bags. Plastic bags were used once, then discarded. Glass fibre tanks were disinfected with a 10% solution of Chloros (Boots, Nottingham, Notts.) between journeys. Water was at ambient temperature unless there was a risk of it exceeding 20°C, in which case ice was added at intervals during a journey to maintain the temperature below 20°C.

SECTION 5. ANAESTHETIZING EXPERIMENTAL FISH

MS222 (Sandoz, Basle, Switzerland) at a concentration of 0.01% was used to sedate or anaesthetize experimental fish. Fish for blood sampling were anaesthetized individually immediately after netting, those for transmission experiments were usually anaesthetized in pairs. A satisfactory level of anaesthesia was normally reached after fish had been immersed in the anaesthetic for 45-60 seconds. Fish which were to be kept alive after the experimental procedure had been carried out were returned to the holding tanks with water flow and aeration increased until recovery was complete.

SECTION 6. COLLECTION AND PROCESSING OF TISSUE SAMPLES

(a) Post-mortem procedure

Individual fish were killed by transecting the spinal cord at the level of the first and second cervical vertebrae. Fish larger than 150 mm in length, unless anaesthetised, were stunned by one or two sharp blows to the top of the cranium prior to transection of the spinal cord.

Although the full dissection procedure is described below, infection was often assessed on gross appearance of internal organs and histological appearance of renal sections alone.

Dissection was performed using standard instruments. An incision was made in the midline 5-10 mm cranial to the cloaca and extended to the level of the pectoral fins, avoiding damage to the viscera. A skin flap was reflected cranially and dorsally to expose the abdominal organs (Fig. 2.6). Gross pathological changes were noted. The alimentary tract, liver and spleen were removed together by transecting the oesophagus at the cardiac end of the stomach and the rectum. The swim bladder was removed from the surface of the kidney capsule. The kidney was examined and anterior and posterior parts removed (Fig. 2.7). An operculum was excised to expose the gill rakers (Fig. 2.6) and part of one raker was removed. The heart was exposed and removed. The head was cut from the body and the skull bisected by a dorsoventral, longitudinal, midline incision, exposing and transecting the brain. Skin and muscle specimens were taken anterior to the cloaca.

Samples for histological examination were placed in 10% neutral buffered formalin (NBF), (Appendix 1a), for at least 24 h prior to processing. Tissues for ultra-structural examination were immersed in 5% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.2) at 4°C immediately after removal from the fish. They were held overnight at

4°C prior to further processing.

(b) Tissue processing for histological examination

Using standard procedures, tissue samples were trimmed to a maximum thickness of 4 mm and processed on a Tissue Tek II Tissue Processor (Miles Laboratories, Slough, Berks), such that samples were dehydrated through graded alcohols from 70-100%, cleared with chloroform, and impregnated with molten paraffin wax. Tissues were then embedded in wax. Sections 4-5 μm thick were cut on a rotary microtome (No.1512 Leitz [Instruments] Ltd., Luton, Beds.), floated out in a water bath at 50°C which incorporated 0.5% albumin for adhesion, and transferred to pre-cleaned glass slides which were subsequently dried in a hot air oven at 45°C for 12-24 h before staining.

(c) Preparation of glass slides

Standard glass slides (Select Micro Slides, Chance Propper Ltd., Smethwick) were used for both tissue sections and smears. These slides were pre-cleaned and washed. No further preparation, except labelling, was required prior to use for histological sections. Slides for smears were cleaned following the guidelines of Baker and Silverton (1978). They were soaked in tap water and then placed in a 5% solution of Decon 90 (Decon Laboratories Ltd., Hove) for approximately 4 h. The slides were thoroughly washed in tap water, followed by distilled water, to remove traces of detergent. They were then dried in a hot air oven at 45°C.

(d) Section staining for histological examination

Sections for routine histological examination were stained with

Gill's haematoxylin (Appendix 1b) and eosin. Gill's haematoxylin was used because of its storage qualities,its speed of nuclear staining and the controllable nature of the staining reaction (Gill, Frost and Miller, 1974). Acidophilic cellular components were stained with 1% aqueous eosin. This combination of stains resulted in demarcation of nuclear and cytoplasmic compartments, and produced a characteristic eosinophilic appearance in the PKX cells.

The staining schedule is detailed in Appendix 2.

(e) Tissue processing for ultra-structural examination

Processing followed the principles described by Meek (1976) and Bancroft and Stevens (1977). Samples were washed for 15 min in each of three changes of 0.1 M cacodylate buffer at 4°C, and then secondarily fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for one hour. After two further 15 min washes in buffer, tissues were dehydrated by passing through graded alcohols (30%, 50%, 70%, 90%, 100%, 100% anhydrous alcohol, 10 min each wash). After being washed in a 50/50 mixture of propylene oxide and absolute anhydrous alcohol for 10 min, then in 100% propylene oxide for 10 min, samples were infiltrated with epoxide resin (EMix, EMscope Laboratories Ltd., Ashford, Kent) of medium hardness, firstly in a 50/50 mixture of propylene oxide and resin for 30min at 37°C, then in 100% resin for one hour at 37°C. Samples were embedded in resin in polythene capsules and polymerized in a hot air oven for 48 h at 60°C. Resin blocks were rough trimmed with a razor blade. Final trimming, semi- and ultra-thin section cutting were performed on an ultramicrotome (OM U3, Reichert-Jung, Slough, Berks) fitted with a glass knife made on an LKB knife maker (BROMMA, Sweden). Semi-thin sections were picked up on glass slides, ultra-thin

sections were floated out onto uncoated copper grids.

(f) Staining of semi-thin and ultra-thin resin sections

Semi-thin sections of 1 μm thickness were stained with alkaline toluidine blue (Bancroft and Stevens, 1977).

Ultra-thin resin sections were double stained in alcoholic uranyl acetate and Fahmy's lead citrate (Lewis and Knight, 1977).

(g) Light and electron microscopy and photography

Slides were routinely viewed with either a Nikon Apophot microscope (Nikon, Nippon Kagaku KK, Japan) or a Reichert-Jung Polyvar wide field photomicroscope (Reichert-Jung, Slough, Berks). On the Polyvar microscope specimens could also be viewed with phase contrast and interference contrast illumination. Material for fluorescence examination was viewed with a Leitz Dialux 20 microscope fitted with a Ploemopak 2.4 fluorescence vertical illuminator.

Electron microscopy was performed using a JEOL-JEM 100 CX electron microscope. Electron micrographs were taken on Ilford technical film (Ilford, Essex).

Photomicrographs were taken on Ilford PAN F 50, 35 mm film. Colour photographs were taken on Kodak Ektachrome (daylight) 200 film (Eastman Kodak Co., Rochester, N.Y.).

SECTION 7. ASSESSMENT OF INFECTION WITH PKD

The presence of PKD and severity of infection were assessed by grading kidneys according to their gross appearance, and by the microscopical appearance of sections and impression smears.

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SECTION 7. ASSESSMENT OF INFECTION WITH PKD

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(a) Macroscopic appearance of the kidney

A kidney grading system was developed to relate the gross appearance of the kidney to clinical signs and histopathological changes of PKD. The system allowed a rapid, though subjective, assessment of severity of infection but was not suitable for determining early stages of infection.

The appearance of the kidney was arbitrarily divided into 6 grades, from grade 0 (kidney with no swelling) to grade 4 (kidney with greatest swelling). A sixth grade (grade 5) was applied to recovering kidneys where swelling was decreasing and lesions were circumscribed.

Grading was as follows:

GRADE 0 - Kidney showing no obvious lesions. The renal capsule was glistening and transparent. The shape of the kidney followed the concavity of the peritoneal cavity ventral to the vertebral column (Fig. 2.7). The renal tissue was a homogeneous dark red.

GRADE 1 - The kidney was slightly enlarged, especially at its posterior end, giving it a convex appearance. The capsule showed some corrugation. The renal tissue was still a dark, homogeneous red (Fig. 2.8).

GRADE 2 - The kidney was obviously enlarged along its length with marked convexity and corrugation of the kidney capsule, which also had crenellated edges. The kidney surface was often mottled red and grey (Fig. 2.9).

GRADE 3 - The kidney was enlarged along its length to approximately six times its normal volume. The ventral surface was markedly corrugated, with the kidney capsule having a characteristic blue sheen. This increase in kidney volume often displaced the swim bladder ventrally and laterally and contributed to the abdominal distension seen clinically. The surface was mottled with diffuse dark red tissue interspersed with pale grey patches and darker brown areas. Some of the grey patches appeared to

have a central dark red focus (Fig. 2.10).

GRADE 4 - Kidneys were more swollen than those with grade 3 changes and were friable. Occasionally the swelling, usually of the posterior part, ruptured the capsule (Fig. 2.11). Clear fluid ran from the cut surface of the kidney, and clotted, gelatinous fluid often adhered to the underside of the capsule. Fish with these changes usually had ascites and were anaemic.

GRADE 5 - This grade included kidneys with discrete grey or pale cream areas and those containing grey or white spherical nodules. These nodules were up to 10 mm in diameter and up to 20 in number. Some protruded at the kidney surface, others were enclosed within the kidney (Fig. 2.12). Renal tissue between the nodules was dark red. The degree of swelling in these kidneys ranged from no swelling to that seen in grade 2 kidneys.

(b) Histological examination of kidney sections

Histological examination of kidney sections prepared as described previously (Section 6) was the method of choice for diagnosing PKD, although the preparation of material was time consuming. Severity of infection was graded by histological changes as follows:

"Infected" - PKX cells present in renal tissue without other obvious histological or gross changes.

"Midly affected" - PKX cells present with evidence of early tissue proliferation. Grade 0 or grade 1 gross changes.

"Moderately affected" - PKX cells present with characteristic lesions (described in detail in Chapter 3) of haemopoietic hyperplasia, diffuse inflammation, vascular aggregations, crystal formation and fibrous whorling. Grade 2 gross changes.

"Severely affected" - Histological changes as for moderately affected fish but with grade 3 or 4 gross changes.

(c) Kidney impression smears

Impression smears were used to provide a rapid confirmation of PKD infection without recourse to preparation and examination of histological sections (Clifton-Hadley, Richards and Bucke, 1983). Although Giemsa, Leishman, Leishman-Giemsa, Methyl green-pyronine, Papanicolaou and Wright's methods of staining were tried, the standard May-Grünwald-Giemsa procedure for blood smears, detailed in Appendix 2, was found most satisfactory in differentiating PKX cells from host cells. The advantages of imprint techniques have been described by Ashley and Smith (1963) and Klontz (1972).

SECTION 8. PREPARATION AND INTRAPERITONEAL INJECTION OF A COARSE KIDNEY TISSUE SUSPENSION

Coarse kidney tissue suspensions from uninfected and PKD-infected rainbow trout were prepared for use in experiments described in Chapters 5 and 6.

Donor fish were killed by transection of the spinal cord. Using sterile instruments, the lateral wall of the abdomen was removed. If the intestine was perforated during dissection the fish was discarded. The viscera were reflected from the kidney. Using a second set of sterile instruments the kidney was removed avoiding contact with the skin or internal organs. It was placed in a universal tube containing sterile phosphate buffered saline (PBS) (Appendix 1c) chilled to 15-16°C, and previously weighed to the nearest 0.1 g. The tube plus kidney tissue was re-weighed and PBS added to give approximately 1 g of kidney tissue

per 10 ml PBS. Using a sterile pair of dressing forceps the kidney tissue was disrupted until most of the interstitial tissue was released from the connective tissue matrix. Samples of the suspension were checked by interference contrast microscopy to ensure that whole PKX cells were present. Often PKX cells were in close association with host cells, forming clumps with a diameter in excess of 40 μm. A piece of kidney was also taken for impression smear preparation or histology as described in Section 6.

Fish for inoculation were anaesthetized as described in Section 5. 0.1 to 0.5 ml of the suspension was injected intraperitoneally using a syringe fitted with a 21 gauge needle. The needle was directed through the midline anteriorly at an angle of 45° to avoid damage to the abdominal organs. Injected fish were placed back in their holding tank to recover.

Table 2.1. River Avon water quality measurements at the experimental site, 1982-1984. (Courtesy of Wessex Water Authority)

Parameter	Value		Number of readings
	Range	Mean	
Dissolved oxygen p.p.m.	6.9 - 14.6	10.1	35
% saturation	65.7 - 139.1	90.3	35
Ammoniacal nitrogen mg/l	< 0.01 - 0.25	0.06	34
Nitrite nitrogen mg/l	0.01 - 0.1	0.04	34
Chloride mg/l	9.0 - 27.0	17.1	34
Silicon mg/l	2.7 - 6.9	5.1	34
pH	7.0 - 8.5	7.8	36
Suspended solids mg/l	< 1.0 - 33.0	10.1	36
Biological oxygen demand mg/l	0.6 - 3.3	1.7	35

Table 2.2. "Omega" trout and salmon food: composition and feeding recommendations.

Description of pellet	Pellet contents: Oil %	Protein %	Fibre %	Vitamin A i.u./kg	Vitamin D i.u./kg	Vitamin E i.u./kg	Selenium mg/kg	Fish size for which pellets are recommended
No. 0	-	-	-	-	-	-	-	Up to 31 mm
No. 1	-	-	-	-	-	-	-	31-46 mm
No. 2	-	-	-	-	-	-	-	46-61 mm
No.3	-	-	-	-	-	-	-	61-92 mm
Fingerling	10.0	50	3.0	18,000	2,000	120	0.3	92-127 mm
No. 4	8.0	47	4.5	18,000	2,000	120	0.3	127-152 mm
No.5	5.5	41	4.5	18,000	2,000	120	0.3	152-203 mm
No. 6	5.5	41	4.5	18,000	2,000	120	0.3	203 mm and above

- Figures not supplied

Table 2.3. Analysis of FDL water supply to the experimental facilities after dechlorination.
(Courtesy of Wessex Water Authority 21.3.80)

	*
pH	7.7
Alkalinity (as $CaCO_3$)	206
Non-carbonate hardness	20
Total hardness (as $CaCO_3$)	226
Calcium hardness (as Ca CO_3)	222
Magnesium hardness (as $CaCO_3$)	4
Total chlorine	0.02
Chloride	21
Fluoride	< 0.1
Nitrate	5.2
Silica	3.7
Metals - copper	< 0.05
iron	< 0.1
lead	< 0.01
manganese	< 0.02
zinc	< 0.02

* mg/l (except pH)

Table 2.4. River Wey water quality measurements near farm A, 1982-1984.

(Courtesy of Wessex Water Authority)

Parameter	Value		Number of readings
	Range	Mean	
Dissolved oxygen p.p.m.	6.5 - 14.4	-	25
% saturation	62 - 112	-	25
Ammoniacal nitrogen mg/l	< 0.01 - 0.99	0.07	28
Nitrite nitrogen mg/l	< 0.01 - 0.10	0.03	28
Chloride mg/l	14.0 - 47	28	28
Silicon mg/l	1.5 - 4.1	3.25	26
pH	7.15 - 8.30	7.70	30
Suspended solids mg/l	< 1.0 - 300	14	30
Biological oxygen demand mg/l	1.0 - 4.7	1.4	28

Fig.2.1. Geographical location of the experimental site and farms supplying experimental fish.

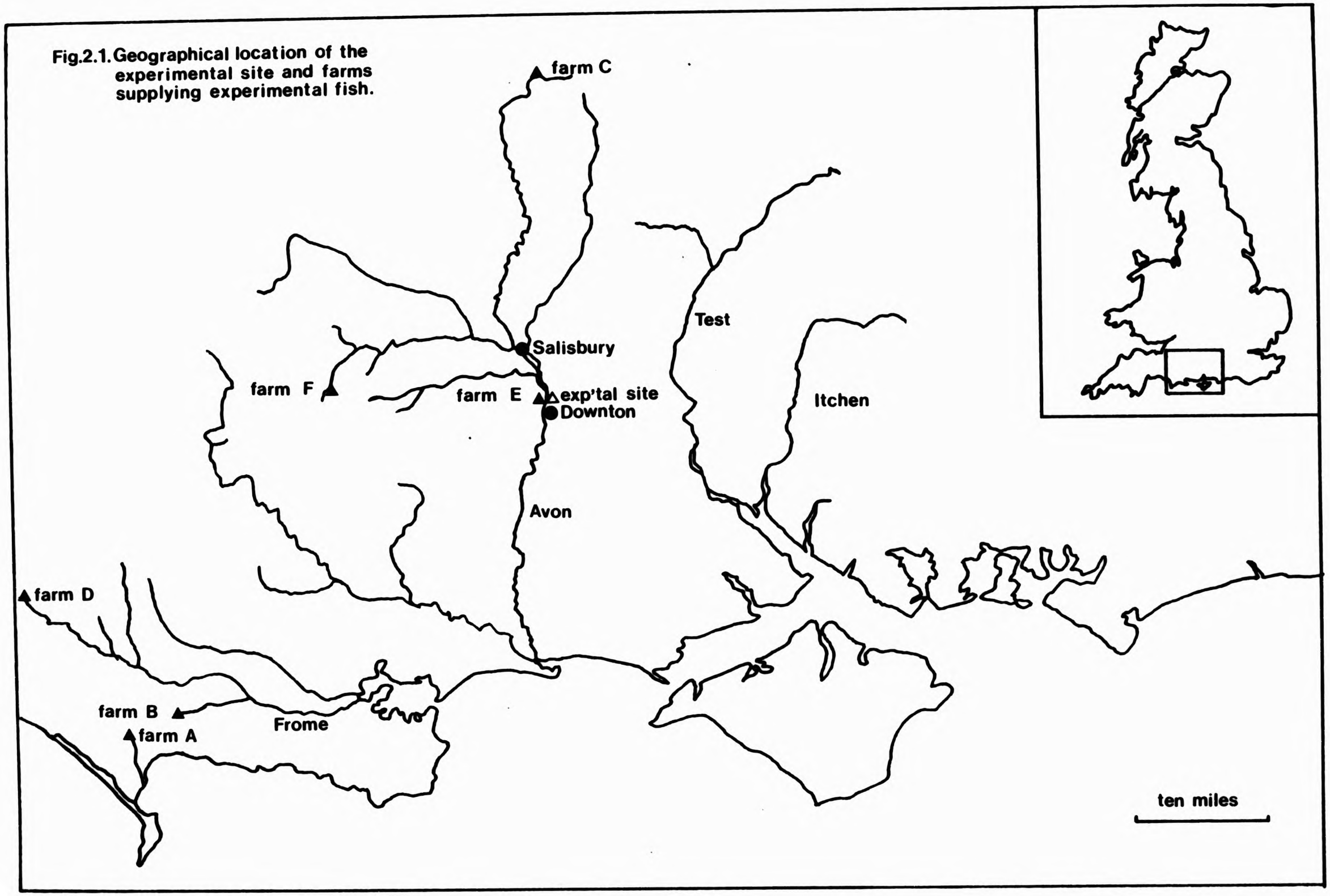


Fig.2.2. Dissolved oxygen measurements, expressed as parts per million and percentage saturation, in the water supply to the experimental site over a two year period during 1982–1984.

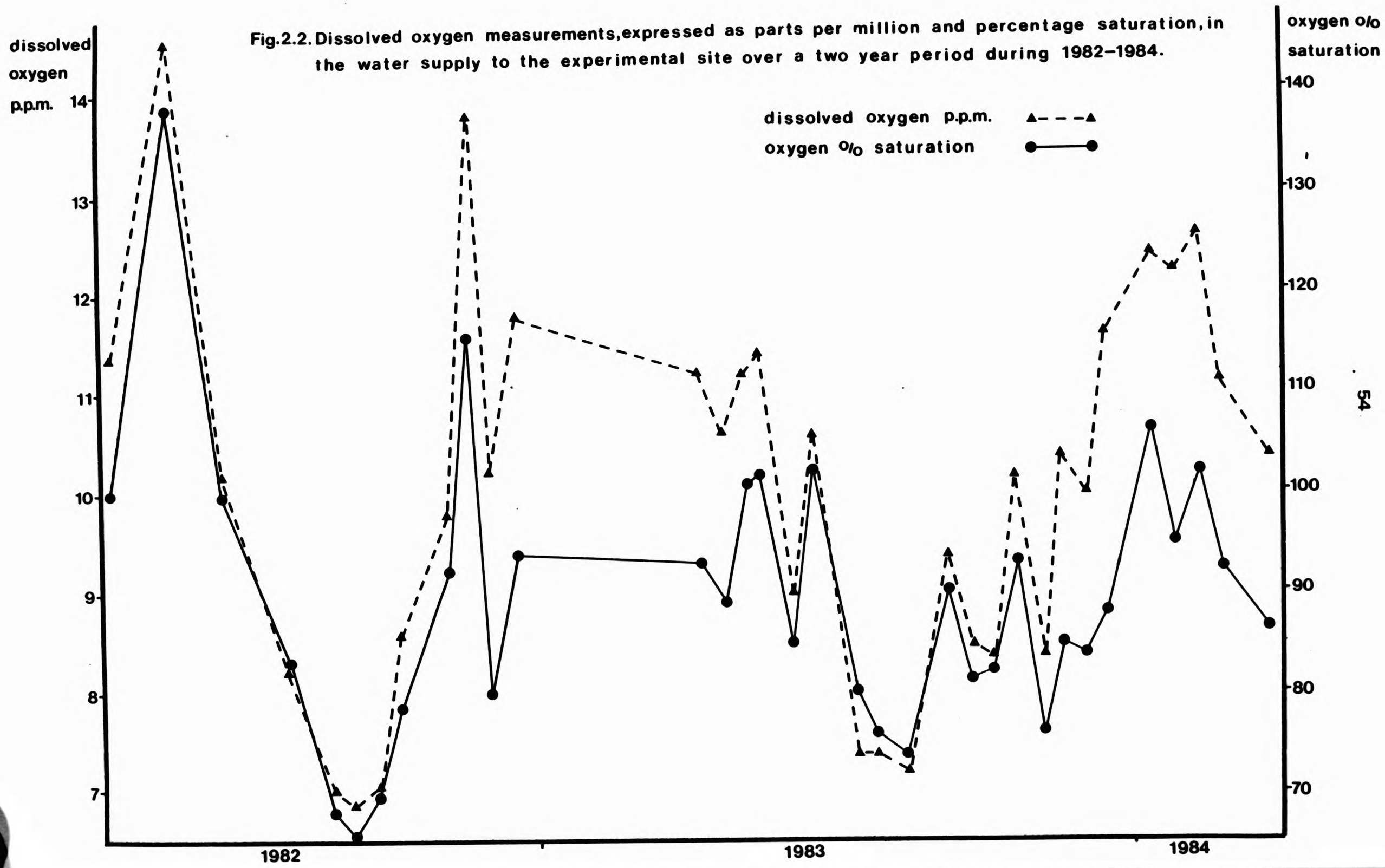


Fig.2.3. Seven day rolling average maximum and minimum water temperatures at the experimental site in 1983.

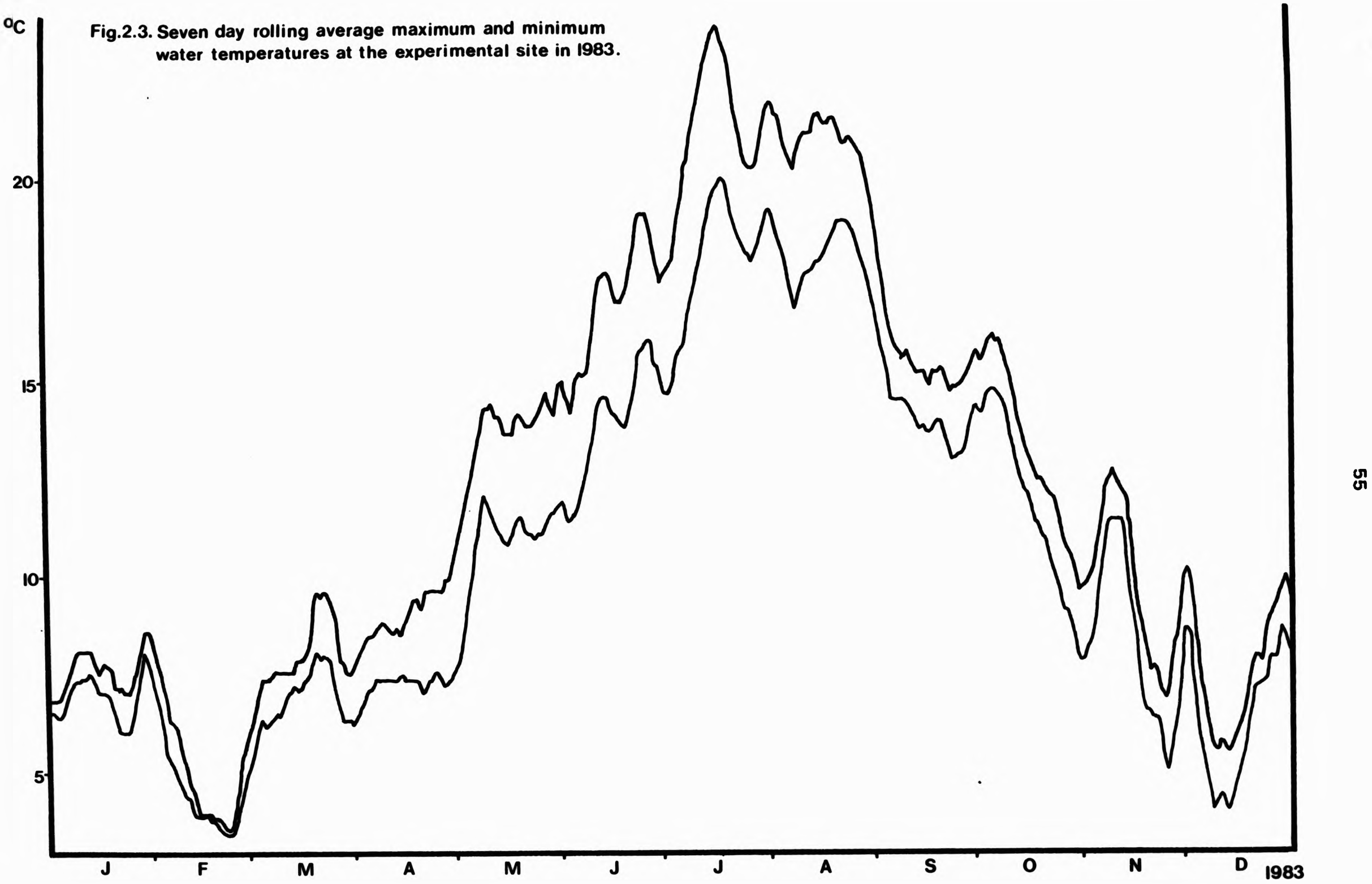


Fig. 2.4. A six metre diameter tank at the experimental site, with central monk (M) and automatic feeder (A).

Fig. 2.5. The closed tank room at FDL, Weymouth, containing small (S), medium (M) and large (L) tanks.

Fig. 2.4. A six metre diameter tank at the experimental site, with central monk (M) and automatic feeder (A).

Fig. 2.5. The closed tank room at FDL, Weymouth, containing small (S), medium (M) and large (L) tanks.

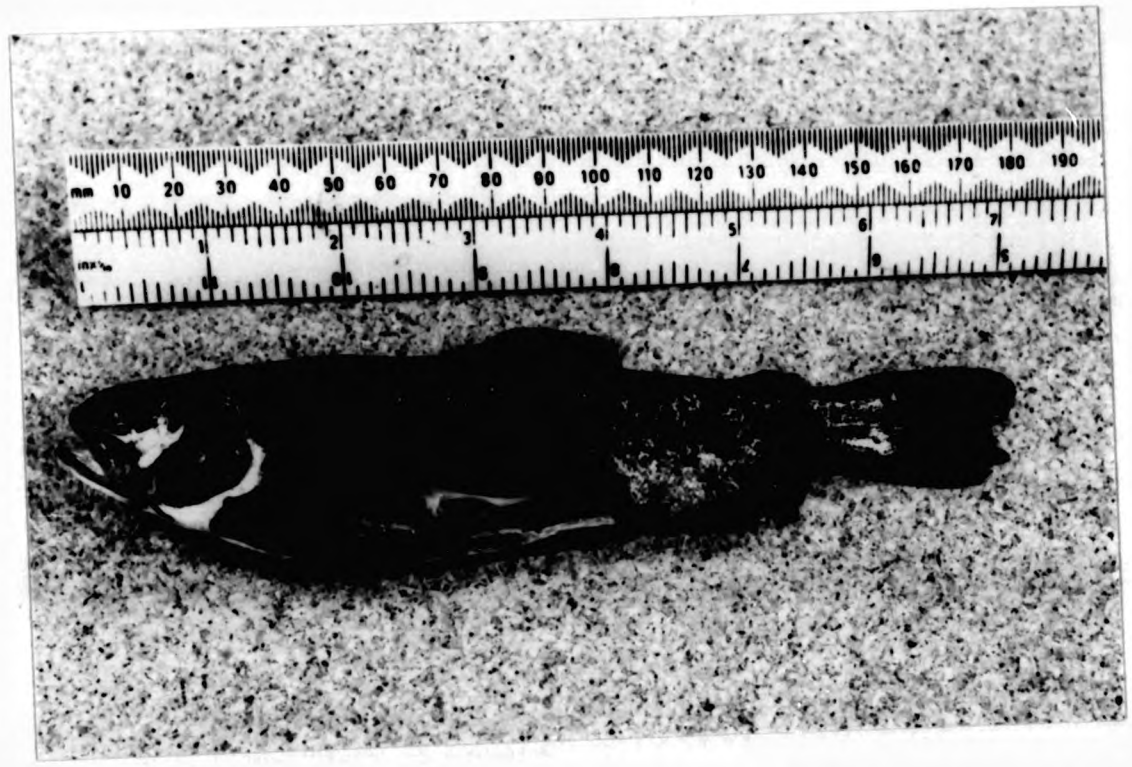


Fig. 2.6. Routine post-mortem procedure. Exposure of abdominal organs and gill rakers.

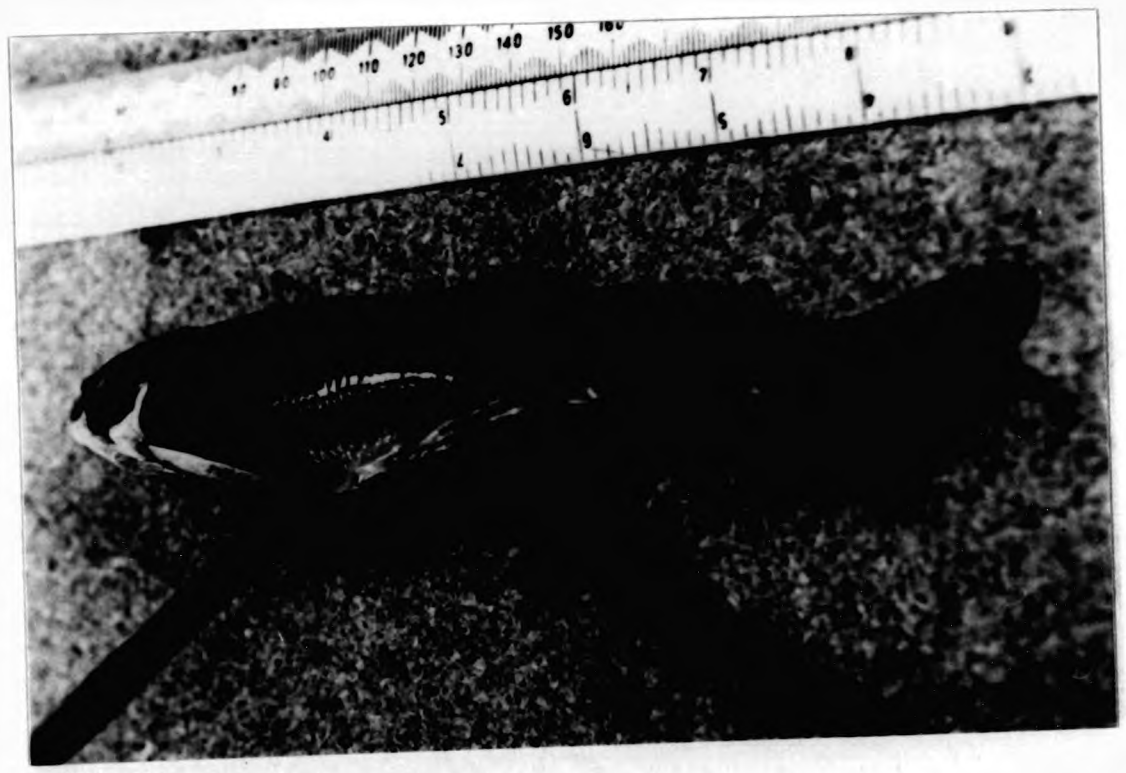


Fig. 2.7. Routine post-mortem procedure. Exposure of kidney (arrowed) after removal of other viscera. The kidney is not diseased and has a concave appearance and glistening capsule, equivalent to grade 0 kidney swelling.

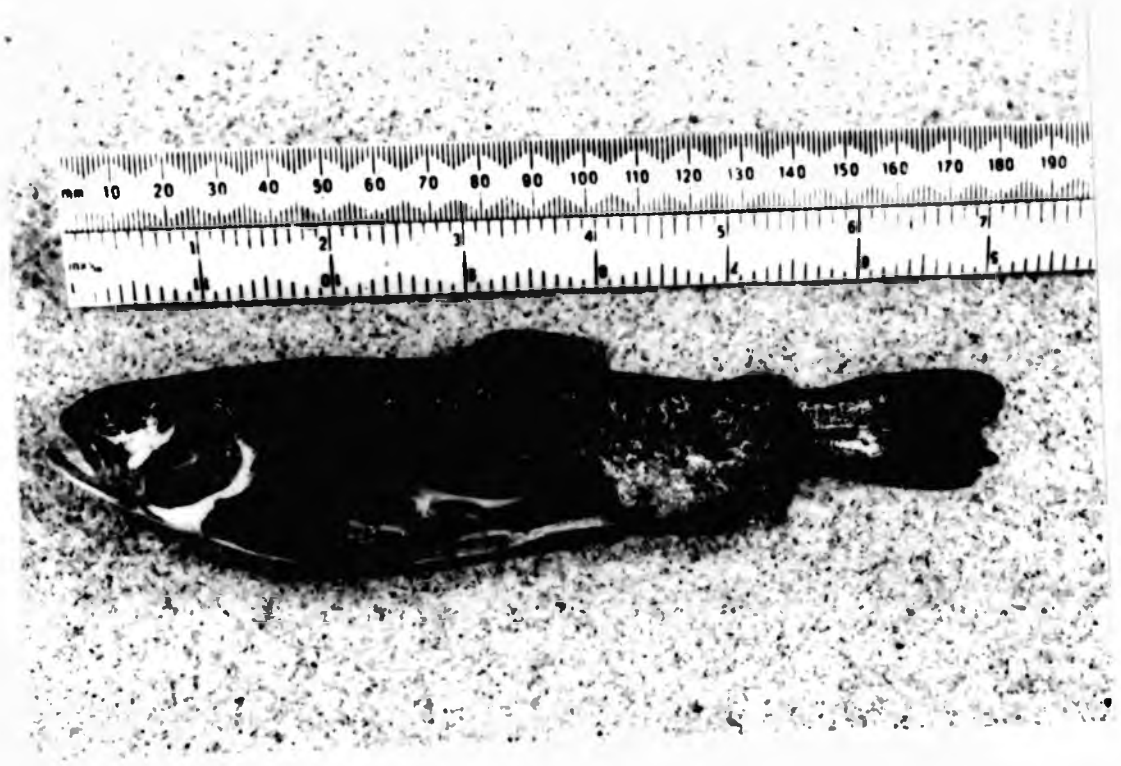


Fig. 2.6. Routine post-mortem procedure. Exposure of abdominal organs and gill rakers.

Fig. 2.7. Routine post-mortem procedure. Exposure of kidney (arrowed) after removal of other viscera. The kidney is not diseased and has a concave appearance and glistening capsule, equivalent to grade 0 kidney swelling.

Fig. 2.8. Grade 1 kidney swelling and appearance.

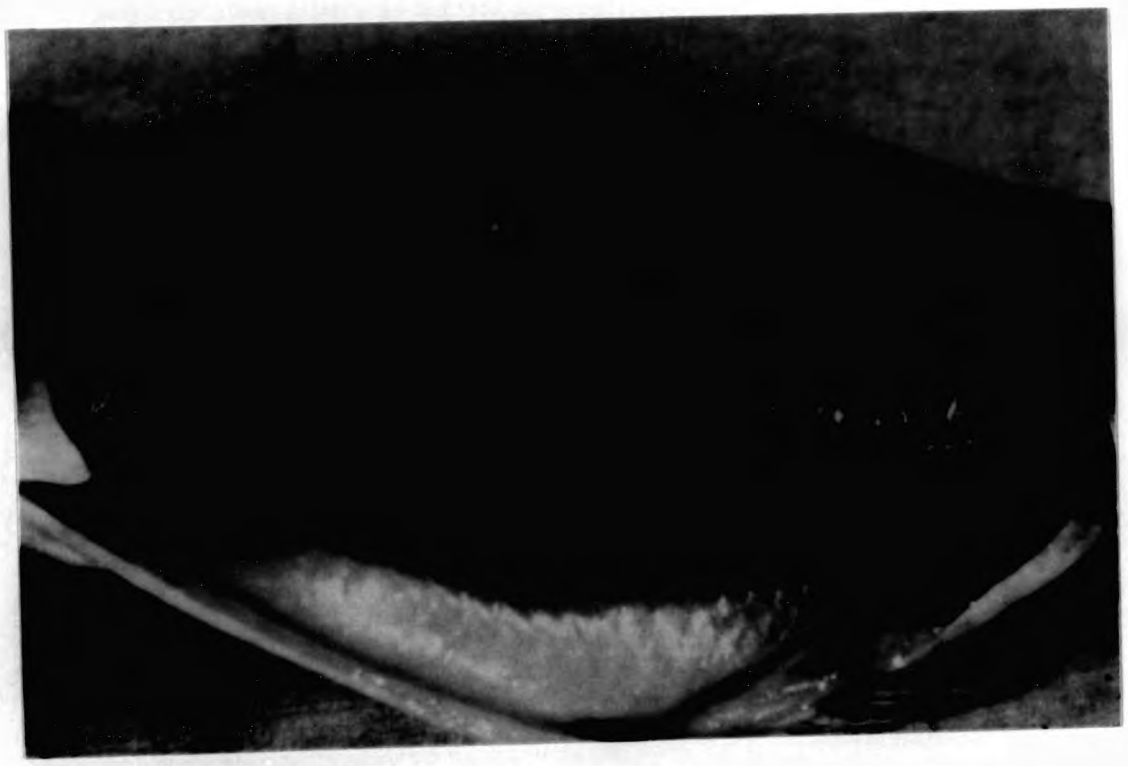


Fig. 2.9. Grade 2 kidney swelling and appearance.

Fig. 2.8. Grade 1 kidney swelling and appearance.

Fig. 2.9. Grade 2 kidney swelling and appearance.

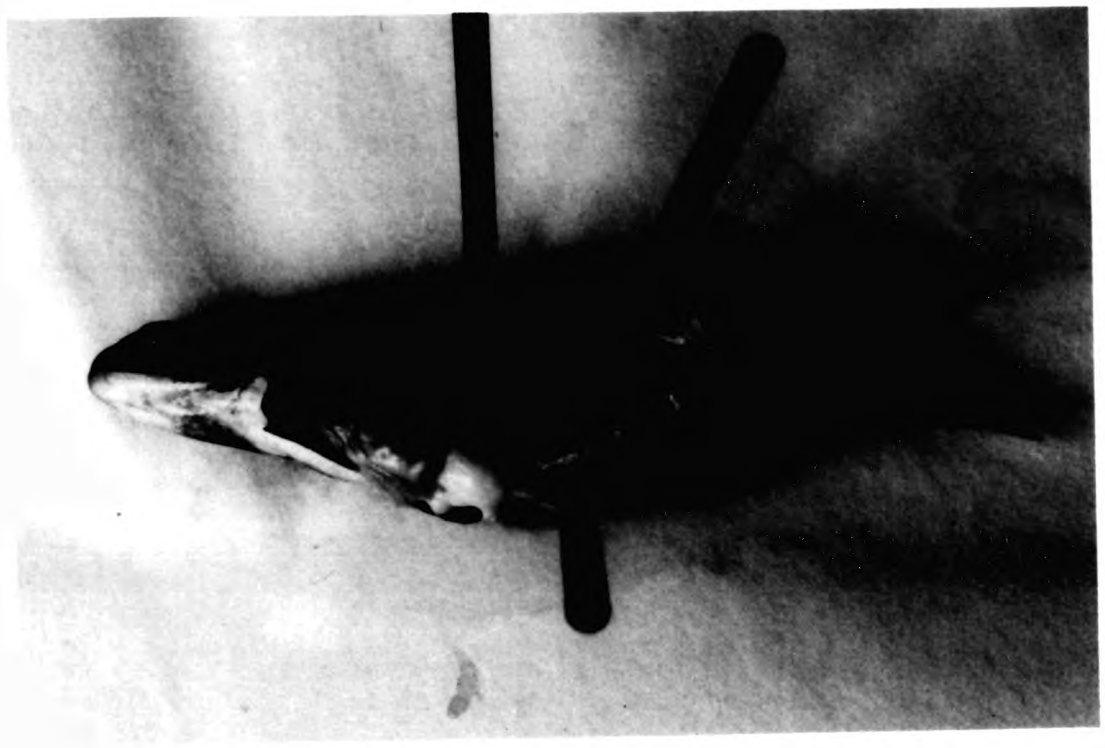


Fig. 2.8. Grade 1 kidney swelling and appearance.

Fig. 2.9. Grade 2 kidney swelling and appearance.

Fig. 2.10. Grade 3 kidney swelling and appearance.

Fig. 2.11. Grade 4 kidney swelling and appearance. This fish also shows gill pallor, a spleen (arrowed) with a roughened surface, and a pale liver (L).

Fig. 2.10. Grade 3 kidney swelling and appearance.

Fig. 2.11. Grade 4 kidney swelling and appearance. This fish also shows gill pallor, a spleen (arrowed) with a roughened surface, and a pale liver (L).

Fig. 2.10. Grade 3 kidney swelling and appearance.

Fig. 2.11. Grade 4 kidney swelling and appearance. This fish also shows gill pallor, a spleen (arrowed) with a roughened surface, and a pale liver (L).

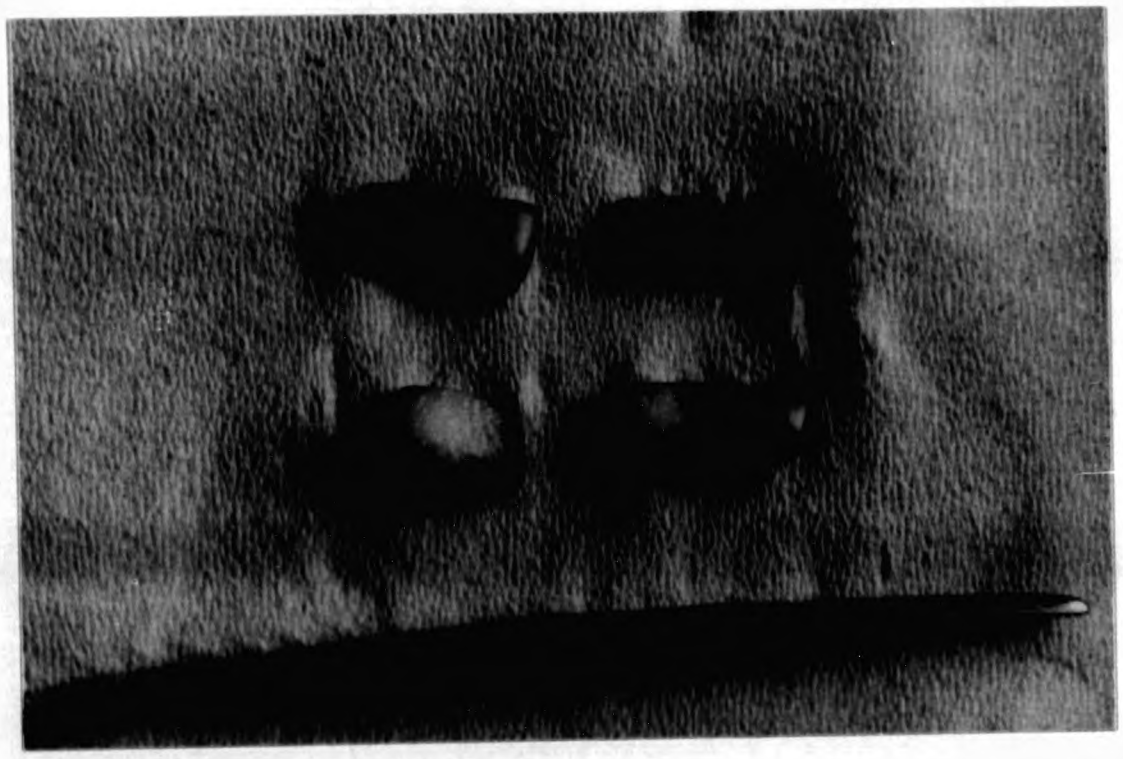


Fig. 2.12. Grade 5 kidney appearance with chronic nodules of inflammation within the renal tissue.

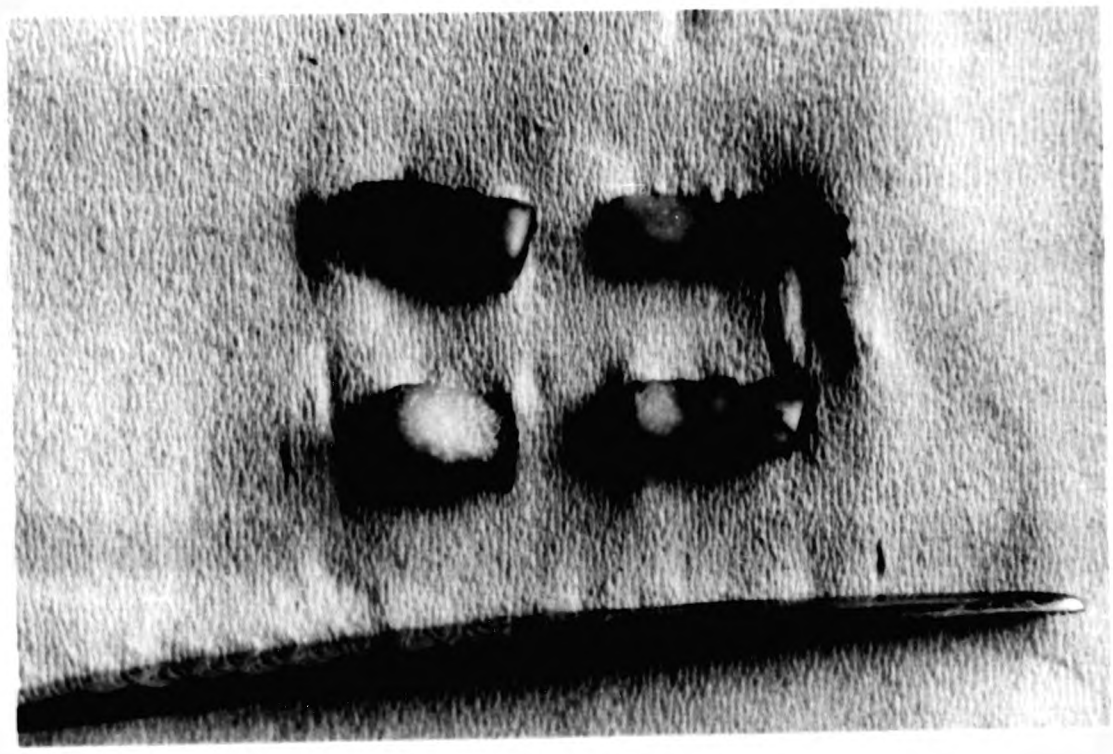


Fig. 2.12. Grade 5 kidney appearance with chronic nodules of inflammation within the renal tissue.

Fig. 2.12. Grade 5 kidney appearance with chronic nodules of inflammation within the renal tissue.

Chapter 3

Studies of the sequential clinical and pathological changes in rainbow trout with PKD

INTRODUCTION

Prior to the work described in this chapter, there were several reports of the clinical signs and pathology of PKD in rainbow trout and these were reviewed in chapter 1. These reports described changes in fish at the height of PKD outbreaks when fish were dying from the disease. However, there were no reports about the sequential development of clinical signs from initial infection to death or recovery, or of the related pathological changes in the tissues of the fish. Furthermore, the number of tissues examined from diseased fish was limited.

The studies described in this chapter were undertaken to investigate the sequential changes occurring in naturally-infected fish at one infected site over a period of two consecutive summers. Their purposes were:

1. To study the development of clinical signs from initial infection to resolution of the disease or death of the fish.
2. To study the tissues affected and the development of lesions in these tissues throughout the course of the disease.
3. To study the relationship between the clinical signs and the gross and microscopical pathological lesions.
4. To compare the pathological changes in the kidneys of these fish with those seen in kidneys from diseased rainbow trout from 16 other PKD-infected sites in the United Kingdom.

It was hoped that, by studying the sequence of changes in infected fish, some light might be thrown on the pathogenesis of the disease.

MATERIALS AND METHODS

Details of the materials and methods were given in chapter 2.

Unexposed control fish

These comprised 1,000 fingerling rainbow trout, average length 80 mm, from farm A. They were grown from eggs which were stripped on 18 December 1981 from 3+ rainbow trout broodstock of the Jorgensen variety and which had started hatching on 19 January 1982. They were transported to the laboratory on 30 April 1982 and kept in a tank in the holding area.

Experimental fish

Fish for these experiments were derived from two farms, A and B, previously free from PKD, and were held and naturally infected at the experimental site. Group A comprised 1,500 rainbow trout fingerlings from the same hatch as the unexposed control fish. They were transported to the experimental site on 30 April 1982 and held there in a 6 m concrete tank.

Group B comprised 300 fingerling rainbow trout, average length 60 mm, from farm B. They were grown from eggs, hatched in December 1982, derived from the farm's own broodstock. On 10 March 1983 they were transferred to the experimental site and held throughout the experimental period in a 1 m^3 cage placed in a 6 m tank.

During the course of the study, 175 fish from group A and 91 fish from group B were examined pre- and post-mortem.

Diseased fish from other PKD-infected sites

Renal samples were examined from 5-15 fish from each of 16 PKD-infected farms in England, Scotland and Wales which had been submitted to FDL, Weymouth, or the Institute of Aquaculture, Stirling, for diagnostic

purposes. Samples were from fish killed between July and September during the years 1978-1984. Lesions were compared with those in the experimental fish.

Sampling schedule

The sample dates of groups A and B fish are detailed in Tables 3.1a and 3.1b, respectively, together with the number of fish in each sampling, their lengths and weights.

One hundred and sixty fish in group A were sampled at the experimental site at 3-13 day intervals from May to September 1982. A further 15 fish were sampled in March 1983. Ninety-one fish in group B, required principally for haematological analysis (chapter 4), were sampled between March and September 1983.

A total of 20 unexposed control fish was sampled on 13 May 1982, 12 August 1982 and 20 September 1982.

Pre-mortem sampling

GROUP A

Throughout the experimental period fish were selected at random. Once netted, they were immediately transferred to a container holding aerated water and examined for external signs of disease. Individual fish were killed by spinal cord transection.

GROUP B

Samples were collected in the morning. Individual fish were randomly netted and immediately transferred to a 0.01% solution of MS222 (Sandoz Ltd., Basle, Switzerland). After noting external signs of disease, a blood sample was taken from the caudal vein as described in chapter 4 prior

to killing the fish by transection of the spinal cord.

UNEXPOSED CONTROLS

These fish were treated as the group A fish.

Post-mortem sampling

The majority of fish sampled at the experimental site were measured to the nearest mm using head to tail fork length, and weighed to the nearest gram (Tables 3.1a and b). Fish were dissected and gross pathological changes noted. Kidney swelling was graded from 0 to 5 in accordance with the schedule detailed in chapter 2. Tissues were removed and fixed for histological and ultra-structural examination. In all fish sampled in group A in 1982 and group B in 1983 tissues for examination included the caecum, gill, kidney, large intestine, liver, pancreas, small intestine, spleen and stomach. In addition, in all group A fish, except sample numbers 21 and 22, brain and heart samples were also taken. In group A, sample numbers 21 and 22, only kidney and gill specimens were taken. In addition, specimens were taken from clinically-affected and recovering fish in groups A and B of eye (5 samples), gonads (5 samples), skin including the lateral line (46 samples), spinal canal (46 samples), striated muscle (46 samples), swim bladder (36 samples), and ureter (46 samples).

Kidney impression smears were prepared as described in appendix 2 from each fish in group A sampled from 5 July 1982 to 23 September 1982, and in group B from 2 June 1983 to 1 September 1983.

In the 20 unexposed control fish, tissues sampled were as for group A fish sampled in 1982.

Processing tissue samples

Tissue specimens from all samplings were paraffin wax embedded and sections prepared and stained for microscopical examination with Gill's haematoxylin and eosin. Selective staining techniques were used for particular cellular and tissue components, pigments, chemical elements and parasites. The techniques and numbers of samples stained by each technique are summarized in Table 3.2. All techniques were applied to NBF-fixed paraffin wax tissue sections except for lipid staining which required NBF-fixed tissue sections cut on a cold microtome cryostat (Pearse HR cold microtome cryostat, SLEE Medical Equipment Ltd., London).

Kidney specimens for ultra-structural examination were collected from group A fish in sample numbers A12 (5 specimens), A15 (5 specimens), A17 (2 specimens) and A19 (1 specimen), and from group B fish in sample numbers B7 (3 specimens), B8, B11, B13 and B15 (1 specimen from each). Semithin and ultrathin resin sections were prepared as described in chapter 2.

Representative examples of each common cell type in lesions were examined ultra-structurally and their identity confirmed by comparison with descriptions of lymphocytes by Weinreb (1963) and Ferguson (1976), macrophages by Ferguson (1976), plasma cells by Bessis(1961), polymorphonuclear leucocytes by Ferguson (1976), red blood cells by Weinreb (1963) and Yamamoto and Iuchi (1975), and thrombocytes by Ferguson (1976).

Expression of results

Results were grouped into seven categories according to macro- and microscopical appearance, using the kidney grading system from 0 to 5 described in chapter 2. Results from fish with grade 0 renal swelling were divided into two categories, those from fish without detectable

renal PKX cells and those with renal PKX cells. Results in category 7 included those from fish with kidneys classed as grade 5 and those from fish whose kidneys contained advanced histological signs of lesion resolution.

RESULTS

Although group A fish were introduced to the field site at the end of April and group B fish in March of the next year, there was little difference between the two groups in the time of year at which histological signs of infection, gross pathology and clinical signs developed. Furthermore, the histopathology in tissues showing particular gross pathology was common to samples from both groups. The results, therefore, of the studies of clinical signs and pathology in the two groups have been combined.

1. Unexposed control fish

Control fish presented no clinical, gross or histopathological signs of PKD throughout the experimental period. Signs of nephrocalcinosis were seen in two fish.

2. Experimental fish

The numbers of fish in groups A and B at each sampling in categories 1-7 are detailed in Tables 3.3a and 3.3b.

CLINICAL SIGNS OF PKD

The number of fish in each category exhibiting particular clinical signs during the experimental period are recorded in Table 3.4. No clinical signs were observed before 15 July 1982 in group A (77 days

after introduction to the experimental site), or before 14 July 1983 in group B (127 days after introduction). Approximately 100 of the fish in group A died between 15 July 1982 and 23 August 1982. Ten of the 300 fish died in group B between 14 July 1983 and 1 September 1983. These fish showed varying degrees of abdominal distension (Fig.3.1) and pallor. Several fish examined in extremis presented a similar external appearance. They also tended to swim erratically, show loss of equilibrium and gasp. Fish with gross abdominal swelling swam slowly, maintaining momentum and balance by use of the pectoral fins since movements of the tail and trunk were impeded by the swelling. Excitation of affected fish exacerbated signs of oxygen deficiency and caused loss of equilibrium. Several fish presented with bilateral exophthalmos and melanosis. Those with melanosis were the smaller fish of a sample batch. One fish with grade 4 renal swelling presented with ecchymoses and one fish with grade 1 swelling presented with bilateral corneal cloudiness. This corneal cloudiness was also seen, together with abdominal distension, in several other fish in group B, which were not sampled. All except three fish with clinical signs had kidney swelling of grades 3 or 4.

GROSS PATHOLOGY OF PKD

Gross pathological changes associated with PKD were present in samples taken between 5 July 1982 and 23 September 1982 (67 to 147 days after introduction to the experimental site) in group A, and in samples taken between 4 July 1983 and 1 September 1983 (117 to 176 days after introduction) in group B. The most obvious changes related to kidney size and appearance (Figs. 2.8-2.12). The numbers of fish at each sampling exhibiting various kidney grades are detailed in Table 3.5. In both groups, the first signs of renal swelling occurred at the beginning of July. Severity of kidney

pathology increased throughout July, but declined during August and September in the majority of fish sampled, as surviving fish began to recover.

Changes in other organs were mainly in fish with renal swelling of grades 3 and 4. They were seen in spleens, which were enlarged with rounded edges or of normal size but with a roughened surface (Fig.2.11), livers, which were often pale or yellow (Fig.2.11), the alimentary tract, which was sometimes empty (occasionally with a correspondingly full gall bladder), and the gills, which were pale (Fig.2.11). In 10 of 34 fish with grade 3 renal swelling, the internal organs showed petechiation. Fish with grade 4 renal swelling were oedematous. The numbers of fish in each category with these gross changes are detailed in Table 3.6.

Apart from these changes, discrete nodules were occasionally found in the spleen and liver. In two longstanding infections individual primary gill lamellae were thickened (Fig.3.2). No gross signs associated with PKD were found in the brain, heart or rudimentary gonads.

HISTOPATHOLOGY OF PKD

Histopathological changes associated with PKD were first seen in the kidneys of fish from both groups A and B sampled in early June. As the disease progressed, changes were noted in a variety of other tissues but the most marked changes occurred in the kidney. These changes, seen during the course of the disease, are described below in sections relating to each tissue examined. Where specific cell types are mentioned, the identity of representative examples was confirmed by ultra-structural examination.

The kidney

Infection with PKX cells was initially diagnosed in sample number A5 in group A fish on 4 June 1982 (36 days after introduction to the experimental site) and in sample number B5 in group B fish on 2 June 1983 (85

days after introduction). Twenty to 80 days after initial diagnosis of infection in these batches, renal samples from all fish examined contained PKX cells. It was found that the lesions developing in the kidney could be divided into three broad types related to haemopoietic hyperplasia, vascular pathology and diffuse inflammation. These are described in detail below. The numbers of fish in each category of renal involvement showing the principal histopathological changes are summarized in Table 3.7.

PRE-CLINICAL CHANGES

Kidney impression smears from most fish sampled at the beginning of June, whether they contained PKX cells or not, included macrophages which were about 20 μm in diameter with irregular, eccentric pink nuclei and pale blue cytoplasm. The cytoplasm of these cells contained light and dark purple, spherical inclusions. Occasionally these macrophages were found in close association with cells having crescent-shaped, dark-staining nuclei (Fig.3.3).

The first observation of PKX cells in group A was in a single fish on 4 June 1982. Similarly in group B the first observation was in a single fish on 2 June 1983. The cells, 10-15 μm in diameter, were usually within peritubular capillaries in the mesonephros and less frequently within the interstitial tissue (Fig.3.4). Strands of pale eosinophilic material, unstained with Alcian blue, were usually associated with these intra-capillary organisms, but no cellular response was apparent. Intra-capillary PKX cells were found in infected kidneys throughout the course of the disease until lesions were organizing and resolving.

As PKX cells became more numerous, their distribution in the kidney was patchy, with most localized in groups in renal blood sinuses. These were sometimes seen without an obvious host response but on other occasions

were associated with small cellular aggregations attached to blood vessel walls and loss of definition of the endothelial lining (Fig. 3.5). These aggregations comprised large and small lymphocytes, and macrophages. The macrophages often contained melanin granules. Cell fragments, irregularly shaped cells of no recognizable type, red blood cells, neutrophils and thrombocytes were also present. There was little fibrin associated with these early aggregations, although serous material was sometimes present. Some PKX cells in these lesions appeared to have one or more cells closely attached (Figs. 3.6, 3.7 and 3.8). Some of these cells had intracytoplasmic granules and a nuclear staining pattern similar to neutrophils. Others were similar to macrophages. The cellular aggregations at this early stage appeared to cause little obstruction of vascular lumina.

From 13 days after initial PKX cell identification in group A and from 28 days after identification in group B, there was a marked increase in interstitial cell numbers particularly of cells similar to the haemopoietic cells, with a diameter of about 7.5 μm, normally surrounding renal tubules (Figs. 3.9 and 3.10). Mitotic figures were often seen in the interstitial tissue as well as mature erythrocytes, macrophages and melanomacrophages. PKX cells were rarely present. As the infection progressed, increasing numbers of neutrophils and plasma cells were seen (Fig. 3.11). As the hyperplasia developed there was an apparent reduction in excretory elements (Fig. 3.9). Tubules and glomeruli in this tissue often appeared hypercellular (Fig. 3.12) with stenosis of affected lumina and reduction of Bowman's space in the glomeruli. In tubules the hypercellularity consisted of lymphocytes and increased numbers of epithelial cells, some showing pyknosis. In glomeruli, the hypercellularity reflected an increase in capillary tuft cells. Sections stained with PAS occasionally showed

thickened basement membranes and capillaries in affected glomeruli, but this was not a constant finding. Affected tubules showed no peritubular fibrosis, although some collecting ducts and mesonephric ducts in both uninfected and infected kidneys had thick, collagenous sheaths.

In the pre-clinical stage of infection the epithelium of many proximal tubules, especially those isolated within the hyperplastic haemopoietic tissue, contained numerous droplets of brightly staining eosinophilic material (Figs.3.12 and 3.13). This material was positively stained by the PAS method (Table 3.2.), and fluoresced brightly when stained by Gomori's rapid one-step trichrome (Table 3.2.).

Four days prior to the appearance of gross pathology in fish in group A the kidneys of five fish sampled on 1 July 1982 contained a diffuse inflammatory reaction which became more prominent as the disease progressed. Unlike haemopoietic hyperplasia, this cellular reaction occurred only where PKX cells were present and was not confined to haemopoietic tissue. Initially it comprised a disorganized collection of different cell types. Many of the cells appeared to be macrophages with large amounts of pale cytoplasm, while a few were lymphocytes. Small fluid-filled spaces were apparent within the reaction (Fig.3.14). Cells with pleomorphic nuclei, showing pyknosis and karyorrhexis were found, especially in close proximity to PKX cells, but erythrocytes and melanomacrophages were scarce. Fibroblasts were occasionally seen, although silver impregnation failed to show the presence of reticulin at this stage. Host cells tended to be arranged around PKX cells in some lesions.

PKX cells were occasionally found between tubule epithelial cells (Fig.3.15) and some organisms appeared to contain within their cytoplasm single, eosinophilic spherical inclusions about 5 μm in diameter and surrounded by a clear "halo" (Fig.3.15). Other PKX cells, especially

in longer standing lesions, contained several inclusions (Fig.3.16). Small amounts of DNA-positive material were demonstrated within the main cell and the inclusions. PKX cells also contained some PAS-positive material but were not stained by oil red O. Granules within the cytoplasm were argyrophilic, autofluoresced and were prominent when viewed with phase contrast microscopy.

LESIONS ASSOCIATED WITH CLINICAL DISEASE

Exaggeration of the cellular changes associated with blood vessels and disorganized inflammation accompanied the development of gross pathology and the appearance of clinical signs.

The diffuse inflammatory reaction around increasing numbers of PKX cells became the dominant, histopathological feature in the majority of fish sampled between 41 and 80 days after initial finding of the PKX cell, and accounted for most of the kidney swelling, seen grossly as grey areas in grade 2, 3 and 4 kidneys. Initially, excretory elements were contained within the reaction (Fig.3.17), but as the reaction progressed, they appeared to lose their integrity (Fig.3.18) until the histological picture showed areas, often several millimetres in diameter, with few tubule structures or glomeruli (Fig.3.19). Some glomeruli and tubules within the reaction contained possible PKX cells (Fig.3.18), but no Dunn-Thompson-positive material. Fluid-filled spaces became more noticeable, especially in kidneys of grade 4 swelling. Cellular necrosis was not a marked feature of the inflammation. Granules of melanin were seen throughout the reactive tissue (Fig.3.18), but melanomacrophages were few compared with those in normal renal tissue. There was no evidence of increased bile pigment. Erythrocytes were scarce, but occasionally foci of erythrocytes were encountered within the inflammatory

tissue perhaps corresponding to the red foci seen grossly within the grey patches. In some lesions, there were more fibroblasts and reticulin, and one specimen for sample number A12, and one from B11, contained lesions with a "whorling" appearance where collagen content was increased (Fig. 3.20).

The haemopoietic hyperplasia described earlier was seen throughout the course of the disease and corresponded to the dark red areas seen grossly. Initially, small areas of inflammation were interspersed between the hyperplastic tissue. However, with development of larger areas of inflammation and destruction of tubules and glomeruli, the haemopoietic response was seen as islands of cells together with excretory elements surrounded by disorganized inflammatory tissue containing PKX cells, accounting for the grey and red mottling seen in grossly swollen kidneys (Figs. 3.17 and 3.19).

The intravascular aggregations described earlier increased in size and sometimes contained small amounts of fibrin. Many had numerous PKX cells (Fig. 3.21), although more advanced lesions occasionally contained no visible parasites. Affected vessels were partially or totally occluded, and some were surrounded by increased amounts of reticulin. Generally, thin-walled vessels were affected but occasionally thicker walled vessels were also found occluded (Figs. 3.22 and 3.23). Vessels affected in this way caused the apparent petechiation seen grossly in certain liver, gill and abdominal fat samples.

From day 34 after initial diagnosis of infection in group A, and from day 46 in group B, fusiform crystals, up to 50 μm in length, were found in association with intravascular lesions. In kidney impression smears, these crystals were blue when stained with May-Grünwald-Giemsa often divided across their width by a reflecting line (Fig. 3.24). Many

crystals, including the largest examples, appeared to be intracellular (Fig.3.25). In electron micrographs the cells appeared to be macrophages (Fig.3.25). In transverse section the crystals were rhomboid (Fig.3.26). They stained eosinophilic with H & E and Gomori's rapid one-step trichrome method, and green with Dunn-Thompson's method for haemoglobin. They were unstained by Stein's, Perls' and Schmorl's methods (Table 3.2). When viewed in unstained sections they were clear and uncoloured. Viewing with polarized light and phase contrast produced negative results. They were electron dense and one sample showed a fine lattice structure.

Apart from being present in many vascular lesions, crystals were also seen throughout areas of diffuse inflammation. Some specimens of kidney, gill, heart, liver and spleen contained blood vessels with crystals apparently free within the lumen in the absence of PKX cells or intravascular aggregations (Fig.3.27). Other vessels contained many intra- and extra-cellular crystals, often orientated at right angles to the endothelium (Fig.3.28).

Sections with areas of diffuse inflammation and vascular lesions contained no increased amounts of Perls'- or Schmorl's-positive material. Positive material was usually associated with melanomacrophages.

ORGANIZATION AND HEALING OF LESIONS

In group A, specimens collected from 41 days after initial infection contained more circumscribed areas of inflammation which were surrounded by areas of apparently normal tissue (Fig.3.19). These circumscribed areas were seen grossly as distinct, circular, grey areas within a framework of dark red tissue. Lesions at different stages of development within the same kidney resulted in areas of diffuse inflammation being

adjacent to these circumscribed lesions. Lesions undergoing organization in this way showed no increase in reticulin but contained PKX cells with loss of cytoplasmic detail, apparently undergoing necrosis (Fig. 3.29). Kidneys with these organizing areas contained increased Schmorl's-positive material, usually associated with melanin granules, sometimes within inflammatory areas but more often within normal tissue. Amounts of melanin were also increased as were the numbers of melano-macrophages, capillaries and erythrocytes.

One kidney section of 23 stained by the Giemsa method contained a single, intraluminal immature spore with two spherical polar bodies. This sample was from a fish with grade 5 renal swelling in sample B15. No similar spore-forms were found in other kidneys with grade 5 swelling or in any kidney smear.

In the majority of surviving fish, organization of inflammatory tissue progressed, such that in 16 fish sampled in September the only signs of previous infection in nine fish were an increase in Schmorl's-positive material, and in haemopoietic tissue.

However, in some kidneys the circumscribed areas of inflammation developed into nodules of chronic inflammation (Fig. 3.31). In these cases the PKX cells, which often appeared to be undergoing necrosis, were central within small granulomata (Fig. 3.32) comprising cellular debris, macrophage-like cells, epithelial cells, lymphocytic cells, and cells containing large globules of eosinophilic material. The granulomata were enclosed in reticulin and collagen. This fibrous tissue sometimes contained many eosinophilic granule cells. Many of these small granulomata were combined to form the intensely white nodules seen grossly (Fig. 2.12).

Renal specimens from group A fish sampled in 1983 (numbers A21 and

A22) contained no evidence of PKD.

The spleen

The numbers of spleens in which PKX cells were seen in each category of fish are detailed in Table 3.8. PKX cells appeared fewer than in comparably-sized sections of mesonephros from the same fish. The tissue response was similar to that seen in the pronephros, with hyperplasia of haemopoietic cells, and a diffuse inflammation centred on PKX organisms and involving the same cell types as described in the kidney (Fig.3.33). Few spleens showed extensive areas of diffuse inflammation comparable to those in the kidney, although fibrosis and cellular necrosis were found and intravascular aggregations, sometimes with accumulations of fibrin, did occur. Occasionally, spleens contained areas of macrophage-like cell proliferation (Fig.3.34). Although there was little melanin in areas of inflammation, where erythrocytes and organisms were numerous, PKX cells would often appear to contain melanin granules (Fig.3.34) and the electron microscopical appearance of these PKX cells suggested increasing electron density and vacuolation (Fig.3.29). As in kidney specimens, where lesions were resolving, there were increased amounts of intracellular Schmorl's-positive material.

Marked increases in Perls'-positive material were present in one spleen from a group A fish with grade 3 renal swelling sampled on 13 September 1982, and in six spleens from group B fish, three with grade 3 and three with grade 4 renal swelling sampled between 18 July 1983 and 29 July 1983. This material appeared to be intracellular and throughout the splenic tissue apart from areas of inflammation. In spleens with roughened surfaces, numbers of lymphocytes and macrophages were increased. In enlarged spleens with rounded edges and a dark red colour, numbers of

erythrocytes appeared increased. Other spleens were enlarged with pale areas of tissue which on histological examination consisted of diffuse inflammation.

The liver

The numbers of livers in which PKX cells were found in each category of fish are detailed in Table 3.8. Samples taken prior to the appearance of PKX cells occasionally exhibited circumscribed areas of increased cellularity, the predominant cell type being similar to small lymphocytes (Fig.3.35). This increased cellularity was seen in samples in groups A and B throughout the experimental period. It became pronounced and diffuse during the period of greatest kidney pathology, and was centred upon blood vessels and bile ducts (Fig.3.36). Blood vessel pathology was similar to that in the kidney and spleen, with disorganised cellular aggregations, including crystals and fibrin, totally or partially occluding vessels (Fig.3.37). The aggregations did not always contain PKX cells. Crystals were frequently found free within blood vessels (Fig.3.38). These vascular obstructions corresponded to the petechial lesions seen grossly. Where PKX cells were within the parenchyma there was often a circular area of inflammatory tissue comprising dying cells, and cells similar to lymphocytes and macrophages.

The gill

The occurrence of PKX cells within gills in each category of fish is summarized in Table 3.8. Gills with and without PKX cells often contained pairs of secondary lamellae fused together and small areas of epithelial hyperplasia.

PKX cells were sometimes associated with epithelial hyperplasia and

were often seen, apparently within capillaries, in secondary lamellae, appearing more elongated than in the kidney specimens. One sample from sample A13, taken 48 days after initial diagnosis of infection, showed epithelial hyperplasia and telangiectasis, with organisms apparently trapped within sequestered blood and fibrin (Fig.3.39). PKX cells were also found within primary lamellae, especially in the central blood vessels, which occasionally contained crystals.

Two specimens from group B collected 55 and 57 days after initial diagnosis of infection contained a primary gill lamella which was grossly thickened with disorganized, fibrous, inflammatory tissue including many PKX cells (Fig.3.2).

The heart

Of 95 hearts examined from PKX-positive fish, 11 from group A fish with kidney swelling from grade 1 to grade 3, sampled between 1 July 1982 and 30 July 1982, showed the presence of PKX cells. The organisms usually occurred singly within the stratum spongiosum of the ventricle (Fig.3.40), although one section contained an intravascular PKX cell in the stratum compactum.

The pancreas

Eleven pancreas samples from groups A and B, detailed in Table 3.8, contained occasional PKX cells within the periacinar fatty tissue, sometimes associated with the type of inflammatory reaction described in the kidney (Fig.3.41).

Many pancreatic specimens before and after PKD infection in group A contained areas of increased cellularity, occasionally with fibrosis, within the lipoid tissue surrounding the Islet and acinar cells.

Infiltration appeared to be with lymphocytes and macrophages. This type of lesion was not seen in group B specimens.

Striated muscle

Of 46 muscle samples from PKX-positive fish in group A, three (two from fish with grade 4 and one from a fish with grade 5 renal swelling) showed the presence of PKX cells, together with a diffuse inflammatory response with fluid collection similar to that seen in the kidney (Fig. 3.42).

The intestine (including stomach, pyloric caeca, small and large intestine)

Of 153 samples from PKX-positive fish, one specimen from a group B fish with grade 3 renal swelling sampled on 2 July 1983 contained PKX cells in the submucosa of the caeca and small and large intestines. Affected villi were thickened due to increased cellularity of the submucosa, comprising macrophage-like cells and eosinophilic granule cells (Fig.3.43).

The brain

Of 95 brains from PKX-positive fish, one from a fish with grade 4 renal swelling from sample A16 contained PKX cells. These were present in a blood vessel of the pia mater and in an adjacent circumscribed area of the stratum fibrosum marginale of the optic tectum.

The ureter

Of 31 ureteral samples from PKX-positive fish collected from group B fish between 18 July 1983 and 1 September 1983, one sample was found to contain a PKX cell within the connective tissue surrounding the ureter.

No organisms were found within the lumina of the ureters.

Eye, gall bladder, gonad and swim bladder

Of 10 eye specimens from sample number B10, one showed fluid-filled spaces within the stromal lamellae of the cornea, without hitological evidence of cellular infiltration or parasitic, bacterial or fungal infection. Ten specimens of both gall bladder and gonad from sample number B10 showed no evidence of PKX cells. No swim bladder specimens from 31 PKX-positive fish collected from group B fish between 18 July 1983 and 1 September 1983 contained PKX cells.

RELATIONSHIP BETWEEN CLINICAL, GROSS PATHOLOGICAL AND HISTOPATHOLOGICAL SIGNS

The chronological relationship between the clinical signs, gross pathological changes and principal renal histopathological changes are summarized in Fig.3.44.

OTHER DISEASES

Nephrocalcinosis

The kidneys of two control fish and 11 group A fish contained histopathological signs of early nephrocalcinosis. Calcium deposits were present in proximal and distal tubules and collecting ducts. Occasional tubules had flattened epithelium and dilatation. The stomach musculature of four fish, two from group A and two from group B, and one kidney from group A contained several calcareous granulomata.

Pansteatitis

Occasional pancreatic specimens from group A fish sampled between 4 May 1982 and 1 July 1982 contained foci of increased cellularity within

the periacinar fat. Similar foci were seen in liver samples from group A fish throughout the experimental period and from group B fish from 23 June 1983 onwards. These changes were consistent with pansteatitis. However, no swim bladder abnormalities were noted, and there was no increase in ceroid-containing cells.

Acanthocephalan infestation

Infestation with an acanthocephalan of the genus Pomphorhynchus was present in fish from group A from 28 days after introduction to the experimental site until the end of the sampling period, and in occasional samples from group B fish. Infected samples presented with several proboscides penetrating the intestinal wall, occasionally numbering more than 50. Some worms were free within the abdominal cavity, whereas others were attached to internal structures such as swim bladder, liver, spleen or abdominal wall. These worms were normally encapsulated and necrotic. Several moribund fish from group A had single or multiple fistulae in the abdominal wall associated with emerging worms.

Histopathological changes related to worm attachment and penetration, and were similar to those described by Hine and Kennedy (1974) for Pomphorhynchus laevis infection in various species, and to those described by McDonough and Gleason (1981) for Pomphorhynchus bulbocolli in the rainbow darter, Etheostoma caeruleum.

Infection with the ciliate Ichthyophthirius multifiliis

Eleven gill specimens from group A fish sampled up to 67 days after introduction to the experimental site and two gill specimens from group B fish sampled on 27 July 1983 were infected with the ciliate Ichthyophthirius multifiliis. Single organisms were encountered between secondary gill

lamellae, often enclosed within hyperplastic epithelium with adjacent tissues showing necrosis. Secondary lamellar fusion with areas of epithelial hyperplasia were found in many specimens where no parasite was seen on histological examination. No clinical or gross pathological changes associated with the parasite were observed.

Infection with a species of the ciliate Trichodina

Eight gill samples from group A fish collected 42-92 days after introduction to the experimental site and 23 gill samples from group B fish collected throughout the experimental period contained occasional examples of a Trichodina species. Early epithelial hyperplasia was associated with the parasite.

Furunculosis

Furunculosis, caused by the Gram-negative bacterium Aeromonas salmonicida was diagnosed by culture of the organism on tryptone soya agar plates in brown trout at the experimental site on 14 June 1982. One group A fish sampled on 10 June 1982 contained histopathological evidence of bacterial infection. Group A fish were treated with food containing Aqualinic powder (Parke-Davis and Co., Pontypool, Gwent) at a concentration of 125 g Aqualinic powder/25 kg food, for 10 days commencing on 17 June 1982. Group B fish were not treated with any antibiotic preparation throughout the experimental period.

3. Diseased fish from other PKD-infected sites

Kidney specimens from fish at 16 other PKD-infected sites exhibited between them all the changes relating to PKD described in renal specimens from groups A and B. However, individual samples showed variation in the extent and number of changes. No histopathological changes which could be

ascribed to PKD were different from those seen in groups A and B.

DISCUSSION

The main purposes of these studies were:

1. To follow the development of clinical signs during the course of PKD from initial infection to recovery or death.

2. To study the tissues affected and the development of lesions in those tissues.

3. To relate the clinical signs to the gross and microscopic pathological lesions.

4. To compare the renal pathology with that of diseased fish from other PKD-infected sites.

With regard to the first purpose, the infection rate in group A was apparently 100% from 24 June 1982 to 23 August 1982 and in group B from 23 June 1983 to 29 July 1983, whereas morbidity, judged from gross renal pathology, was 100% in group A from 15 July 1982 and in group B from 14 July 1983. These rates agree with figures reported by Ferguson and Ball (1979) and Seagrave et al. (1981). It is interesting to note that, although group B was exposed 51 days earlier in the year than group A, PKX cells were identified in both groups for the first time in the first week of June, and the first sample to show 100% infection was collected on 24 June 1982 from group A and on 23 June 1983 from group B. This implies that the infective organism was not present in the water supply or that fish were intractable to infection or development of disease until a similar date in both years. This aspect will be discussed in more detail in chapter 5.

Clinical signs were evident from mid-July. Abdominal distension was the most frequent sign followed by exophthalmos, melanosis, corneal

cloudiness and ecchymoses. These signs are similar to those described by Plehn (1924), Ghittino et al. (1977), de Kinkelin and Gérard (1977a,b) and Ferguson and Needham (1978). Only 10 of the 25 fish with abdominal distension were dropsical, suggesting that ascites and oedema are not necessary sequelae of gross renal swelling. This supports the observation of de Kinkelin and Gérard (1977b) that ascites was found only when the disease was advanced. Severely affected fish and those recently dead were lighter in colour than normal, supporting the original description of Plehn (1924). However, melanosis has also been considered a presenting sign in PKD (Roberts and Shepherd, 1974; de Kinkelin and Gérard, 1977a,b; Ferguson and Needham, 1978). In the present study, only four fish were melanotic, and these were runts with severe acanthocephalan infestation. Therefore, it was not clear whether this infestation or PKD caused the melanosis. The bilateral corneal cloudiness noted in several ascitic fish was histologically similar to corneal oedema described by Lee, Roberts and Shepherd (1976). This might be expected to occur where fluid balance control was failing.

The mortality figures for groups A (6.5%) and B (3.3%) were low compared with those of most reported occurrences of PKD, despite the concurrent disease problems encountered during the experimental period. However, they do compare with the lower estimates in 0+ rainbow trout of 10% reported by Ferguson and Ball (1979) and 7% reported by Smith et al. (1984), and with the figures in Atlantic salmon parr of 1.3-9.1% and in brown trout of 8% reported by Ellis et al. (1985). The mortality figures may reflect the low stocking density of the experimental fish, being about a third of that found on many production sites. They may also have been due to the fish being subjected to few stress factors shown by Seagrave et al. (1981) to be associated with high mortalities caused by PKD. In

group A it was noticeable during the period of greatest pathology that many fish when netted showed marked signs of oxygen deficiency and may have died if this stimulus had been maintained for more than a few seconds.

With regard to the development of lesions, gross signs of disease were first seen at the beginning of July. Apart from various degrees of renal enlargement, the signs included empty alimentary tracts, distended gall bladders, gill pallor, pale, yellow livers, oedema and ascites, petechiation, and enlarged or roughened spleens. The only constant finding in affected fish was some degree of renal swelling. Of the other signs, aphagia, which resulted in gall bladder distension, has been described by Plehn (1924) and Ghittino et al. (1977), and gill pallor and oedema, with or without ascites, has been noted by these authors, as well as by de Kinkelin and Gérard (1977b) and Ferguson and Needham (1978). Splenic enlargement has been described by de Kinkelin and Gérard (1977b) and Ghittino et al. (1977) while spleens, smaller than normal, and perhaps equivalent to those seen in the present study with roughened surfaces, have been noted by Ferguson and Needham (1978). Previously unreported findings include the petechiation in gills and the gross thickening of individual primary lamellae, as well as the intrarenal white nodules of chronic inflammation in long-standing PKD cases. It would have been desirable to sample more fish with chronic inflammatory nodular lesions not only to determine how quickly these resolve and to follow the stages of resolution, but also to study the PKX cell, since the form of the organism seen in the granulomatous lesions may develop into a further stage of the parasite. However, it appears that only a small percentage of fish exposed during the early summer develop this type of lesion and findings in group A fish sampled in spring 1983 indicate that the organism was apparently cleared from infected fish by February.

The majority of fish sampled had intestinal contents, as found by Ferguson and Needham (1978). However, some fish with grades 3 and 4 renal swelling had pale, yellow livers and empty alimentary tracts or tracts containing few, mucoid faeces. This implies, as mentioned above, that prior to sampling the fish had been aphagic. However, the histological evidence suggested that the fish had not suffered prolonged inappetance, since in starved rainbow trout there is an increase in melanin and melano-macrophages in both kidney and spleen from one week after starvation commences (Agius and Roberts, 1981). This was not seen in these fish.

Histologically, the first possible indication of infection was in the kidney with the finding of macrophages containing inclusions (Fig.3.5) which could represent the infective stage of the PKX cell. No ultra-structural examples were found to investigate this possibility. PKX cells were first recognised in the kidney and then in the spleen. With increasing numbers of organisms other tissues were seen to be infected. These tissues were never infected with the number of PKX cells seen in the kidney. It appeared that in mild disease the infection only established in the kidney and spleen, whereas in severe disease infection was generalized. The same picture was noted by Ferguson and Needham (1978).

The principal changes which developed in the kidney as the disease progressed were those of haemopoietic hyperplasia, diffuse inflammation, glomerulus and tubule destruction, vascular obstruction, and crystal formation. As recovery started, changes were associated with organization of the diffuse inflammation and vascular lesions.

The increase in interstitial haemopoietic tissue which occurred two to four weeks after initial appearance of the PKX cell may be a generalized response to infection by the PKX cell analogous to bone marrow hyperplasia seen during many infections in man (Miale, 1982). The diffuse

inflammation around the PKX cells developed after this hyperplasia and was distinct from it. Associated with the diffuse inflammation was a decrease in tubule and glomerulus numbers, noted previously by Schäperclaus (1954), O'Brien et al. (1977) and Ferguson and Needham (1978). However, there were no large areas of necrosis such as were described by Schäperclaus (1954), but rather a progressive replacement of excretory elements by inflammatory tissue, with hypercellularity of tubules within affected tissue. This hypercellularity was sometimes due to lymphocytes, but more often due to increased numbers of epithelial cells, some with pyknotic nuclei. Affected tubules showed no peritubular fibrosis, again suggesting little tissue necrosis, although collagenous sheaths surrounded some collecting and mesonephric ducts. However, these sheaths have been described in normal rainbow trout kidneys (Yasutake and Wales, 1983). The sclerosis of glomeruli described by Ferguson and Needham (1978) was an infrequent finding and calcium deposition within glomeruli was not found even in the most severe infections. These changes are more characteristic of nephrocalcinosis (Harrison and Richards, 1979), and early signs of this condition were seen in some experimental fish perhaps explaining the occasional example of periglomerular fibrosis, increased glomerular cellularity and thickened glomerular basement membranes.

The eosinophilic droplets in proximal tubule epithelium may, by analogy with findings in other animals (Brewer and Eguren, 1962), be proteinaceous. They were found on histological examination in both apparently healthy and infected fish, although neither Anderson and Loewen (1975) or Yasutake and Wales (1983) describe similar droplets in their histological studies of kidneys from normal trout. The amount of hyaline material was accentuated in proximal tubules within hyperplastic interstitial tissue. This may imply dysfunction of the tubular epithelium or

decreased glomerular filtration efficiency, allowing a greater protein load in the filtrate than normal. Similar histological findings were reported by Daoust and Ferguson (1984) in fingerling rainbow trout subjected to ammonia stress. It was suggested that the ammonia caused a diuresis, with the increased glomerular filtration rate resulting in an increase in plasma proteins being presented for tubule epithelium re-absorption. The histopathological picture may also imply that the protein load presented to the glomerulus was greater than normal. A proteinuria with increased tubular epithelial hyaline material was described by Ellis, Hastings and Munro (1981) following intramuscular injection of extracellular products of Aeromonas salmonicida into rainbow trout which caused muscle liquefaction.

Unlike the haemopoietic hyperplasia, the diffuse inflammatory response was centred around PKX cells. It contained little connective tissue initially, but as the disease progressed the lesions assumed the characteristics of chronic inflammation.

The vascular pathology presented at least two distinct forms, one with the organism present, the other without. The early lesions appeared to result from the presence of the parasite within blood vessels causing cellular aggregation on the endothelium but little fibrin formation. Blood vessel margins adjacent to the aggregations were indistinct, indicating possible endothelial damage with subsequent thrombocyte aggregation. This may be analogous to platelet adherence and aggregation during mammalian haemostasis where subendothelial tissue is exposed (Born, 1980).

As the vascular lesions became more extensive, fusiform crystals formed and these, together with small numbers of cells, were found in blood vessels in most tissues, often without the presence of PKX cells. The staining properties of the crystals suggested a haem fraction, but

negative staining by Stein's method indicated that they were not crystallized bile pigments. These pigments might have been expected to increase in quantity with increased haem catabolism. The crystals were unstained by Perls' method for ferric iron, suggesting that any iron was tightly bound to other components, as occurs in haem and haemoglobin.

Although haemoglobin crystallization has been recorded in several species of fish (Dawson, 1932; Kisch, 1949; Conroy and Rodriguez, 1965; Knight, 1964), the crystals in these reports have been described as intra- rather than extra-erythrocytic and of various shapes but not fusiform with light-reflecting, transverse lines as was found in the rainbow trout with PKD. Many of the crystals were apparently phagocytosed by macrophages perhaps prior to recycling for haemopoiesis.

During organization and healing of lesions the majority of fish had an increase in the amount of Schmorl's positive material in macrophages, especially in the kidney. This material was probably lipofuscin which is a product of processing dead and dying cells. PKX cells appeared to be dying and there was little evidence of fibrosis. In some fish, however, PKX cells were not destroyed but were surrounded by large amounts of connective tissue as part of a granulomatous response. These chronic nodules were found especially in fish with long-standing infections sampled as water temperatures were decreasing from the summer peaks. The immune response to antigenic stimulus in poikilotherms at low ambient temperatures has been reviewed by Avtalion, Wojdani, Malik, Shahrabani and Duczyminer (1973) who showed depression or abolition of the immune response at low temperatures. A similar depression, despite prolonged antigenic stimulus by PKX cells, may be occurring in these long-standing cases.

Despite the severity of renal pathology in PKD, few fish showed

oedema and ascites. This was similar to the findings of Harrison and Richards (1979) in severe nephrocalcinosis. It suggests that other organs, probably the gill and intestine, maintain ionic and fluid balance in most cases until organization of affected renal tissue begins. At this stage many immature tubules and glomeruli were seen in histological examination, suggesting that the kidney is capable of rapid regeneration and return of functional efficiency once PKX cells have been destroyed or contained.

With regard to the third purpose of these studies, From Fig.3.44 relating clinical signs, gross pathology and histopathology it can be seen that no clinical signs or gross pathological changes occurred for at least a month after initial appearance of renal PKX cells. The majority of these signs developed in fish with grade 3 or 4 renal swelling. Signs of anaemia occurred once vascular pathology, including haemoglobin crystal formation, was pronounced.

With regard to the fourth purpose of these studies, the results of the histopathological examination of kidneys from 16 positive sites in England, Scotland and Wales indicated that the severity of lesions varied between farms, Samples collected earlier in the summer generally showed fewer changes. Lesions were similar to those seen in groups A and B. The progression of pathological changes in groups A and B can probably be applied to other infected sites, although the time course will vary. This aspect of PKD will be considered in more detail in chapter 5.

It was hoped that studies of the pathology of PKD would help elucidate the pathogenesis of the disease. Several findings may be relevant in this context.

Ferguson and Needham (1978) suggested that infection may occur via the ureters since they found PKX cells in some renal tubules. In the present study there was no evidence of the site of entry of the organism.

However, recognisable PKX cells were found initially within renal peritubular capillaries and not within the tubules. This perhaps suggested that PKX cells arrived in the kidney via the circulation from the site of entry rather than via the ureters. The kidney is supplied with blood by the renal artery from the dorsal aorta. This supply serves the glomeruli initially via the afferent arterioles and the peritubular capillaries secondarily via the efferent arterioles. Therefore, if PKX cells were arriving in the renal artery, it might be expected that they would be found in the glomeruli. This was a rare finding. However, the peritubular capillaries receive a second supply from the caudal vein forming a renal portal system, and this appeared to be the supply carrying PKX cells to the kidney. It is interesting to speculate on whether the kidney was actively filtering PKX cells from the circulation. Renal uptake of denatured human serum albumin was demonstrated in chars by Dannevig and Berg (1978), and similar active renal filtration was described in plaice, Pleuronectes platessa L., by Ellis (1980) and in mirror carp, Cyprinus carpio L., by Secombes and Manning (1980). In rainbow trout, macrophages in close association with the endothelium of peritubular capillaries were shown to filter bacteria from the circulation by Ferguson (1984).

It was noticeable in the early stages of infection that large areas of kidney were apparently free from PKX cells, whereas localized areas contained several organisms. This perhaps suggests that preferential routes for parasite migration existed within the kidney or that PKX cells were multiplying in situ. This aspect will be considered further in chapter 5.

Ferguson and Needham (1978) also suggested that the causative organism of PKD may be released via the urinary tract. In the present studies, PKX

cells were found within renal tubules in small numbers from the beginning of July and one spore form similar to possible Myxosporidan spores described by Hedrick et al. (1984) was seen in one fish with healing lesions. However, no organisms similar to the PKX cell, their inclusions or spores were seen in ureteral sections from fish with severe infection. The release of the organism will be considered further in chapter 6.

Foci of increased cellularity within livers were found without PKX cells in many infected fish. Similar foci, centred on bile ducts, have been described by Roberts (1978) as metastatic haemopoietic tissue. This type of lesion has also been described in pansteatitis by Roberts, Richards and Bullock (1979), a condition associated with diets containing low vitamin E levels and high concentrations of unsaturated fatty acids. This perhaps indicates that a dietary factor may be of importance in the exacerbation of the effects of PKD. It is interesting that lipoid degeneration of the liver, described by Davis (1953), was characterized by some signs similar to those of PKD, including pale, yellow livers sometimes with petechiation, anaemia, exophthalmos, ascites, and empty intestines apart from pale yellow fluid. This condition was found especially where diets contained excess fat.

There was evidence of extracellular haemoglobin crystallization in the majority of fish with grades 3 and 4 renal swelling, and some of these fish showed splenic haemosiderosis. It is interesting to speculate on whether the intravascular aggregations seen during the development of PKD resulted in haemostasis and subsequent haemolysis. That more evidence of haemolysis, such as haemoglobinuria, was not found perhaps indicated that released haem was rapidly recycled during haemopoiesis. However, with many blood vessels obstructed, newly-formed erythrocytes may not have been freely released into the circulation. This may have contributed to

the anaemic appearance of affected fish and provided a further stimulus to haemopoiesis.

Table 3.1a. Sampling schedule of naturally-infected rainbow trout in group A.

Sample date	Sample number	Number of fish in sample	Lengths of sample fish (mm)		Weights of sample fish (g)	
			Mean	Range	Mean	Range
4.5.82	1	10	ND		ND	
14.5.82	2	10	80	77-84	8	7-9
19.5.82	3	10	83	77-89	9	6-12
27.5.82	4	10	88	86-92	12	10-13
4.6.82	5	10	104	92-114	16	12-20
10.6.82	6	10	100	88-113	14	9-21
17.6.82	7	10	107	70-123	19	4-27
24.6.82	8	10	123	97-138	29	11-40
1.7.82	9	10	137	105-150	39	18-51
5.7.82	10	5	148	127-162	48	31-62
8.7.82	11	10	138	120-150	42	28-58
15.7.82	12	10	150	117-166	55	23-72
22.7.82	13	10	145	100-172	49	12-78
30.7.82	14	5	162	117-182	68	23-99
9.8.82	15	5	161	137-201	60	33-106
16.8.82	16	5	162	152-179	66	49-90
23.8.82	17	5	172	154-185	73	50-87
31.8.82	18	5	179	168-195	74	60-87
13.9.82	19	5	191	169-218	90	59-132
23.9.82	20	5	194	159-215	103	60-131
10.3.83	21	10	ND		ND	
25.3.83	22	5	ND		ND	

ND - not done

Table 3.1b. Sampling schedule of naturally-infected rainbow trout in group B.

Sample date	Sample number	Number of fish in sample	Lengths of sample fish (mm)		Weights of sample fish (g)	
			Mean	Range	Mean	Range
25.3.83	1	5	ND		ND	
20.4.83	2	5	ND		ND	
6.5.83	3	5	ND		ND	
20.5.83	4	5	ND		ND	
2.6.83	5	5	153	143-158	45	40-50
13.6.83	6	5	157	151-165	51	42-57
23.6.83	7	5	173	164-188	68	56-81
30.6.83	8	5	180	173-190	94	78-109
4.7.83	9	10	171	158-184	73	55-92
14.7.83	10	5	191	177-205	ND	
18.7.83	11	10	183	132-200	92	33-123
21.7.83	12	5	175	164-182	75	60-89
27.7.83	13	10	184	166-204	92	57-126
29.7.83	14	5	182	165-201	84	61-115
1.9.83	15	6	182	137-204	79	26-101

ND - not done

Table 3.2. Selective staining methods and their application.

Component stained	Method and original source	Immediate source of method *	Total number of specimens stained by each method		
			Kidney	Spleen	Liver
Acid mucopolysaccharides	Lison's alcian blue method (1954)	1	4	-	-
Bile pigment	Stein's method (1935)	3	4	-	-
Calcium salts	Von Kossa's method (1901)	2	3	1	-
Carbohydrates	Periodic acid Schiff (PAS) method of McManus (1946)	3	10	-	2
Collagen	Picro-fuchsin method of Van Gieson (1889)	2	16	1	1
Deoxyribonucleic acid (DNA)	Feulgen and Rossenbeck's method (1924)	3	7	-	-
Elastin	Aldehyde fuchsin method of Scott (1952)	1	4	-	-
Ferric iron	Perls' method (1867)	1	45	53	23
Fibrin	Picro-Mallory method of McFarlane (1944)	1	20	6	5
Haemoglobin	Dunn and Thompson's method (1945)	3	3	-	2
Lipids	Oil Red O method of Lillie and Ashburn (1943)	3	5	-	-
Lipofuscin	Schmorl's method modified by Lillie (1954)	1	24	24	1
Protozoa	Giemsa's method (1902)	3	23	23	23
Reticulin	Gordon and Sweets' method (1936)	3	8	2	-

* 1 = Disbrey and Rack (1970); 2 = Drury and Wallington (1973); 3 = Bancroft and Stevens (1977)

- = none

Table 3.3a. Number of experimental fish in group A in categories 1 to 7 at each sampling.

Sample number	Number of fish in category: (1) Grade 0 kidney swelling PKX negative	(2) Grade 0 kidney swelling PKX positive	(3) Grade 1 kidney swelling	(4) Grade 2 kidney swelling	(5) Grade 3 kidney swelling	(6) Grade 4 kidney swelling	(7) Grade 5 kidney swelling and advanced histological signs of recovery	Total	Sample date
A 1-4	40	0	0	0	0	0	0	40	4.5.82-27.5.82
A 5	9	1	0	0	0	0	0	10	4.6.82
A 6	4	6	0	0	0	0	0	10	10.6.82
A 7	2	8	0	0	0	0	0	10	17.6.82
A 8	0	10	0	0	0	0	0	10	24.6.82
A 9	0	10	0	0	0	0	0	10	1.7.82
A 10	0	3	1	1	0	0	0	5	5.7.82
A 11	0	3	3	3	1	0	0	10	8.7.82
A 12	0	0	3	5	2	0	0	10	15.7.82
A 13	0	0	0	6	4	0	0	10	22.7.82
A 14	0	0	0	2	3	0	0	5	30.7.82
A 15	0	0	0	0	2	0	3	5	9.8.82

Table 3.3a (continued)

Sample number	Number of fish in category: (1) Grade 0 kidney swelling PKX negative	(2) Grade 0 kidney swelling PKX positive	(3) Grade 1 kidney swelling	(4) Grade 2 kidney swelling	(5) Grade 3 kidney swelling	(6) Grade 4 kidney swelling	(7) Grade 5 kidney swelling and advanced histological signs of recovery	Total	Sample date
A 16	0	0	0	1	0	1	3	5	16.8.82
A 17	0	0	0	0	0	1	4	5	23.8.82
A 18	0	0	0	0	1	0	4	5	31.8.82
A 19	0	0	0	0	1	0	4	5	13.9.82
A 20	0	0	0	0	0	0	5	5	23.9.82
A 21	10	0	0	0	0	0	0	10	10.3.83
A 22	5	0	0	0	0	0	0	5	25.3.83
Total	70	41	7	18	14	2	23	175	

Table 3.3b. Number of experimental fish in group B in categories 1 to 7 at each sampling.

Sample number	Number of fish in category: (1) Grade 0 kidney swelling PKX negative	(2) Grade 0 kidney swelling PKX positive	(3) Grade 1 kidney swelling	(4) Grade 2 kidney swelling	(5) Grade 3 kidney swelling	(6) Grade 4 kidney swelling	(7) Grade 5 kidney swelling and advanced histological signs of recovery	Total	Sample date
B 1-4	20	0	0	0	0	0	0	20	25.3.83-20.5.83
B 5	4	1	0	0	0	0	0	5	2.6.83
B 6	4	1	0	0	0	0	0	5	13.6.83
B 7	0	5	0	0	0	0	0	5	23.6.83
B 8	0	5	0	0	0	0	0	5	30.6.83
B 9	0	7	3	0	0	0	0	10	4.7.83
B 10	0	0	5	0	0	0	0	5	14.7.83
B 11	0	0	0	6	4	0	0	10	18.7.83
B 12	0	0	0	0	5	0	0	5	21.7.83
B 13	0	0	0	0	8	2	0	10	27.7.83
B 14	0	0	0	0	3	2	0	5	29.7.83
B 15	0	0	0	0	0	1	5	6	1.9.83
Total	28	19	8	6	20	5	5	91	

Table 3.4. Clinical signs in naturally-infected rainbow trout in groups A and B.

Category of fish	Number of fish in each category	Number of fish in each category exhibiting: Abdominal distension	Corneal cloudiness	Ecchymoses	Exophthalmos	Melanosis	Total number of fish with clinical signs
1. Grade 0 renal swelling; PKX negative	98	0	0	0	0	0	0
2. Grade 0 renal swelling; PKX positive	60	0	0	0	0	0	0
3. Grade 1 renal swelling	15	0	1	0	0	0	1
4. Grade 2 renal swelling	24	2	0	0	1	2	2
5. Grade 3 renal swelling	34	17	0	0	6	2	20
6. Grade 4 renal swelling	7	6	0	1	3	0	6
7. Grade 5 renal swelling and advanced histological signs of recovery	28	0	0	0	0	0	0
Total	266	25	1	1	10	4	29

Table 3.5. Kidney grading in naturally-infected rainbow trout in groups A and B.

Fish group	Sample date	Days after first PKX positive sample	Number of fish at each sampling exhibiting kidney swelling of grade: 0	1	2	3	4	5
A 10	5.7.82	31	3	1	1	0	0	0
11	8.7.82	34	3	3	3	1	0	0
12	15.7.81	41	0	3	5	2	0	0
13	22.7.82	48	0	0	6	4	0	0
14	30.7.82	56	0	0	2	3	0	0
15	9.8.82	66	0	0	0	2	0	3
16	16.8.82	73	0	0	1	0	1	3
17	23.8.82	80	0	0	0	0	1	4
18	31.8.82	88	0	0	0	1	0	4
19	13.9.82	101	0	0	0	1	0	4
20	23.9.82	111	0	0	0	0	0	5
B 9	4.7.83	32	7	3	0	0	0	0
10	14.7.83	42	0	5	0	0	0	0
11	18.7.83	46	0	0	6	4	0	0
12	21.7.83	49	0	0	0	5	0	0
13	27.7.83	55	0	0	0	8	2	0
14	29.7.83	57	0	0	0	3	2	0
15	1.9.83	91	0	0	0	0	1	5

No gross kidney pathology was seen at other samplings.

Table 3.6. Gross pathological changes in naturally-infected rainbow trout in groups A and B.

Category of fish (No. in category)	Number of fish in each category exhibiting: alimentary tract empty	gall bladder full	gill pallor	liver pale/ yellow	oedema and/or ascites	petech-iation	spleen enlarged	spleen with roughened surface	Total No. of fish with gross changes
1. Grade 0 renal swelling; PKX negative (98)	0	0	0	0	0	0	0	0	0
2. Grade 0 renal swelling; PKX positive (60)	0	0	0	1	0	0	0	2	3
3. Grade 1 renal swelling (15)	0	0	0	0	0	1	0	1	2
4. Grade 2 renal swelling (24)	0	0	0	1	2	0	1	1	5
5. Grade 3 renal swelling (34)	4	2	3	5	1	10	7	6	23
6. Grade 4 renal swelling (7)	4	0	5	7	7	1	1	1	7
7. Grade 5 renal swelling; recovering (28)	1	1	0	2	0	0	1	1	4
Total (266)	9	3	8	16	10	12	10	12	44

Table 3.7. Principal renal histopathological changes in naturally-infected rainbow trout in groups A and B.

Category of fish	No. of samples	Number of renal specimens in each category showing: haemo-poietic hyper-plasia	diffuse inflamm-ation	glomerular and tubule destruction	vascular aggreg-ations	crystals	organizing diffuse inflamm-ation	increased lipo-fuscin	Total No. of renal samples with changes
1. Grade 0 renal swelling; PKX negative	98	0	0	0	0	0	0	0	0
2. Grade 0 renal swelling; PKX positive	60	42	13	8	23	0	0	0	42
3. Grade 1 renal swelling	15	14	12	10	11	3	1	1	15
4. Grade 2 renal swelling	24	21	24	19	17	12	2	2	24
5. Grade 3 renal swelling	34	32	34	29	28	29	4	3	34
6. Grade 4 renal swelling	7	7	7	7	6	7	0	1	7
7. Grade 5 renal swelling recovering	28	17	6	6	1	7	10	23	27
Total	266	133	96	79	86	58	17	30	149

Table 3.8. Distribution of PKX cells in the internal organs of naturally-infected rainbow trout in groups A and B.

Category of fish	Number of samples in each category	Number of samples containing PKX cells in: Gill	Liver	Pancreas	Spleen
1. Grade 0 renal swelling; PKX negative	98	0	0	0	0
2. Grade 0 renal swelling; PKX positive	60	2	2	1	31
3. Grade 1 renal swelling	15	9	6	0	15
4. Grade 2 renal swelling	24	15	13	5	21
5. Grade 3 renal swelling	34	24	17	1	33
6. Grade 4 renal swelling	7	7	7	2	7
7. Grade 5 renal swelling and advanced histological signs of recovery	28	1	2	0	5
Total	266	58	47	9	112

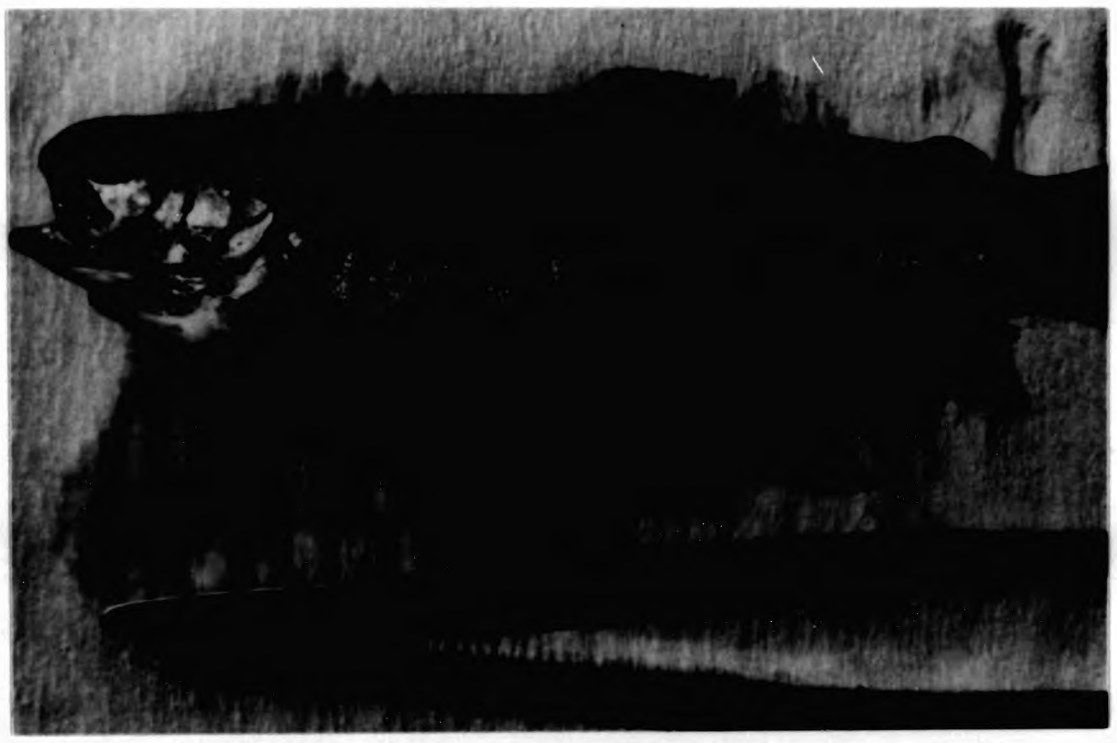


Fig. 3.1. Clinical PKD. Rainbow trout with abdominal distension and exophthalmos.

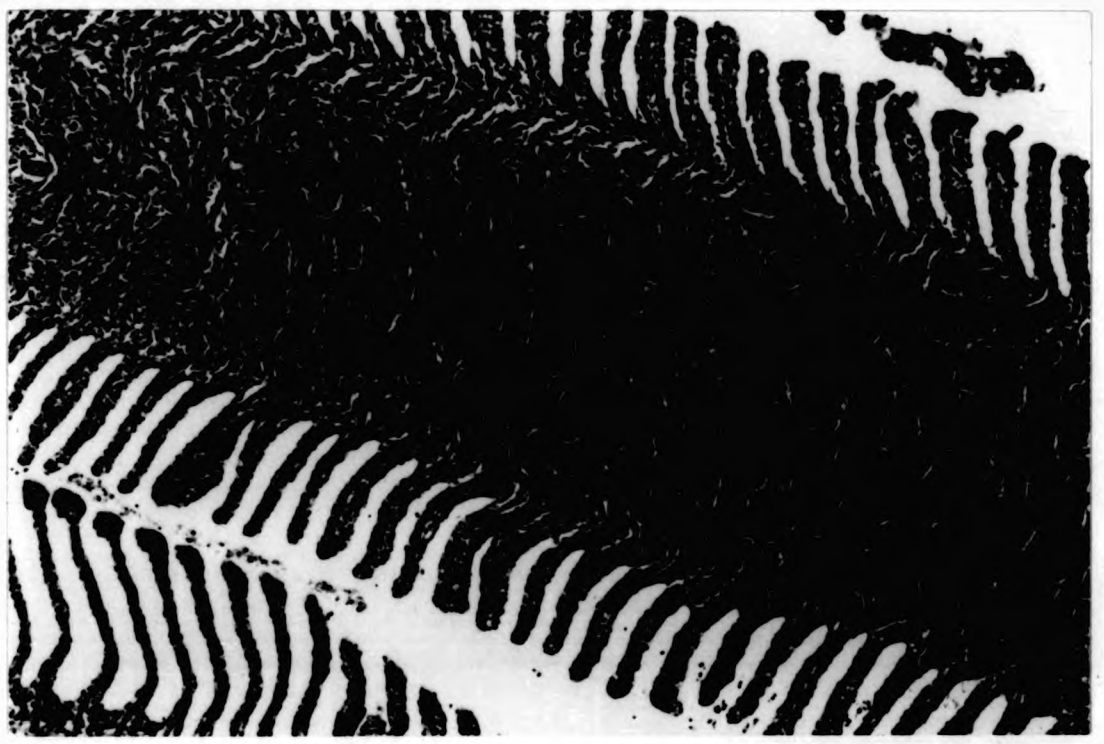
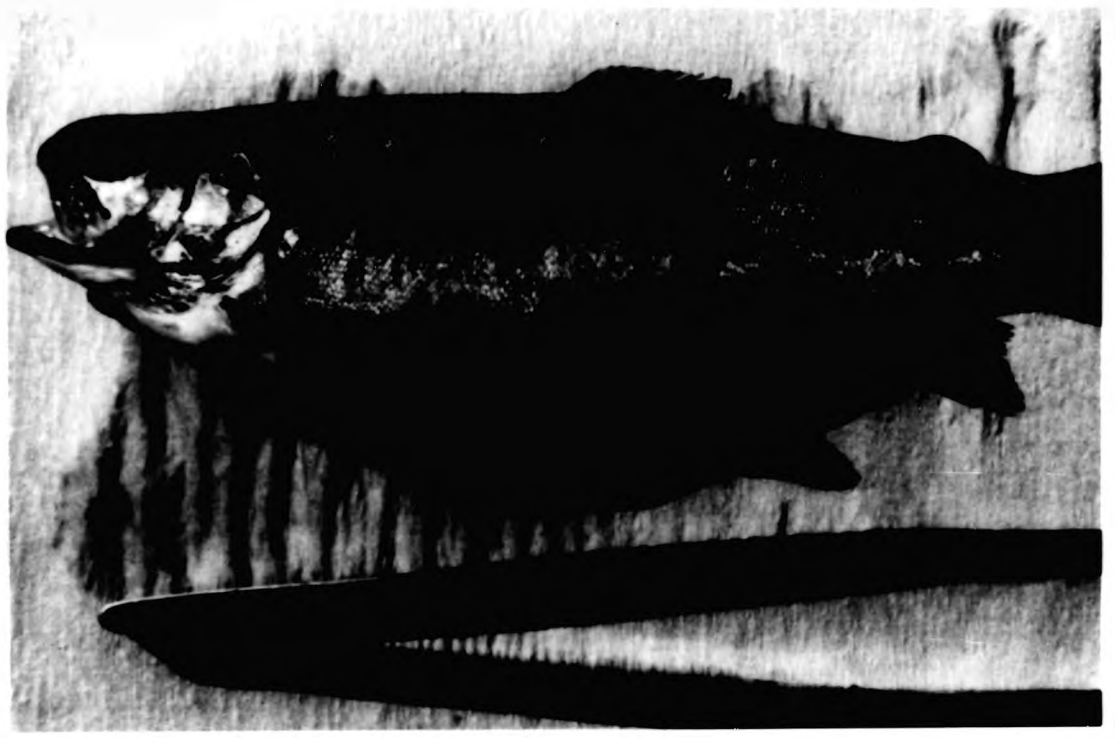


Fig. 3.2. Pathology of PKD. Thickened primary lamella in gill of rainbow trout. H & E x 85.



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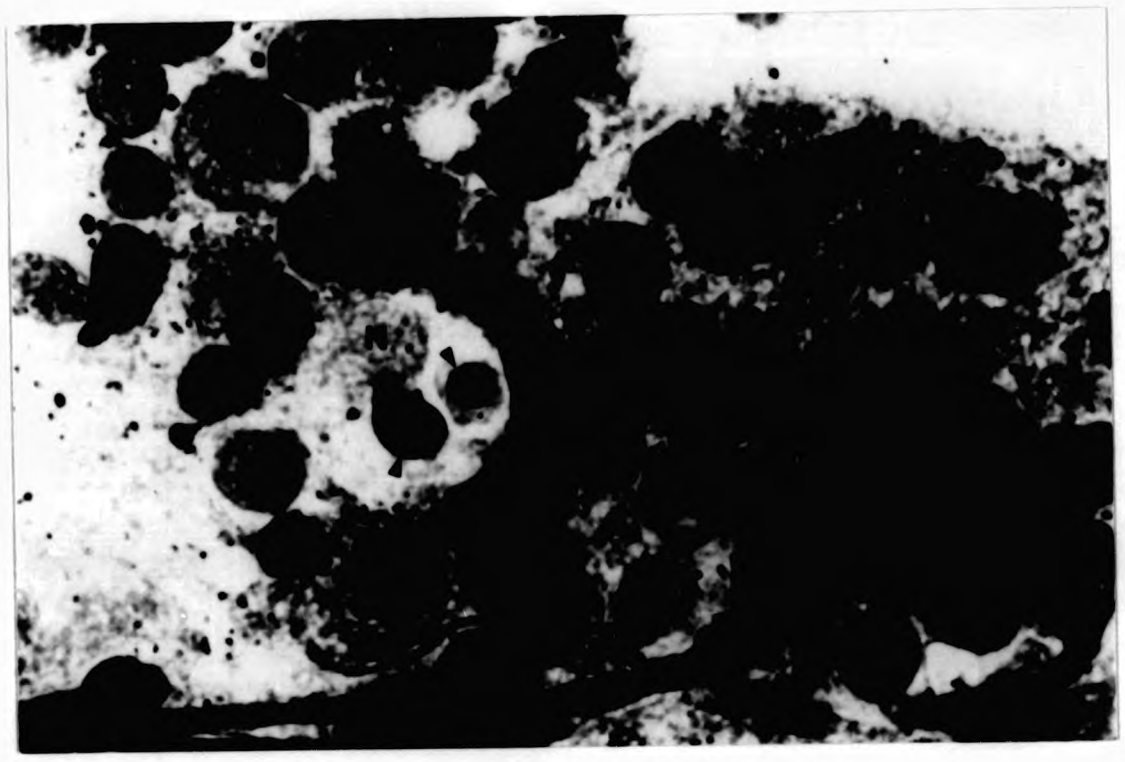

Fig. 3.3. Macrophage with pale nucleus (N) and two intracytoplasmic inclusions (arrowed). Several cells (L) are apparently closely associated with the cell. Kidney smear stained with May-Grünwald-Giemsa. x 1240.

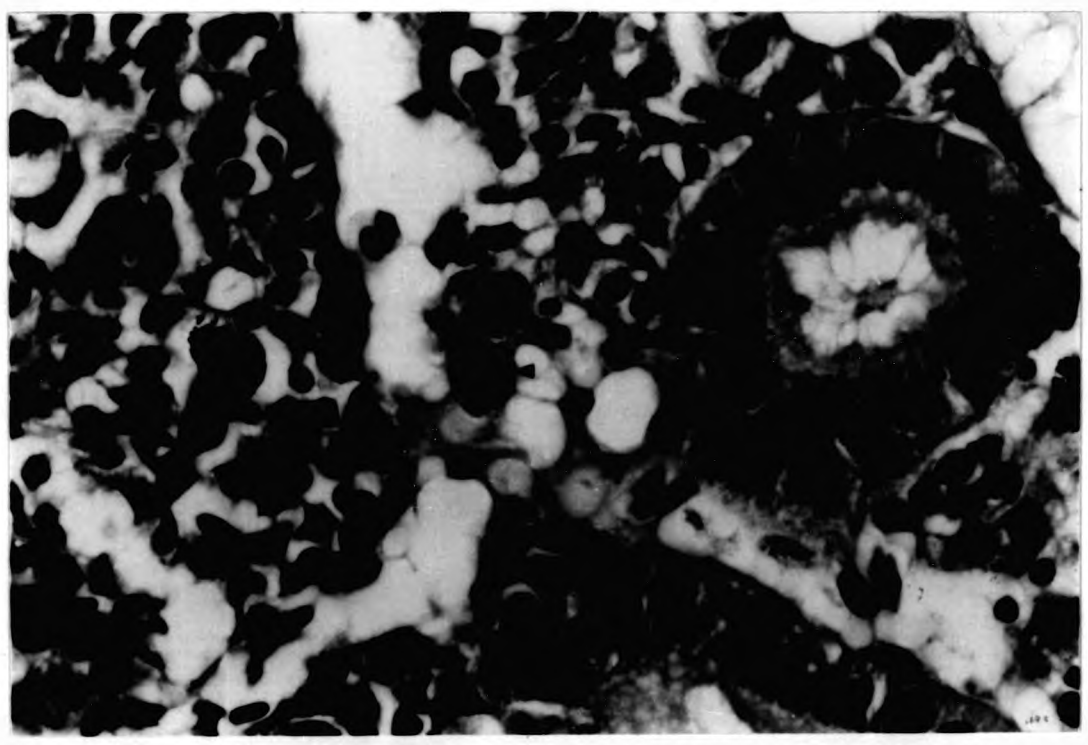

Fig. 3.4. PKX cell (arrowed) within a renal peritubular capillary. H & E x 745.

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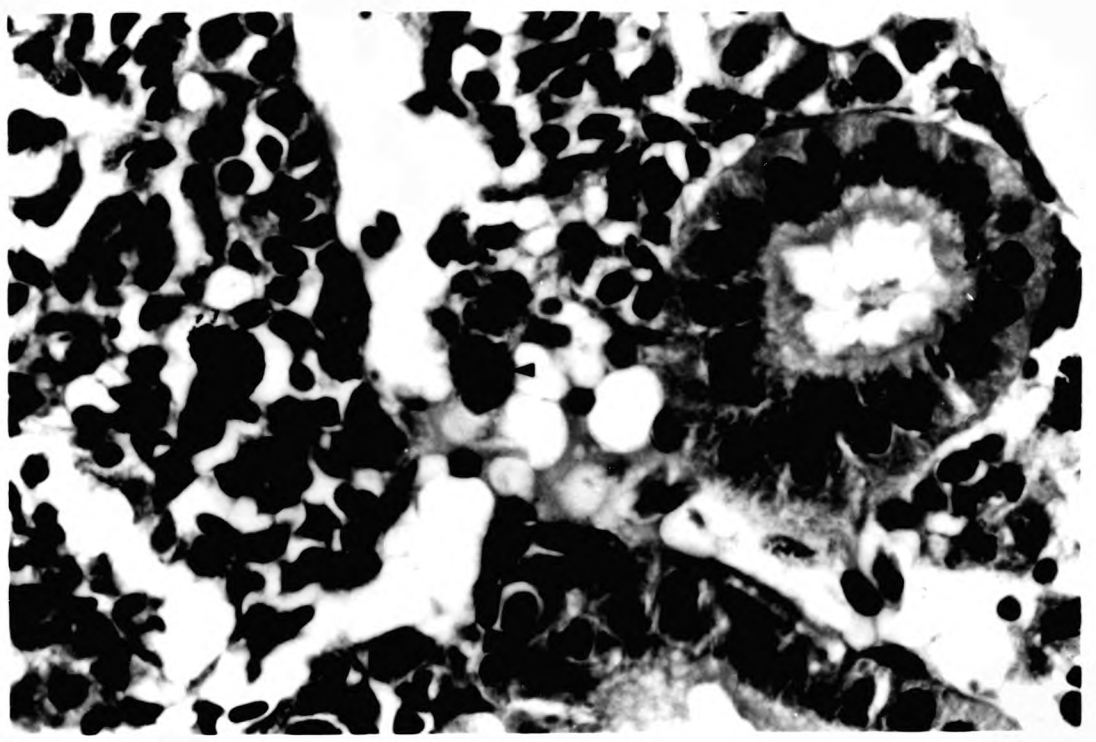

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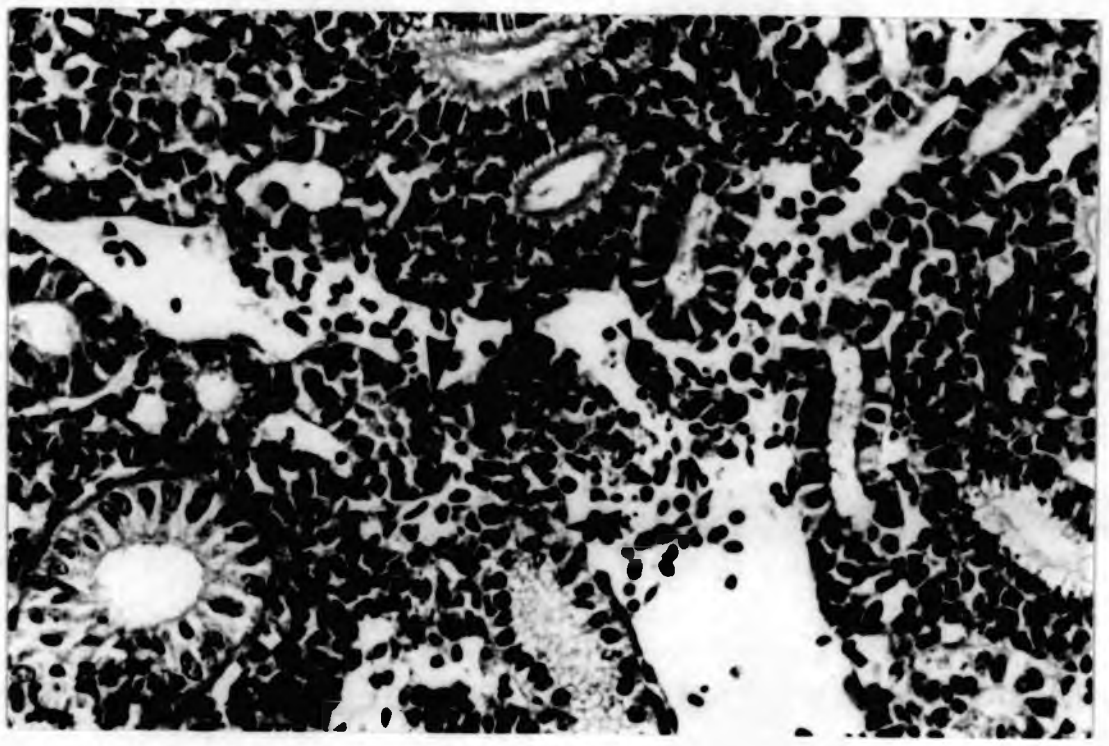


Fig. 3.5. Intrarenal sinus containing cellular aggregation, with PKX cells (small arrows) attached to vessel wall, with apparent loss of definition of endothelial lining (between large arrows). H & E x 325.

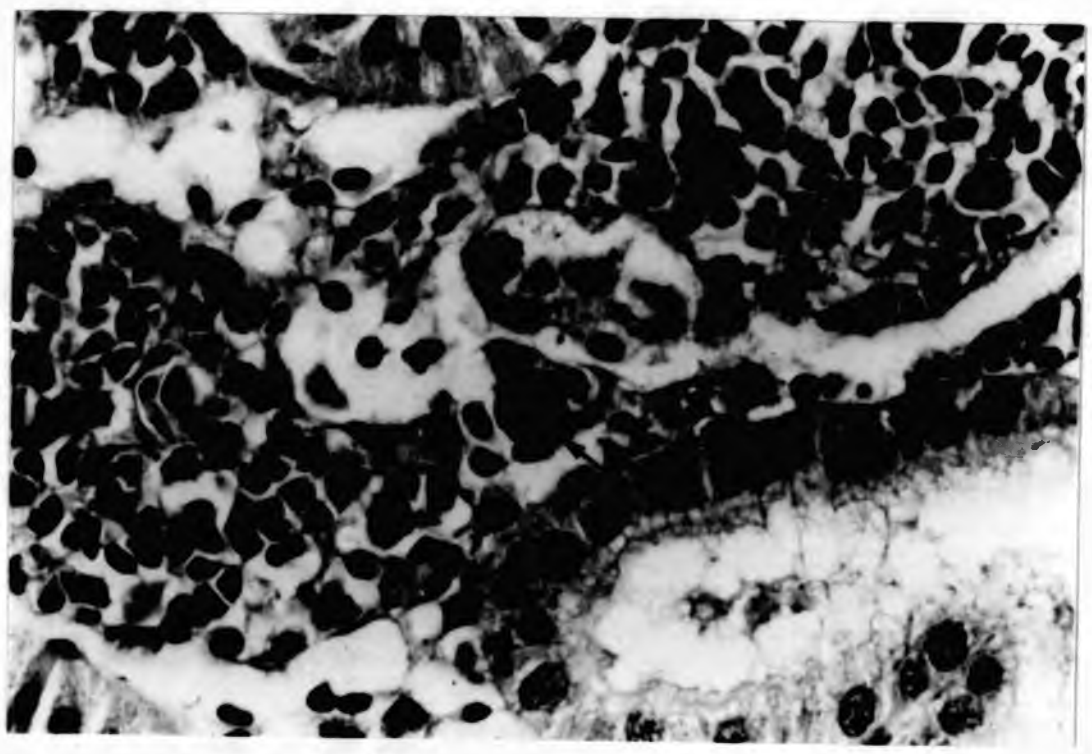
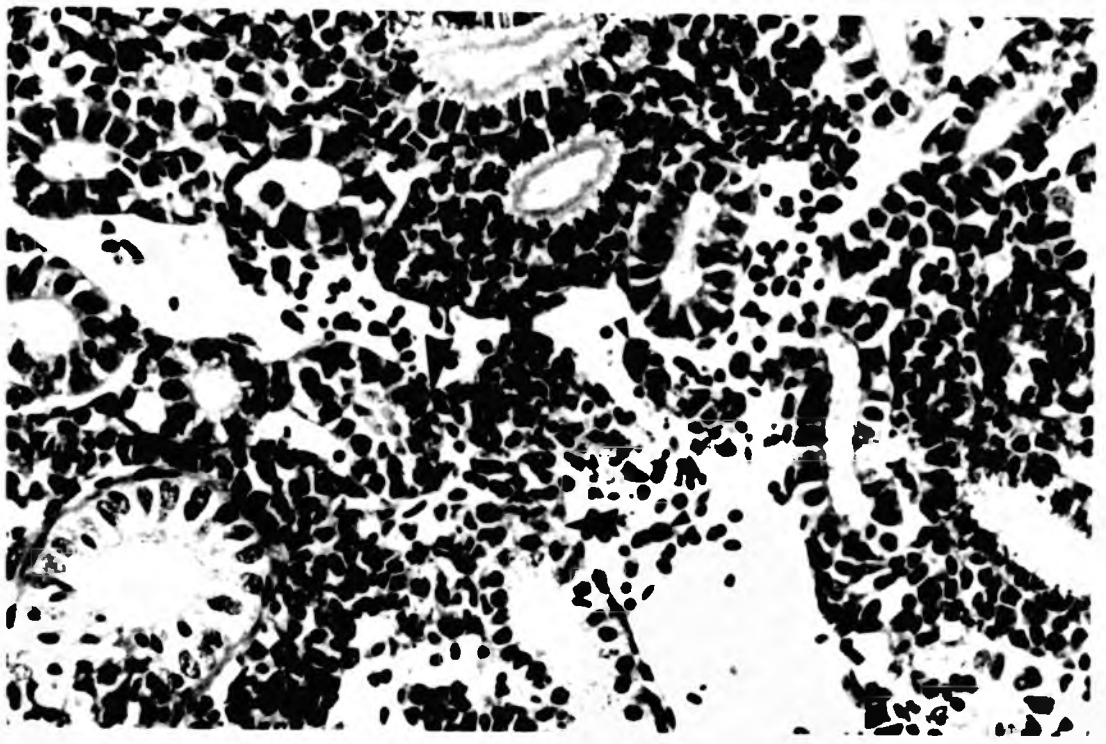


Fig. 3.6. PKX cell (arrowed), apparently in close association with three host cells, within peritubular capillary. H & E x 745.



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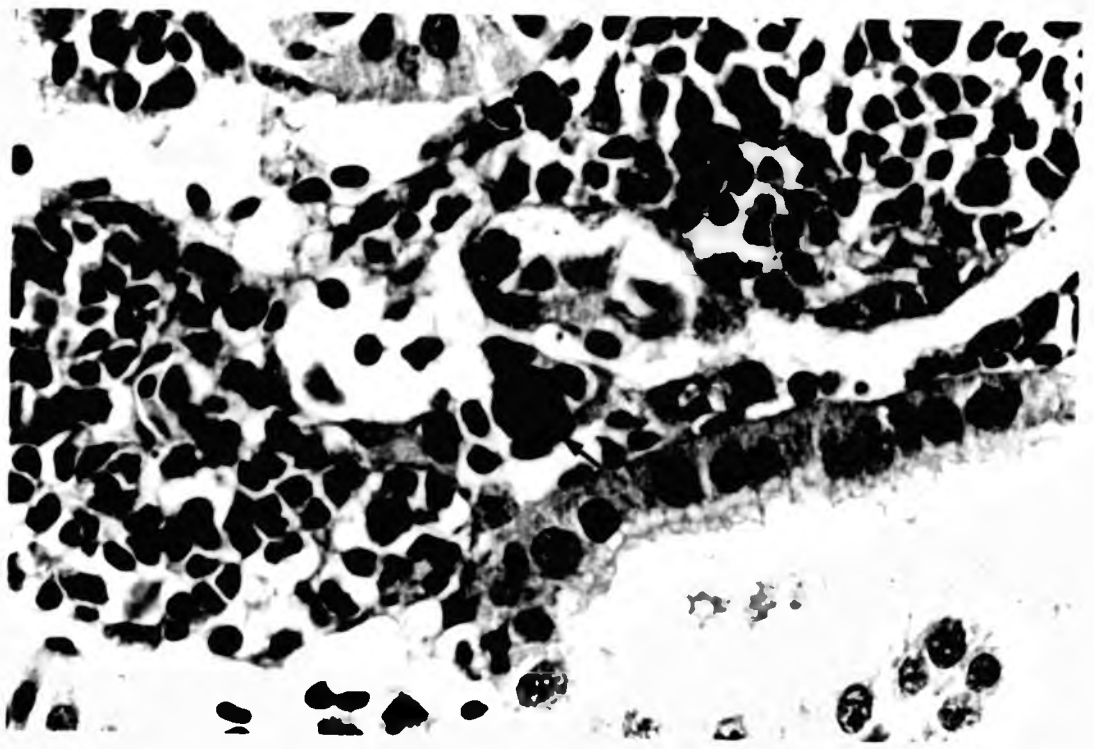

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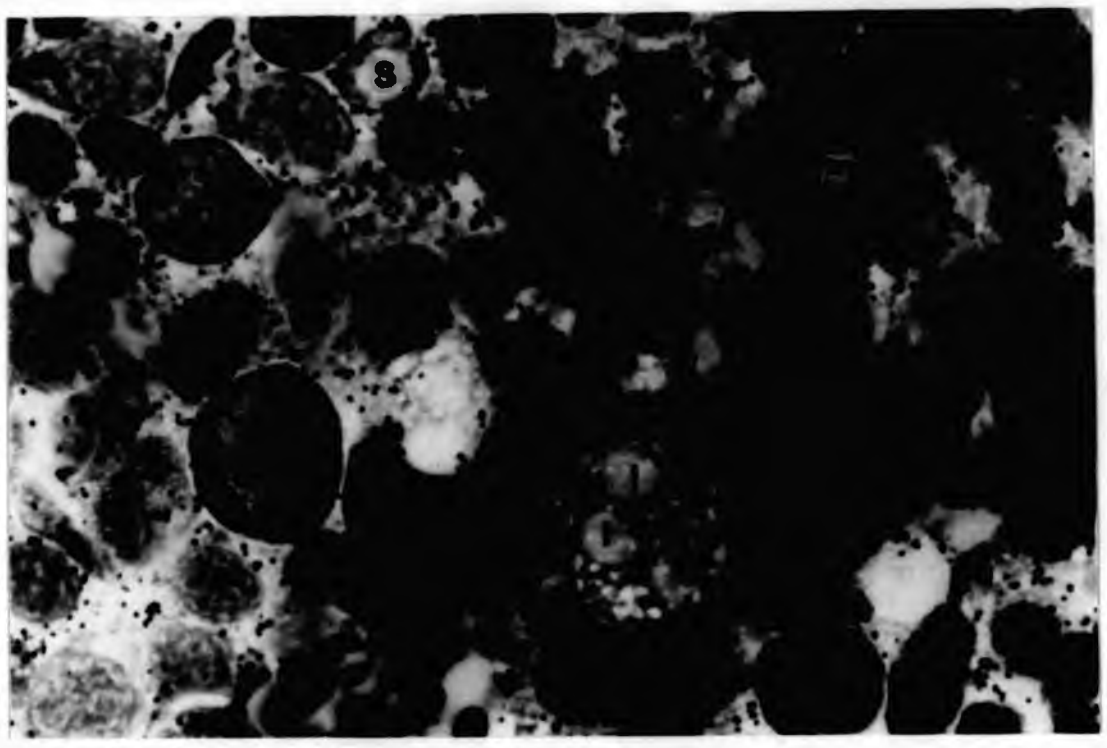


Fig. 3.7. PKX cell (P) containing at least two inclusions (I) with three host cells in close apposition (L). Possible small PKX cell (S). Kidney smear stained with May-Grünwald-Giemsa. x 1210.

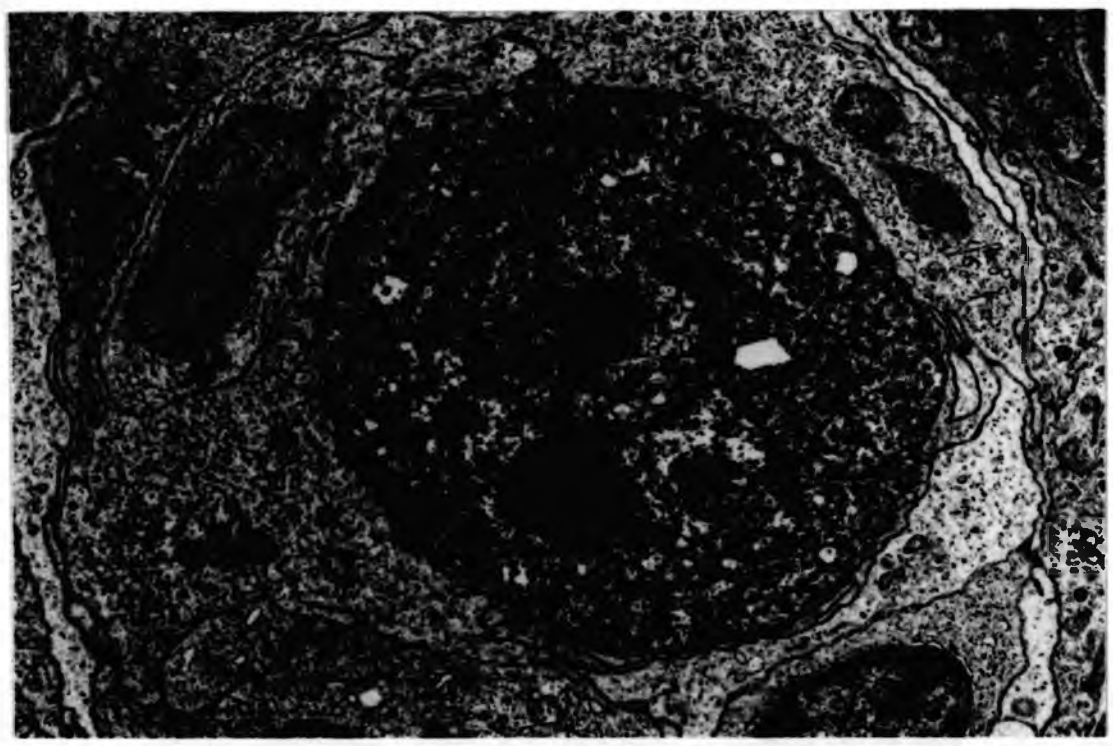
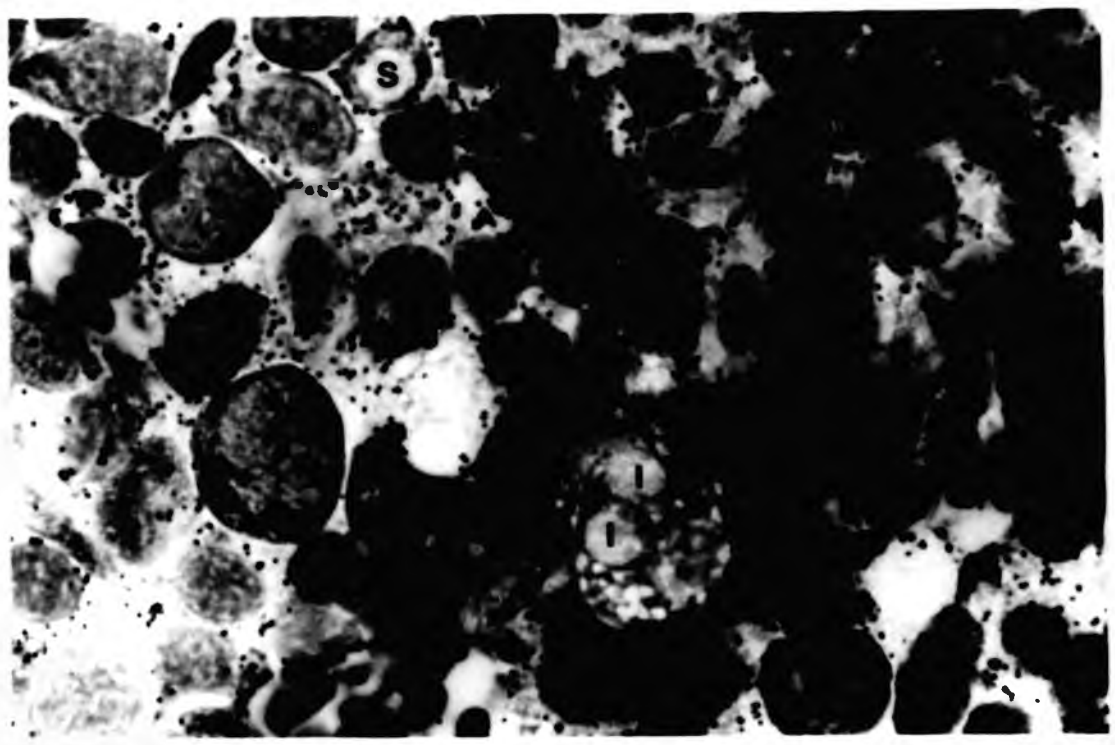


Fig. 3.8. Electron-micrograph of PKX cell (P) containing many "haplosprosomes", occasional mitochondria (arrowed) and an inclusion (I) in cytoplasm. Surrounded by cytoplasm (C) of (?) three host cells, two including nuclei (N). Uranyl acetate and lead citrate. x 3,300.



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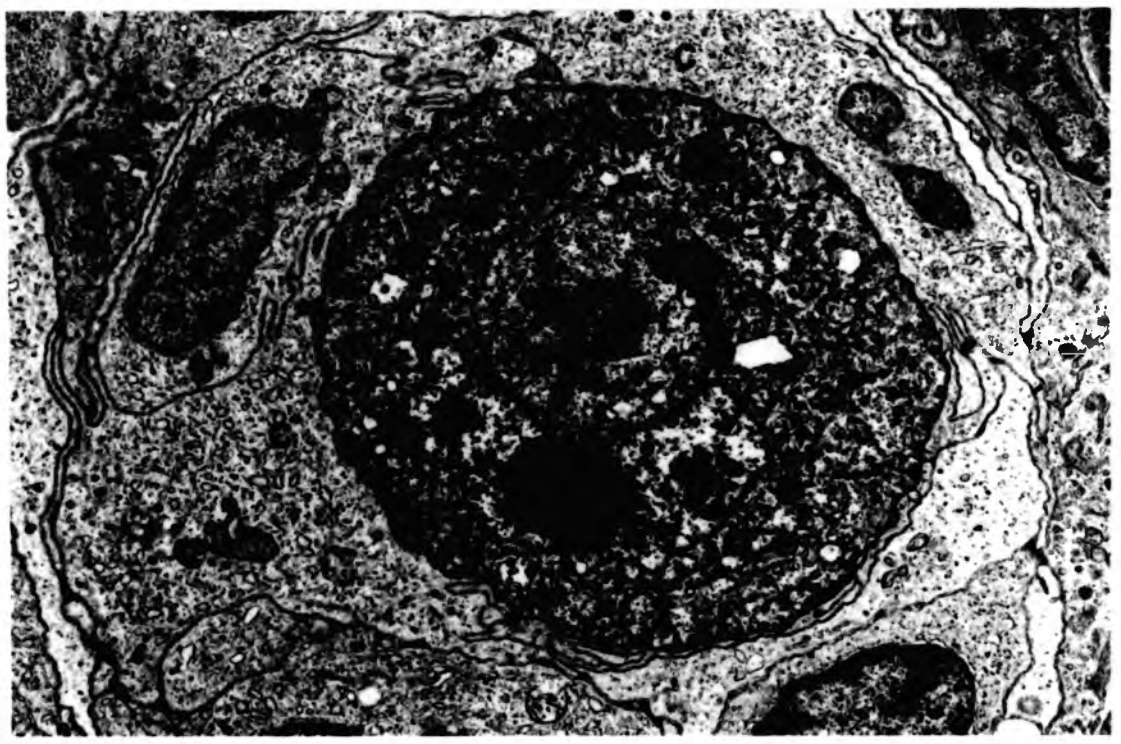

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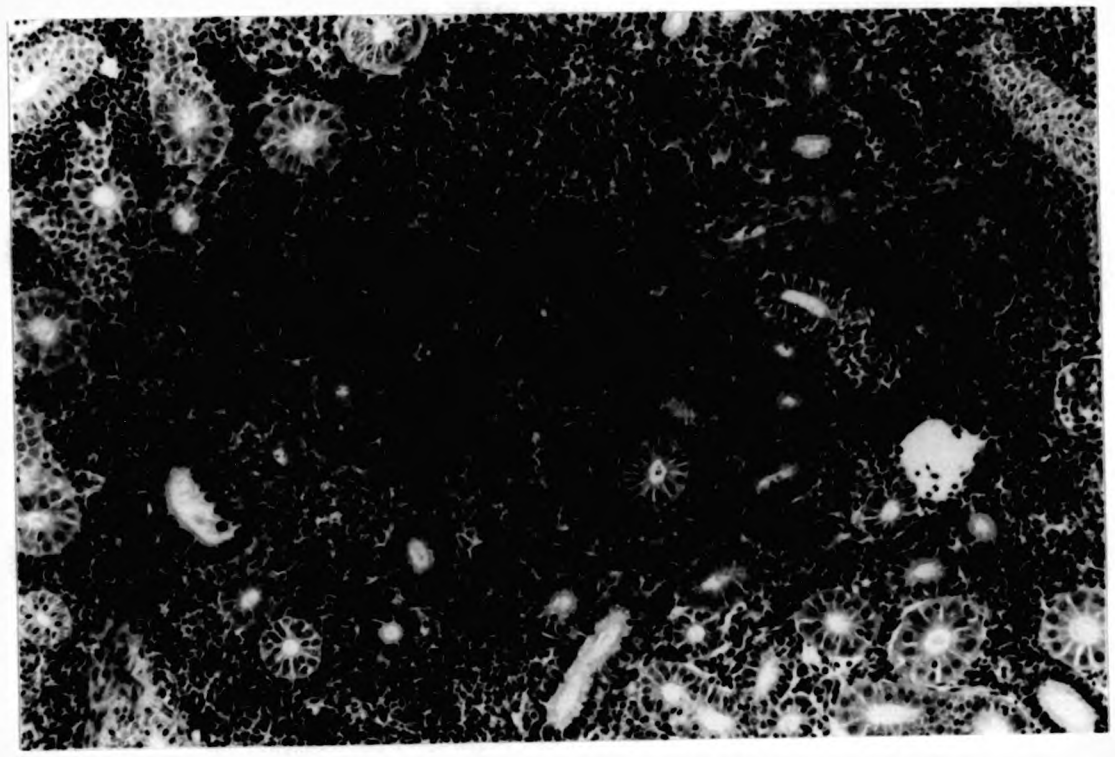


Fig. 3.9. Area of hyperplastic renal interstitial tissue displacing tubules. Melano-macrophages present (M). H & E x 120.

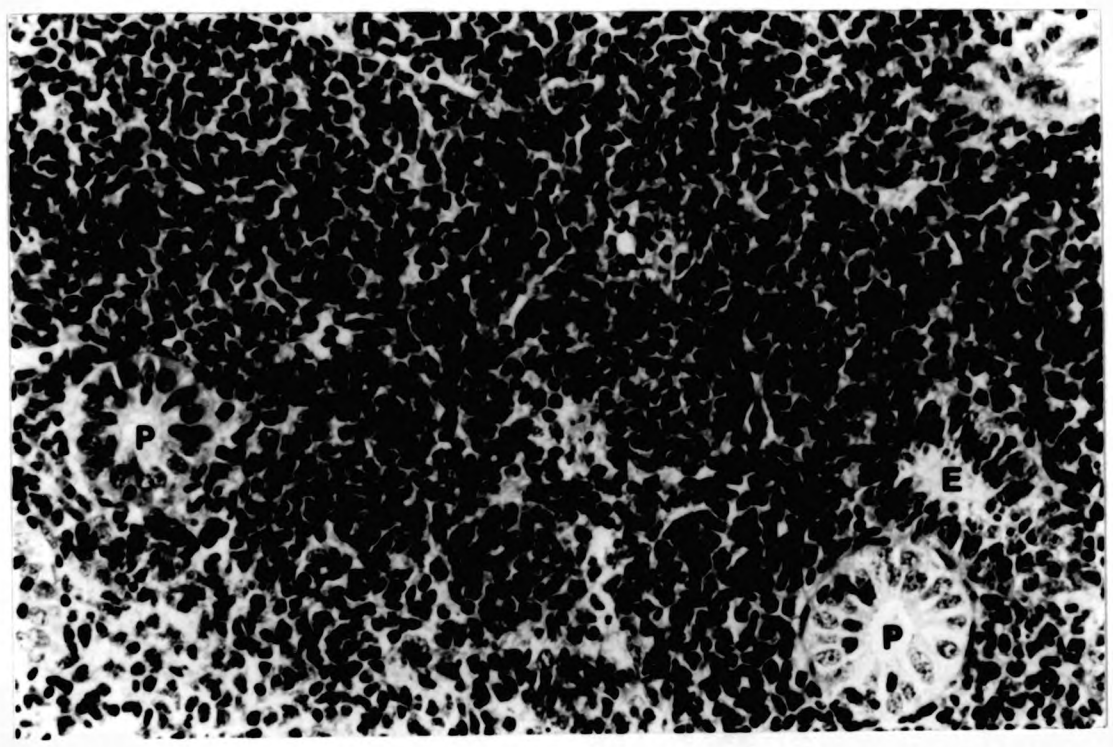
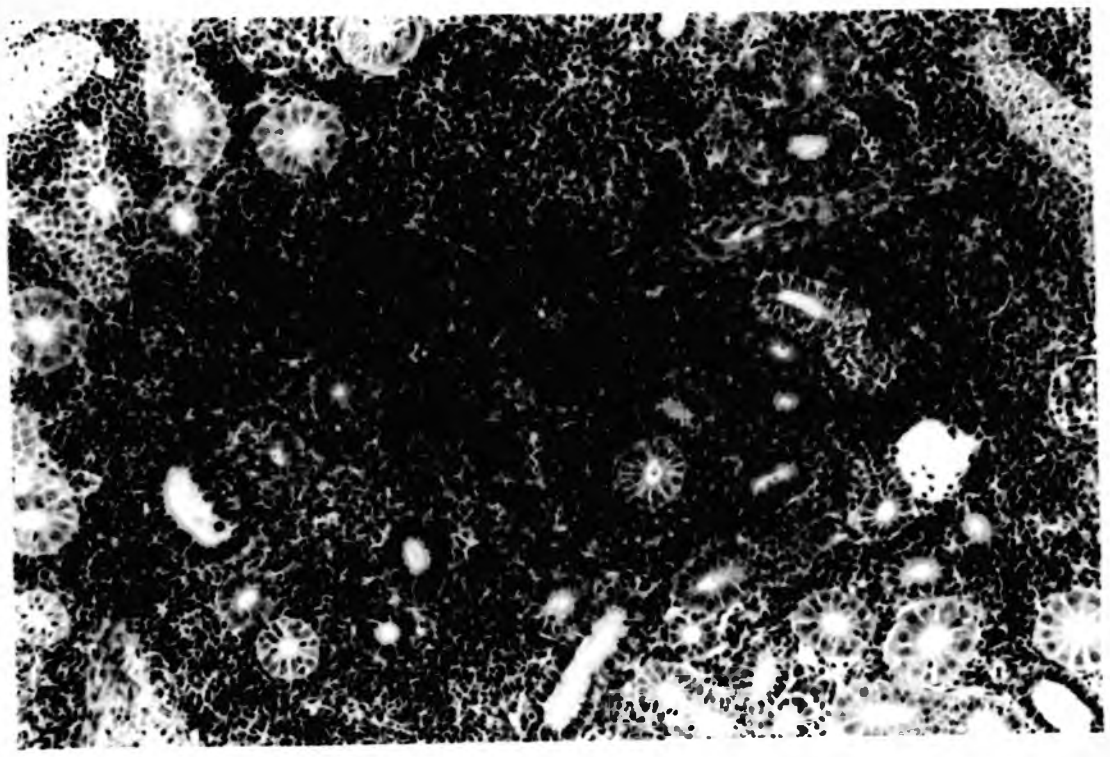


Fig. 3.10. Detail of Fig. 3.9. Proliferation of haemopoietic cells. Separation of proximal convoluted tubules (P), one with droplets in epithelial cell cytoplasm (E). H & E x 290.



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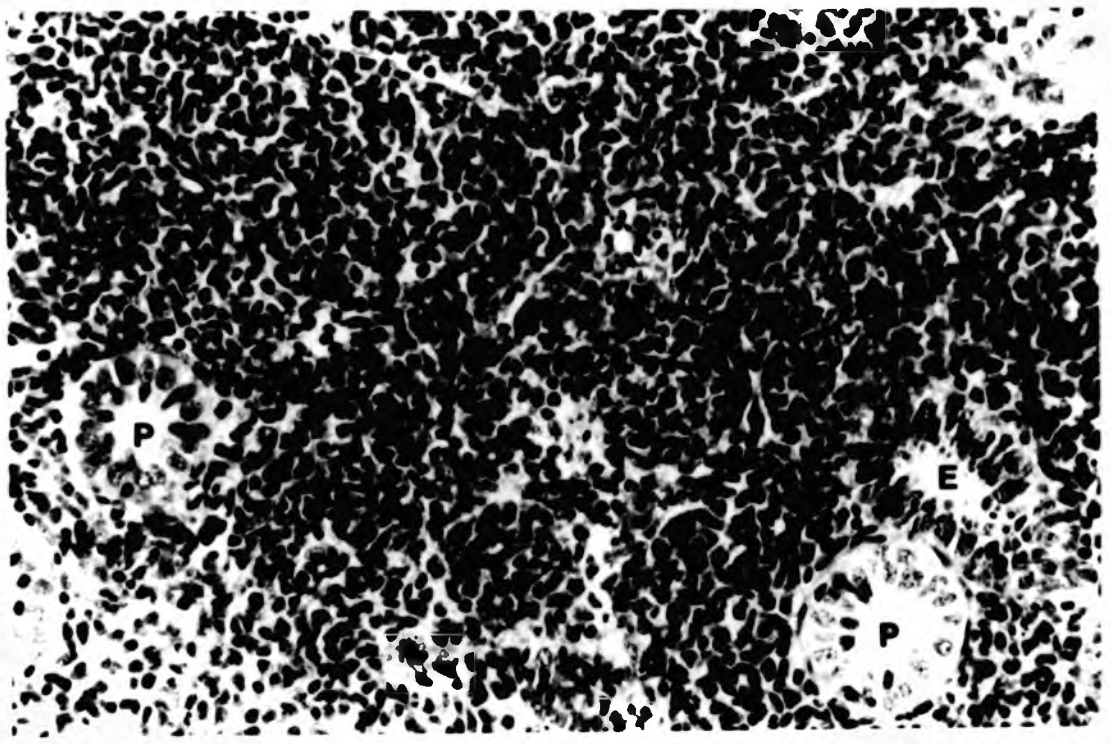

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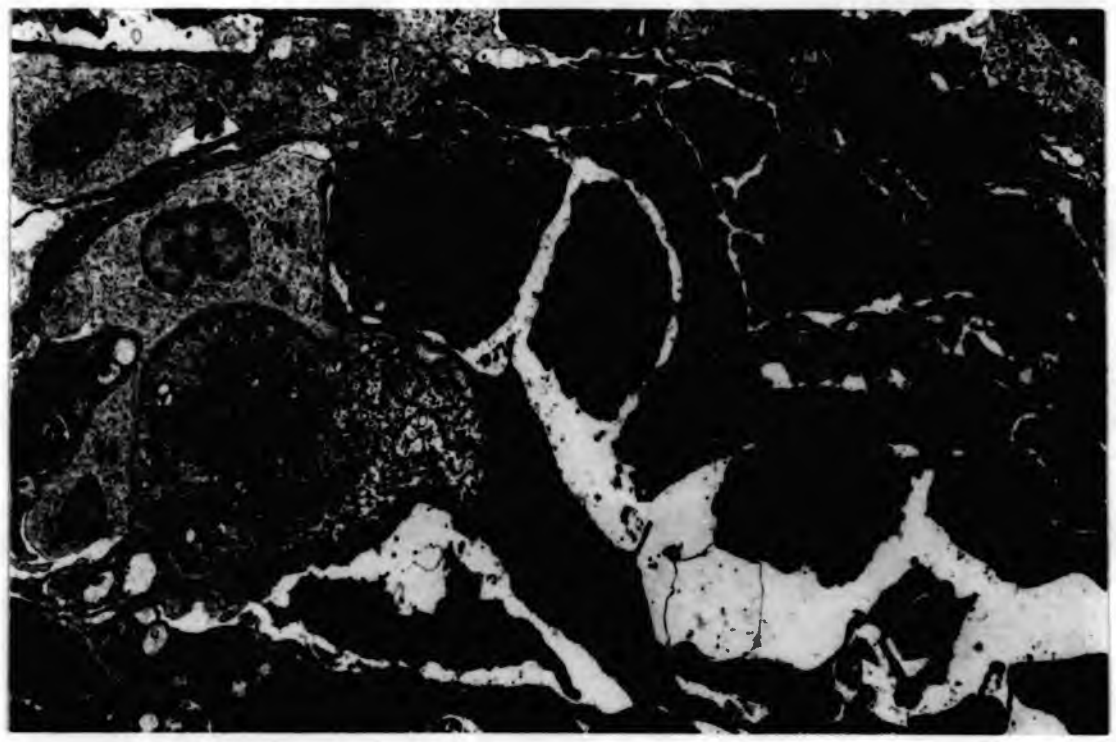


Fig. 3.11. Electron-micrograph showing renal blood vessel containing PKX cell (P) surrounded by two neutrophils (N) and a lymphoid cell (L). Endothelial (E) cells and plasma (PL) cells also present. Uranyl acetate and lead citrate. x 1,600.

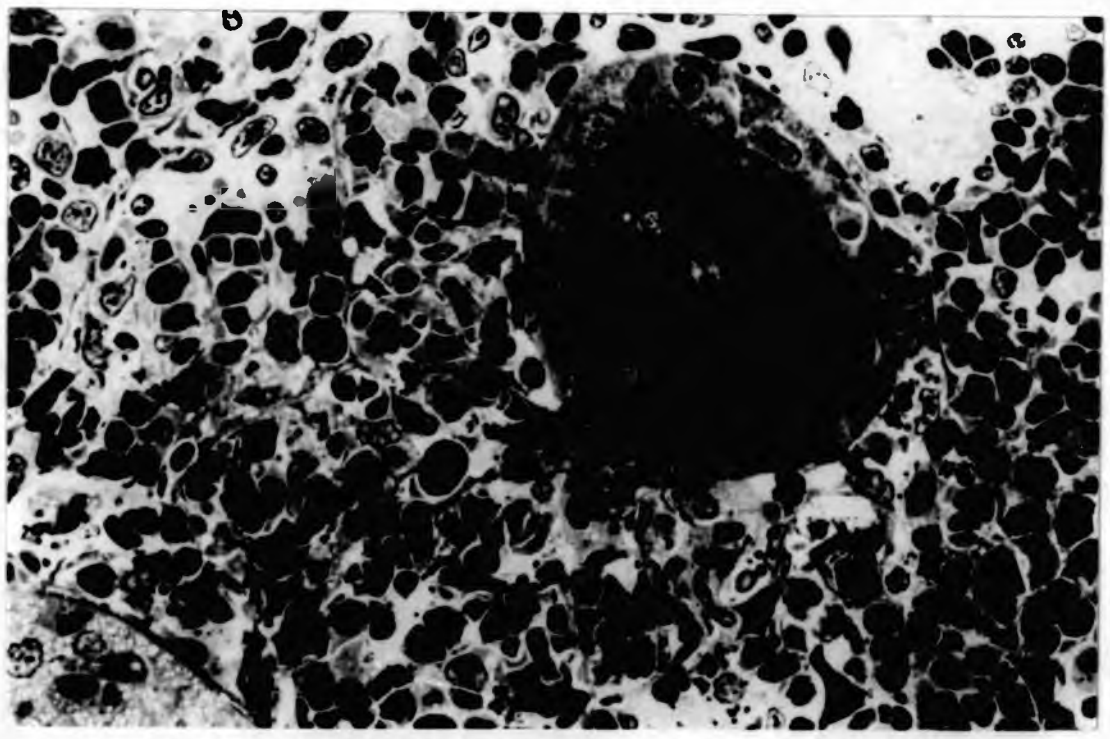
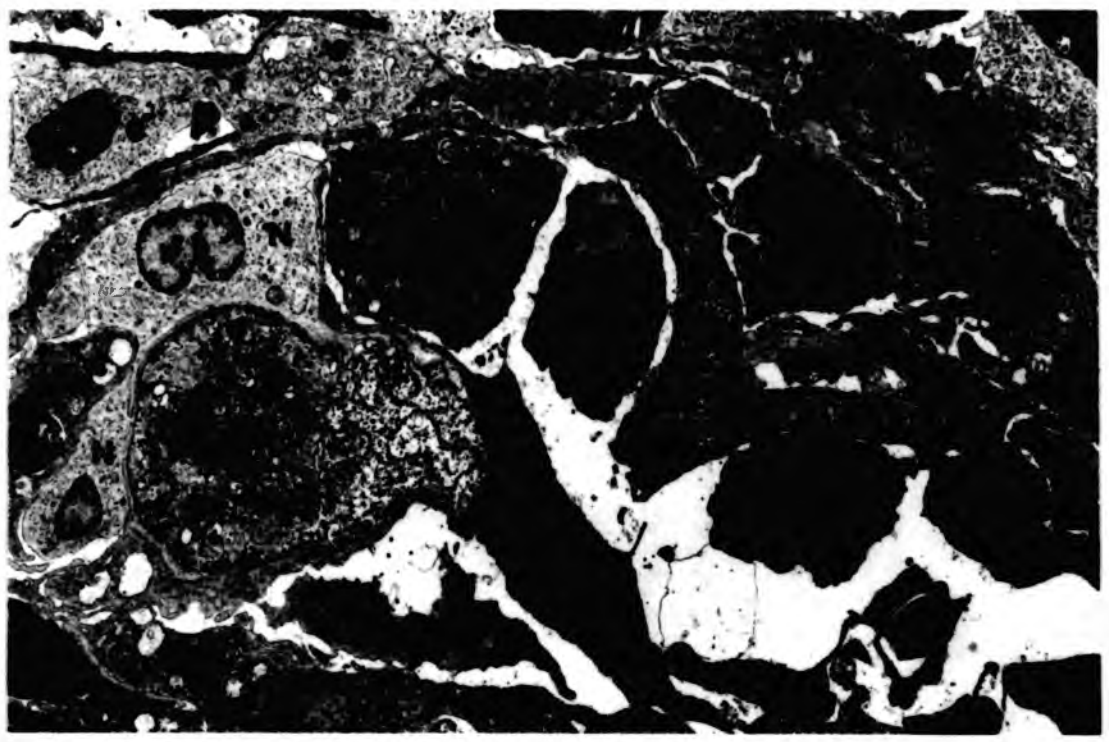


Fig. 3.12. Hypercellularity of proximal convoluted tubule (P) with apparent stenosis of lumen with area of interstitial cell hyperplasia. 2 µm section stained with toluidine blue. x 635.



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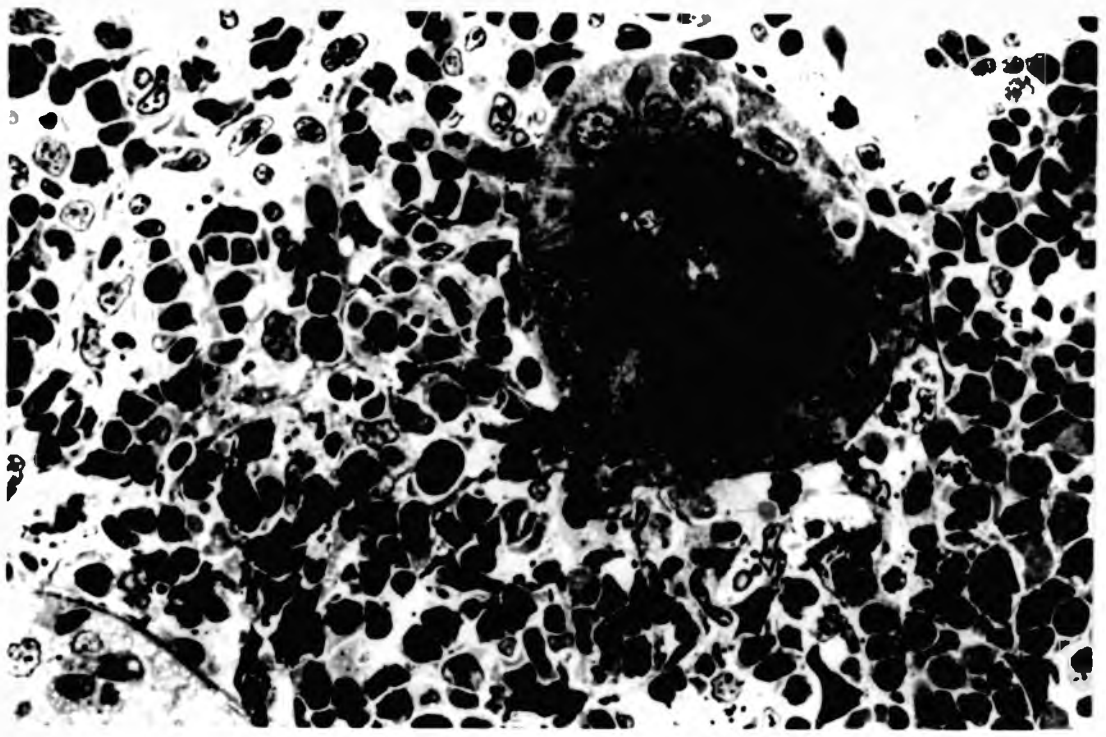

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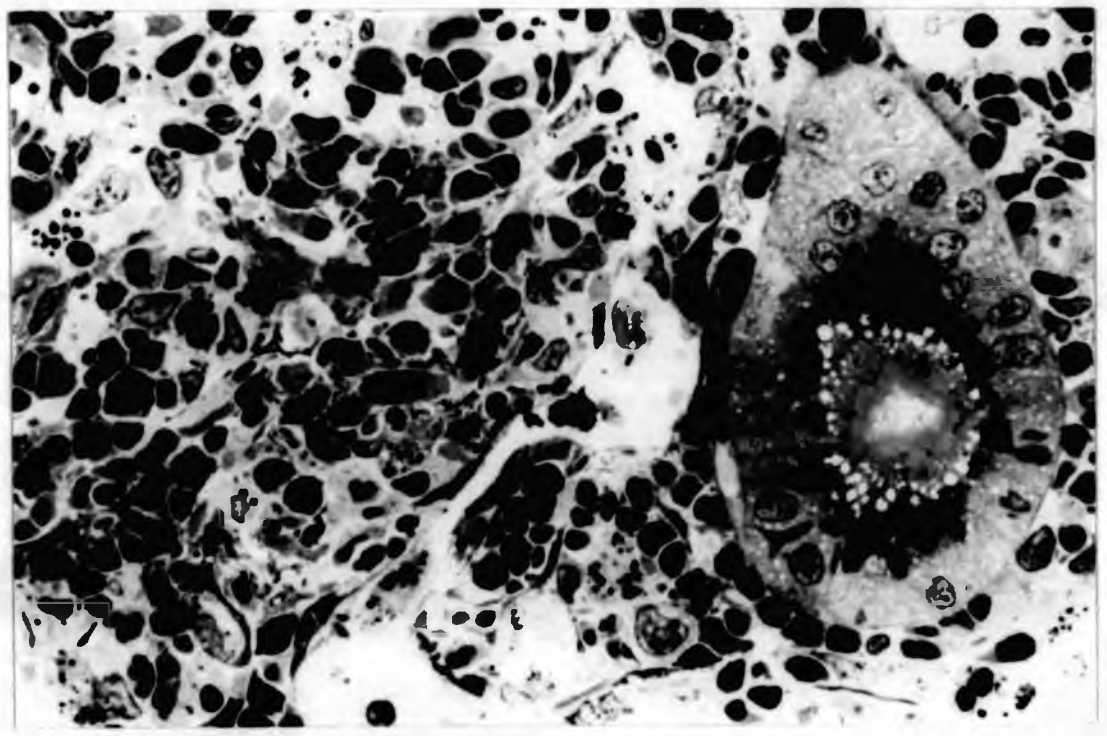


Fig. 3.13. Proximal convoluted tubule containing many droplets within epithelial cell cytoplasm. 2 µm section stained with toluidine blue. x 635.

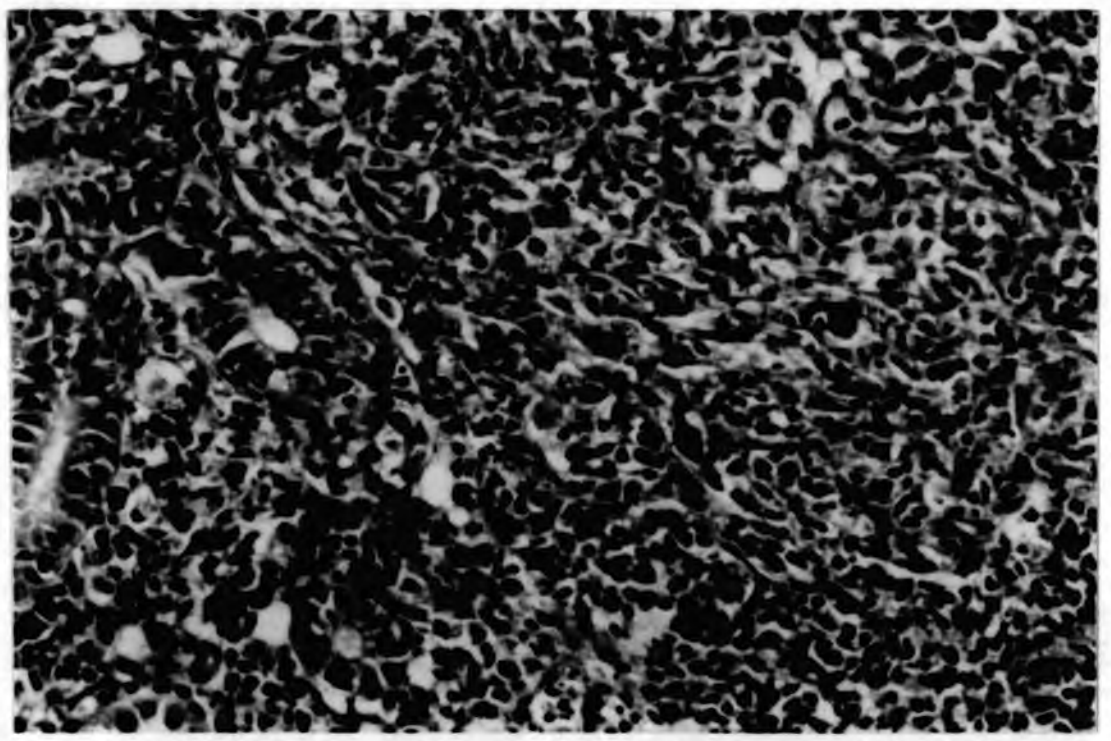
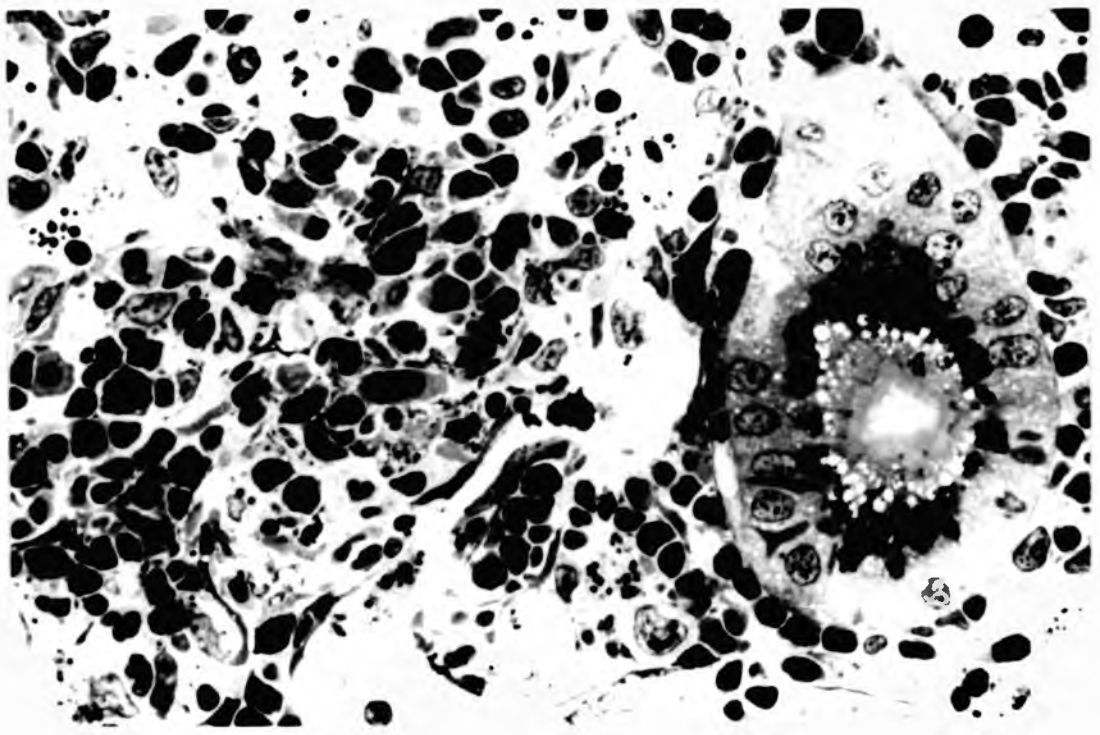


Fig. 3.14. Diffuse inflammatory changes within the kidney. Many small, fluid-filled spaces evident. PKX cells (arrowed) present but few melano-macrophages. H & E x 290.



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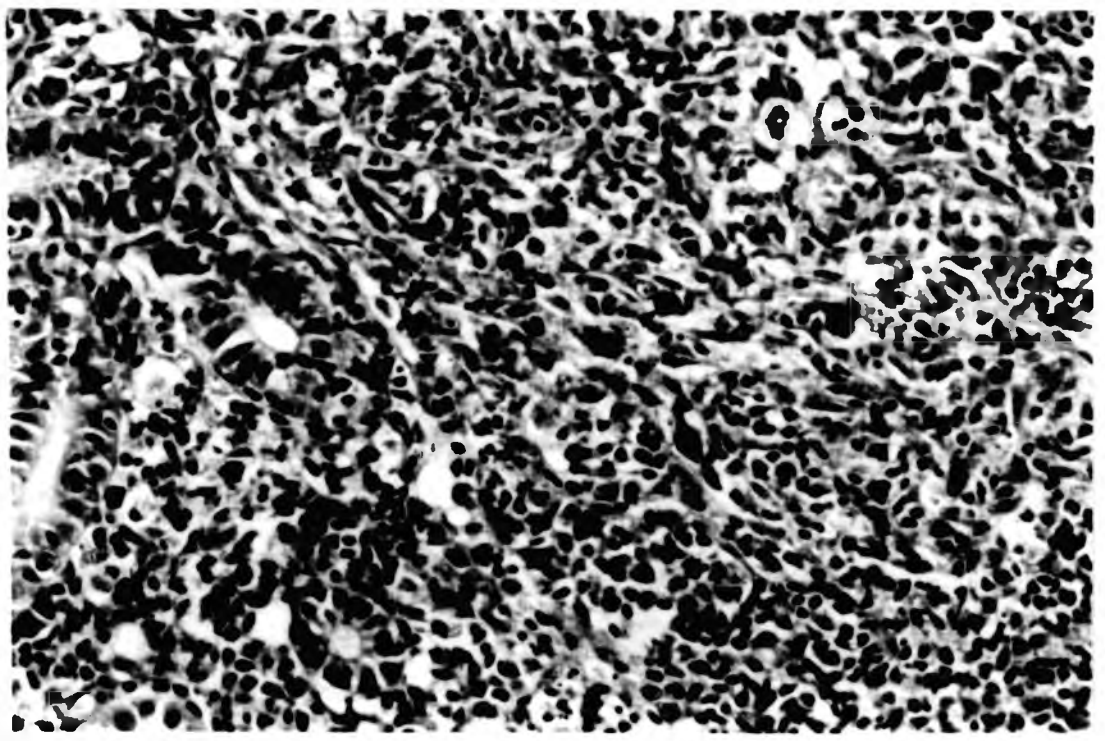

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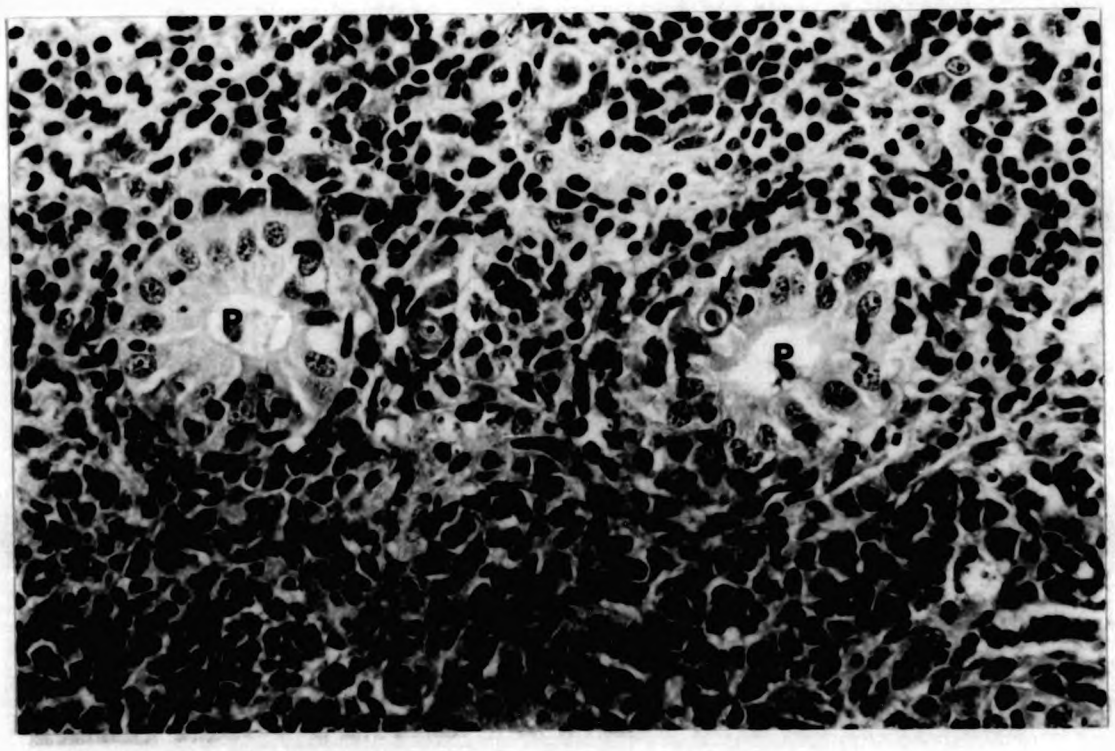


Fig. 3.15. Hypercellular proximal convoluted tubules (P), one with PKX cell between epithelial cells with "haloed" inclusion (arrowed). H & E x 360.

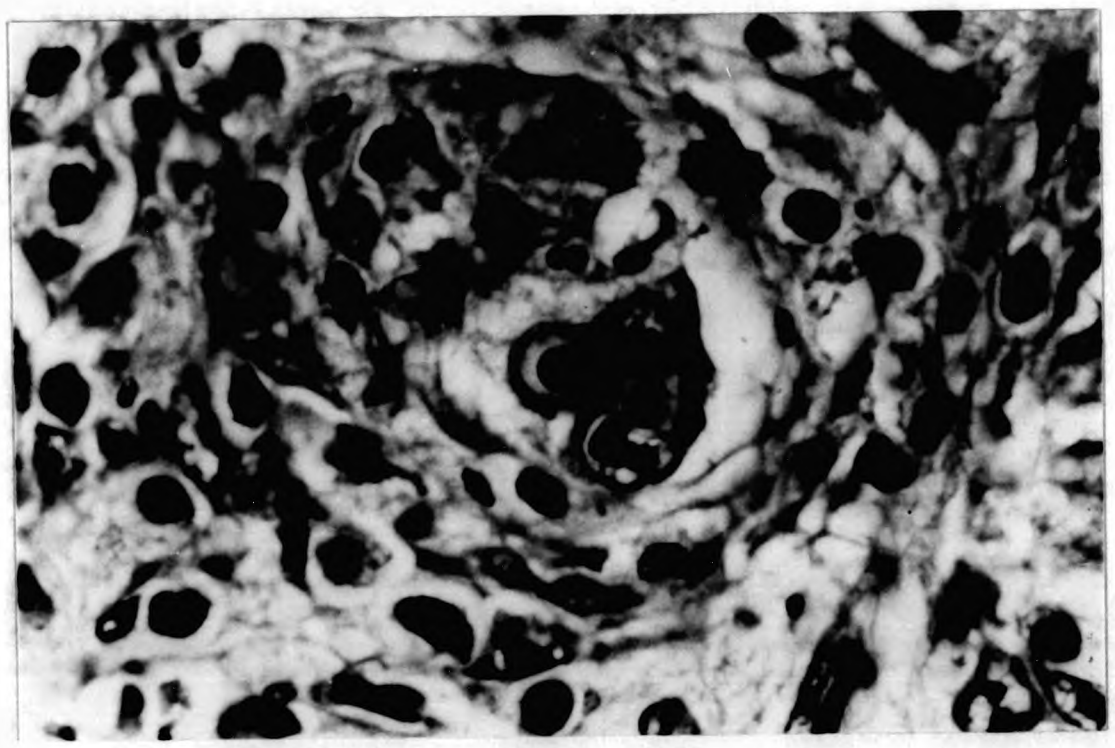
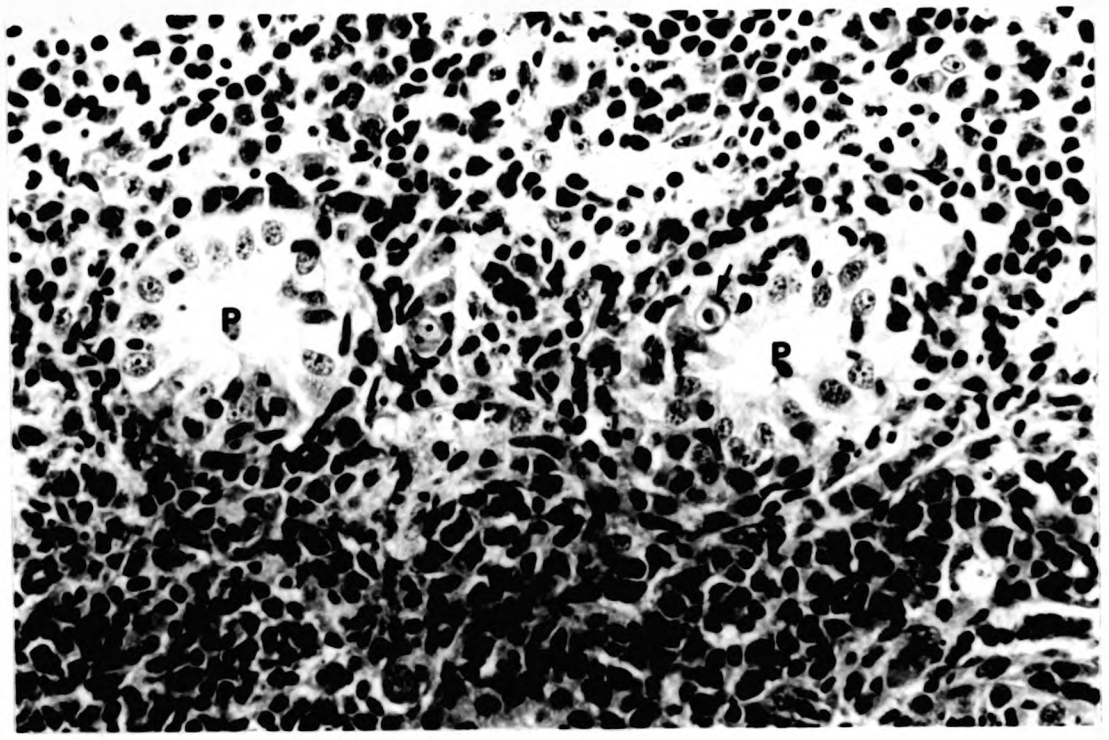


Fig. 3.16. Irregularly-shaped PKX cell, with several distinct inclusion cells (I) in renal lesion where fibrosis has commenced. H & E x 1,240.



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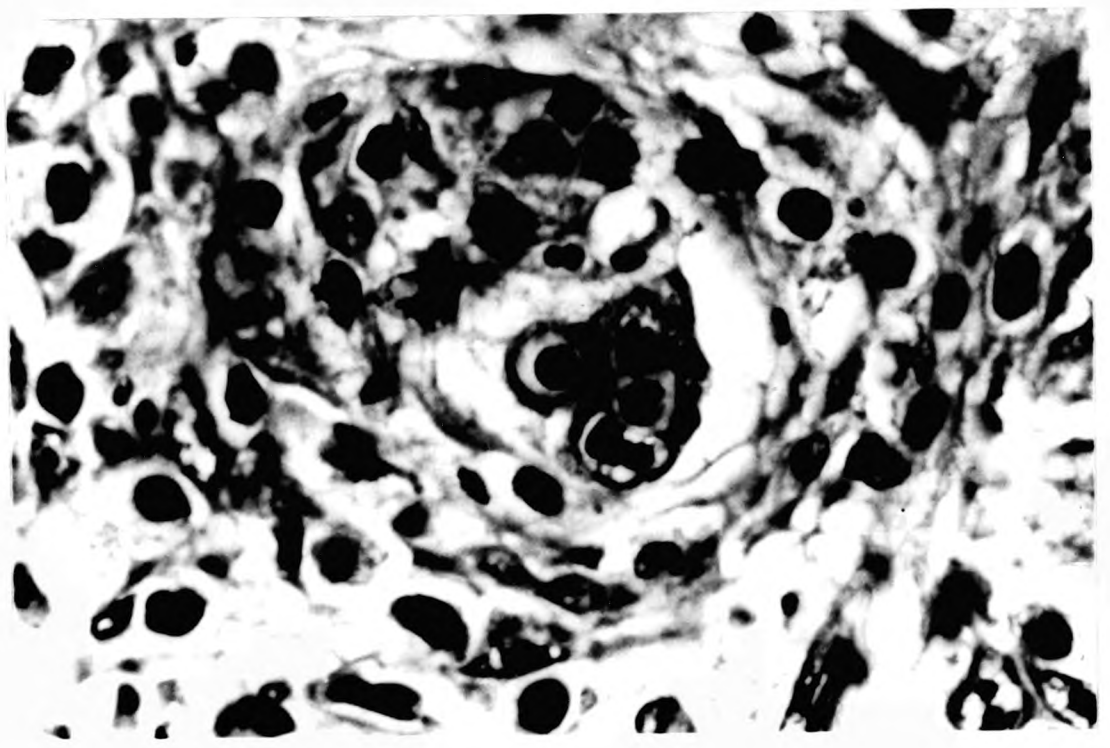

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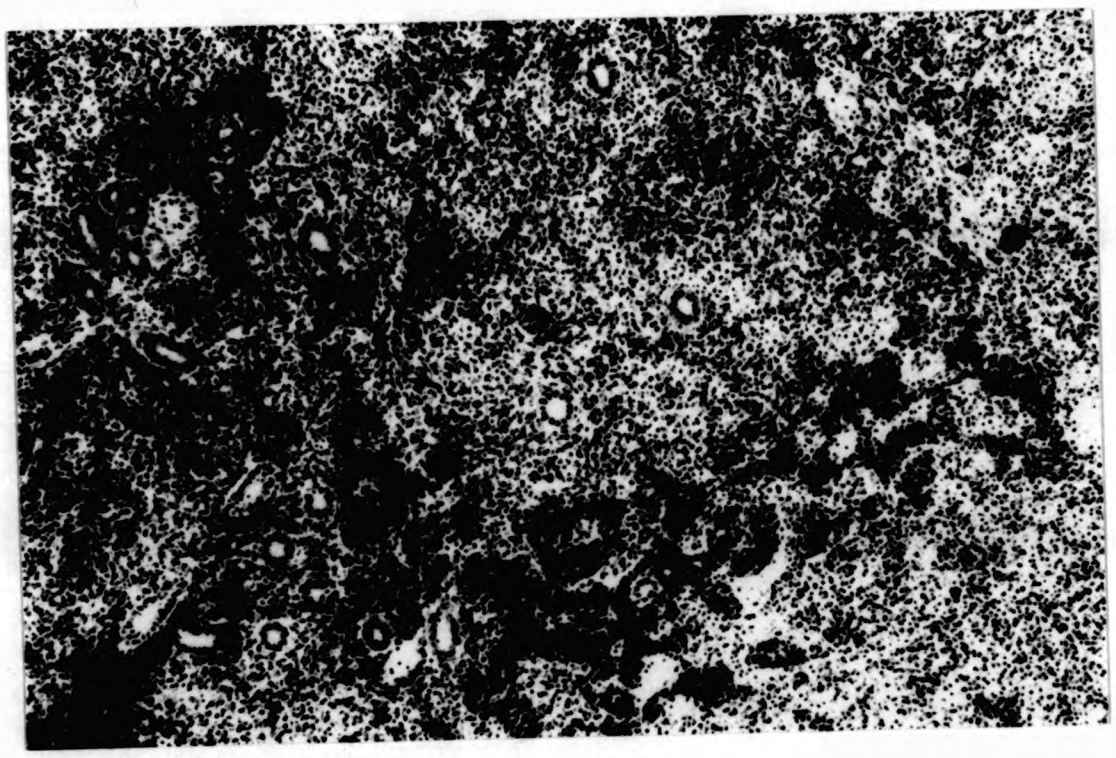


Fig. 3.17. Lightly-staining diffuse inflammation with few tubules interspersed with darker haemopoietic tissue. H & E x 95.

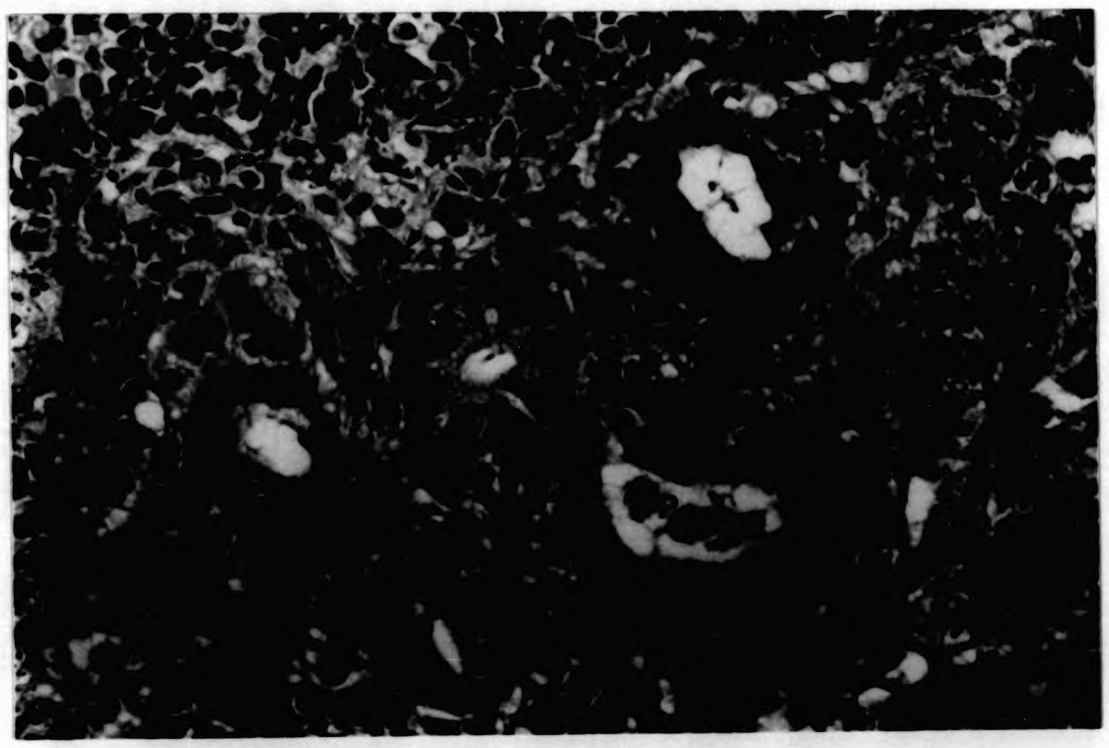


Fig. 3.18. Renal tubule destruction in area of diffuse inflammation. One tubule with intraluminal PKX cells (P). Scattered melanin granules (arrowed). H & E x 370.

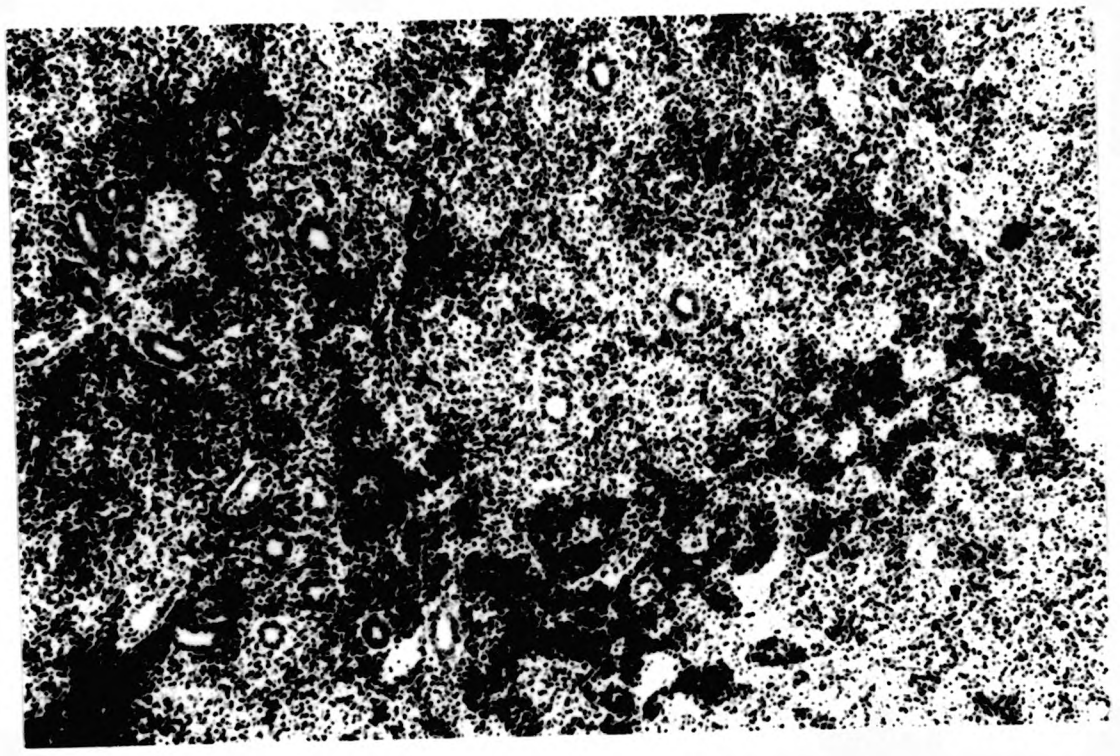


Fig. 3.17. Lightly-staining diffuse inflammation with few tubules interspersed with darker haemopoietic tissue. H & E x 95.

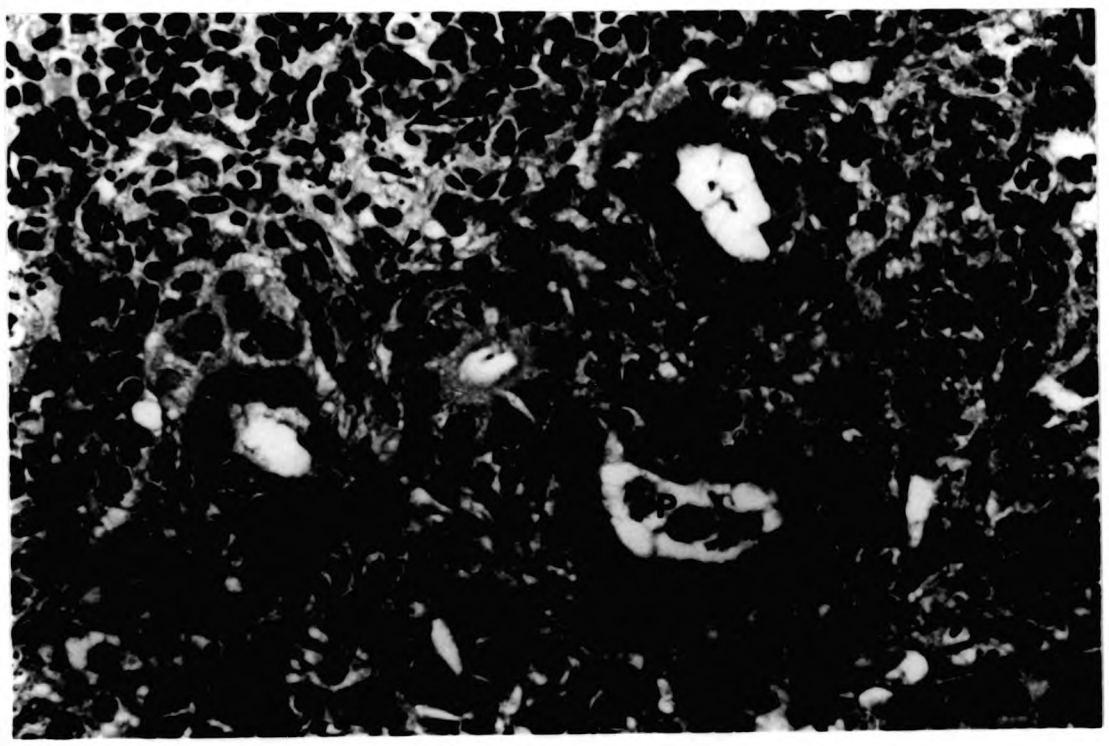


Fig. 3.18. Renal tubule destruction in area of diffuse inflammation. One tubule with intraluminal PKX cells (P). Scattered melanin granules (arrowed). H & E x 370.

Fig. 3.19. Large area of inflammation (circumference arrowed) containing few tubule structures or haemopoietic tissue, surrounded by more normal renal tissue. H & E x 40.

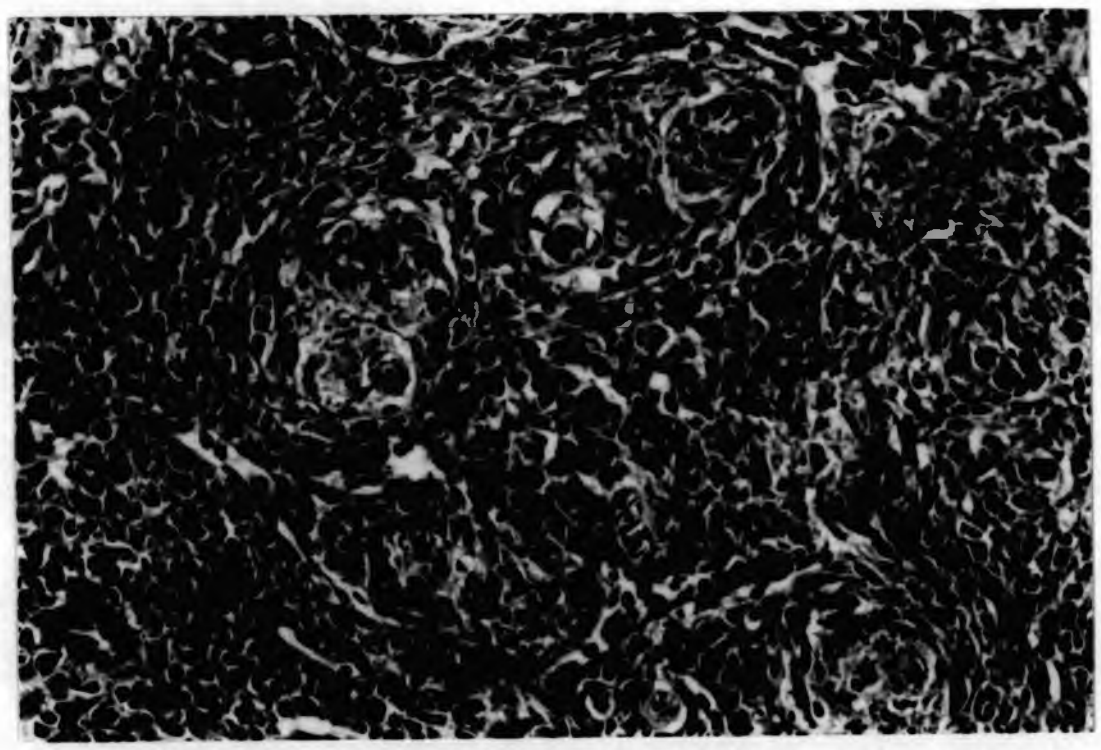


Fig. 3.20. "Whorling" effect of cellular arrangement around PKX cells (arrowed), some apparently undergoing necrosis in renal specimen. H & E x 225.

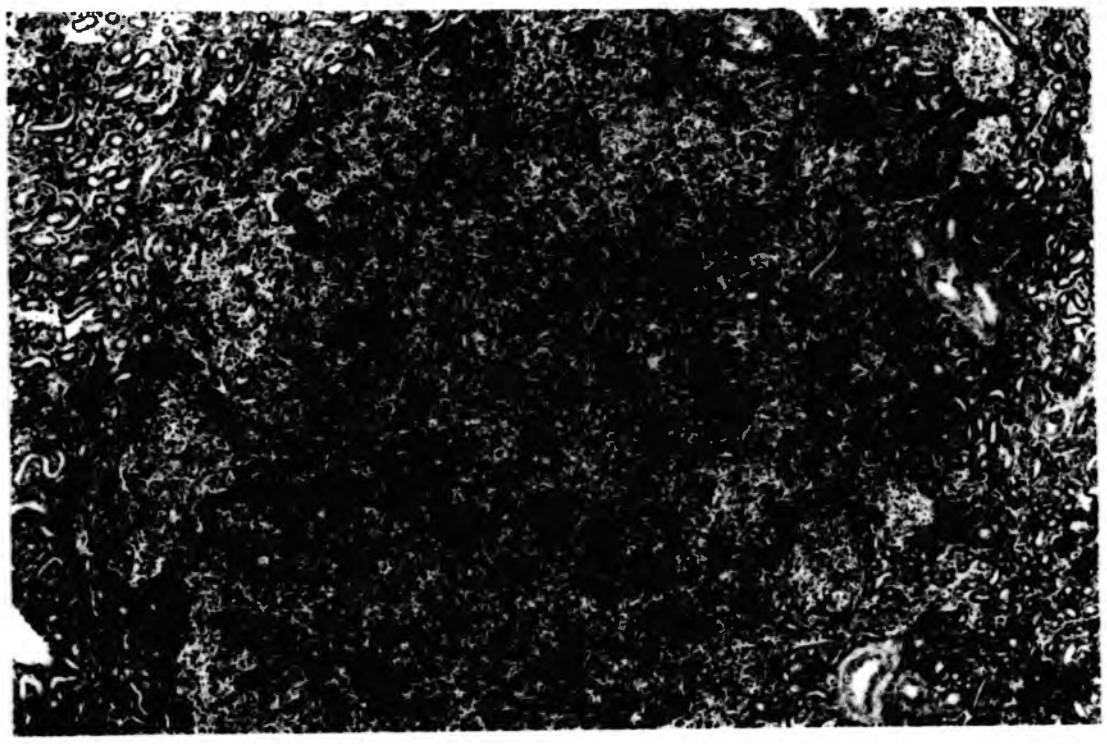
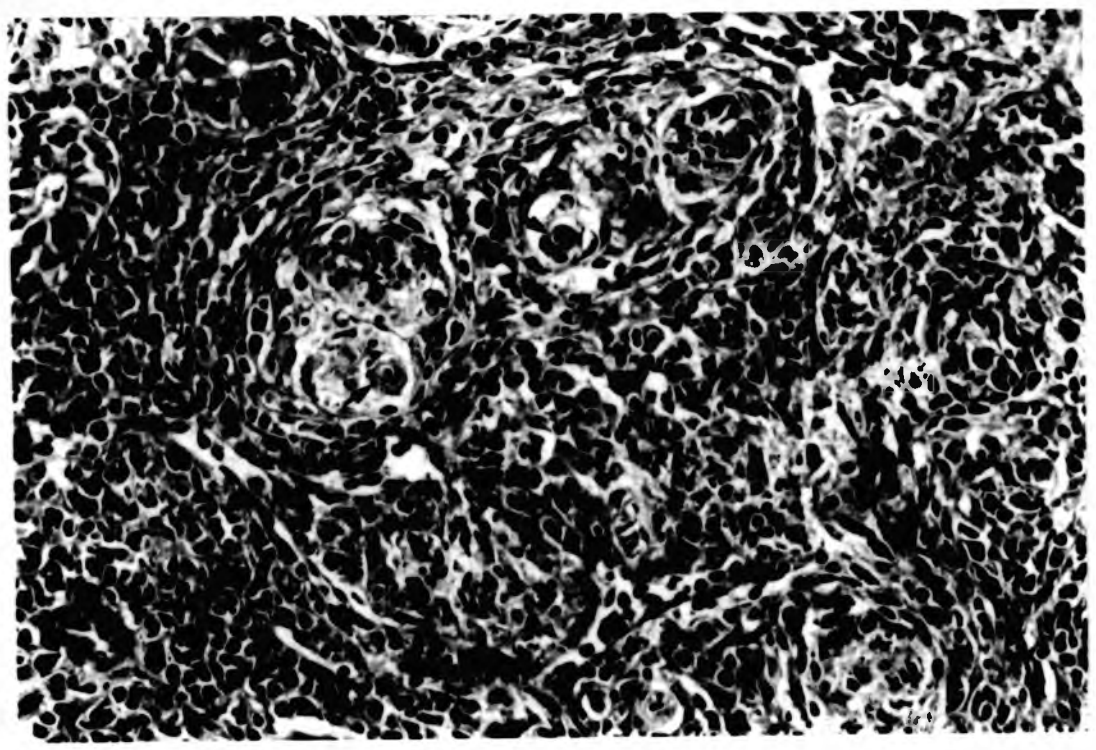


Fig. 3.19. Large area of inflammation (circumference arrowed) containing few tubule structures or haemopoietic tissue, surrounded by more normal renal tissue. H & E x 40.



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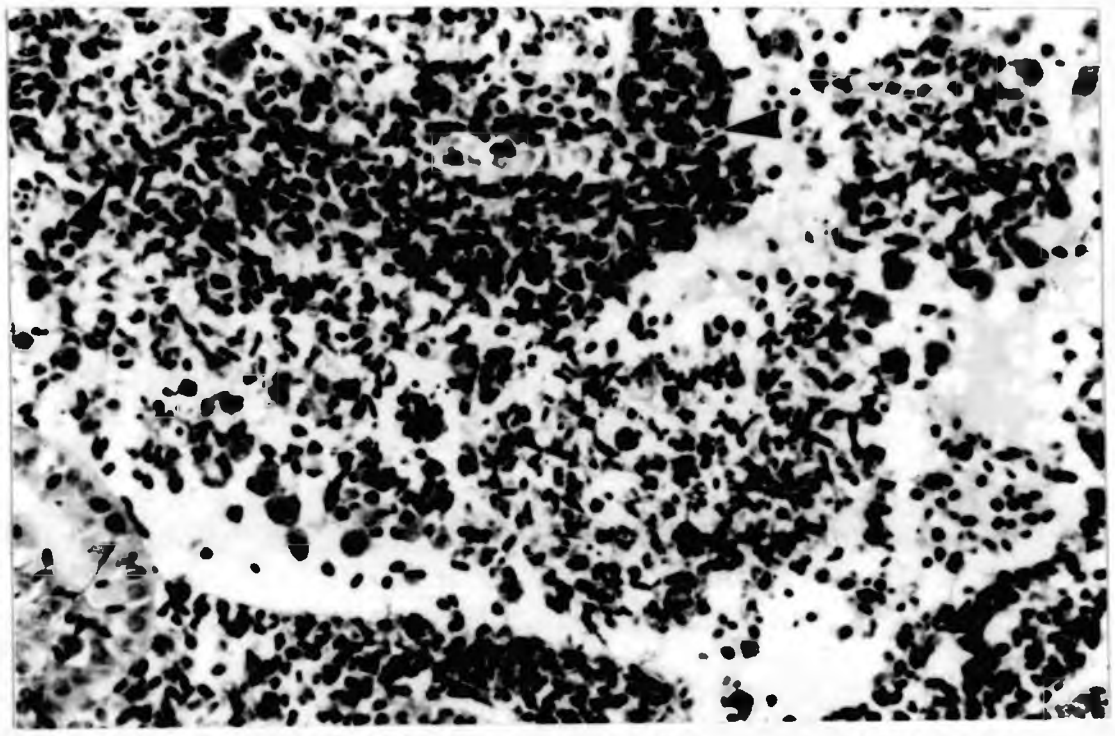


Fig. 3.21. Intravascular aggregation, including many PKX cells, partially occluding a renal sinus. Loss of endothelial definition (between large arrows). Tubule containing PKX cell (small arrow). H & E x 300.

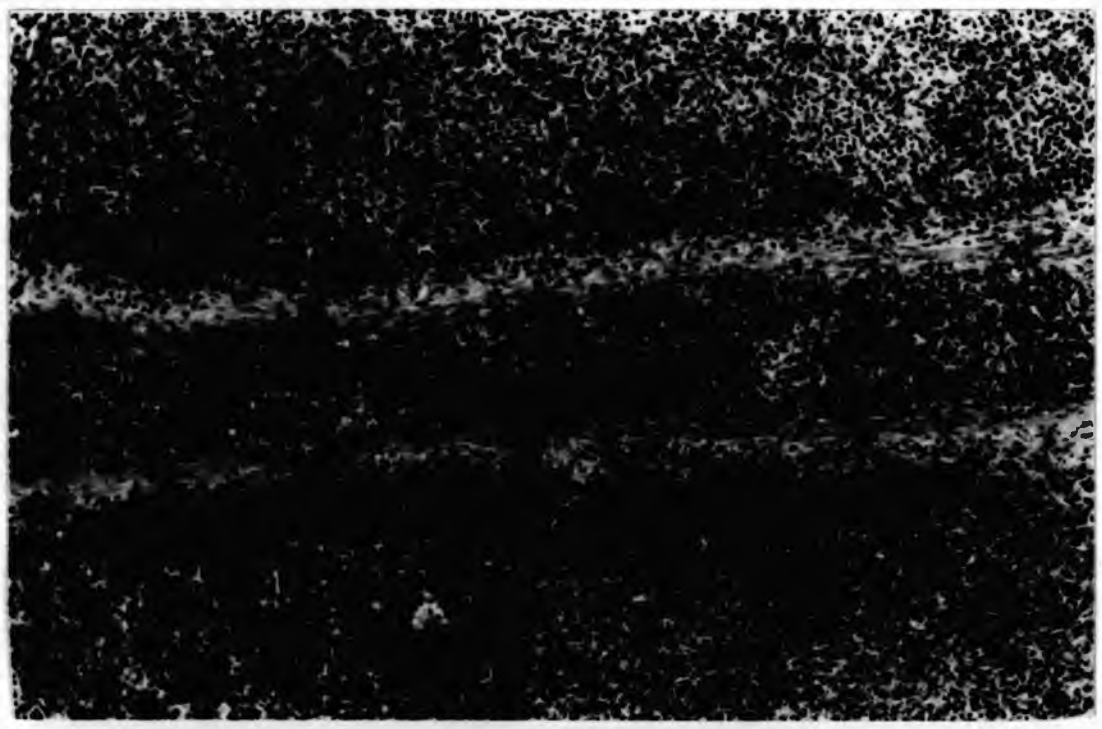
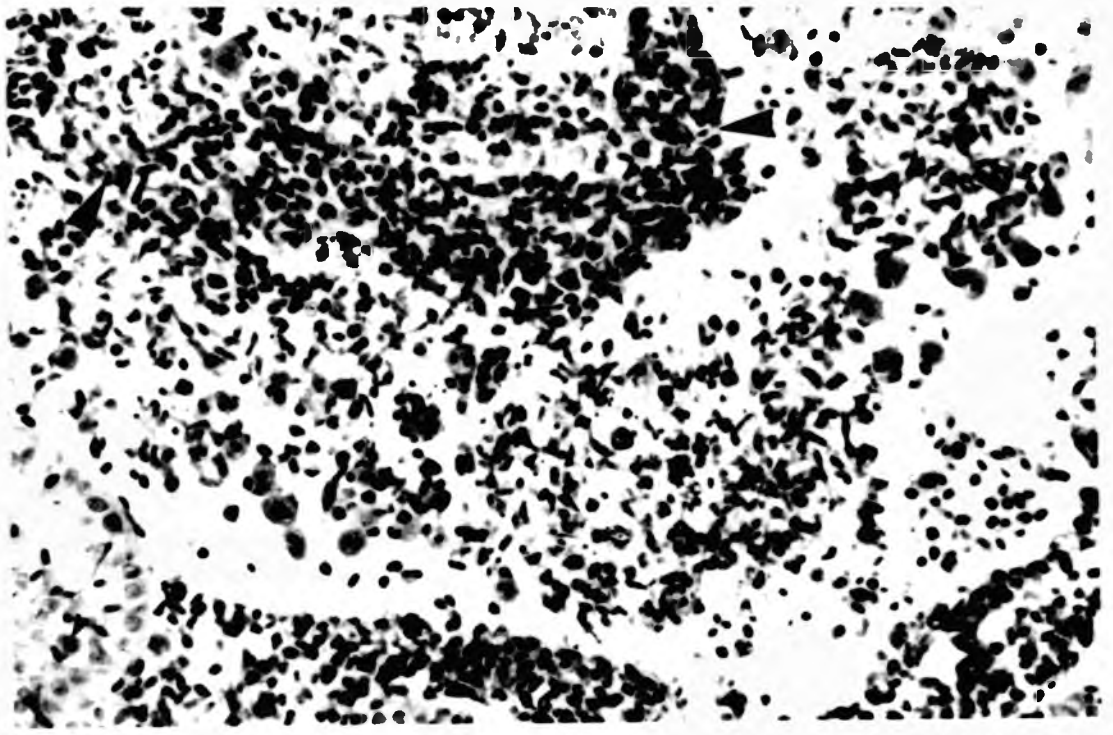


Fig. 3.22. Occlusion of intrarenal blood vessel with cellular debris (C) and aggregations of pleomorphic cells. Smooth muscle of the tunica media (M) still apparently intact. H & E x 95.



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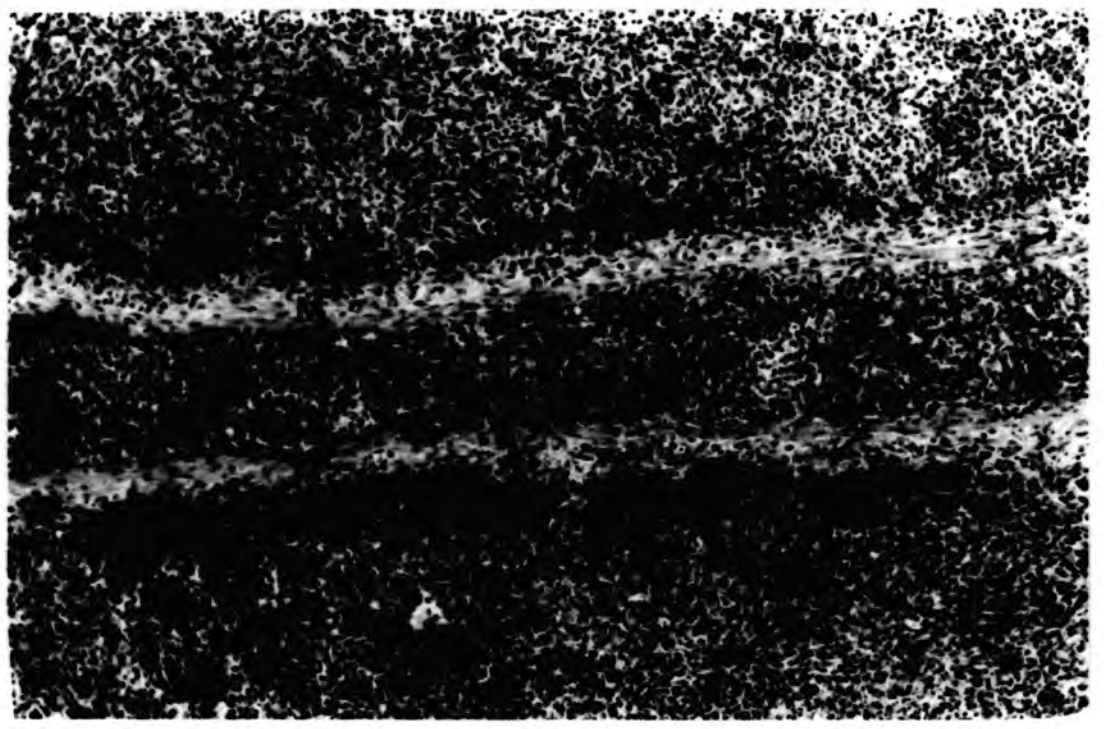

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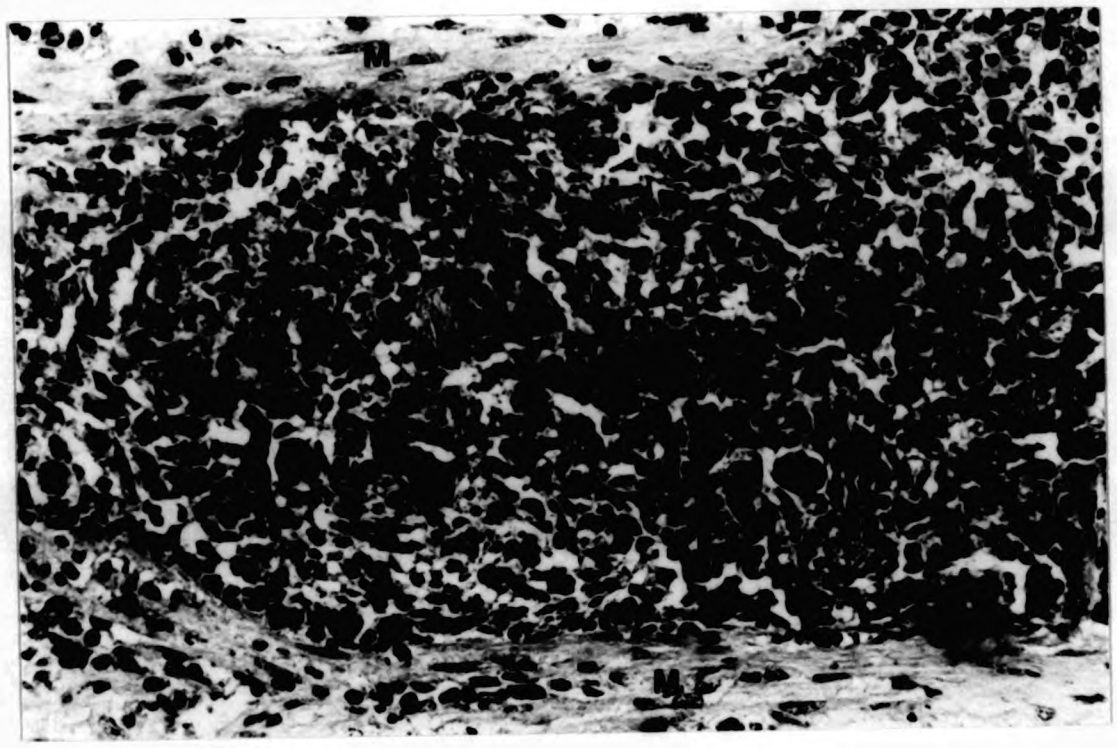


Fig. 3.23. Detail of Fig. 3.22. Aggregation containing PKX cells, fusiform crystals, pleomorphic cells. Vessel walls marked (M). H & E x 335.

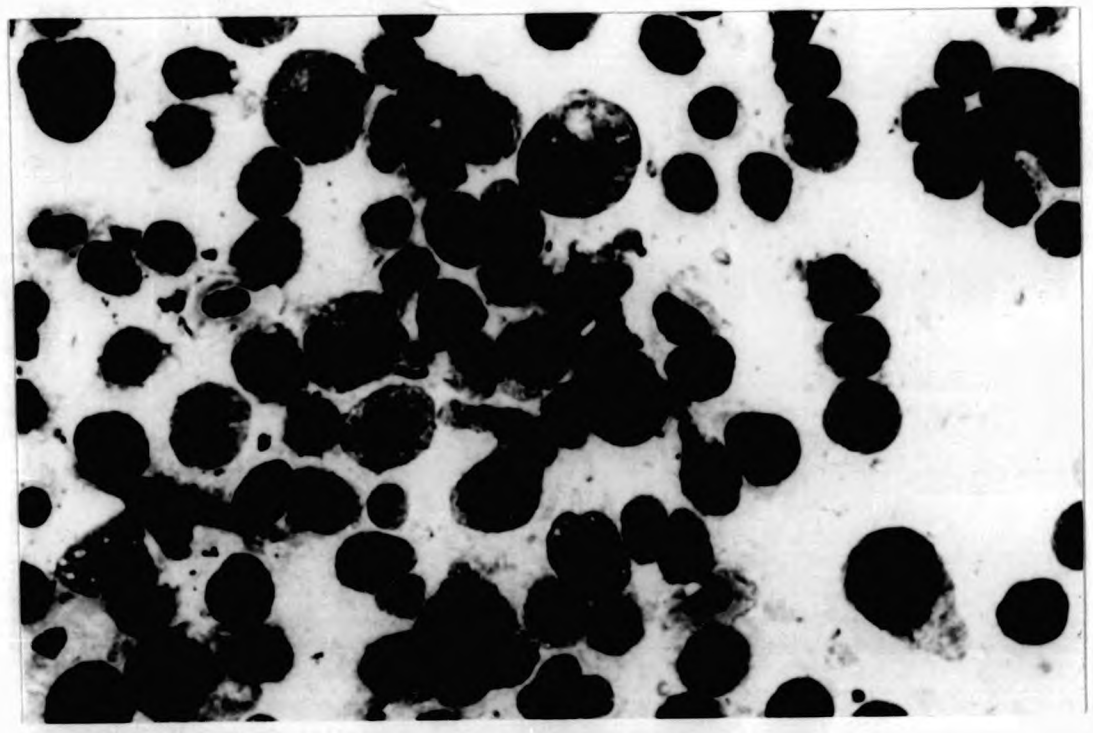
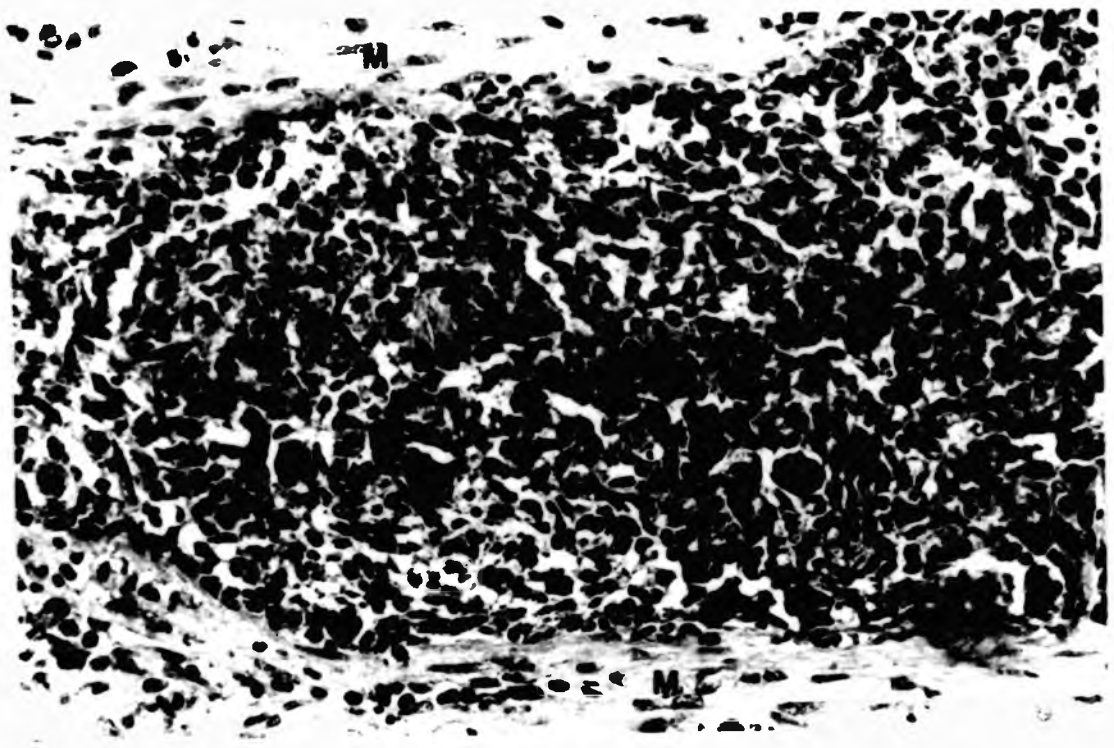


Fig. 3.24. Fusiform crystals, one with light-reflecting transverse line. Kidney smear stained with May-Grünwald-Giemsa. x 965.



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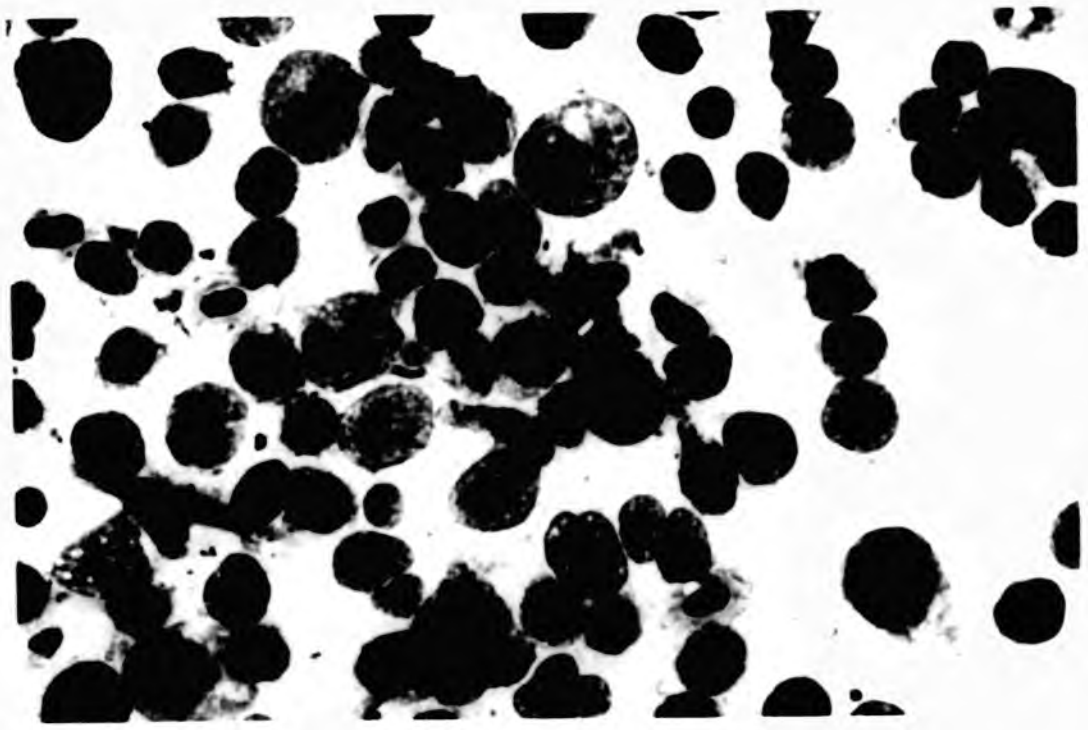

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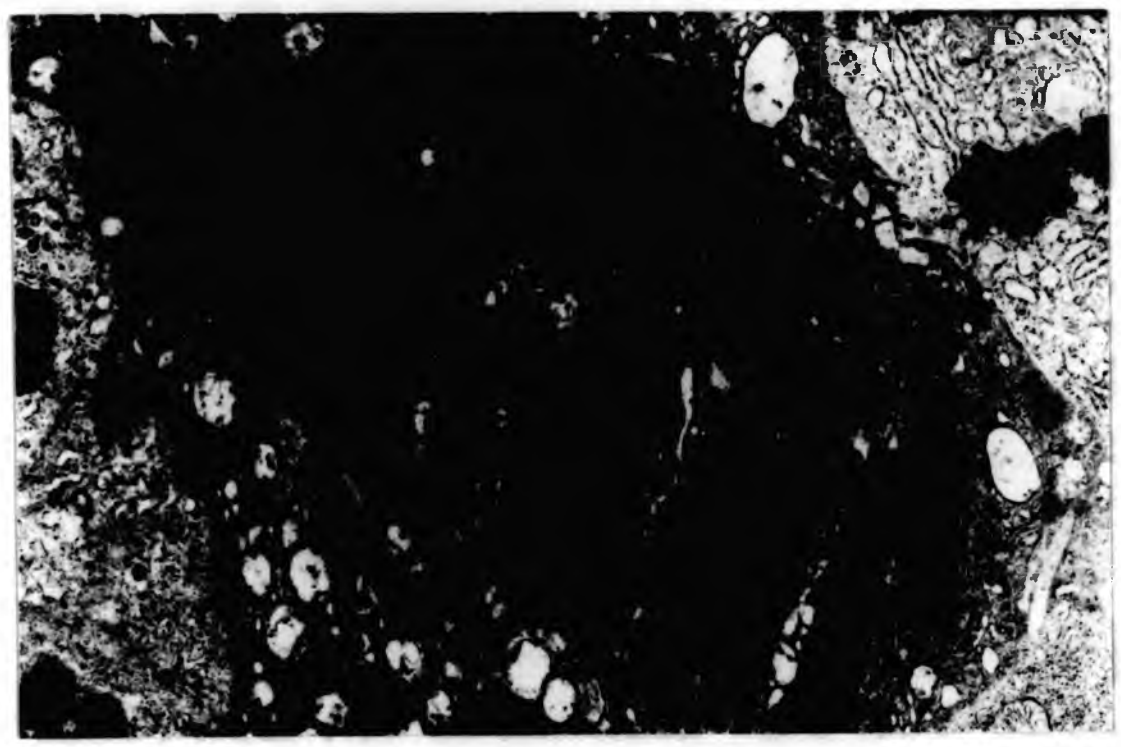


Fig. 3.25. Electron-micrograph of macrophage-like cell containing many possible haemoglobin crystals from kidney with grade 4 swelling. Uranyl acetate and lead citrate. x 6,600.

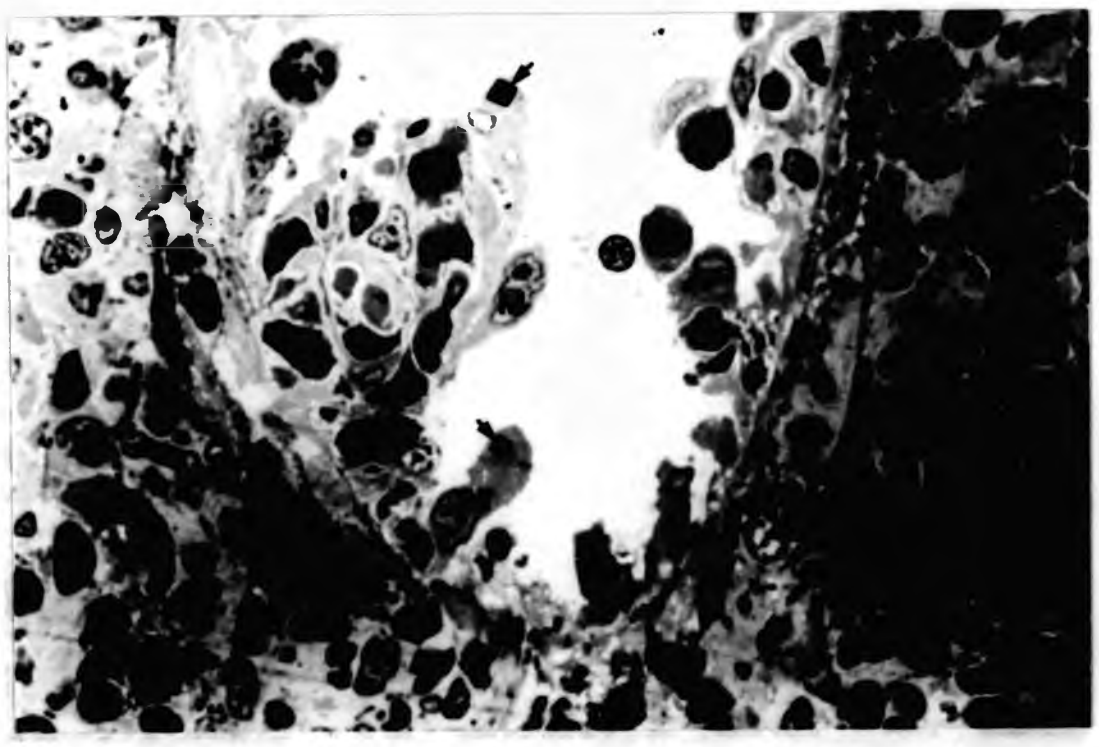
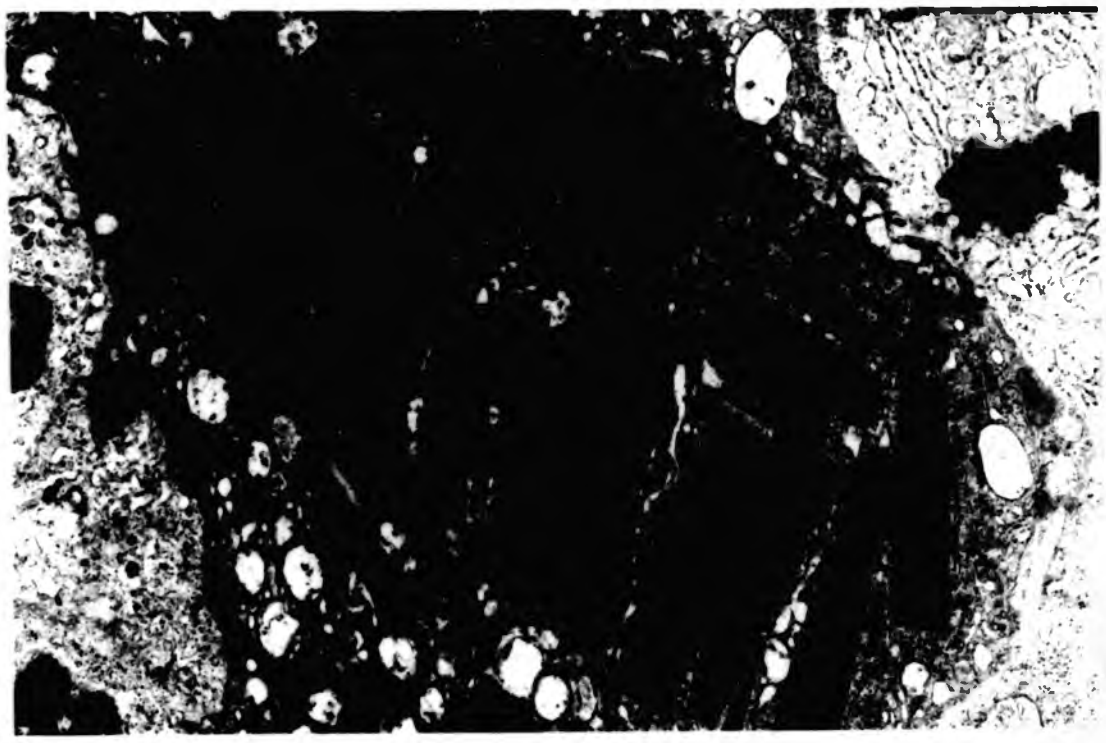


Fig. 3.26. Crystals (arrowed) in transverse section within a blood vessel containing a small cellular aggregation attached to an area of the blood vessel wall where endothelium is apparently damaged. Lumen contains neutrophils (N) and macrophages (M) perhaps containing the crystals. 2 μm section stained with toluidine blue. x 905.



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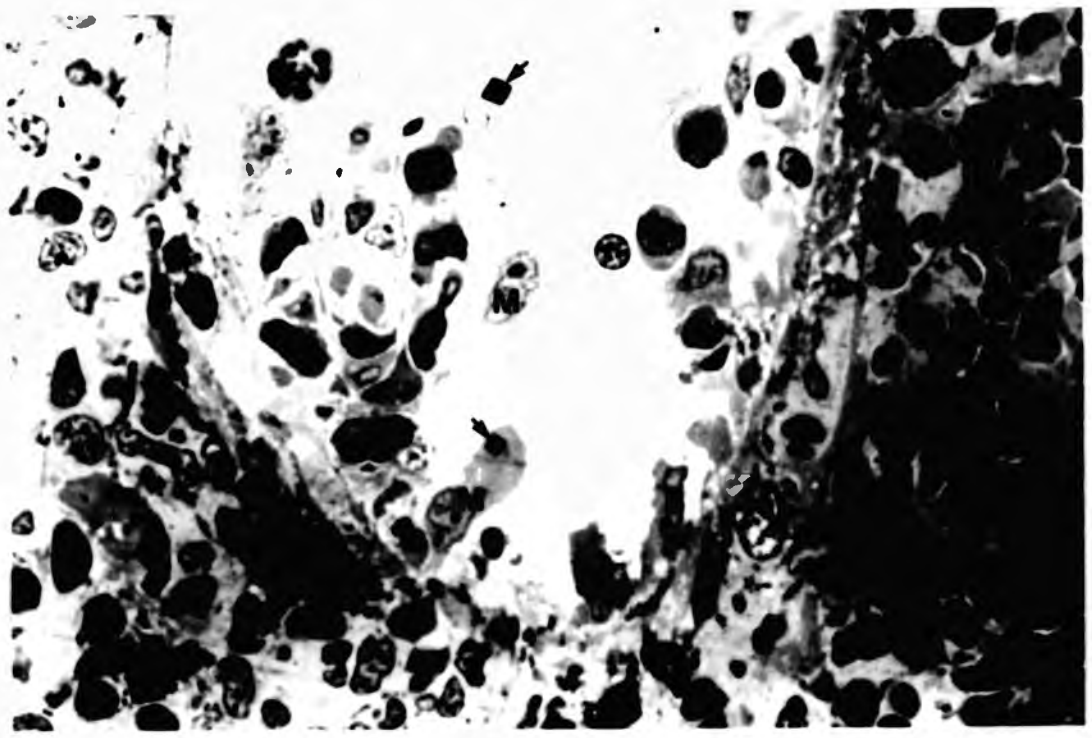

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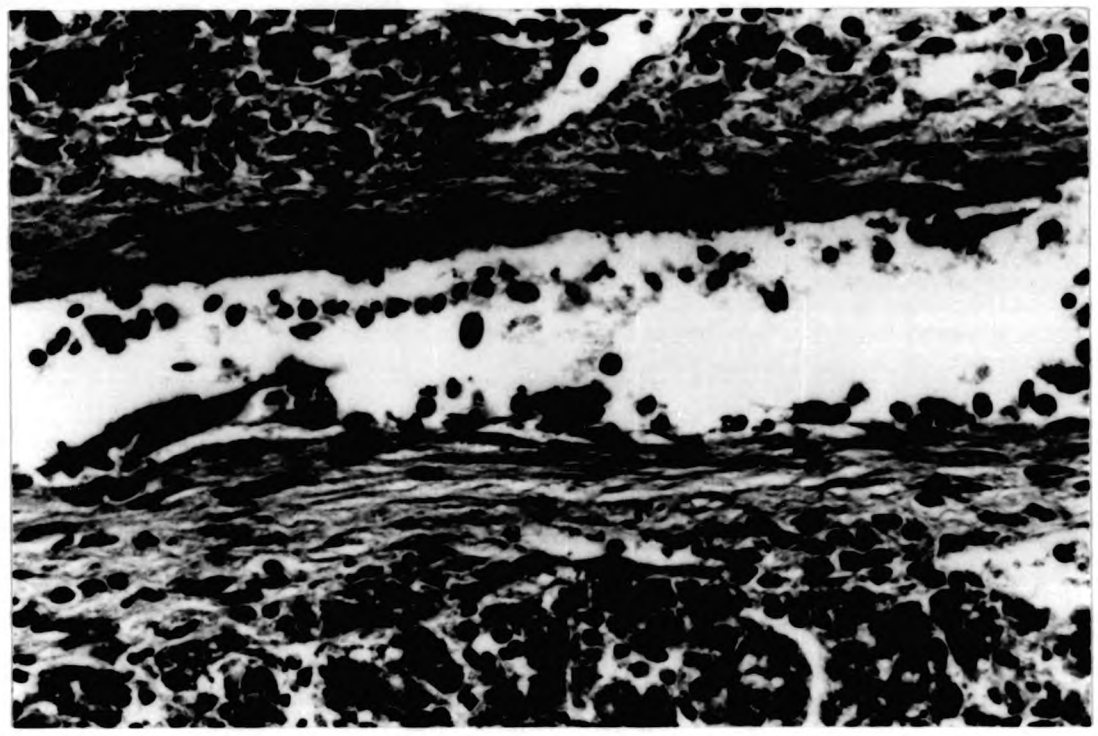


Fig. 3.27. Crystals apparently free within hepatic artery. H & E x 375.

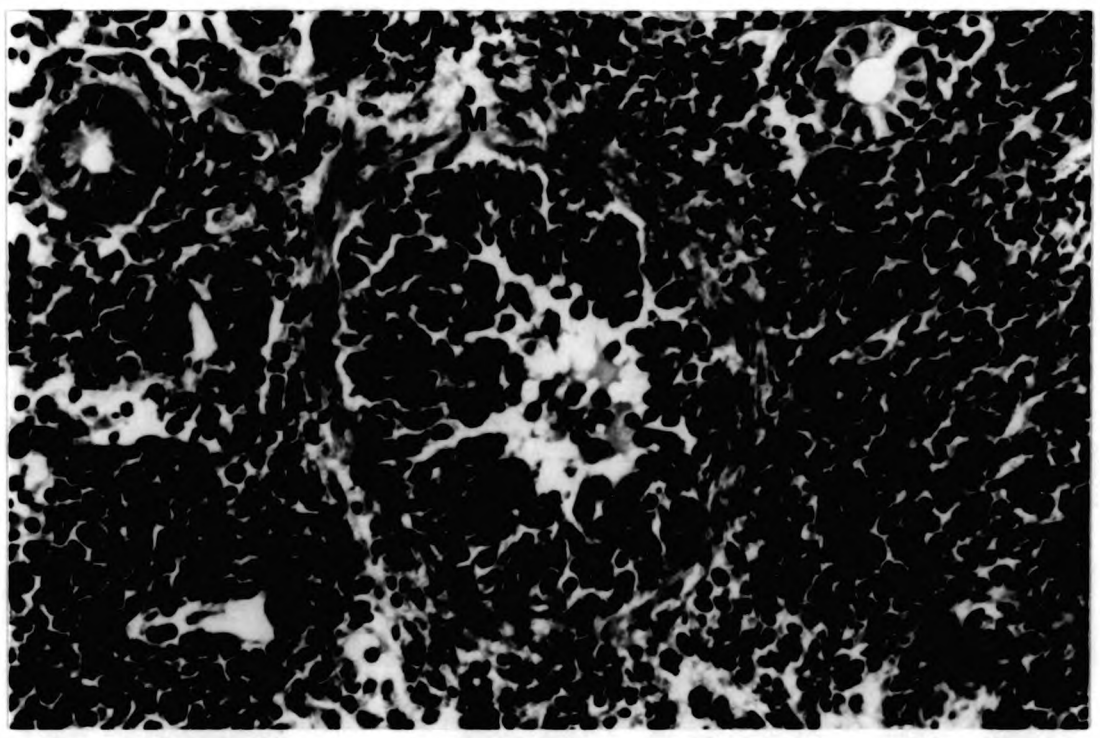
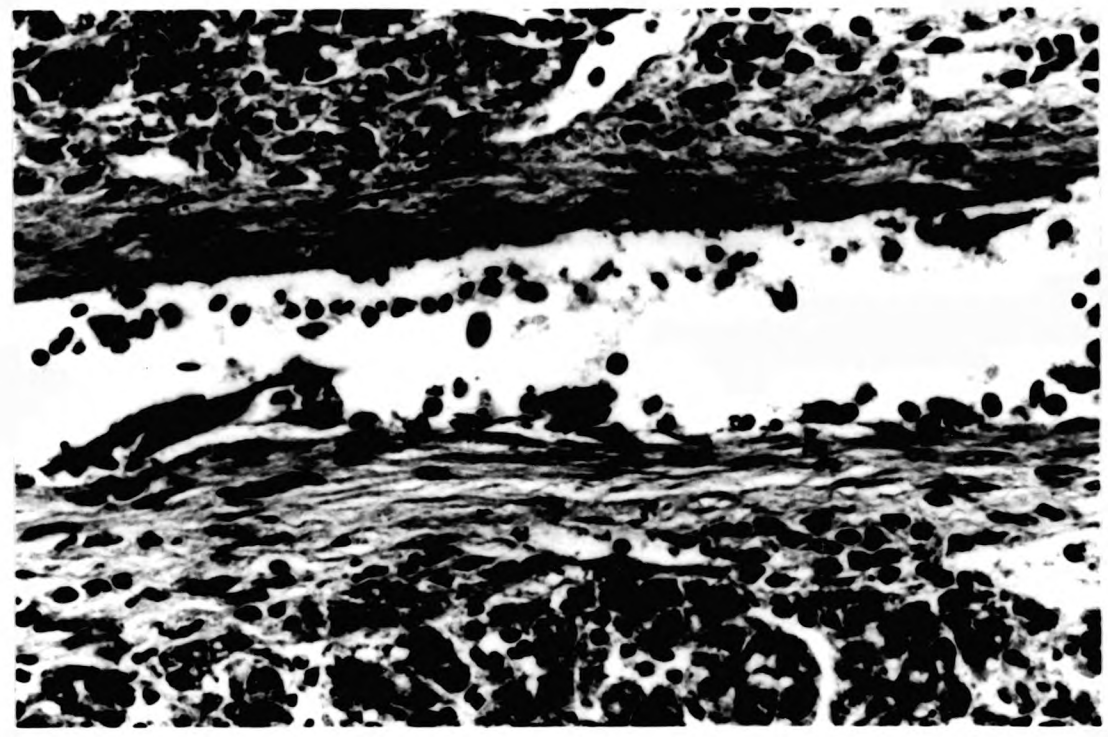


Fig. 3.28. Intra-renal blood vessel partially occluded with cells and crystals, many possibly intra-cellular, orientated at right-angles to the vessel wall. The vessel is surrounded by hyperplastic haemopoietic tissue. H & E x 385.



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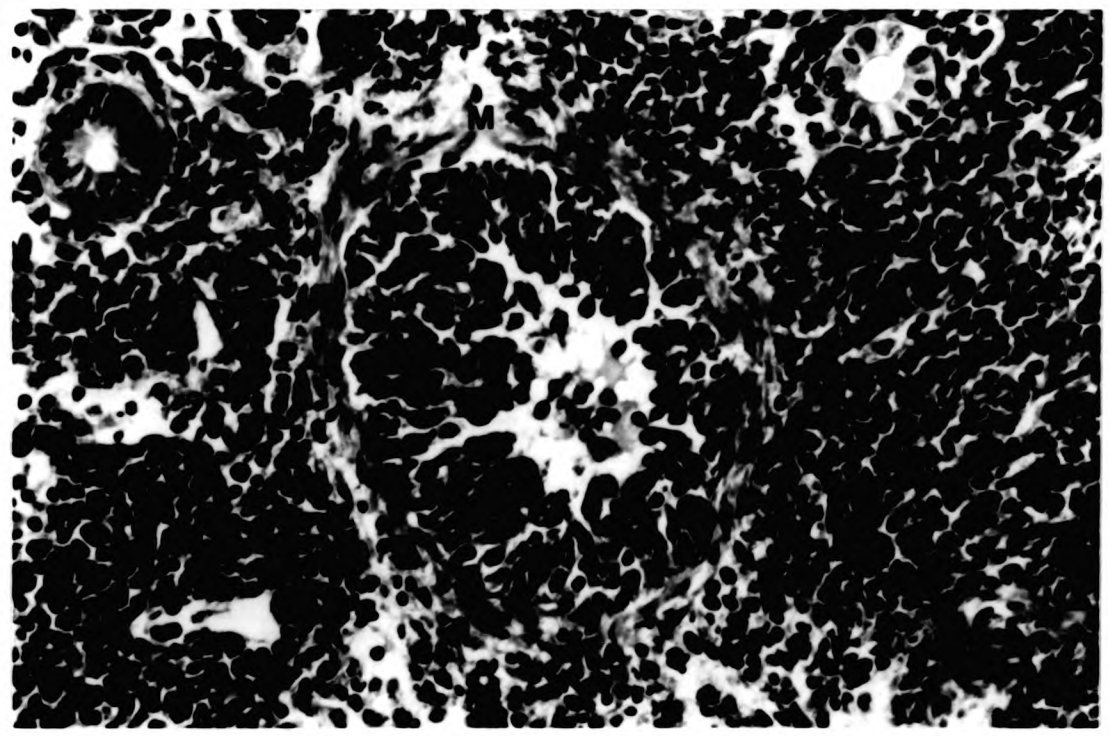

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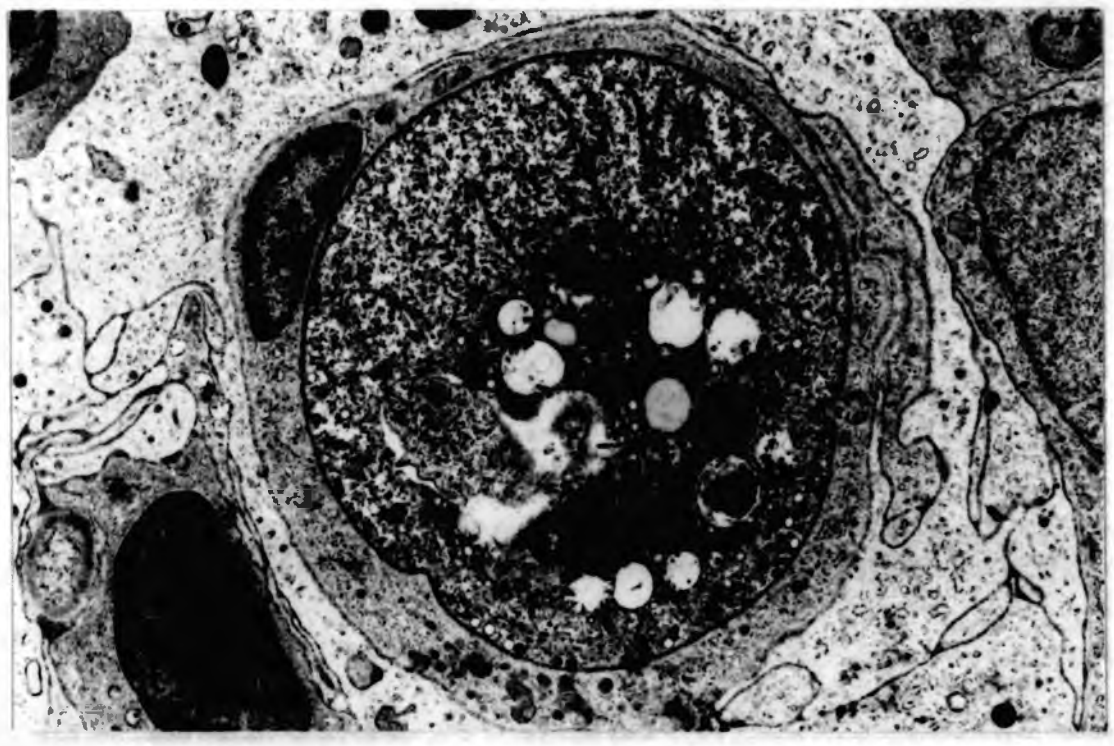


Fig. 3.29. Electron-micrograph of PKX cell (P) showing loss of cytoplasmic detail, increased vacuolation and decreased number of haplosporosomes, surrounded by phagocytic cell (L). Uranyl acetate and lead citrate. x 3,300.

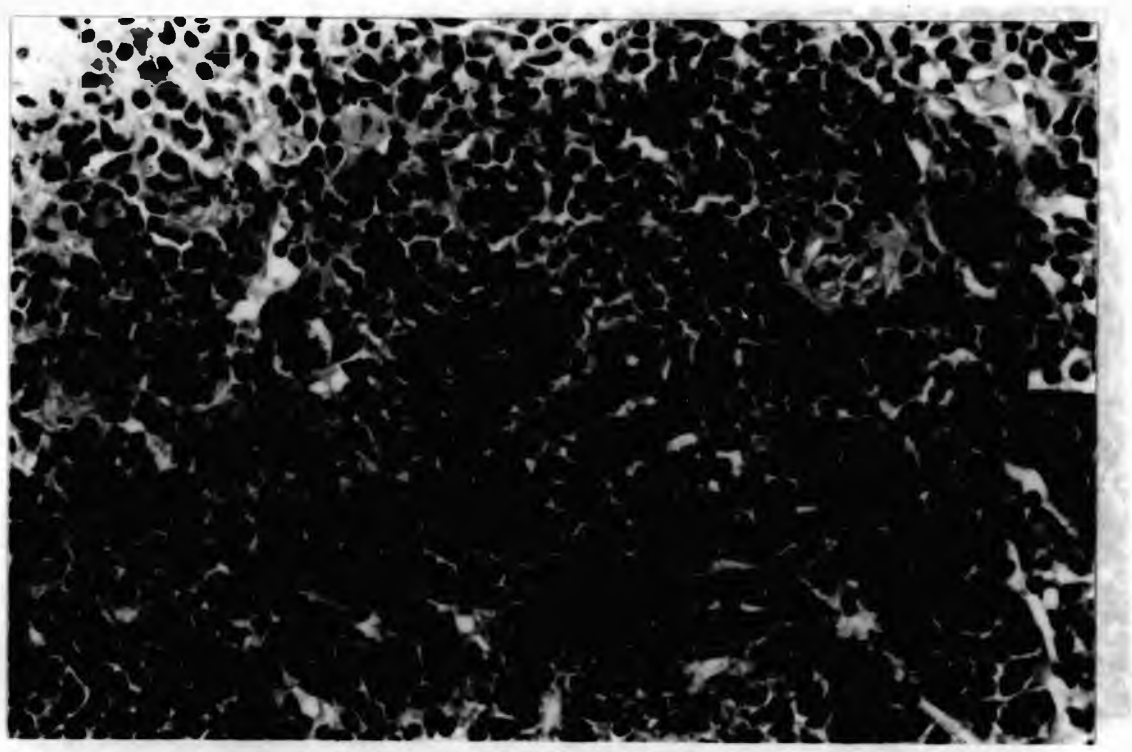
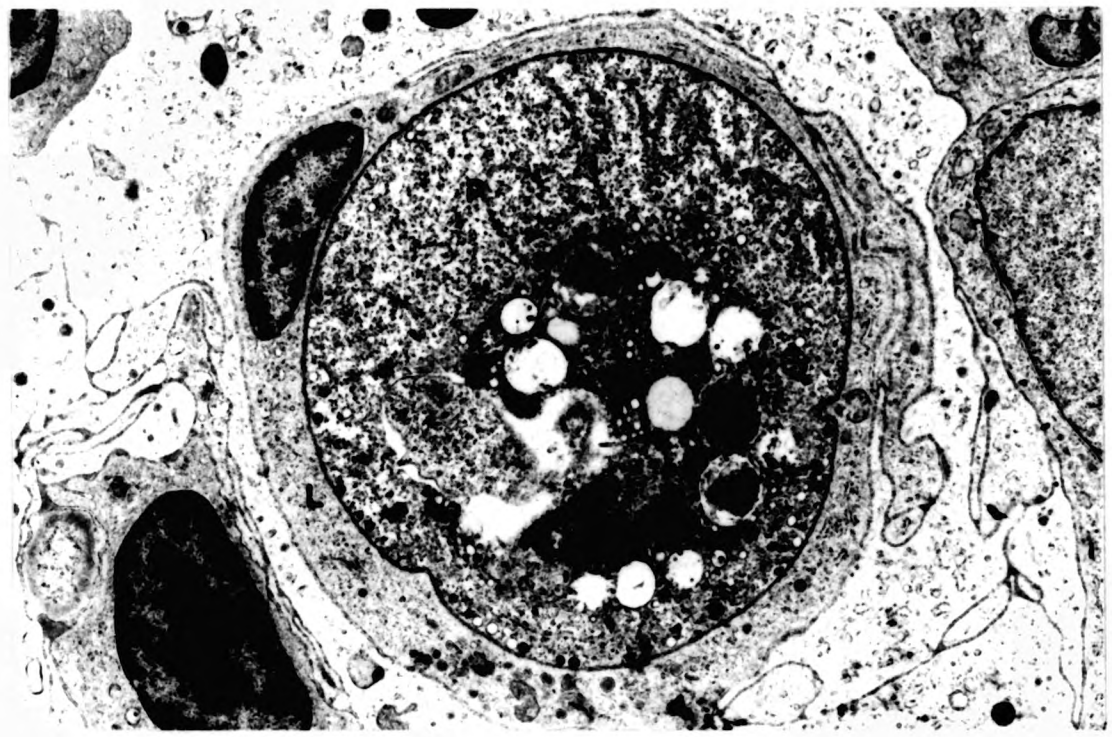


Fig. 3.30. Increase in cells containing Schmorl's positive material (arrowed), including melanin, within renal interstitial tissue. H & E x 375.



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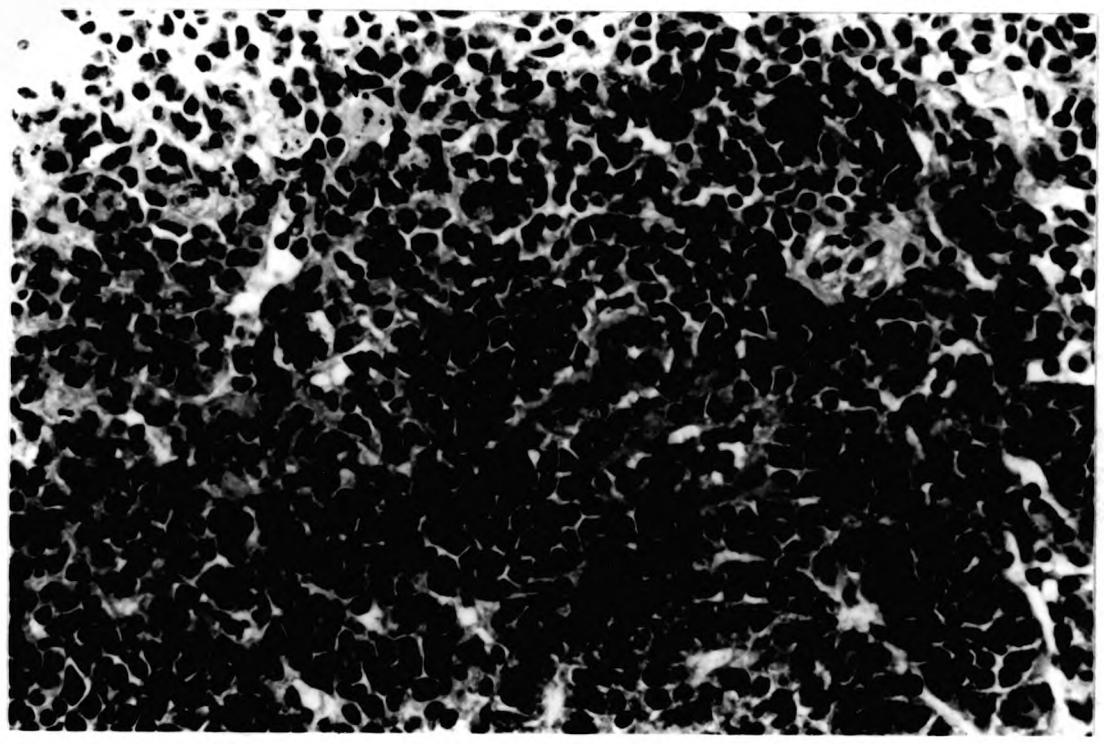

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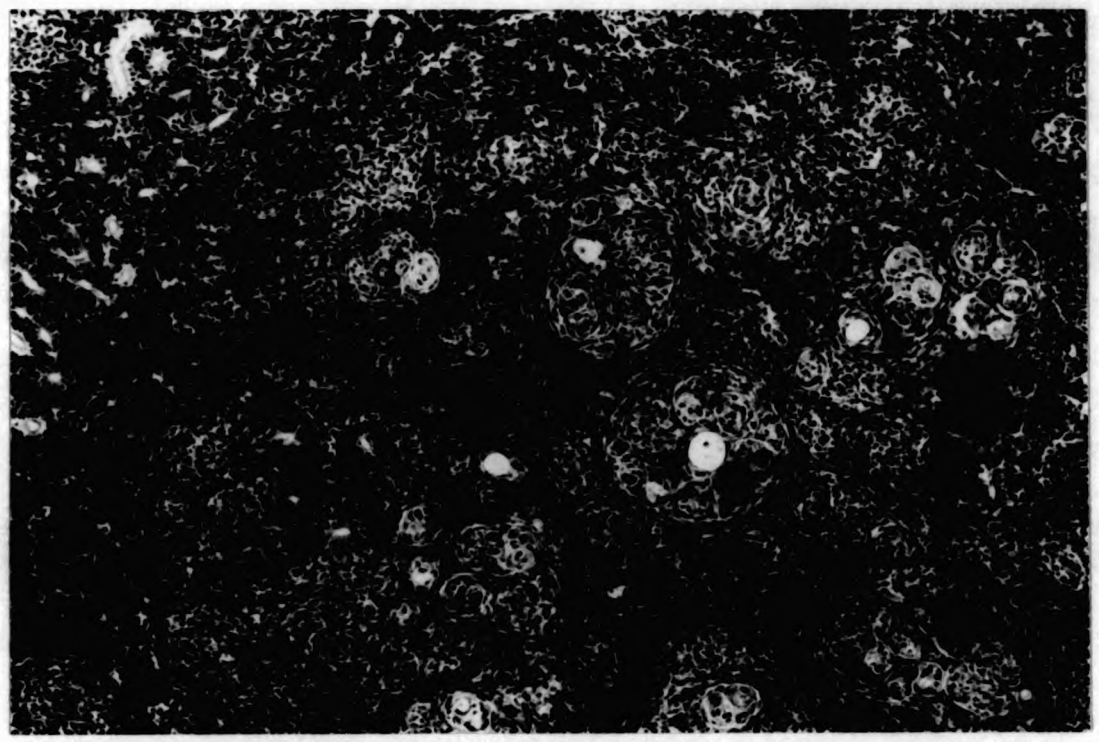


Fig. 3.31. Nodule of chronic inflammation containing several granulomata (G) surrounded by "normal" renal tissue (N). H & E x 90.

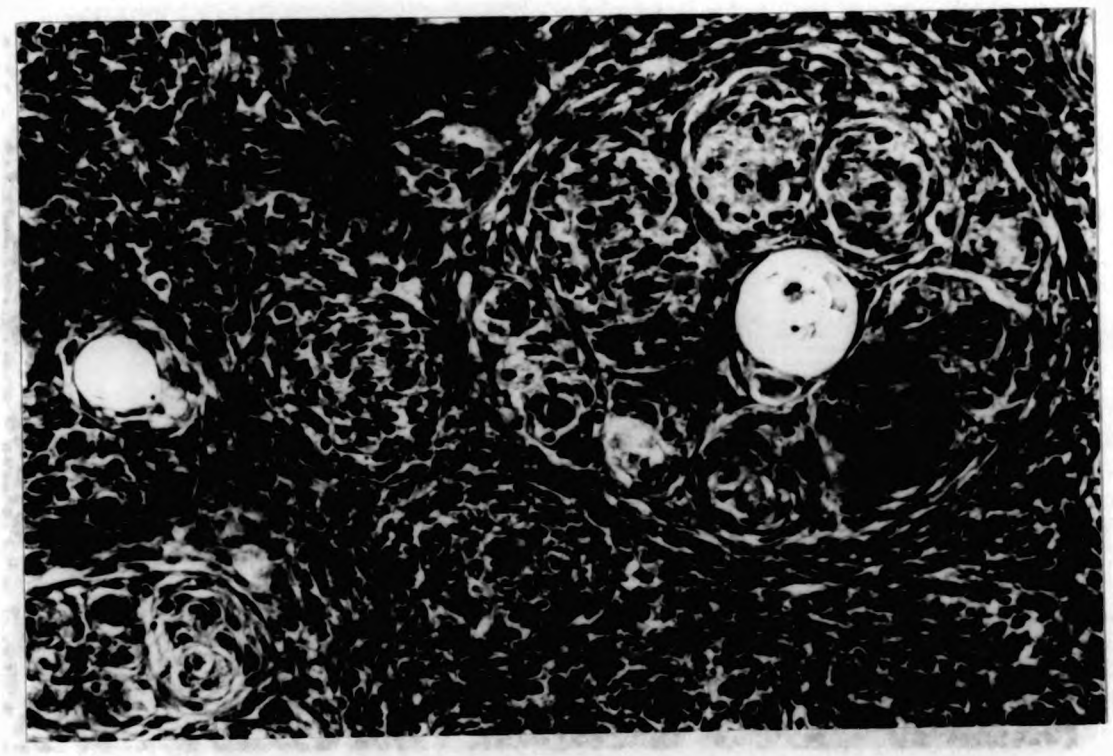
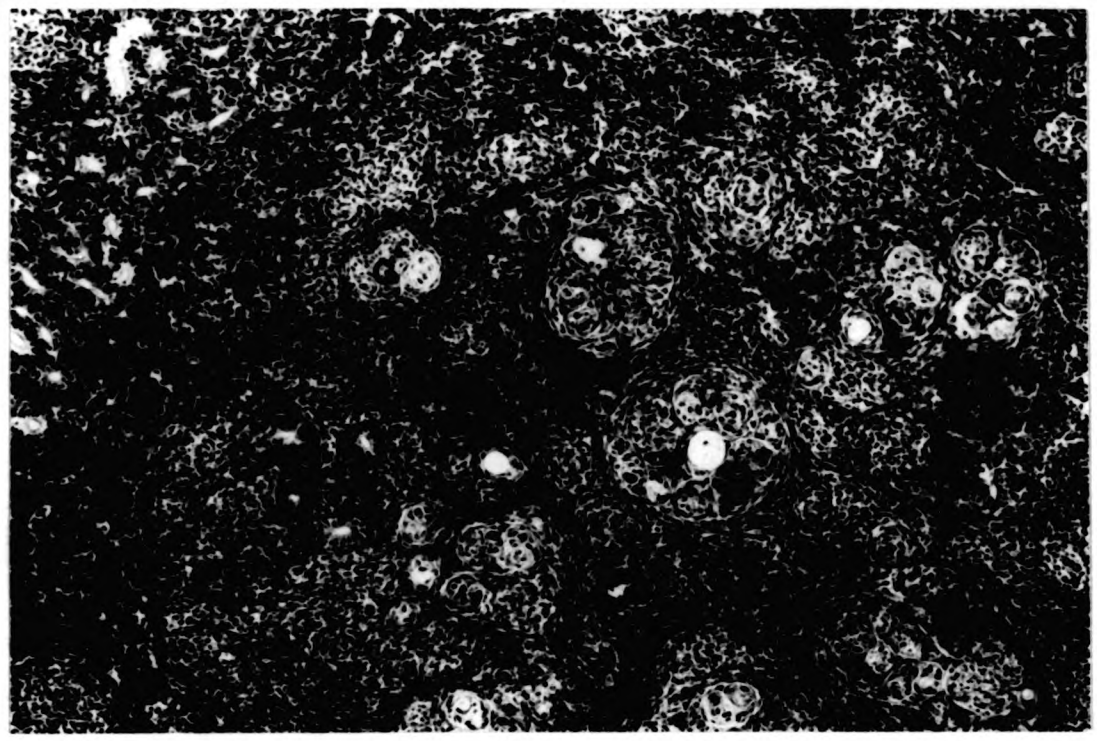


Fig. 3.32. Detail of Fig. 3.31. Granulomata within nodule of chronic inflammation often centred on PKX cells (arrowed) and necrotic material. H & E x 280.



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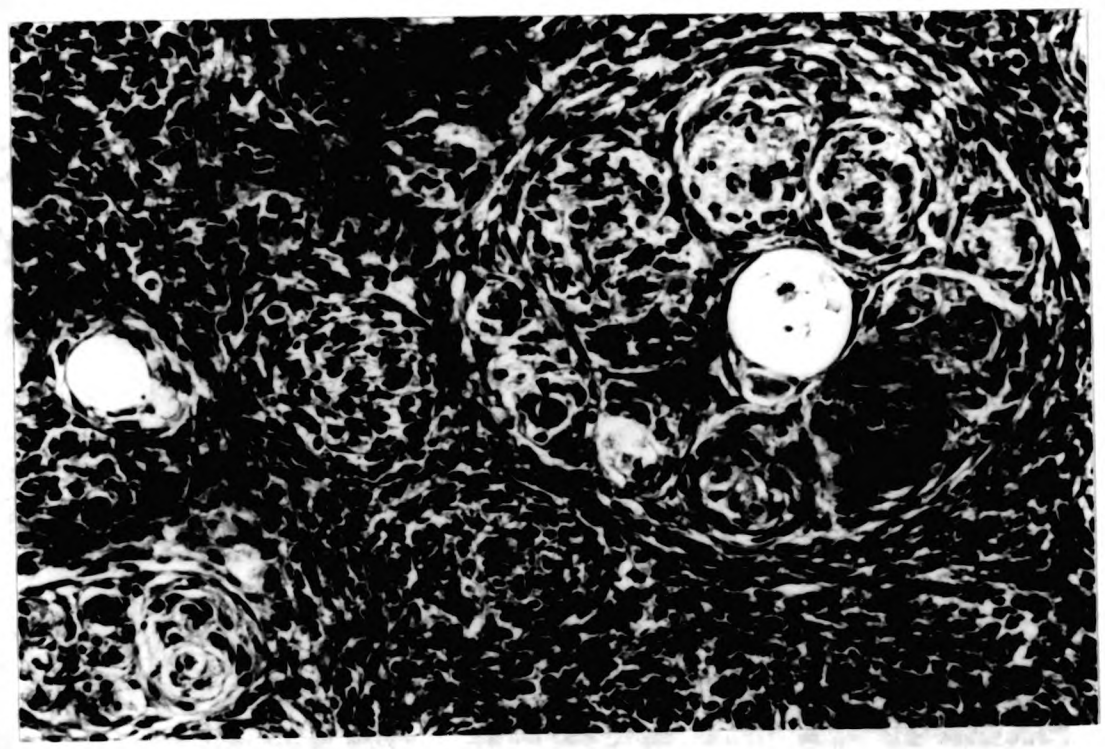

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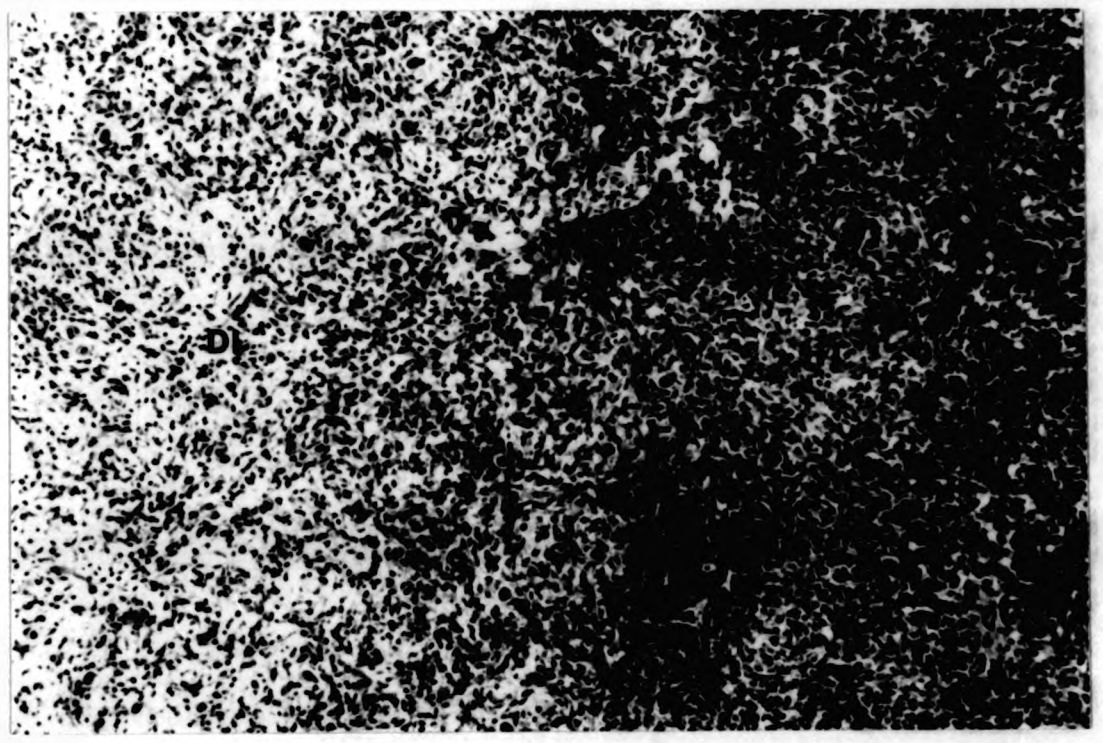


Fig. 3.33. Area of diffuse inflammation (DI) with many fluid-filled spaces and PKX cells interspersed with darker haemopoietic tissue (HT) in a spleen. H & E x 105.

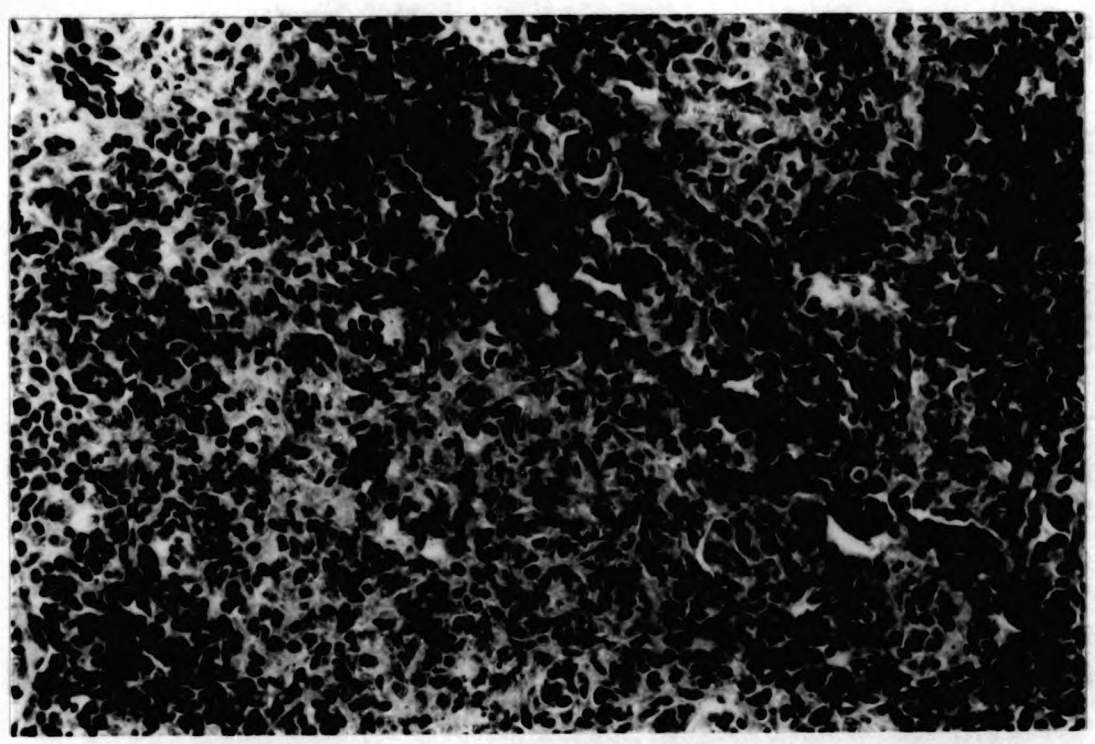
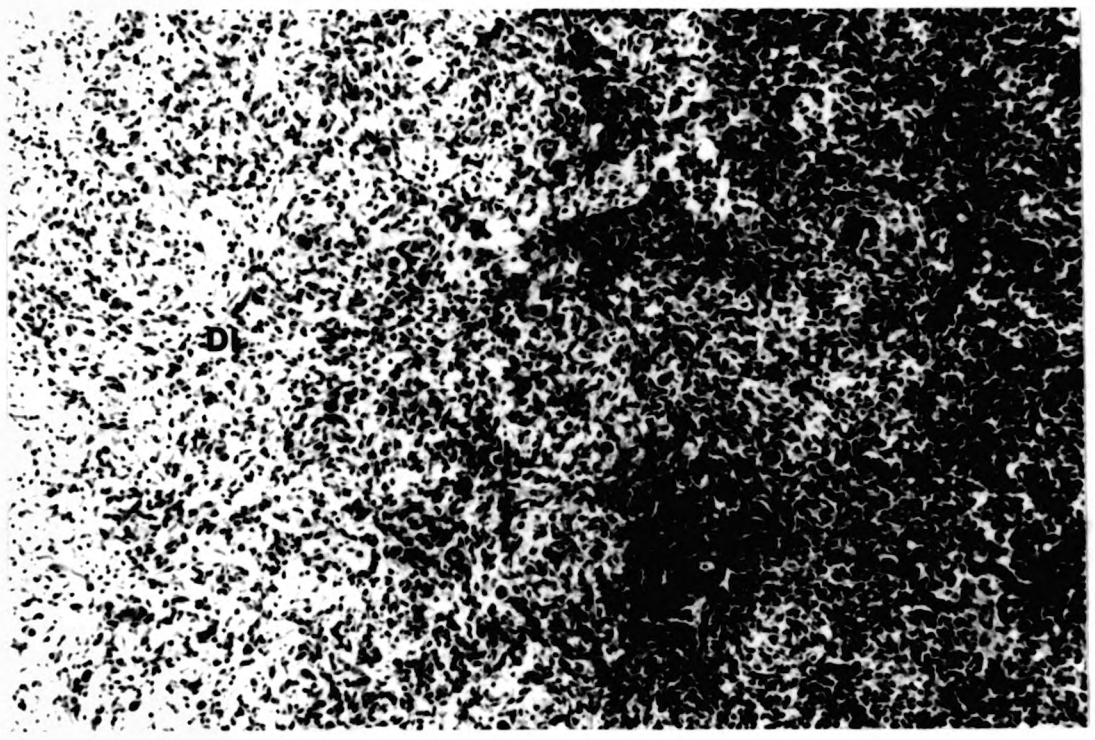


Fig. 3.34. PKX cells (arrowed) in spleen, apparently containing melanin granules. Possible macrophage proliferation (M). H & E x 260.



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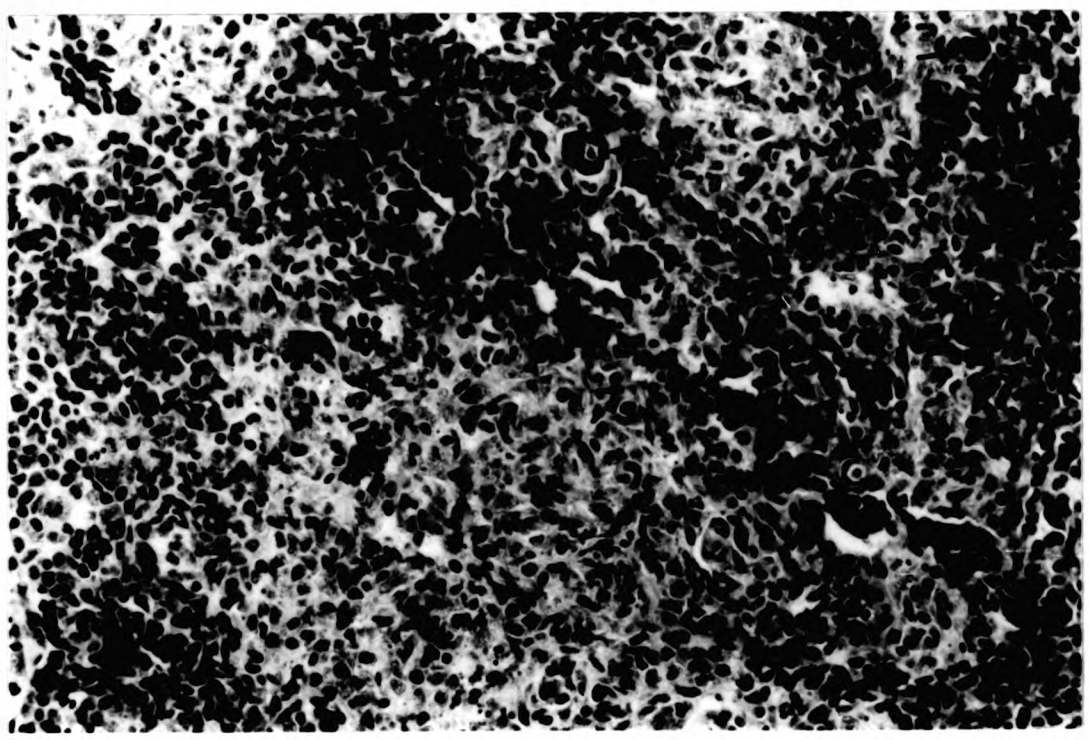

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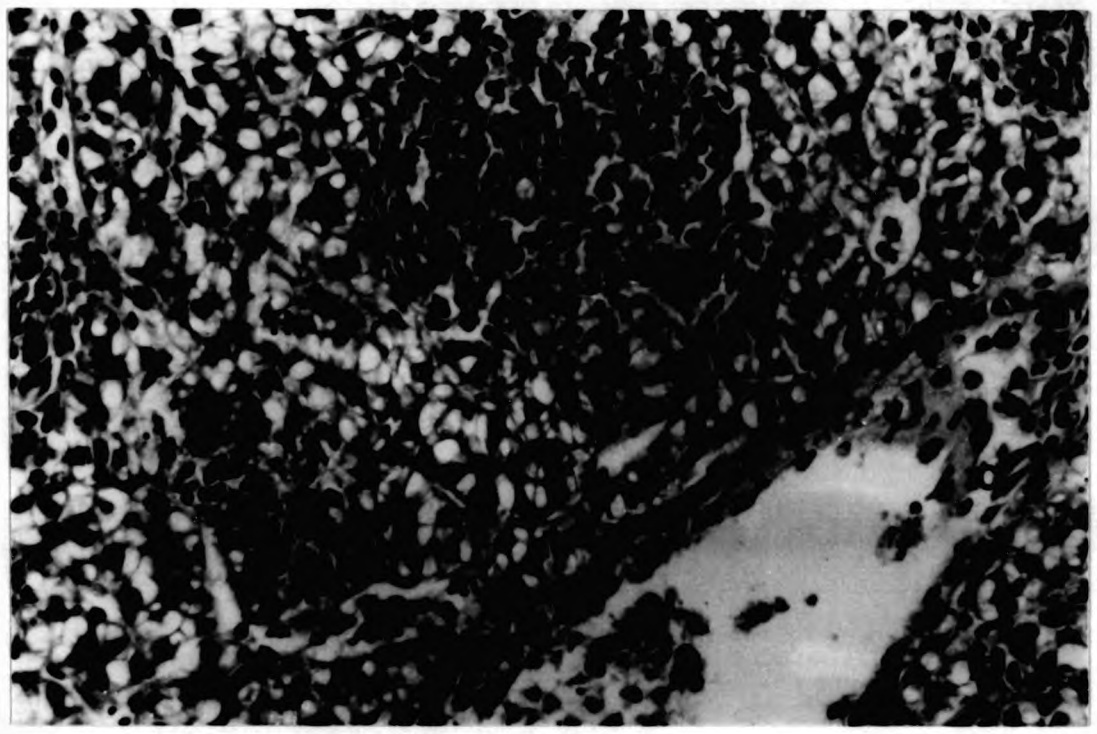


Fig. 3.35. Histological appearance of yellow liver, showing vacuolated hepatocytes (H), foci of increased cellularity (IC) and blood vessel with crystals (C). H & E x 310.

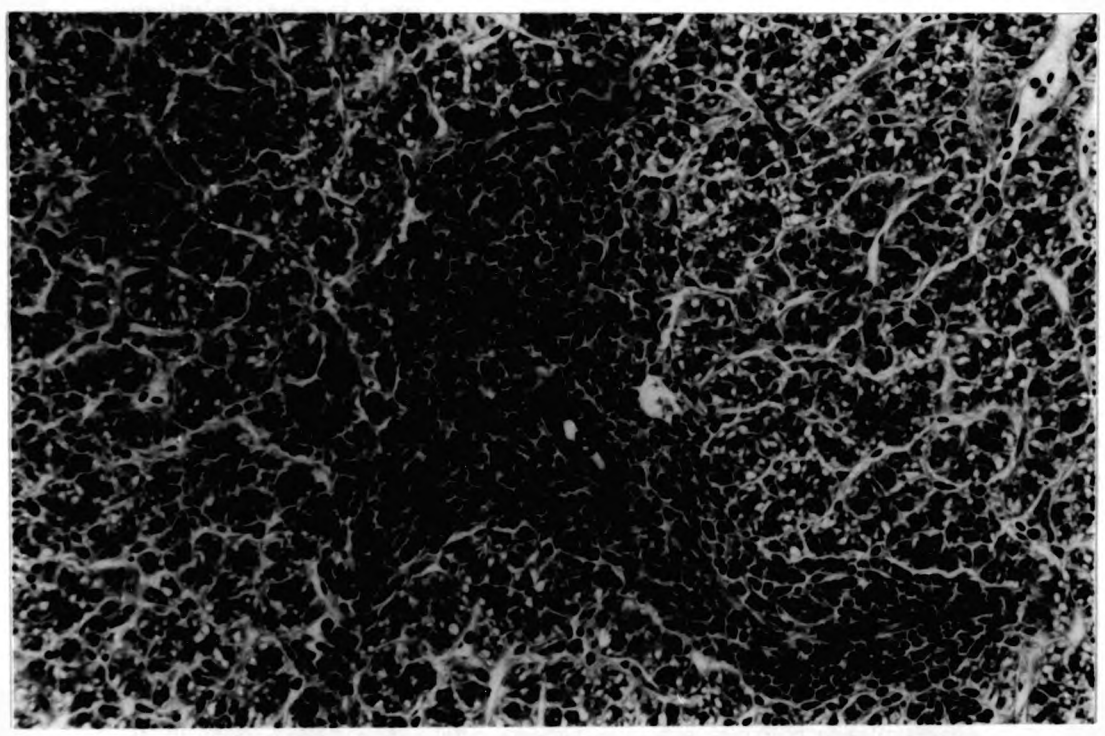
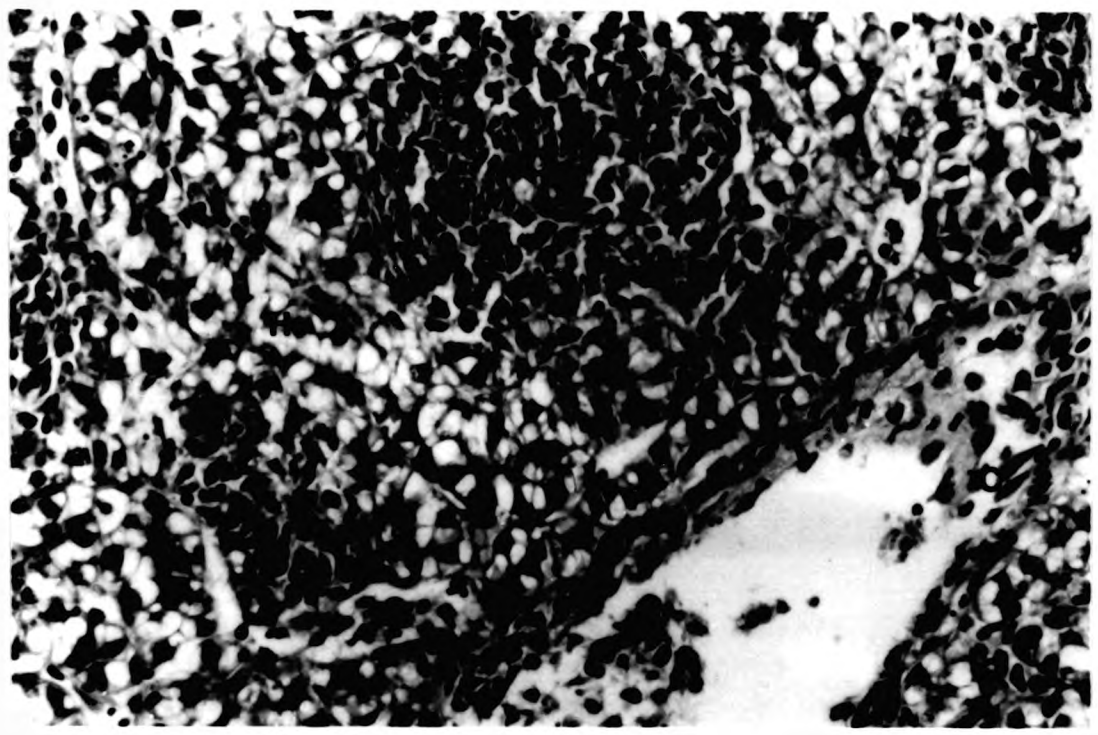


Fig. 3.36. Area of possible hepatic metastatic haemopoiesis (M) centred on bile ducts (B). Hepatocytes (H) with some vacuolation. H & E x 155.



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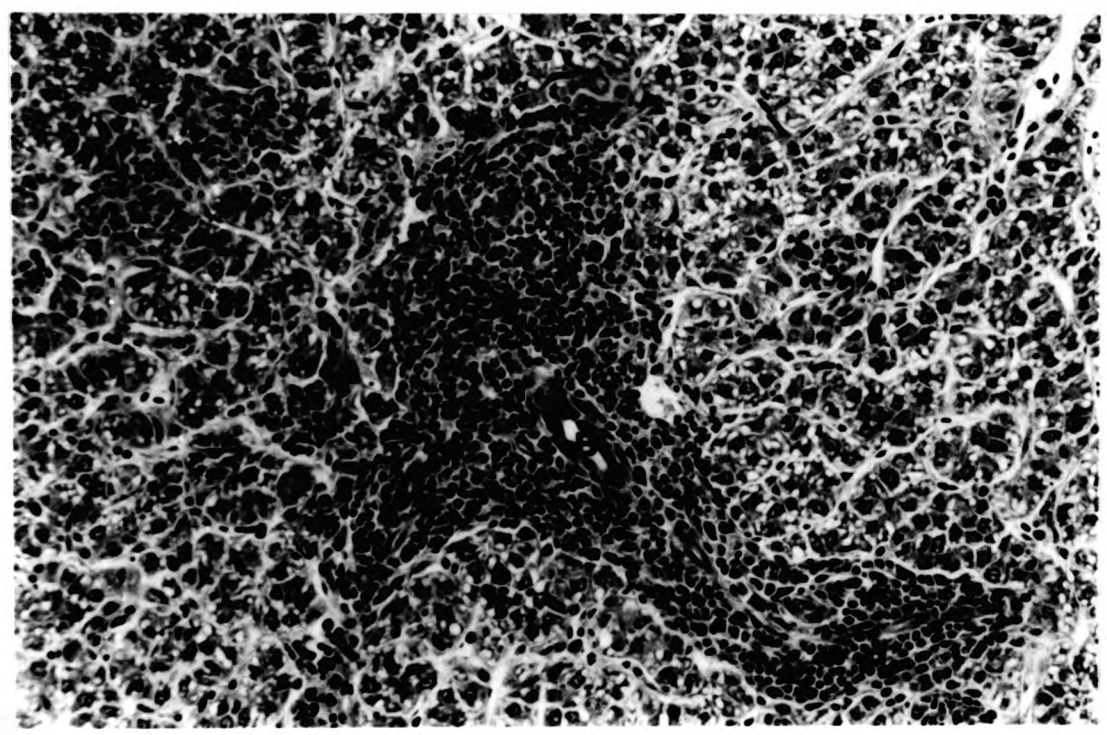

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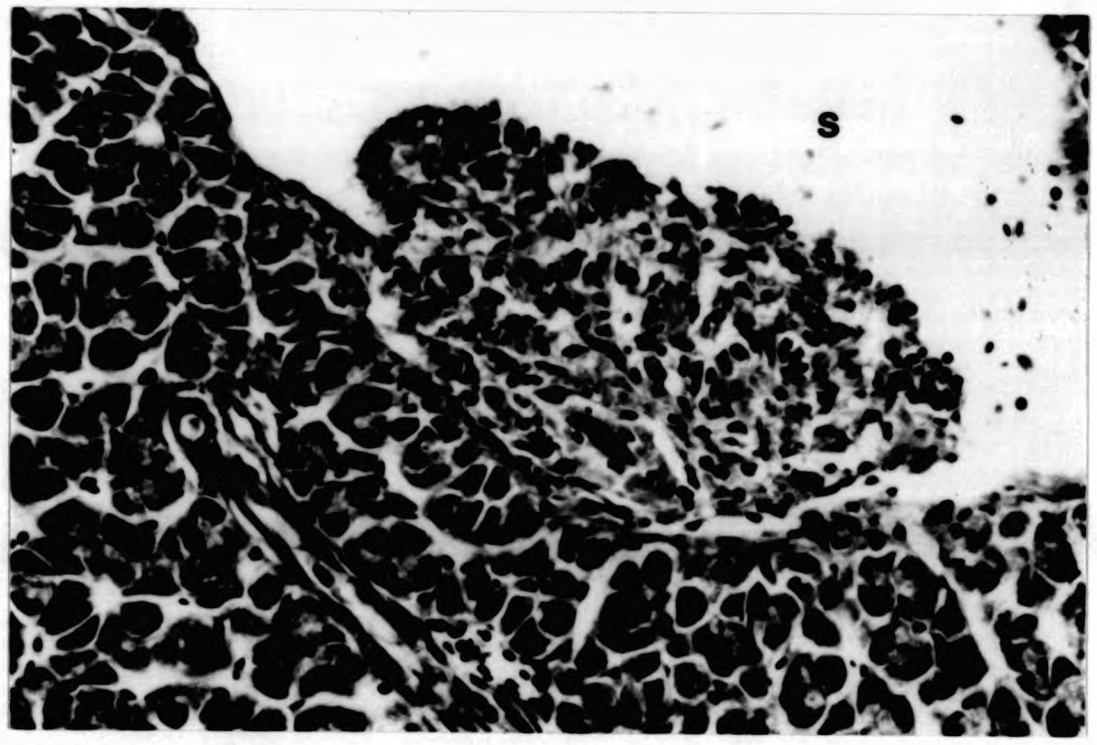


Fig. 3.37. Hepatic sinusoid (S) with partial occlusion due to cellular aggregation containing fibrin. H & E x 310.

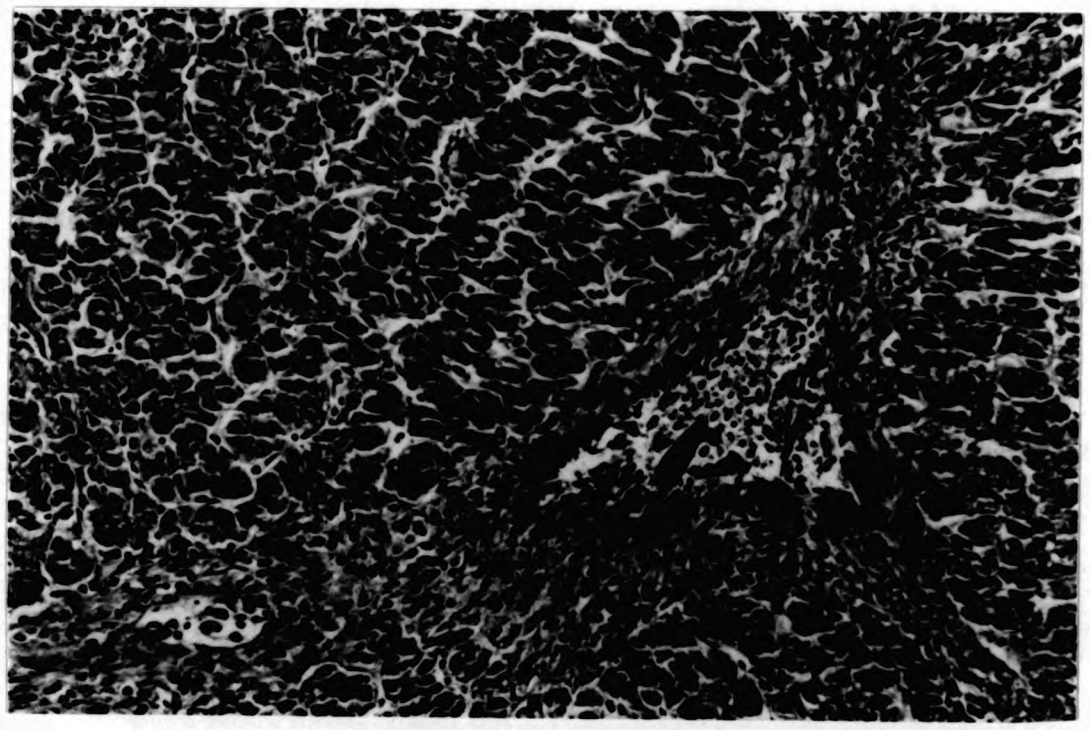


Fig. 3.38. Hepatic blood vessel containing many crystals, some apparently free within vessel lumen. H & E x 160.

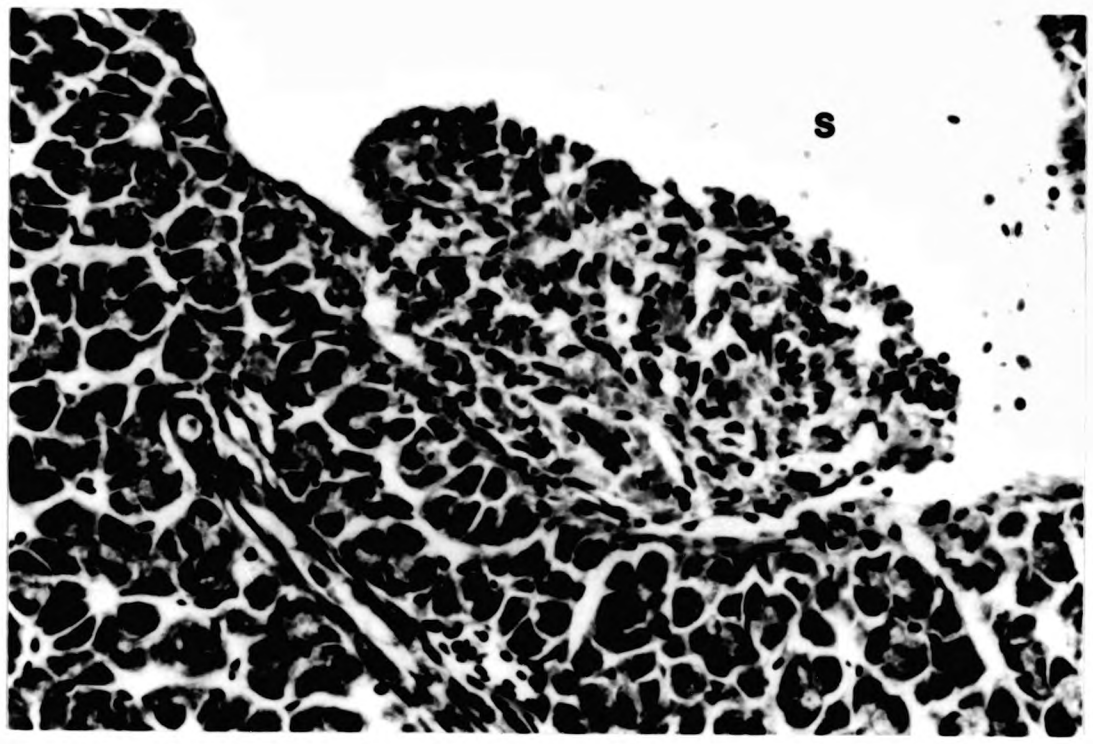


Fig. 3.37. Hepatic sinusoid (S) with partial occlusion due to cellular aggregation containing fibrin. H & E x 310.

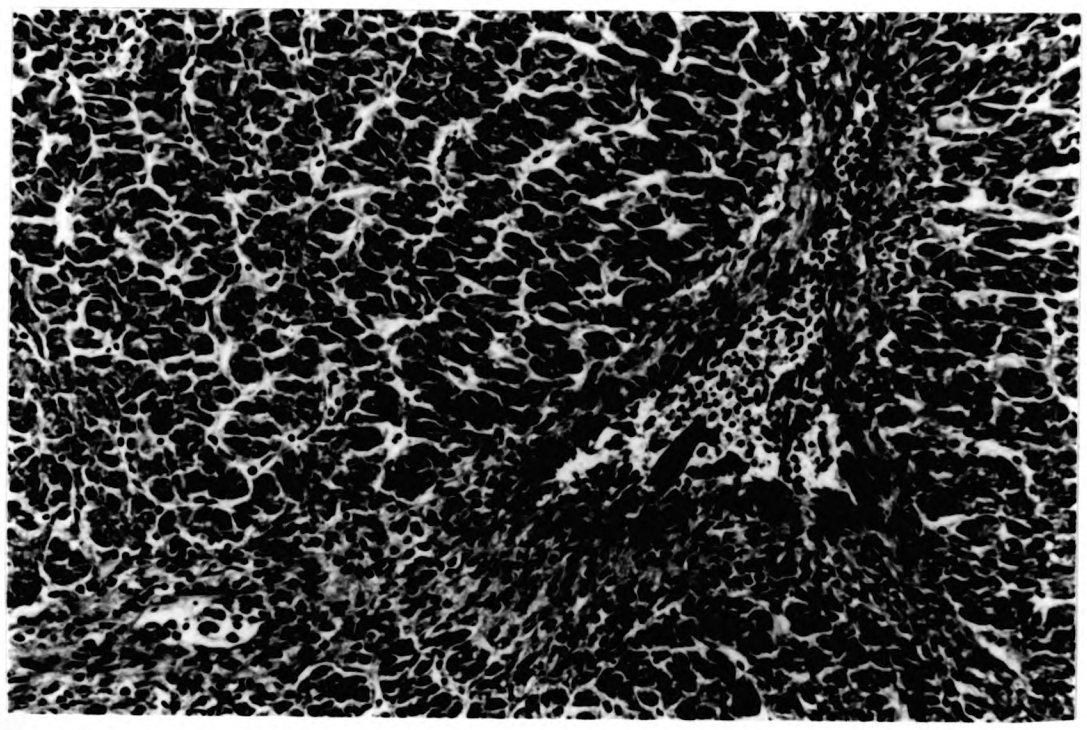


Fig. 3.38. Hepatic blood vessel containing many crystals, some apparently free within vessel lumen. H & E x 160.

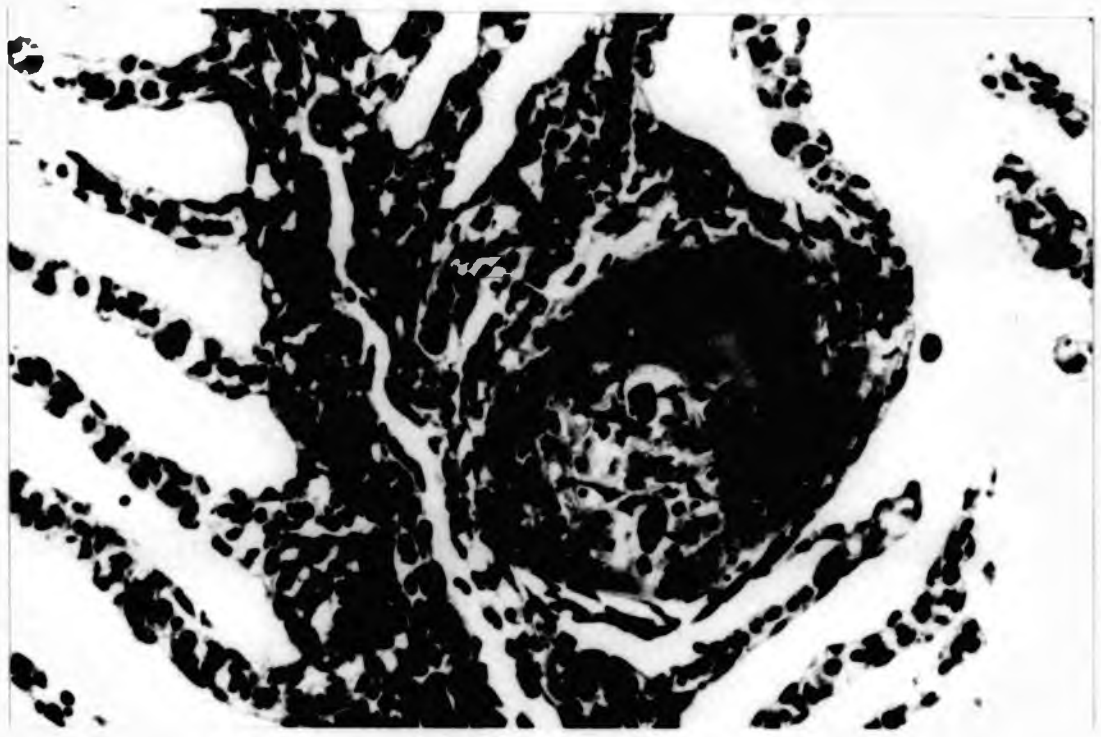


Fig. 3.39. Telangiectasis within secondary lamella of the gill. PKX cells (arrowed) within the clotted blood. H & E x 385.

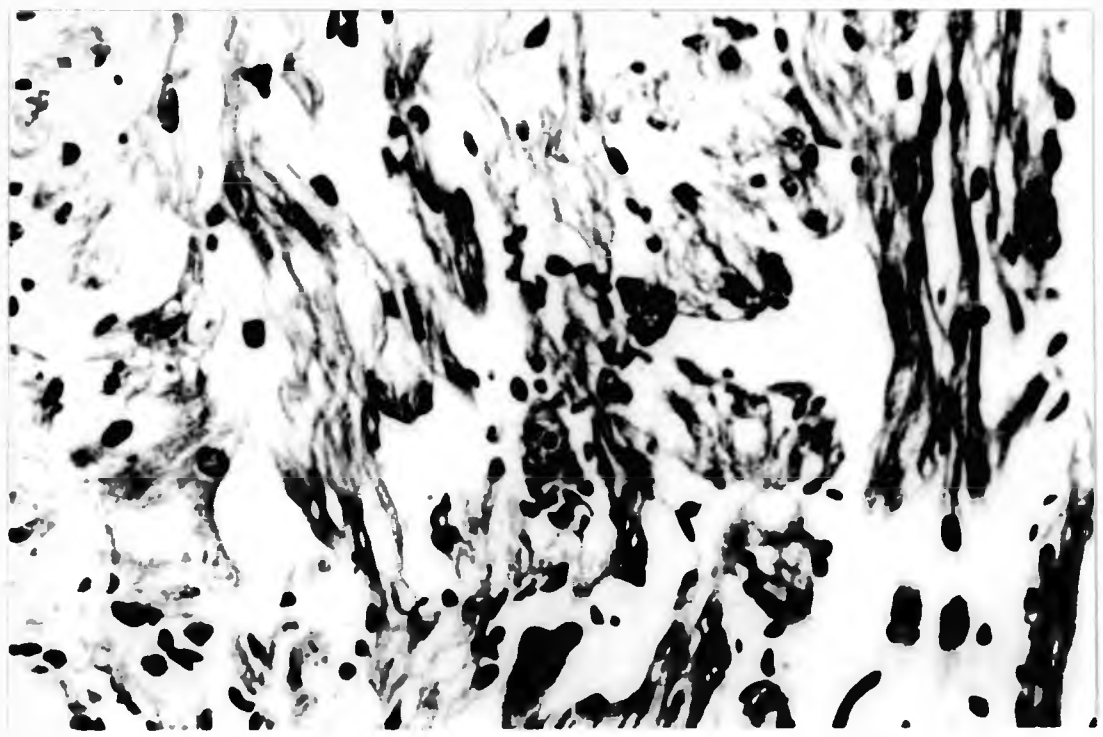
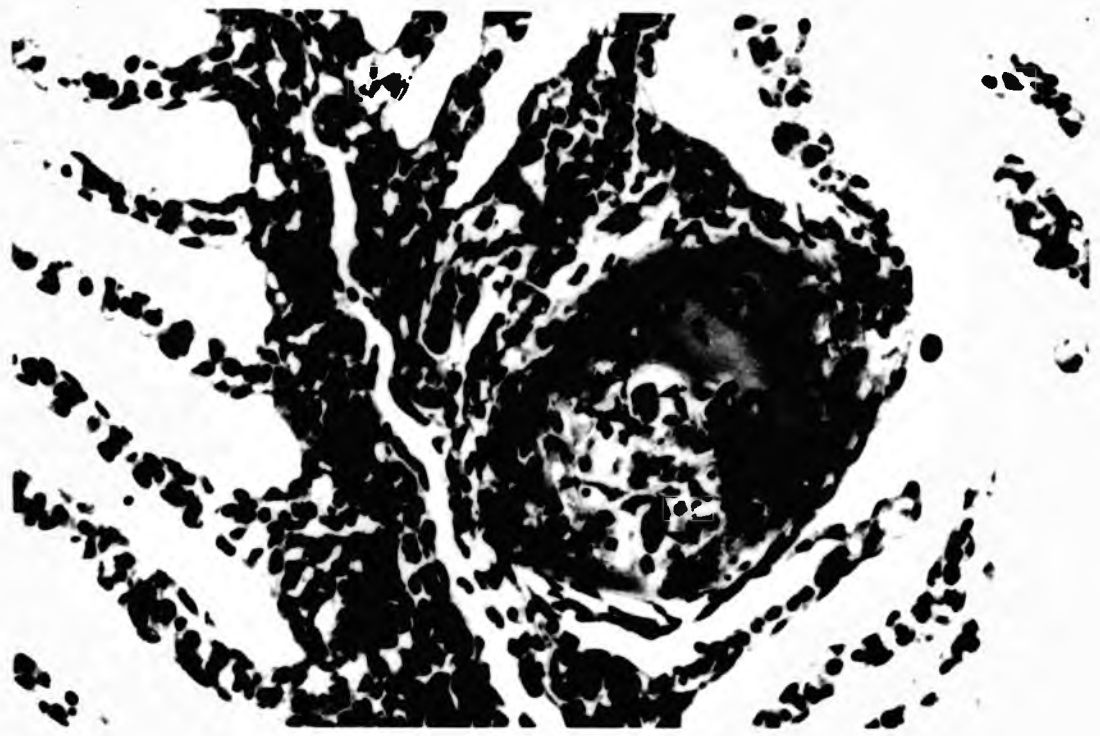


Fig. 3.40. Stratum spongiosum of the cardiac ventricle with two PKX cells (P). H & E x 420.



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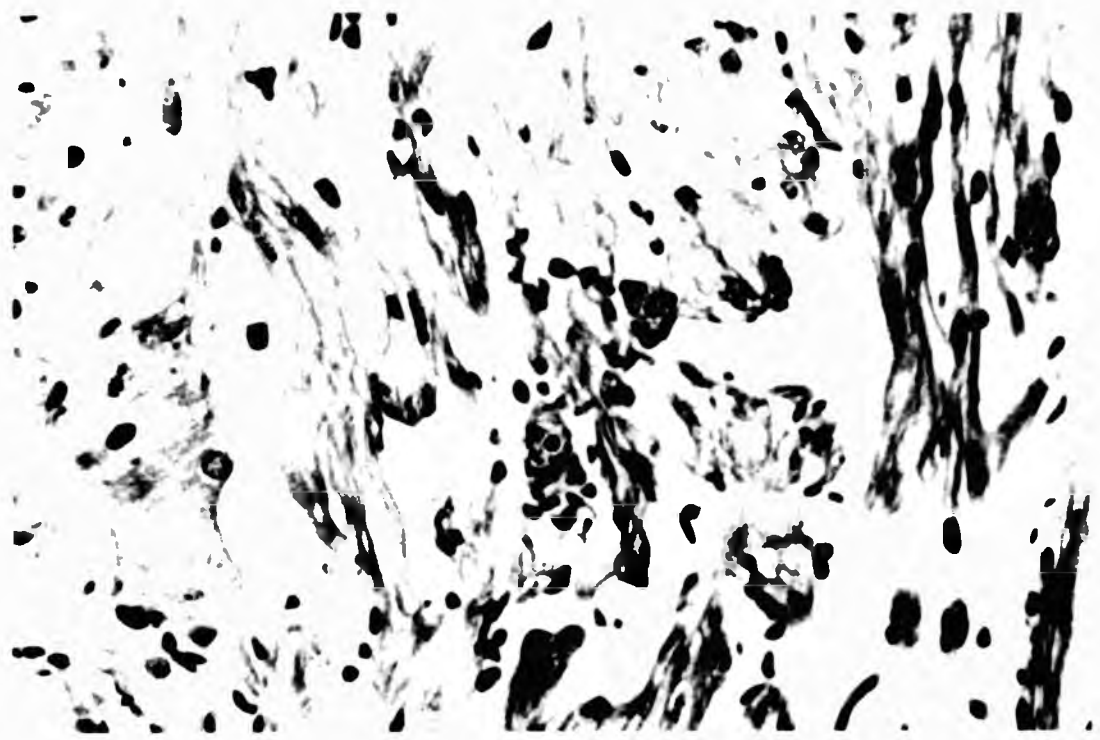

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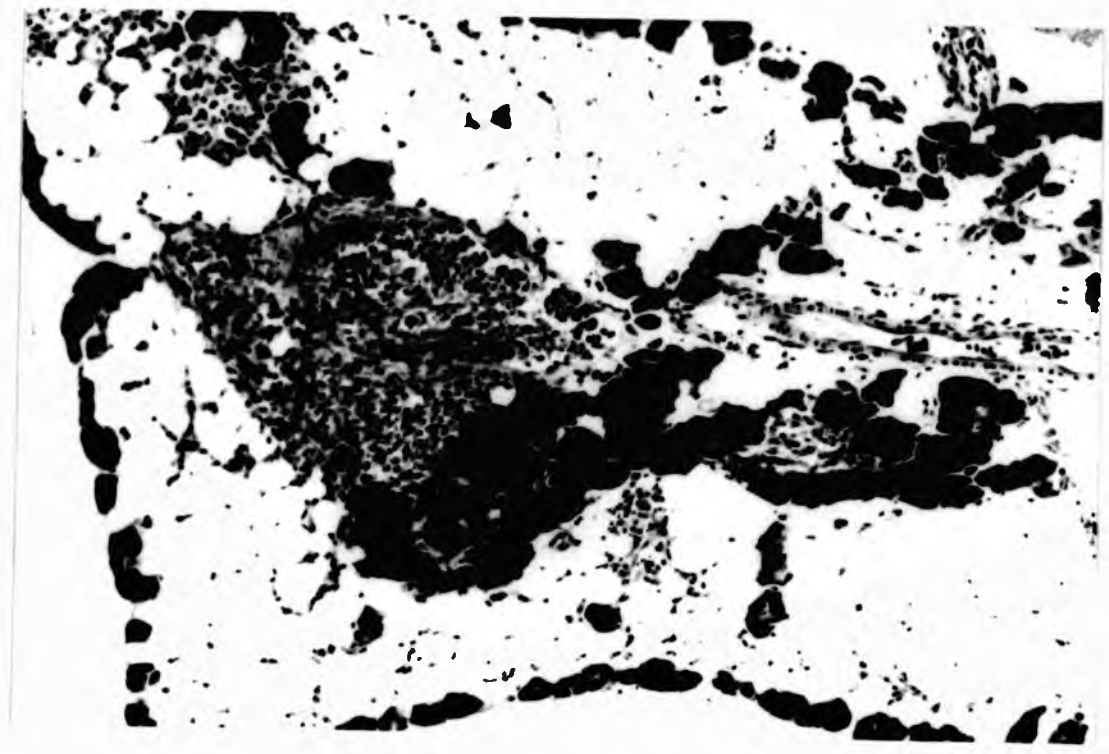


Fig. 3.41. Nodule of inflammation in peri-acinar lipoid tissue. Blood vessel (BV) distended and occluded by cellular debris and PKX cells. H & E x 110.

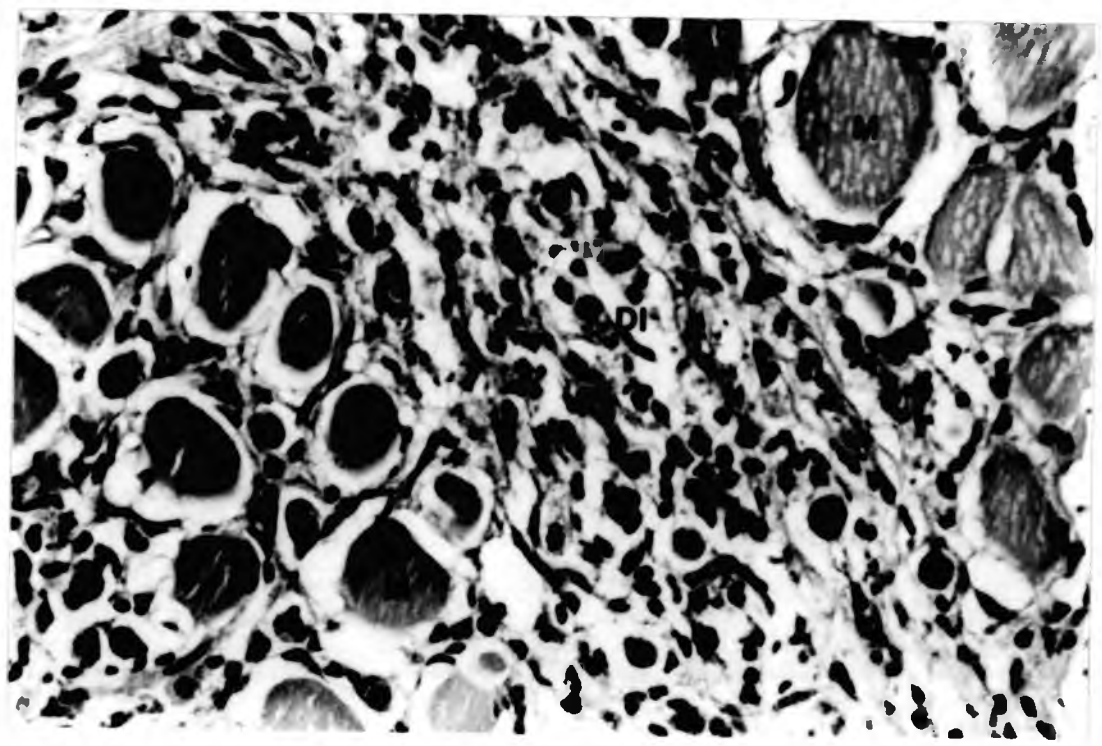
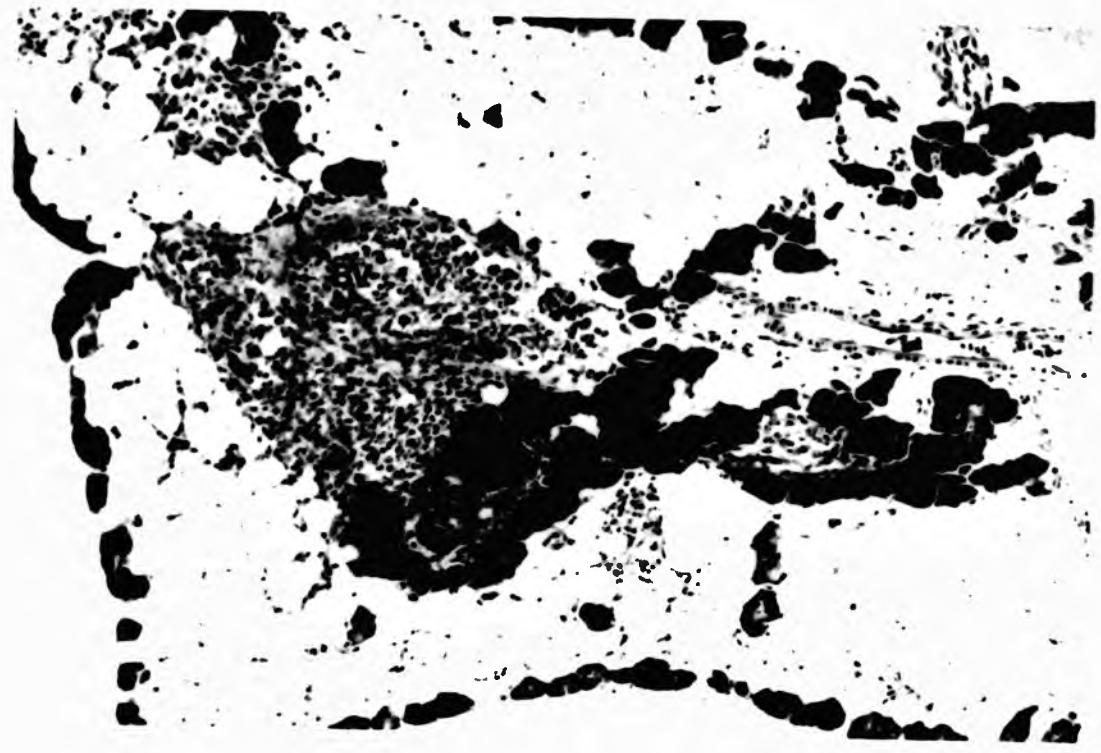


Fig. 3.42. Diffuse inflammation (DI) with several PKX cells between striated muscle fibres (M).
H & E x 350.



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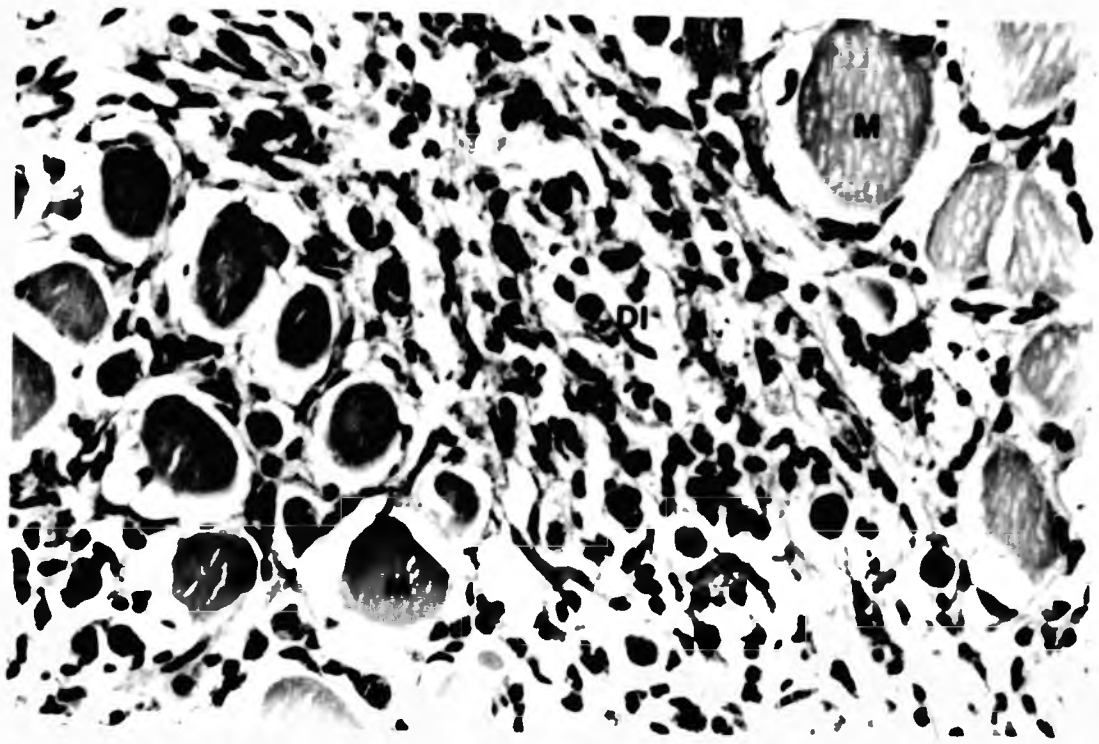

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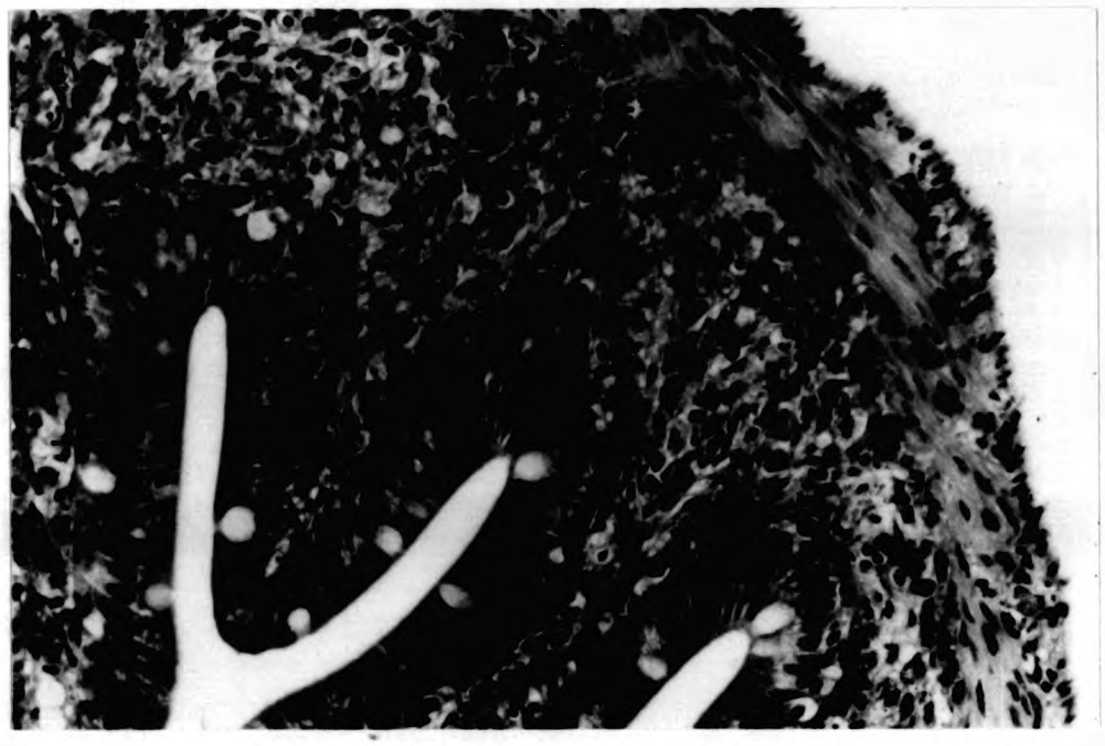
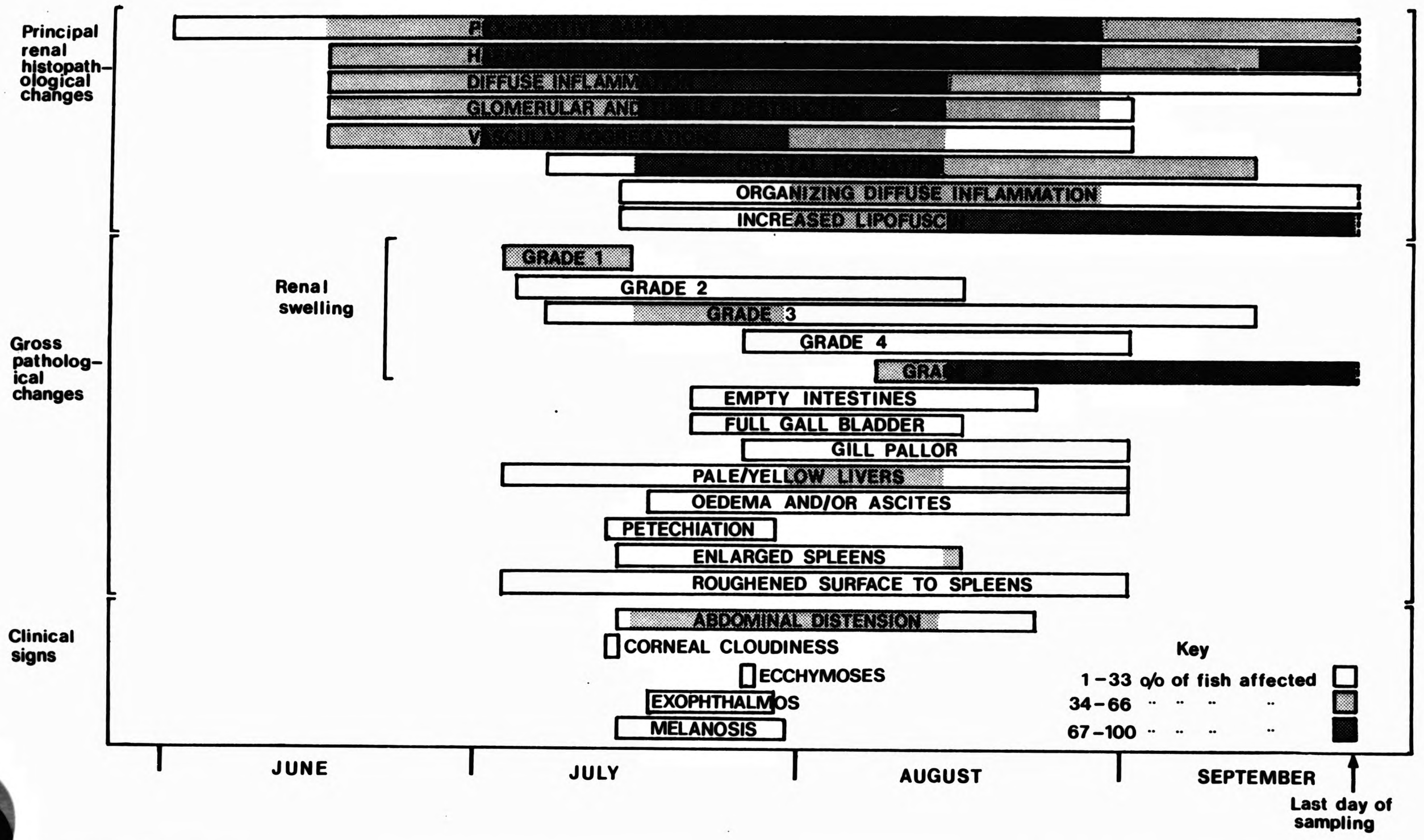


Fig. 3.43. Thickened caecal submucosa containing PKX cells (arrowed), areas of macrophage-like cells (M) and eosinophilic granule cells (E). H & E x 280.

Fig. 3.43. Thickened caecal submucosa containing PKX cells (arrowed), areas of macrophage-like cells (M) and eosinophilic granule cells (E). H & E x 280.

Fig.3.44. The chronological relationship between appearance and duration of clinical signs, gross pathological changes and principal renal histopathological changes in rainbow trout sampled in groups A and B.



Chapter 4

The haematology of PKD

INTRODUCTION

It has long been recognised that fish clinically-affected with PKD appear anaemic (Plehn, 1924; Ghittino et al., 1977; de Kinkelin and Gérard, 1977b; Ferguson and Needham, 1978; Roberts, 1978; Smith et al., 1984). As described in chapter 1, several workers have studied blood parameters in such fish, including haematocrits, total red and white blood cell counts, appearances of red blood cells, haemoglobin levels, urea levels and total serum protein concentration (Ghittino et al., 1977; Ferguson and Needham, 1978; Hoffmann and Lommel, 1984; Scott, 1984). Only Hoffmann and Lommel (1984) studied several parameters in the same fish. Discrepancies were evident between the results of different workers. There have been no previous reports on the haematology of sub-clinically-affected fish. Only serum protein values have been reported in recovered and re-challenged fish (Scott, 1984).

From the experiments described in chapter 3, histological findings suggested that haemopoiesis was stimulated in the early stages of PKD. Only with the development of diffuse inflammation were renal and splenic haemopoietic tissue and erythrocytes reduced. This was accompanied by stimulation of secondary centres of haemopoiesis. The main purposes of the work described in this chapter were:

1. To study in more detail the cellular and biochemical changes in the blood of naturally-infected fish:

(a) Sub-clinically infected with PKD.

(b) Clinically affected with PKD.

(c) Recovering from PKD.

(d) Exposed after recovery to infected water for a second year.

2. To relate these changes to other pathological changes in the fish.

It was hoped that these studies would help resolve the discrepancies between previous reports of haematological findings in PKD-affected fish and contribute to the understanding of the pathogenesis of the disease.

MATERIALS AND METHODS

Experimental fish

Samples were derived from five groups of fish. Four groups, A-D, were naturally infected at the experimental site. One uninfected group, E, was held at the laboratory. Group A comprised 300 fingerling rainbow trout, average length 65-75 mm, from farm B, and was exposed at the experimental site from 10 March 1983. Group B comprised part of a batch of 1,500 fingerling rainbow trout, 50-75 mm in length, from farm C and was moved to the site on 26 May 1983. Group C comprised 400 fingerling rainbow trout similar to group B. This group was transferred in two equal batches to the site on 2 December 1983 and 5 January 1984. Group D comprised 300 yearling rainbow trout, derived as fingerlings from farm A, and were moved to the experimental site on 30 April 1982. Groups A-D were managed at the site as described in chapter 2. Group E comprised 500 fingerling rainbow trout, approximately 50 mm in length, which were hatched and reared at farm A and transferred to the laboratory on 16 May 1983, where they were kept in the holding area as described in chapter 2.

Sampling schedule

Details of the sampling schedule are given in Table 4.1.

Fish in group A were sampled in their first summer on infected water to provide a sequential haematological and histopathological record of PKD until recovery from the disease was occurring. Haematocrit values,

haemoglobin concentrations, total red and white blood cell counts, erythrocytic indices, differential white cell counts, and total protein, albumin and urea concentrations were estimated from these fish.

Fish in group B were sampled in their first summer on infected water when they showed advanced clinical signs of PKD. Haematocrit values, total cell counts, and protein, albumin, globulin and urea concentrations were estimated for these fish.

Fish in group C were also sampled in their first summer on infected water to investigate serum or plasma albumin, serum bilirubin and serum globulin values.

Fish in group D were sampled during their second summer on infected water following recovery from and re-challenge with PKD. The same parameters as in group A were studied and, in addition, serum globulin concentration was estimated.

Fish in group E were unexposed to PKD and were sampled to assess normal plasma or serum total protein, albumin, bilirubin, globulin and urea values.

Pre-mortem sampling

Blood collection was performed during the morning. Without prior fasting, fish were netted individually and transferred to an aerated 0.01% solution of MS222 (Sandoz, Basle, Switzerland). Clinical signs were noted and, after approximately 60 sec, fish were removed from the anaesthetic solution. After drying the upper side of the fish, a 1.0-5.0 ml blood sample was taken from the caudal vein using a sterile plastic syringe fitted with a 21 gauge needle. The vein was located ventral to the vertebral column in the mid-line by inserting the needle 2-5 mm ventral to the lateral line at the level of the vent and at right-angles

to the body wall. After removing the needle from the syringe, the blood was ejected into a heparinized polystyrene blood specimen tube (Sterilin Ltd., Ashford, Kent), which was then slowly rotated by hand for about 60 sec to ensure adequate mixing. Tubes were filled approximately half full to give a final heparin concentration of 4 mg/ml of blood. If there was sufficient blood, 0.5-2.5 ml was also put into sterilized 2.5 ml glass vials containing no anti-coagulant. Seventy-five mm heparinized microhaematocrit tubes ("Volac", J. Poulter Ltd., Barking, Essex) were filled from the heparinized blood sample and sealed at one end with Sigillum (Modulohm 1/S, Copenhagen, Denmark). A blood smear was made from a single drop of heparinized blood on a clean glass slide (chapter 2) as described by Dacie and Lewis (1975) and left to air dry. Blood samples were refrigerated at 4°C. Heparinized samples were discarded if there was evidence of clotting.

Fish were then killed as described in chapter 2.

Post-mortem sampling

Fish were measured and weighed as described in chapter 3. Values are recorded in Table 4.1. They were dissected and kidney samples were taken for histological examination. Kidney impression smears were prepared from each fish sampled in groups A and D. Grade of kidney swelling was noted with other gross abnormalities.

Sample processing

Kidney specimens were processed and sections prepared and stained for histological examination as described in chapter 2.

Blood smears were fixed for 5 min in 100% ethanol, and were stained within 24 hr with May-Grünwald-Giemsa (appendix 2). This staining procedure was found to give good differentiation of erythrocyte cytoplasm

and different types of leucocyte. Other staining methods applied to kidney impression smears (chapter 2) were also evaluated for blood smears, but were not found to be as reliable as May-Grünwald-Giemsa staining. Some blood smears were also stained by the Feulgen method (Table 3.2) for DNA.

Blood specimen handling

Blood samples were transferred to the laboratory in chilled boxes. Heparinized samples were removed for haemoglobin estimation and total cell counts. The remaining heparinized blood and the clotted samples were spun for 10 min at 2,500 g. Plasma or serum was pipetted into sterilized glass tubes which were held at 4°C if samples were to be analysed within 24 hr, otherwise they were stored at -20°C until required.

Measurement of blood parameters

TOTAL CELL COUNTS

Total cell counts were made using a Neubauer improved haemocytometer, a standard red blood cell diluting pipette and modified Dacie's fluid (appendix 1). Cells were counted following the principles of Dacie and Lewis (1975). Values were multiplied x 10^{10} to give the total number of cells within one litre of blood.

TOTAL RED AND WHITE BLOOD CELL COUNTS

Because of the difficulty experienced in differentiating nucleated red blood cells from leucocytes in a haemocytometer, total red and white blood cell counts were calculated indirectly using blood smears and total cell counts. Choosing an area of the blood smear where cells were evenly distributed, away from the edge of the smear and where there was little

overlapping of cells, approximately 1,500 cells were counted under oil at a magnification of x 1000. These cells were differentiated into immature, mature and senile erythrocytes, large and small lymphocytes, neutrophils, monocytes, eosinophils, lymphoid cells and thrombocytes. Immature erythrocytes included erythroblasts and pro-erythrocytes, but cells with nulcear and overall shape similar to mature cells but with some cytoplasmic basophilia were counted with the mature cells, although the polychromasia was noted. From the relative percentages of erythrocytes and leucocytes (including thrombocytes) and the total cell counts it was possible to calculate the total number of erythrocytes and leucocytes within one litre of blood.

DIFFERENTIAL WHITE CELL COUNT

With the blood smears under oil at a magnification of x 1000, approximately 400 leucocytes (including thrombocytes) were counted while traversing areas of cells which were evenly distributed, avoiding the head, tail and edge areas of the smear. Leucocytes were differentiated into small and large lymphocytes, neutrophils, eosinophils, monocytes, lymphoid cells and thrombocytes. Lymphocytes were classified as "small" if their diameter was less than 10 μm, and "large" if more than 10 μm. Some cells, having more cytoplasm than mature lymphocytes, with the nucleus eccentrically placed, were classified as lymphoid cells, and represented immature lymphocytes. From the relative percentages of these cell types and the total leucocyte counts, absolute numbers of each type of leucocyte, including thrombocytes, were calculated per litre of blood.

PCV MEASUREMENT

The microhaematocrit capillary tubes, filled at the time of taking

the blood samples, were spun immediately after the last blood sample of a batch was taken, using a Hawksley microhaematocrit centrifuge (Gelman Hawksley Ltd., England) set at 12,000 g for 5 min. PCV values were read using a Hawksley microhaematocrit reader (Gelman Hawksley Ltd., England).

HAEMOGLOBIN ESTIMATION

Haemoglobin was estimated by the cyanmethaemoglobin method. Heparinized whole blood was thoroughly mixed before pipetting 16 µl of blood into 4 ml of Drabkin's fluid (appendix 1) in a macrodisposable cuvette, pathlength 10 mm (Camlab, Cambridge). The cuvette was sealed with parafilm (Marathon Products, Wisconsin, U.S.A.) and mixed by vigorous shaking. The solution was left for 15-20 min at room temperature. The optical density of the solution was read at 540 nm in a Pye Unicam SP6-400 UV spectrophotometer (Pye Unicam Ltd., Cambridge), after zeroing with a distilled water blank. This reading was used to estimate the haemoglobin concentration in g/100 ml blood by comparing with a calibration curve constructed using cyanmethaemoglobin standards (Cyanmethaemoglobin Standard Set, Boehringer Manheim GmbH, Diagnostica).

ERYTHROCYTE INDICES

The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were derived from total red cell counts, haemoglobin concentration and haematocrit values applying the following formulae:

(a) $MCV = \frac{\text{Haematocrit value x 1000}}{\text{erythrocyte count } (\times 10^{12}/1)}$ value in cubic microns.

(b) $MCH = \frac{\text{Haemoglobin in g/100 ml blood x 10}}{\text{erythrocyte count } (\times 10^{12}/1)}$ value in pico grams

(c) MCHC = $\frac{\text{Haemoglobin in g/100 ml blood}}{\text{haematocrit value}}$ value in %.

TOTAL PLASMA AND SERUM PROTEIN

The Lowry method (Lowry, Rosebrough, Farr and Randall, 1951) was used to estimate protein in serum or plasma. All steps were conducted at ambient temperature. Duplicate samples were prepared from all specimens.

Procedure:

0.9 ml of 0.9% NaCl was added to 0.1 ml of test serum/plasma in 25 ml glass bottles.

0.2 ml of this solution was added to 2.5 ml distilled water

+ 0.2 ml N/5 NaOH

+ 0.3 ml Folin and Ciocalteu's phenol reagent (BDH Chemicals Ltd., Poole, Dorset) in a glass bijou. A further 2.5 ml of distilled water was added to mix the solution. This was left for at least 5 min. Optical density readings were made in a Pye Unicam SP6-400UV Spectrophotometer (Pye Unicam Ltd., Cambridge) at 640 nm against a distilled water blank. These readings were compared with a standard curve constructed each time test samples were analysed, plotting optical density against g % protein. This curve was derived using different dilutions of bovine serum albumin (Sigma Chemical Co., St Louis, Missouri), the protein concentrations being 0.5, 1.0, 2.0, 4.0 and 8.0 g %.

TOTAL SERUM/PLASMA ALBUMIN ESTIMATION

Total serum or plasma albumin was estimated using the colorimetric test kit of Sigma (Sigma Chemical Co., St Louis, Missouri) following Sigma Technical Bulletin Number 630.

TOTAL SERUM GLOBULIN

Total serum globulin was estimated using the colorimetric test kit of Sigma (Sigma Chemical Co., St Louis, Missouri) following Sigma Technical Bulletin Number 560. Duplicate tests were performed for each serum sample.

TOTAL SERUM UREA

Serum urea was estimated by the Test-Combination kit of Boehringer Mannheim (Cat. No. 124,770) following the instructions supplied, except that twice the recommended serum sample volume was used.

SERUM BILIRUBIN

Total bilirubin concentration was estimated by the colorimetric test kit of Sigma (Sigma Chemical Co., St Louis, Missouri) following Sigma Technical Bulletin Number 605. Single estimations were made for each sample since 0.6 ml serum was required for each test.

Expression of results

Results from infected fish were grouped according to the age of the fish, the macroscopical appearance of the kidney assessed by using the kidney grading system described in chapter 2, and the microscopical appearance of the kidney assessed in sections and smears. 0+ rainbow trout exposed to PKD (groups A, B and C) were divided into seven categories:

1. Fish on infected water without histological changes associated with PKD, except for occasional renal PKX cells in some fish.
2. Fish with renal PKX cells plus haemopoietic hyperplasia, but without renal swelling.

3. Fish with grade 1 renal swelling.

4. Fish with grade 2 renal swelling.

5. Fish with grade 3 renal swelling.

6. Fish with grade 4 renal swelling.

7. Fish with advanced histological signs of recovery from PKD.

1+ fish exposed for a second summer to PKD (group D) were divided into those without renal PKX cells, or haemopoietic hyperplasia, and those with renal PKX cells with haemopoietic hyperplasia.

The total numbers of fish sampled in each category are detailed in Table 4.2. Results for each parameter are given in standard units and are recorded as mean values ($\bar{x}$), and standard deviations (σn) for each category of fish. Standard deviation values have been derived from the equation $\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n}$ where n = number of samples in the batch.

x = sample value.

Σx^2 = sum of the squares of the sample values.

Significance of mean values has been assessed using "Student's" t test and has been expressed as the probability (p) of the test sample mean being similar to that of the control means. Differences have been considered as significant in the present study if this probability (p) was numerically less than 0.05. Control means have been derived from the results of 0+ fish category 1, described above, except for values relating to total serum or plasma protein, albumin, globulin and urea which had been derived from the results of uninfected group E fish held at the laboratory.

RESULTS

The principal renal histopathological changes associated with each category of fish are detailed in Table 4.2. In 0+ fish with haemopoietic

hyperplasia but no renal swelling (category 2) there was evidence of vascular lesions and diffuse inflammation in only two of 17 renal samples. These changes became more pronounced as kidney swelling developed, and the majority of samples from fish with grade 3 renal swelling (category 5) contained extensive areas of diffuse inflammation, intravascular aggregations with occlusion of vessels, evidence of glomerular and tubule destruction, and crystal formation. The same changes were found in fish with grade 4 renal swelling but only eight of 14 samples contained areas of haemopoietic hyperplasia. In recovering fish, crystals were still present in three of nine samples, although there was no evidence of vascular lesions. Renal samples from 1+ fish with PKX cells contained areas of haemopoietic hyperplasia, and the majority also contained evidence of diffuse inflammation and vascular lesions, but only three of 12 contained crystals.

Total erythrocyte counts are detailed in Table 4.3. Counts from 0+ fish with grade 1 renal swelling (category 3) were significantly higher than those from fish with no renal swelling (categories 1 and 2), but those from fish with grades 3 and 4 renal swelling were significantly lower, despite four of 15 fish with grade 3 renal swelling having total erythrocyte counts above $1 \times 10^{12}/l$.

Immature erythrocytes were present in samples from all categories of fish, with maximum percentages seen in PKD-challenged fish prior to the development of histological changes (Table 4.3). The ratios of immature to mature erythrocytes in 0+ fish with renal swelling of grades 3 and 4 remained similar, despite significant decreases in total erythrocyte counts, to those in 0+ fish in categories 2 to 4.

The numbers of blood smears containing erythrocytes with various staining appearances in each category of fish are detailed in Table 4.4.

Anisocytosis and polychromasia were found in samples from all categories whereas poikilocytosis was seen in individual smears in only four categories and hypochromasia in only one smear. Mature erythrocytes were classified as "malformed" if they had a figure-of-eight appearance, deeply notched nuclei or pyknotic, eccentric nuclei. These cell types were found in all categories of fish but were more frequent in those with renal swelling of grades 2-4. Occasional erythrocytes in smears from fish with grades 3 and 4 renal swelling had intracytoplasmic, single, spherical inclusions of 1 μm diameter which stained positively for DNA. Immature erythrocytes from these fish often exhibited poikilocytosis.

Haematocrit and haemoglobin values, summarized in Table 4.5, progressively decreased in samples from fish with grade 2 to grade 4 renal swelling (categories 4-6) compared to those from 0+ fish in categories 1-3. However, the haemoglobin values in 0+ fish in categories 2 and 3 were significantly above those in 0+ fish of category 1. In 1+ fish with haemopoietic hyperplasia, haematocrit values were significantly less than those of both 0+ and 1+ fish challenged with PKD but without related histopathological changes, although there was no similar significant change in either haemoglobin value or total erythrocyte count.

Values for erythrocytic indices are detailed in Table 4.6. MCV values were significantly decreased in 0+ fish with grade 1 renal swelling and in both categories of 1+ fish. MCH values were significantly raised in 0+ fish with haemopoietic hyperplasia without renal swelling and in 1+ fish with haemopoietic hyperplasia. MCHC values in all categories except 0+ fish in categories 4 and 7 were significantly higher than those in 0+ fish in category 1.

Values for leucocyte counts, excluding thrombocytes, and thrombocyte counts are detailed in Table 4.7. Significant decreases in leucocyte

numbers were seen in all categories except 0+ fish categories 5 and 7, compared with values in 0+ fish category 1 and significant decreases in thrombocyte numbers were found in all categories except 0+ fish categories 2 and 7. Differential leucocyte counts detailing percentages of large and small lymphocytes, lymphoid cells and neutrophils are recorded in Tables 4.8 and 4.9. Significant decreases in percentages of small lymphocytes were found in 0+ fish with grades 3 and 4 renal swelling, whereas increases in lymphoid cells and neutrophils, especially juvenile segmented forms, were encountered in the same groups. Monocytes and eosinophilic granulocytes were seen in all categories of fish, never representing more than 1.2% and 0.5% of the total leucocyte population respectively.

Plasma and serum protein values are detailed in Table 4.10. Significant decreases compared with values in group E fish were noted, especially in 0+ fish with grade 4 renal swelling, but not in fish with grade 3 renal swelling.

Plasma and serum albumin and serum globulin values are detailed in Table 4.11. A marked decrease in albumin values compared to those in group E fish was seen in fish with grade 4 renal swelling, whereas significant increases in globulin levels were noted in 0+ fish PKX-positive without kidney swelling, in those with grade 2 renal swelling, and in those recovering from PKD.

Serum urea values are recorded in Table 4.12. No significant increases compared with values in group E were found in 0+ fish categories 2-7, although an increase was noted in uninfected 1+ fish.

Serum bilirubin values are also recorded in Table 4.12. Values in 0+ fish categories 4-7 were all significantly lower than those in group E fish.

DISCUSSION

When assessing the results of haematological studies in fish, two causes of variation of values for a given parameter should be considered; firstly, the effects of environmental and physical factors both on healthy and diseased fish and, secondly, the lack of technique standardization in the collection and analysis of fish blood.

Variation due to environmental and physical factors

Haematological values in healthy fish vary not only between species, whether marine or freshwater, but also between members of the same species. The evidence of Barnhart (1969) suggested that haematological values for particular parameters varied between different strains of rainbow trout, although McCarthy, Stevenson and Roberts (1975), when comparing the Kamloops and Shasta varieties of rainbow trout, found that differences in the parameters measured were probably within accepted normal ranges.

Sano (1960a) reported that blood values in rainbow trout were influenced by the time of year, and Denton and Yousef (1975) found seasonal differences in haemoglobin and haematocrit values. Sano (1960b) and McCarthy *et al*. (1975) also considered that variation occurred depending on the age of fish. Although Barnhart (1969) found no significant variation in values between male and female rainbow trout, possibly because he was using immature fish, McCarthy *et al*. (1975) described significant differences in the ranges of values for both haematocrits and erythrocyte counts in sexually mature male and female rainbow trout of the Shasta variety. Barnhart (1969) also implicated diet, the manner in which fish were handled prior to sampling, and the method sampling as causes of blood value variation.

In the present study, to minimize some of these causes of variation,

numbers were seen in all categories except 0+ fish categories 5 and 7, compared with values in 0+ fish category 1 and significant decreases in thrombocyte numbers were found in all categories except 0+ fish categories 2 and 7. Differential leucocyte counts detailing percentages of large and small lymphocytes, lymphoid cells and neutrophils are recorded in Tables 4.8 and 4.9. Significant decreases in percentages of small lymphocytes were found in 0+ fish with grades 3 and 4 renal swelling, whereas increases in lymphoid cells and neutrophils, especially juvenile segmented forms, were encountered in the same groups. Monocytes and eosinophilic granulocytes were seen in all categories of fish, never representing more than 1.2% and 0.5% of the total leucocyte population respectively.

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In the present study, to minimize some of these causes of variation,

the same diet was fed to all fish groups, and all fish were held in the same tanks except for group E. At sampling, all fish, once netted, were handled similarly and all samples were taken using the same procedure, at approximately the same time of day to minimize the effects of diurnal variation in water temperature. Although several reports mention pre-sampling fasting, usually overnight (Phillips, 1958; Sano, 1960a; Klontz, 1972), this practice was not followed in the present study since Sano (1957) found significant differences in blood sugar, creatinine and urea values in two groups of healthy Japanese eel, Anguilla anguilla Temminck and Schlegel, one group starved for two days, the other for four to six hours prior to sampling, implying that no prolonged period of fasting should occur before collection of blood.

Variation due to collection and analysis techniques

Although attempts have been made to standardize routine fish blood analytical methodology (Blaxhall and Daisley, 1973; Warner, Tomb and Diehl, 1979), and to establish normal values for selected haematological parameters in a variety of fish species, there is little agreement in the literature as to the most reliable techniques of blood collection and analysis. In the present study all samples were collected and analysed in a similar fashion, whatever the limitations of the technique used. Therefore, it was hoped that differences in the results within the study would be meaningful, although the results might not be comparable with absolute values in other reports.

Before blood samples were taken, fish were anaesthetized with un-buffered tricaine methanesulphonate (MS222 Sandoz, Sandoz Ltd., Basle, Switzerland). This form of the agent had been assessed for use in haematological studies by Wedemeyer (1970) and Smit, Hattingh and Burger

(1979). Wedemeyer (1970) found that with increasing exposure times yearling rainbow trout anaesthetized with unbuffered tricaine methane-sulphonate at a concentration of 0.008% showed increased blood urea nitrogen and cholesterol levels, while vitamin C levels decreased. Wedemeyer (1970) considered these changes resulted from the stress of anaesthesia caused by the sulphonic acid constituent of MS222 Sandoz since these disturbances did not occur when buffered MS222 Sandoz and benzocaine were used. However, changes in values did not occur provided the period of anaesthesia was limited to 3-4 min. Smit et al. (1979) also considered that buffered MS222 Sandoz decreased any haematological changes due to the anaesthetic agent and suggested a concentration be used "to anaesthetize the fish in the shortest time". In the present study a concentration of 0.01% was chosen since at this concentration fish were anaesthetized within 60 sec. The buffering effect of the water supplies both at the laboratory and at the experimental site were sufficient to counteract the possible effects of the sulphonic acid moiety of the anaesthetic agent.

Various methods of taking blood samples from fish have been tried. Cardiac puncture with or without previous surgical exposure of the heart was recorded as the method of choice in several reports, especially if repeat bleeding was intended (Field, Elvehjem and Juday, 1943; Schiffman and Fromm, 1959; Sano, 1960a; Barnhart, 1969; Klontz, 1972; Blaxhall and Daisley, 1973; Soivio, Nyholm and Westman, 1973; Alexander, 1977; Hoffmann and Lommel, 1984), although Klontz (1972) considered fish had to be a minimum of 100-150 mm long if this method was employed. Samples have also been taken from the caudal artery after severing the tail posterior to the anal and adipose fins (Watson, Guenther and Royce, 1956; Snieszko, 1960; Larsen and Snieszko, 1961; Wedemeyer, 1970),

although this method risks contamination of the sample from vent and tissue fluids and was considered by Hesser (1960) to be the method of choice only in fish less than 150 mm in length. Furthermore, increased erythrocyte counts, haemoglobin and haematocrit values were obtained using this method compared with those after cardiac puncture (Hoffmann, Lommel and Riedl, 1982). Because of these drawbacks, samples in the present study were removed from the caudal vein by venipuncture, a technique found to be consistently easy and quick to perform in rainbow trout (McGregor, 1982; Ellis, 1983), using plastic rather than glass syringes, since clotting times were longer in the former (Barnhart, 1969).

Heparin was chosen as the anticoagulant throughout the study. Although Klontz (1972), Blaxhall (1973) and Blaxhall and Daisley (1973) preferred ethylenediamine tetra-acetic acid (EDTA), Hesser (1960) considered heparin more satisfactory since lysis and crenation of erythrocytes was minimal using this anticoagulant. Furthermore, in comparing the effects of disodium EDTA and heparin at different concentrations on pH, pCO_2, pO_2 and haematocrit values in blood from carp, Smit, Hattingh and Schoonbee (1977) decided that heparin was the anticoagulant of choice since increased pCO_2 and pO_2 values, with acidification of blood occurred at concentrations of EDTA sufficient to prevent clotting. Hille and Pöllnitz (1980) also found EDTA unsuitable for blood protein analysis. A concentration of approximately 4 mg heparin/ml blood was found satisfactory and, although this is high compared with the concentration of 0.4-0.6 mg/ml blood suggested by Hesser (1960), the findings of Barham, Smit and Schoonbee (1979) were taken into account. They studied the effects of heparin concentrations of 1-4 mg/ml blood on pH, pCO_2, pO_2, haematocrit and plasma chloride concentration values in rainbow trout blood and found that a concentration of 4 mg/ml blood resulted in the least change on most parameters with time, despite haematocrit values

increasing significantly over a 2 h period at this concentration.

Dacie's fluid (Dacie and Lewis, 1975) modified with brilliant cresyl blue was used in the present study as the diluting fluid for total blood cell counts, being found satisfactory by Blaxhall and Daisley (1973) and recommended by Roberts (1978) in order to help standardize procedures in comparative haematology. A 1:200 dilution was used for both red and white blood cells following Hesser (1960), although a 1:100 dilution was employed by Wedemeyer and Yasutake (1977). Since difficulties were experienced in differentiating erythrocytes and thrombocytes, total white cell counts, including thrombocytes, were derived indirectly from blood smears as described by McKnight (1966) and Amend and Smith (1975). This indirect method of total leucocyte counting had previously been dismissed by Natt and Herrick (1952) because the assumption was made that blood cells were evenly and equally distributed throughout a smear, whereas neutrophils were known to be concentrated at the edges of a smear. However, the indirect method was recommended by Lucas and Jamroz (1961) despite inaccuracies with both techniques in avian blood and was found to be more precise than the direct method by McKnight (1966) when studying the haematology of the mountain whitefish, Prosopium williamsoni.

Haematocrit values were obtained using heparinized samples taken into pre-heparinized microhaematocrit tubes. This procedure avoided the problem encountered by Snieszko (1960), who found that, due to partial clotting, blood samples taken from fish directly into heparinized capillary tubes gave values approximately 7-18% higher than those treated with anticoagulant prior to being put in the tubes. Samples were spun down within 1 h of collection to reduce inaccuracies caused by erythrocyte swelling. This problem was investigated by Soivio et al. (1973), who found that under anaerobic conditions, haematocrit values increased over

a 2 h period up to 30% above original readings taken at the time of sampling.

Although several methods for haemoglobin estimation are suitable for human blood, results have been inconsistent for trout blood. Larsen and Snieszko (1961) in a detailed comparison of the acid-haematin, oxyhaemoglobin and cyanmethaemoglobin methods of haemoglobin estimation considered the acid-haematin method unsuitable for trout blood unless a correction factor, accurate only at normal haemoglobin levels, was incorporated to adjust for suspended erythrocyte nuclei increasing spectrophotometric readings. Spinning out the nuclei also removed haemoglobin, probably accounting for the low haemoglobin values reported by Schiffman and Fromm (1959). Hesser (1960) found this method inaccurate since both time and temperature affected the rate of haemoglobin conversion to acid haematin, the colour change was stable for a limited time, and the colour development was altered by plasma protein and lipid levels. Larsen and Snieszko (1961) considered the oxyhaemoglobin method satisfactory if any gelatinous mass which formed was removed prior to a spectrophotometric reading being made. However, since stable oxyhaemoglobin standards are difficult to prepare (Dacie and Lewis, 1975), the cyanmethaemoglobin method was used in the present study, with reagents both stable and easily obtainable. However, there is some variation in the dilutions of blood employed by different authors, with Blaxhall and Daisley (1973) adding 0.02 ml blood to 4 ml Drabkin's solution, whereas Wedemeyer and Yasutake (1977) and Roberts (1978) suggest 0.02 ml blood in 5 ml Drabkin's solution. Furthermore, there is also variation in the amount of potassium ferricyanide recommended in Drabkin's solution. Dacie and Lewis (1975) and Roberts (1978) used 20 mg of potassium ferricyanide in every litre of distilled water, whereas Wedemeyer and Yasutake (1977)

following Kampen and Zijlstra (1961) suggested 200 mg/l. In the present study, having compared these concentrations and found them to give similar results, the lower concentration of potassium ferricyanide was adopted.

Of the erythrocyte indices, MCHC values should be accurate since errors in haematocrit and haemoglobin estimation can be limited. However, MCV and MCH values will be less precise since erythrocyte counts by manual methods have inherent errors which reduce their accuracy by at least ± 8.8% (Dacie and Lewis, 1975).

As described previously, serum and plasma protein estimation was by the Lowry method (Lowry et al., 1951). This method was chosen because of its sensitivity and because of the close correlation of results obtained from rainbow trout sera using the Lowry method, and automated and manual Biuret methods (Alexander and Ingram, 1980). Refractometry was not used although Alexander and Ingram (1980) considered this method produced results which correlated most consistently with those obtained by five other methods, including the Lowry and Biuret methods, since Wedemeyer and Yasutake (1977) had found refractometry particularly sensitive both to lipids and haemolysis. However, the Lowry method cannot be considered an absolute one since widely differing colour values are obtained for different proteins, depending especially on their tyrosine and tryptophan content (Herbert, Phipps and Strange, 1971).

Other techniques in the present study were used without modification of the test kit suppliers' instructions except for serum urea estimation where double the recommended sample volume was tested , since urea values in fish are generally at least 50% less than in man (McKay and McKay, 1927; Phillips, 1958). Differences in binding affinities of proteins to test reagents may exist between man and fish (Evans, 1985), and this

may explain fish albumin levels being low in all categories of fish.

Blood parameters

From the results it can be seen that the most marked changes in the blood picture during PKD relate to the erythrocyte picture in 0+ rainbow trout. A progressive decrease in erythrocyte numbers, complemented by falls in haemoglobin and haematocrit values, occurred as kidney swelling increased from grade 2 to grade 4. The values for erythrocyte counts and haematocrit in fish with grade 4 kidney swelling were similar to those reported by Ferguson and Needham (1978), but were less than 50% of those recorded by Hoffmann and Lommel (1984) whose values correspond with the results obtained in the present study from fish with grade 3 swelling. This indicates the importance of relating the haematological picture to the severity of the disease at sampling.

Although haemopoietic hyperplasia was apparent on histological examination of kidneys in 0+ fish of all categories except category 1, an increase in circulating erythrocyte numbers was only found in fish with grade 1 kidney swelling, with the MCV value in this group suggesting that the erythrocytes were generally smaller than usual. This may correlate with the progressive development of vascular aggregations and occlusions and the appearance of fusiform crystals in kidneys with grade 1 to grade 4 swelling, perhaps indicating that although new cells were being produced they were unable to enter the circulation. This may also explain why, despite haemopoietic hyperplasia, there was no detectable absolute or relative increase in immature erythrocytes as the disease progressed. This is in contrast to the results of Ghittino *et al*. (1977) and Hoffmann and Lommel (1984), and suggests that vascular pathology may not have been so marked in their experimental fish.

In the present study, haemoglobin values did not decrease in proportion to the measured falls in haematocrit and total erythrocyte counts, but appeared higher than expected. In contrast, Hoffmann and Lommel (1984) found decreased haemoglobin values were more than expected given the recorded falls in haematocrits and erythrocyte counts. To some extent this apparent imbalance may be explained if the crystals, seen as the disease progressed, were derived from haemoglobin and were measured, together with intracellular haemoglobin, by the cyanmethaemoglobin method of analysis. This might also partially explain why the MCH and MCHC values did not fall, contrary to the results of Hoffmann and Lommel (1984). However, the present results suggest that haemoglobin synthesis was unimpaired and this is further supported by the lack of hypochromasia in any of the 0+ fish blood smears, whereas this was a prominent feature of the findings of Hoffmann and Lommel (1984).

In blood smears, anisocytosis and polychromasia, noted also by Hoffmann and Lommel (1984), were frequent findings in all categories of fish, whereas poikilocytosis was an infrequent finding. However, erythrocytes classified as "malformed" were present in smears from all categories of fish, but were more frequent in fish with grades 2-4 renal swelling. Figure-of-eight cells have been described by Lehmann and Sturenberg (1975) as erythrocytes undergoing amitosis which occur occasionally in the peripheral blood of normal rainbow trout. Similar forms have been noted by Ellis (1984) in a plaice injected ten times over a 20-week period with bovine serum albumin, and by Benfey and Sutterlin (1984) in a deformed Atlantic salmon alevin. Erythrocytes with pyknotic nuclei, centrally or eccentrically placed, were also noted by Ellis (1984), similar to those in the present study. These apparent abnormalities were considered by Ellis (1984) as possible signs of advanced senescence

with the spleen failing to remove effete erythrocytes. A similar situation may exist in PKD where splenic inefficiency may result from tissue changes similar to those occurring in the kidney. This may also explain the finding of intracytoplasmic DNA-positive bodies in some erythrocytes similar to Howell-Jolly bodies in mammalian erythrocytes which develop in haemolytic anaemias, splenic dysfunction and after splenectomy.

Haemopoietic hyperplasia was a less prominent feature of the histological appearance in renal tissue from fish with grade 4 renal swelling than in that from other categories, and this perhaps indicates a decrease in blood cell production at this stage of the disease, whereas compensation for kidney damage apparently was successful even in some fish with grade 3 swelling.

The type of anaemia developing in PKD will be discussed in chapter 7 in conjunction with the findings in chapter 3.

In 1+ rainbow trout, although mature erythrocytes were smaller and the numbers of immature erythrocytes fewer both in uninfected and infected fish than in 0+ fish, the overall erythrocyte picture was similar to that in uninfected 0+ fish. This suggests that, although re-infection occurs in survivors of PKD, it does not have the same effect on blood production as in the naive fish.

In 0+ rainbow trout the total leucocyte counts varied through the course of PKD. However, an apparent leucopenia, despite haemopoietic hyperplasia, developed early in the disease process with a possible compensation occurring in fish with grade 3 renal swelling followed by a further decrease in numbers in fish with grade 4 swelling. The leucopenia may reflect the development of vascular pathology, as described previously, preventing new leucocytes entering the circulation. However,

the changes in proportions of lymphocytes and lymphoid cells indicate that at least some newly-formed cells were entering the circulation. Neutrophils increased in proportion to other leucocytes in fish with grades 3 and 4 renal swelling. Therefore, the leucocyte picture overall presented as a leucopenia rather than a leucocytosis, despite the results in fish with grade 3 renal swelling. A neutrophilia also developed during clinical disease. These results differ from those of Hoffmann and Lommel (1984), who found only slight changes in leucocyte values between healthy and PKD-affected fish, but agree with the conclusions of Ghittino et al. (1977). The picture may be compared to that reported by Hines and Spira (1973) in mirror carp, Cyprinus carpio L., with advanced infection with the protozoan, Ichthyophthirius multifiliis, where similar leucocyte changes were found.

Total leucocyte and thrombocyte counts were significantly lower in 1+ fish, whether uninfected or infected, with or without haemopoietic hyperplasia, than in control 0+ rainbow trout, suggesting that this could have been a function of the age of fish rather than related to disease status. In 0+ fish, thrombocyte counts decreased significantly as renal swelling occurred and this perhaps reflected the development of vascular pathology.

Plasma and serum protein values in uninfected and infected fish were lower than those recorded by Scott (1984), perhaps reflecting different holding conditions and diet. Although some decrease was found in experimental fish with grades 0 and 2 renal swelling, marked reductions in protein values, especially in the albumin fraction, were found only in fish with grade 4 renal swelling, agreeing with values given by Hoffmann and Lommel (1984). This implies that renal filtration function and liver function were maintained during the course of PKD, although anaemia was developing, until ascites and oedema were present. Globulin levels were

maintained throughout the infection except in fish with grade 4 renal swelling, with increased values at the beginning of infection and during recovery. It is not known if these increases in globulin level indicate a response to the PKX cell or the associated tissue response.

Although blood urea levels in affected fish were found elevated by Hoffmann and Lommel (1984), this was not so in the present study. However, in fish with grade 4 renal swelling, the results for other parameters indicate that haemodilution was probably occurring as ascites and oedema were developing, implying that urea concentrations may also have been diluted. Bilirubin levels also did not increase through the course of the disease, implying that haemolysis was not occurring to any great extent.

In conclusion, during the present study, in sub-clinically infected 0+ fish the earliest renal histopathology was haemopoietic hyperplasia, followed by vascular and inflammatory lesions. These changes were accompanied in fish with minimal (grade 1) renal swelling by erythrocytosis, leucopenia and thrombocytopenia .

In clinically-affected fish, accompanying the development of extensive renal vascular pathology and inflammation, there was a progressive decrease in erythrocyte numbers to less than 50% of control values, with concurrent declines in haemoglobin and PCV values. As the disease became more severe there was also an increase in malformed erythrocytes. Although there was an apparent leucocytosis in fish with grade 3 swelling, the preclinical leucopenia was seen again in oedematous fish with grade 4 renal swelling. The proportion of immature lymphocytes increased. Clinically-affected fish also showed a neutrophilia and thrombocytopenia. Total protein and albumin levels only decreased in advanced clinical cases.

In fish recovering from PKD, most values were similar to those of control fish. However, globulin concentration was significantly increased.

In recovered, re-challenged fish the most obvious changes were a decrease in erythrocyte numbers, PCV and haemoglobin values in infected compared with uninfected fish.

These results vary in several instances from those of other authors, especially those of Hoffmann and Lommel (1984). The lower erythrocyte values, neutrophilia and leucopenia may reflect a more advanced stage of disease at sampling than that in fish studied by Hoffmann and Lommel (1984). Other differences, such as the increase in immaturity and hypochromasia of erythrocytes reported by Hoffmann and Lommel (1984) may indicate that haemolysis was more pronounced in their fish. The characterization of the anaemia in PKD will be considered in greater detail in chapter 7.

Table 4.1. Sampling schedule of rainbow trout in groups A-E.

Fish group	Sample date	No. of fish in sample	Lengths of sample fish (mm)		Weights of sample fish (g)	
			mean	range	mean	range
A	13.6.83	5	157	151-165	51	42-57
	23.6.83	5	173	164-188	68	56-81
	30.6.83	5	180	173-190	94	78-109
	4.7.83	10	171	158-184	73	55-92
	14.7.83	5	191	177-205	ND	
	18.7.83	10	183	132-200	92	33-123
	27.7.83	10	184	166-204	92	57-126
	29.7.83	5	182	165-201	84	61-115
	1.9.83	6	182	137-204	79	26-102
B	29.7.83	5	ND		ND	
	11.8.83	15	ND		ND	
	25.8.83	5	ND		ND	
C	25.6.84	5	200	186-213	69	47-89
	12.7.84	5	190	185-205	77	66-86
	23.7.84	20	213	200-226	123	90-144
	30.7.84	20	206	178-240	111	75-180
	6.8.84	5	199	190-208	105	88-127
D	26.5.83	5	271	238-291	263	154-372
	30.6.83	5	304	280-320	353	260-423
	4.8.83	10	287	251-340	309	182-490
	15.8.83	5	285	257-310	297	180-405
	19.9.83	5	328	294-340	421	200-515
E	6.3.84	5	209	195-225	112	90-139
	9.8.84	5	ND		ND	

ND = not done.

Table 4.2. Renal histopathology in fish sampled from groups A, B, C and D.

Category of fish	Total no. of fish sampled	No. of renal samples examined	No. of renal samples PKX positive	Number of renal samples in each category with histological evidence of: haemopoietic hyperplasia	diffuse inflammation	vascular aggregations	glomerular and tubule destruction	fusiform crystals
1. PKX positive or negative; no histological changes	10	10	6	0	0	0	0	0
2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	17	17	17	17	2	2	1	0
3. Grade 1 renal swelling	15	12	12	12	10	6	4	0
4. Grade 2 renal swelling	22	16	16	15	16	11	10	6
5. Grade 3 renal swelling	35	18	18	18	18	16	14	16
6. Grade 4 renal swelling	31	14	14	8	14	13	14	13
7. Recovering from PKD	11	9	2	6	1	0	0	3

0+ (groups A, B and C)

Table 4.2. (continued)

	Category of fish	Total no. of fish sampled	No. of renal samples examined	No. of renal samples PKX positive	Number of renal samples in each category with histological evidence of: haemopoietic hyperplasia	diffuse inflammation	vascular aggregations	glomerular and tubule destruction	fusiform crystals
1+ (group D)	1. PKX negative; no histological changes	18	18	0	0	0	0	0	0
	2. PKX positive with renal haemopoietic hyperplasia	12	12	12	12	7	8	0	3
	Total	171	126	97	88	68	56	43	41

Table 4.3. Total erythrocyte counts and immature erythrocyte percentages in each category of fish from groups A and D.

	Category of fish	No. of samples	Total erythrocyte counts ($\times 10^{12}/l$) $\bar{x}$	σn	p	Immature erythrocytes as % of total erythrocyte counts $\bar{x}$	σn	p
0+ (group A)	1. PKX positive or negative; no histological changes	10	1.089	0.229	NA	6.6	1.5	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	1.059	0.178	NS	4.2	2.1	< 0.01
	3. Grade 1 renal swelling	8	1.214	0.104	< 0.05	3.5	1.5	< 0.001
	4. Grade 2 renal swelling	6	0.952	0.247	NS	2.7	1.1	< 0.001
	5. Grade 3 renal swelling	15	0.823	0.261	< 0.01	3.4	2.0	< 0.001
	6. Grade 4 renal swelling	5	0.455	0.179	< 0.001	3.1	2.7	< 0.05
	7. Recovering from PKD	5	1.309	0.234	NS	1.7	1.3	< 0.01
1+ (group D)	1. PKX negative; no histological changes	13	1.189	0.220	NS	1.3	1.1	< 0.001
	2. PKX positive with renal haemopoietic hyperplasia	12	0.961	0.261	NS	1.0	1.3	< 0.001
	Total	86						

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.4. Mature erythrocyte appearance in each category of fish from groups A and D.

	Category of fish	No. of samples	Number of blood smears in each category containing evidence of: aniso-cytosis	poly-chromasia	malformed erythro-cytes	poikilo-cytosis	hypo-chromasia
0+ (group A)	1. PKX positive or negative; no histological changes	10	7	2	3	1	0
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	6	11	3	1	0
	3. Grade 1 renal swelling	8	5	8	4	0	0
	4. Grade 2 renal swelling	6	6	5	6	0	0
	5. Grade 3 renal swelling	15	13	8	13	0	0
	6. Grade 4 renal swelling	5	5	3	5	1	0
	7. Recovering from PKD	5	4	4	4	0	0
1+ (group D)	1. PKX negative; no histological changes	13	5	8	2	0	0
	2. PKX positive with renal haemopoietic hyperplasia	12	8	6	1	1	1
	Total	86					

Table 4.5. Haematocrit and haemoglobin values in each category of fish from groups A, B, C and D.

	Category of fish	Haematocrit: No. of samples	Haematocrit: $\bar{x}$	Haematocrit: σn	Haematocrit: p	Haemoglobin estimation (g/100 ml blood): No. of samples	Haemoglobin estimation (g/100 ml blood): $\bar{x}$	Haemoglobin estimation (g/100 ml blood): σn	Haemoglobin estimation (g/100 ml blood): p
0+ (groups A, B and C)	1. PKX positive or negative; no histological changes	10	0.38	0.07	NA	10	6.7	0.9	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	17	0.39	0.08	NS	12	7.8	1.2	< 0.01
	3. Grade 1 renal swelling	12	0.36	0.02	< 0.01	8	7.7	1.0	< 0.05
	4. Grade 2 renal swelling	22	0.30	0.07	< 0.001	6	5.9	1.1	NS
	5. Grade 3 renal swelling	35	0.26	0.08	< 0.001	15	5.2	1.3	< 0.001
	6. Grade 4 renal swelling	26	0.12	0.07	< 0.001	5	3.4	1.0	< 0.01
	7. Recovering from PKD	14	0.36	0.09	NS	5	8.3	2.2	NS
1+ (group D)	1. PKX negative; no histological changes	18	0.37	0.06	NS	18	8.5	2.1	< 0.01
	2. PKX positive with renal haemopoietic hyperplasia	12	0.29	0.09	< 0.01	12	7.2	1.7	NS
	Totals	166				91			

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.6. Erythrocytic indices in each category of fish from groups A and D.

	Category of fish	No. of samples	Mean corpuscular volume, MCV (μm^3)			Mean corpuscular haemoglobin, MCH (pico g)			Mean corpuscular haemoglobin concentration, MCHC (%)		
			$\bar{x}$	σn	p	$\bar{x}$	σn	p	$\bar{x}$	σn	p
0+ (group A)	1. PKX positive or negative; no histological changes	10	355	41.5	NA	63	9.6	NA	17.9	2.8	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	337	32.4	NS	75	11.2	< 0.01	22.3	3.4	< 0.001
	3. Grade 1 renal swelling	8	299	16.5	< 0.001	64	9.1	NS	21.4	2.8	< 0.01
	4. Grade 2 renal swelling	6	315	42.2	NS	64	12.9	NS	20.3	3.8	NS
	5. Grade 3 renal swelling	15	331	74.7	NS	67	16.0	NS	20.5	3.4	< 0.05
	6. Grade 4 renal swelling	5	337	68.5	NS	77	16.2	NS	23.1	1.9	< 0.01
	7. Recovering from PKD	5	320	37.5	NS	63	8.5	NS	19.6	2.7	NS
1+ (group D)	1. PKX negative; no histological changes	13	320	38.7	< 0.01	66	6.2	NS	20.8	3.0	< 0.01
	2. PKX positive with renal haemopoietic hyperplasia	12	309	39.8	< 0.01	77	10.1	< 0.001	25.2	3.9	< 0.001
	Total	86									

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.7. Leucocyte counts (excluding thrombocytes) and thrombocyte counts in each category of fish from groups A and D.

	Category of fish	No. of samples	Leucocyte counts, excluding thrombocytes (x 10^9/l) $\bar{x}$	σn	p	Thrombocyte counts (x 10^9/l) $\bar{x}$	σn	p
0+ (group A)	1. PKX positive or negative; no histological changes	10	45.4	16.6	NA	26.8	14.1	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	28.3	10.1	< 0.001	24.4	12.6	NS
	3. Grade 1 renal swelling	8	31.8	10.2	< 0.01	17.2	7.6	< 0.01
	4. Grade 2 renal swelling	6	30.9	11.5	< 0.05	11.1	3.5	< 0.001
	5. Grade 3 renal swelling	15	41.4	23.3	NS	12.9	7.6	< 0.001
	6. Grade 4 renal swelling	5	20.3	16.2	< 0.05	12.1	10.0	< 0.05
	7. Recovering from PKD	5	67.4	39.2	NS	24.2	14.1	NS
1+ (group D)	1. PKX negative; no histological changes	13	28.4	11.9	< 0.001	14.4	9.6	< 0.001
	2. PKX positive with renal haemopoietic hyperplasia	12	26.9	8.0	< 0.001	14.7	5.0	< 0.001
	Total	86						

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.8. Differential leucocyte counts in each category of fish from groups A and D - lymphocytes.

	Category of fish	No. of samples	Small lymphocytes (%) $\bar{x}$	σn	p	Large lymphocytes (%) $\bar{x}$	σn	p
0+ (group A)	1. PKX positive or negative; no histological changes	10	94.0	5.4	NA	3.7	2.9	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	93.3	3.6	NS	3.7	2.0	NS
	3. Grade 1 renal swelling	8	91.9	2.8	NS	2.0	0.5	< 0.001
	4. Grade 2 renal swelling	6	94.7	1.8	NS	2.9	1.1	NS
	5. Grade 3 renal swelling	15	73.5	16.6	< 0.001	3.3	2.2	NS
	6. Grade 4 renal swelling	5	67.7	16.5	< 0.05	7.1	3.6	NS
	7. Recovering from PKD	5	88.6	7.5	NS	4.5	3.9	NS
1+ (group D)	1. PKX negative; no histological changes	13	92.7	5.0	NS	2.9	2.1	NS
	2. PKX positive with renal haemopoietic hyperplasia	12	90.4	6.6	NS	2.6	3.5	NS
	Total	86						

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.9. Differential leucocyte counts in each category of fish from groups A and D - lymphoid cells and neutrophils.

	Category of fish	No. of samples	Lymphoid cells (%) $\bar{x}$	σn	p	Neutrophils (%) $\bar{x}$	σn	p
0+ (group A)	1. PKX positive or negative; no histological changes	10	0	0	NA	2.3	2.8	NA
	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	12	0	0	NS	2.8	2.5	NS
	3. Grade 1 renal swelling	8	0	0	NS	5.9	3.2	< 0.05
	4. Grade 2 renal swelling	6	0.2	0.5	NS	2.0	1.1	NS
	5. Grade 3 renal swelling	15	15.4	12.5	< 0.001	7.6	11.6	NS
	6. Grade 4 renal swelling	5	10.0	8.3	NS	15.0	7.0	< 0.05
	7. Recovering from PKD	5	1.5	1.5	NS	5.5	2.7	NS
1+ (group D)	1. PKX negative; no histological changes	13	0	0	NS	4.4	5.2	NS
	2. PKX positive with renal haemopoietic hyperplasia	12	0.1	0.4	NS	6.7	5.4	< 0.05
	Total	86						

NA = not applicable NS = not significantly different from 0+ category 1 mean

Table 4.10. Plasma and serum protein estimation in each category of fish in groups A, B, C, D and E.

	Category of fish	No. of samples in each category	Plasma protein estimation (g %) No. of samples	$\bar{x}$	σn	p	Serum protein estimation (g %) No. of samples	$\bar{x}$	σn	p
0+ (groups A, B and C)	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	5	ND				5	4.8	0.6	< 0.05
	3. Grade 1 renal swelling	9	5	5.1	0.7	NS	4	4.7	1.2	NS
	4. Grade 2 renal swelling	18	6	4.6	1.0	< 0.05	12	5.4	0.5	< 0.05
	5. Grade 3 renal swelling	20	15	5.7	1.6	NS	5	4.4	1.5	NS
	6. Grade 4 renal swelling	22	9	3.8	1.4	< 0.01	13	3.0	1.2	< 0.001
	7. Recovering from PKD	11	5	5.6	0.5	NS	6	5.6	0.6	NS
1+ (group D)	1. PKX negative; no histological changes	14	14	6.1	1.4	NS	ND			
	2. PKX positive with renal haemopoietic hyperplasia	11	11	5.0	1.2	NS	ND			
Group E fish		10	5	5.8	1.1	NA	5	5.8	1.1	NA
	Totals	120	70				50			

NA = not applicable NS = not significantly different from group E mean values

ND = no samples taken

Table 4.11. Albumin and globulin values in each category of fish in groups A, B, C, D and E.

	Category of fish	Plasma or serum albumin (g %)				Serum globulin (g %)			
		No. of samples	$\bar{x}$	σn	p	No. of samples	$\bar{x}$	σn	p
0+ (groups A, B and C)	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	5	1.5	0.14	< 0.05	5	2.3	0.33	< 0.01
	3. Grade 1 renal swelling	9	1.7	0.43	NS	4	1.7	0.27	NS
	4. Grade 2 renal swelling	18	1.6	0.26	NS	11	2.0	0.38	< 0.01
	5. Grade 3 renal swelling	19	1.7	0.52	NS	5	1.7	0.32	NS
	6. Grade 4 renal swelling	22	0.8	0.64	< 0.001	14	1.4	0.39	NS
	7. Recovering from PKD	11	1.8	0.29	NS	11	2.2	0.34	< 0.001
1+ (group D)	1. PKX negative; no histological changes	13	2.1	0.36	< 0.01	8	1.8	0.31	NS
	2. PKX positive with renal haemopoietic hyperplasia	12	1.7	0.59	NS	ND			
Group E fish		10	1.7	0.29	NA	10	1.6	0.19	NA
	Totals	119				68			

NA = not applicable NS = not significantly different from group E mean value

ND = no sample taken

Table 4.12. Serum urea and bilirubin values in each category of fish in groups A, B, C, D and E.

	Category of fish	Serum urea (mg %) No. of samples	$\bar{x}$	σn	p	Total serum bilirubin (mg %) No. of samples	$\bar{x}$	σn	p
0+ (groups A, B and C)	2. PKX positive with renal haemopoietic hyperplasia. Grade 0 renal swelling	4	6.2	0.7	NS	ND			
	3. Grade 1 renal swelling	4	5.2	0.7	< 0.05	ND			
	4. Grade 2 renal swelling	12	9.2	4.7	NS	11	0.3	0.16	< 0.001
	5. Grade 3 renal swelling	9	6.9	2.7	NS	5	0.2	0.06	< 0.001
	6. Grade 4 renal swelling	21	7.4	3.5	NS	14	0.3	0.17	< 0.001
	7. Recovering from PKD	11	6.9	4.2	NS	11	0.6	0.41	< 0.01
1+ (group D)	1. PKX negative; no histological changes	9	8.2	2.1	< 0.05	ND			
	2. PKX positive with renal haemopoietic hyerplasia	10	6.8	4.5	NS	ND			
Group E fish		10	6.7	2.1	NA	5	1.0	0.24	NA
	Totals	90				46			

NA = not applicable NS = not significantly different from group E mean value

ND = no samples taken

Chapter 5

The epidemiology of PKD

GENERAL INTRODUCTION

Prior to the work described in this dissertation, various factors had been considered to affect the development and course of PKD. These were reviewed in chapter 1. With regard to environmental factors, field observations had suggested a correlation between rising water temperatures and the development of PKD and between falling water temperatures and the decline of the disease (Ferguson and Ball, 1979; Ellis et al., 1982). It had also been noted that the course of PKD was prolonged where water temperatures were maintained at 15°C (Smith et al., 1984) and at 15-18°C (Schlotfeldt, 1983b). From further studies of the temperature effect, it was concluded by Ferguson (1981) that decreased water temperatures prevented the disease progressing and enhanced recovery. With regard to host susceptibility, PKD had been reported in both 0+ and 1+ rainbow trout (Plehn, 1924; Ferguson and Ball, 1979; Hoffmann and Dangschat, 1981). Although naive 1+ rainbow trout were shown to be susceptible to PKD (Ferguson and Ball, 1979), evidence for resistance among 1+ rainbow trout surviving disease in the previous year was scanty (Ferguson and Ball, 1979). Circumstantial evidence suggested that Atlantic salmon were less susceptible than rainbow trout to PKD (Ferguson and Needham, 1978). However, clinical PKD in both Atlantic salmon and brown trout was reported by Ellis et al. (1982, 1985).

Studies described in this chapter were undertaken to investigate further the effects of water temperature, age, previous exposure and species of host on the development of PKD. Their purposes were as follows:

1. To investigate the time course and infective period of PKD at the experimental site over a two-year period and relate these to water

temperatures at the site.

2. To investigate at the laboratory the effects of different water temperatures on:

(a) The initiation of PKD.

(b) The course of PKD in naturally-infected, sub-clinically-affected rainbow trout.

(c) The course of PKD in naturally-infected, clinically-affected rainbow trout.

3. To investigate the effect of age and previous infection on the susceptibility of rainbow trout to PKD.

4. To investigate the susceptibility of brown trout and Atlantic salmon to natural infection with PKD at the experimental site.

Each study is described and discussed separately and results from all the studies are considered together in the general discussion at the end of the chapter.

STUDY 1. INVESTIGATION OF THE TIME COURSE AND INFECTIVE PERIOD OF PKD AT THE EXPERIMENTAL SITE AND RELATED WATER TEMPERATURES

Introduction

In previous years, 0+ rainbow trout taken to the experimental site in May developed clinical PKD from late July to September. It was not known whether batches of fish introduced earlier or later in the year would become infected and if so whether they would develop clinical disease. This study was undertaken to investigate these possibilities. In addition, maximum and minimum water temperatures were monitored daily during 1983 and related to the experimental findings.

Materials and Methods

Details of the materials and methods relating to the farms supplying experimental fish; the holding and management of fish at the experimental site and at the laboratory; the assessment of infection and severity of disease; and the recording of water temperatures are described in chapter 2.

Four groups of 0+ rainbow trout, designated A, B, C and D, were used. They were derived from fish hatched and reared at sites with no previous history of PKD and were assessed to be free from PKX cells by histological examination of kidney tissue from some fish at the start of each experimental period. Groups A and B were the same as those used in chapter 3 and comprised 1,500 80 mm and 300 60 mm fish, respectively. Group C comprised six batches, Ci-Cvi, each of 25 75 mm fish derived from farm A. Group D comprised five batches, Di-Dv, each of 200 75 mm fish from farm C. Serial batches of fish were taken to and held at the experimental site as shown in Table 5.1. They were sampled at intervals as shown in Table 5.1 to follow the course of the infection after introduction to the site at different times of the year. Serial batches of fish from groups A, B and D were also transferred from the experimental site to the laboratory as shown in Table 5.2. These were held in small or medium tanks supplied with aerated, dechlorinated tap water at 16°C and sampled at intervals as shown in Table 5.2 for the presence of infection with PKX cells.

Evidence of infection and severity of disease were assessed in sampled fish from clinical signs, gross renal pathology and renal histology in sections and impression smears.

Maximum and minimum water temperatures at the experimental site were recorded daily throughout 1983.

Results

PKX cells were first identified in the kidneys from fish in each batch at the experimental site and the laboratory as shown in Tables 5.1 and 5.2. Group A fish at the experimental site were PKX-positive in early June, 36 days after exposure, with kidney swelling developing in July after a further 31 days. Of batches Ai-Avi at the laboratory, batch Ai, removed from the experimental site in May after 11 days and sampled 60 days later, was PKX-positive with renal haemopoietic hyperplasia but no renal swelling. Batches Aii-Avi were also infected, with several fish from each batch developing some degree of renal swelling.

As with group A, group B fish at the experimental site were found PKX-positive in early June, 85 days after exposure, with kidney swelling developing in July, 32 days later. Of group B batches at the laboratory, batches Bi-Biii (moved in March and April) appeared to be uninfected, while fish from batches Biv-Bvi (moved in May) were positive for PKX cells.

In group C, all batches became infected. Batch Civ was shown to be infected 26 days after exposure with gross renal swelling after a further 28 days. Batch Cv was histologically positive after 19 days. Grade 4 renal swelling was seen in fish in batches Ci-Ciii, but no swelling greater than grade 1 was seen in fish in batches Civ-Cvi.

In group D, batch Di was infected on the farm after 91 days (in January), but no further samples from this batch were positive until 274 days after initial introduction (in the following July). The first batch of fish from Di taken to the laboratory was PKX-positive, with some fish developing renal swelling. Neither the second batch from Di nor fish from batches Dii-Dv at the laboratory showed evidence of infection.

Maximum and minimum water temperatures at the experimental site are recorded as 7 day rolling averages in Fig. 5.1.

Discussion

The main aim of these experiments was to introduce fish to the experimental site at different times of the year and to sample them to see when infection could first be detected and what the subsequent course of that infection would be. It was recognised, however, that fish could become infected at the experimental site before such infection would become histologically detectable. For this reason, fish were moved to the laboratory at various intervals and held under conditions thought to favour the progress of an infection to a detectable level (Ferguson, 1981). It was also recognised that infection on the farm might not progress at certain times of the year due to environmental factors. Fish were also transferred to the laboratory, therefore, to investigate whether any infection detected could progress to clinical disease if conditions were favourable.

Results from fish in group A moved to the laboratory 11 days after introduction to the experimental site demonstrated that in 1982 the infective agent was present at the farm between 30 April and 11 May. Results from fish in group B moved to the laboratory showed that in 1983 infection occurred after 20 April and before 4 May. Of fish in group D moved to the farm between October 1983 and February 1984, only the October batch became infected. It would seem, therefore, that at the experimental site in 1983 fish became infected from the third week in April until October. This compares with an infective period from May to November in California (Hedrick et al., 1985b).

Fish in groups A and B held at the experimental site showed no evidence of infection until June (Table 5.1), i.e. 3 to 4 weeks after results from laboratory-held fish (Table 5.2) had shown them to be positive.

Fish introduced in August (group C, batch v) became detectably infected on the farm sooner than fish introduced earlier in the year (groups A, B and C i-iv). They were shown to be positive after 19 days. This may imply a more rapid multiplication of the organism at this time and/or a greater number of organisms entering the fish. These results differ from those of Ferguson and Ball (1979), who found in their studies that May was the optimum time for contracting the disease, and from those of Hedrick et al. (1985b) and Olesen (1985), who concluded that risk of infection was greatest in June.

With regard to the course of the disease at the experimental site, renal swelling was apparent in groups A, B and C 28 to 32 days after infection was detected. This agreed with the findings of Ferguson and Ball (1979). Fish introduced after 8 July (group C, batches iv-vi) did not develop kidney swelling greater than grade 1, while those introduced earlier developed swelling up to grade 4. In contrast, infection in the first batch of group D fish moved to the farm in October did not progress. Yet fish from this batch moved to the laboratory in November did develop PKD. It appeared, therefore, that some environmental factors were inhibiting the development of the disease from July onwards. It is interesting to note that fish from the first batch of group D moved to the laboratory in February did not develop PKD, suggesting that the October infection had not established in fish on the farm during the winter months.

Relating these findings to water temperatures measured in 1983 (Fig. 5.1) it seemed that fish did not become infected in the spring until water temperatures increased from 6.5°C minimum/7.5°C maximum on 20 April to a minimum 11°C/13°C maximum on 3 May. Fish infected in the autumn did not maintain the infection when the water temperatures decreased from 15.5°C maximum/14°C minimum on 6 October to 12.5°C maximum/10.5°C minimum

on 3 November. However, if moved to the laboratory and held at 16°C they did develop PKD. These findings agree with the observations of Ferguson and Ball (1979), Ferguson (1981) and Ellis et al. (1982) that PKD increases with rising water temperatures and decreases with falling water temperatures. The effects of water temperature on the development of PKD will be considered further in the following section.

STUDY 2. INVESTIGATION OF THE EFFECT OF WATER TEMPERATURE ON THE INITIATION, DEVELOPMENT, COURSE AND SEVERITY OF PKD

Introduction

The results of Study 1 suggested that fish introduced to the experimental site would only become infected with the PKX organism between April and October. It was not known whether outside this period fish were resistant to infection or whether infective PKX organisms were absent. From the water temperatures taken throughout 1983 (Fig. 5.1) it was shown that when fish could not be infected at the experimental site (November to April) water temperatures generally remained below 10°C. The first experiment described in this section, therefore, was designed to investigate whether fish held at 9°C were susceptible to infection with PKD.

In infected fish at the experimental site, only in those infected before the beginning of July did the disease progress beyond grade 1 renal swelling. In October-infected fish, the infection was abortive. It seemed possible that these findings were related to the water temperature. The second and third experiments in this section, therefore, were designed to investigate the effects of different water temperatures on the development, course and severity of PKD in naturally-infected sub-clinically-and clinically-affected fish, respectively.

Experiment 1. Effect of water temperature on the susceptibility of rainbow trout to experimental infection with PKD.

MATERIALS AND METHODS

Details of the materials and methods are given in chapter 2.

Forty-five uninfected 0+ rainbow trout, average length 80 mm, which had been hatched at the laboratory, were placed in equal-sized groups in three small tanks designated A, B and C. Tanks A and B were supplied with water at 9°C and tank C with water at 16°C.

The material for inoculation was a fresh suspension of infected kidney tissue from three rainbow trout with grades 2 or 3 renal swelling which had been held at 9°C for 7 days prior to being killed. Approximately 1.5 g of tissue was dispersed in 15 ml PBS chilled to 9°C and held at this temperature until injected into the test fish. Counts of the number of PKX cells per ml of suspension were made in an improved Neubauer haemocytometer using interference microscopy.

Eight days after being placed in the tanks, the 45 test fish were anaesthetized and injected intraperitoneally with 0.2 ml aliquots of the chilled suspension.

The temperature of the water to tank A was maintained at 9°C, that to tank B was raised over the 7 days following inoculation to 16°C and that to tank C was maintained at 16°C throughout.

Fish were sampled at 49 and 85 days after inoculation. PKD infection was assessed from the degree of renal swelling and the histological appearance of the kidney.

RESULTS

Each fish was inoculated with approximately 30,000 PKX cells.

Results of histological examinations are summarized in Table 5.3.

Three fish from tank C, sampled at 85 days, contained swollen kidneys, two with grade 1 and one with grade 2 swelling. No other fish contained swollen kidneys. Some fish from all three groups contained PKX cells.

DISCUSSION

As fish had to be maintained at different and constant temperatures in these experiments, it was not possible to infect fish naturally at the experimental site. For this reason, fish were experimentally inoculated intraperitoneally at the laboratory. Results from fish held at 16°C throughout (tank C) showed that 11 of 15 fish sampled 7 and 12 weeks after inoculation had detectable PKX cells in their kidneys and three developed renal swelling. This suggested that the method was successful in establishing a kidney infection and producing lesions in fish held at this temperature.

Two of 15 fish held at 9°C throughout had detectable PKX cells in their kidneys, demonstrating that fish held at 9°C are not intractable to experimental infection. It was not possible to say whether such fish would also be susceptible to natural infection.

Although the numbers were small there seemed to be a difference in infection rate between fish held at 16°C (11 of 15) and fish held at 9°C (2 of 15). It may be that in the field fish at temperatures lower than 9°C (as occurs during the winter months - Fig.5.1) are intractable to infection. Unfortunately, it was not possible to reliably maintain tanks for prolonged periods at, for example, 5°C or less to check that possibility.

It was interesting to note that a smaller proportion of fish inoculated at 9°C then warmed to 16°C had detectable PKX cells in their kidneys (4 of 15) than fish inoculated and held at 16°C throughout (11 of 15).

It could be argued that the haemopoietic hyperplasia seen in 29 of the experimental fish was due to a low grade peritonitis rather than a direct response to infection with PKX cells. Only fish held at 16°C developed other changes attributable to PKD.

It would seem, therefore, that PKX cells derived from rainbow trout held for at least 7 days at 9°C were viable, survived a temperature change from 9°C to 16°C and were able to cause clinical disease when injected intraperitoneally into rainbow trout held at 16°C. However, although constant temperatures of 9°C did not destroy the PKX cells, it did appear to decrease their ability to induce clinical disease at that temperature.

Experiment 2. Effects of different water temperatures on sub-clinically-infected fish.

MATERIALS AND METHODS

In order to study the course of infection in naturally-infected, sub-clinically-affected fish held at four different temperatures, 1,500 50-70 mm rainbow trout from farm C were transported to the experimental site on 26 May 1983 and maintained in a 6 m circular concrete tank as described in chapter 2. They were sampled 18 days after introduction. Then 28 days after initial exposure 500 fish were transferred to the laboratory, divided into four groups, one of 200 and three of 100 fish, and held in medium tanks as described in chapter 2. Over a 9-day period to 2 July 1983 the water temperatures of the four tanks were adjusted to 9°C, 12°C, 15°C and 18°C, respectively, with the largest group being in the 9°C tank. Samples were taken from these fish as shown in Table 5.4. One hundred and eighty days after initial exposure half the remaining fish in the 9°C tank were moved to a tank at 16°C. These were sampled after a further

23 and 85 days.

Tissues taken at each sampling (except 18 and 43 days after introduction when only kidney specimens were collected) included gill, intestine (including pyloric caeca), kidney, liver, pancreas and spleen. Skin, striated muscle and ureter samples were also taken except at samplings 63, 74 and 83 days after initial exposure.

Development of PKD was monitored by grading of kidney swelling, by histological examination of tissue sections and by examination of kidney smears as described in chapter 2.

Fish in both the 15°C and 18°C tanks developed ichthyophthiriasis. They were treated with malachite green/formalin flushes, as described in chapter 2, 69 and 72 days after initial exposure. The 15°C tank was further treated 97 and 100 days after initial exposure. Aeromonas hydrophila infection was also diagnosed in the 15°C tank. This was treated with a 10-day course of 10 mg oxolinic acid/kg body weight of fish daily from 83 days after initial exposure.

RESULTS

None of the 5 fish sampled at the farm 18 days after introduction were detectably infected.

The development of renal swelling in fish at the four temperatures is represented in Fig. 5.2. In the 9°C tank, 25 of 55 fish sampled up to 140 days after initial exposure at the experimental site developed grade 1 renal swelling. None of the 25 fish sampled 160 to 225 days after initial exposure showed renal swelling. In the 12°C tank, 50 of 70 fish sampled 43 to 188 days after initial exposure had grade 1 or 2 renal swelling. Five of 55 fish sampled 83 to 225 days after initial exposure showed renal swelling of grades 3 and 4. Seven of 30 fish

sampled 126 to 203 days after initial exposure had grade 5 renal swelling. In the 15°C tank, 24 of 30 fish sampled 68 to 126 days after initial exposure had grade 3 or 4 renal swelling, and in the 18°C tank 24 of 34 fish sampled 55 to 74 days after initial exposure had grade 3 or 4 swelling.

The numbers of kidney specimens at each sampling from each tank showing histological changes associated with PKD are detailed in Tables 5a, b and c. In fish from the 9°C tank, the principal histopathological change in PKX-positive kidneys throughout the experimental period was haemopoietic hyperplasia. In fish from the 12°C and 15°C tanks, all renal histopathological lesions associated with PKD (described in chapter 3) developed. Those in fish from the 15°C group developed earlier and were more severe. In fish held at 18°C, only lesions associated with the developing phase of PKD were found. Vascular aggregations, glomerulus and tubule destruction and diffuse inflammation developed from 55 days after initial exposure and extensive areas of tissue destruction were seen in samples taken between 60 and 74 days after initial exposure.

As far as distribution of PKX cells was concerned, in fish from the 9°C tank, PKX cells were only seen in the kidney and spleen. In some fish from the 12°C tank sampled from 68 days after initial exposure gill, intestine, liver and pancreas samples were also infected. In fish from the 15°C and 18°C tanks gills and livers were shown to be infected from 55 days after initial exposure, and pancreas and intestine from 68 days. No striated muscle, skin or ureter samples from fish at the four temperatures were found to be infected throughout the experimental period.

Fish from the 9°C tank transferred to a tank at 16°C 180 days after initial exposure were negative for PKX organisms 23 days later, but three of five sampled 266 days after initial exposure were PKX-positive. The

kidneys from these fish showed two with lesions of haemopoietic hyperplasia and three with areas of diffuse inflammation. One had grade 1 swelling.

Although no precise counts were made of PKX organisms in kidney smears, it was apparent that the numbers increased several hundred-fold between grade 1 and grade 4 swelling. In the 15°C tank, numbers of organisms appeared to decline after samples taken 68 days after initial exposure.

DISCUSSION

Thirty-nine of 40 fish sampled 15 days after transfer from the farm to the laboratory, 43 days after initial exposure, were infected with PKX cells and many of the kidneys from these fish showed haemopoietic hyperplasia (Tables 5.5a, b and c). This suggested that almost all the fish transferred to the laboratory had become infected. Despite this, fish subsequently held at 9°C failed to develop clinical disease and PKX cells appeared to be confined to the kidney and spleen. However, the organism was not eliminated from these fish. It was demonstrated in fish held at 9°C for 132 days (160 days after initial exposure at the experimental site) and was still present in fish moved after 152 days at 9°C to a tank at 16°C. The finding that the organism can persist for at least 5 months in fish held at 9°C raises the question of whether the organism can overwinter in some fish on the farm and perhaps provide a source of infection for susceptible fish in the following year. In this regard, it is interesting to note that 10 fish in batch Dib described earlier in Study 1, infected at the farm in October then moved to the laboratory the following February where they were held at 16°C, had no demonstrable PKX organisms in the kidneys by April.

Fish moved from 9°C to 16°C 180 days after initial exposure at the experimental site still harboured PKX cells which were detectable 85 days after moving the fish. Gross pathology was present in one of these fish. This suggests that the presence of the organism in the fish even for prolonged periods does not necessarily induce a protective response.

Fish held at 12°C, 15°C and 18°C developed clinical PKD. The disease developed more rapidly and was more severe in fish held at 15°C and 18°C than in those held at 12°C (Fig. 5.2).

The finding that in fish which developed clinical disease, the number of organisms increased markedly with the development of pathology suggested that the organism could be multiplying within the host. A similar increase was not seen in kidney smears from fish held at 9°C. The start of the apparent decrease in organism numbers in the 15°C fish coincided with the initiation of malachite and formalin treatment for ichthiophthiriasis.

With regard to the original purpose of this experiment, water temperature appeared to affect the progress of sub-clinical infection with PKD. The disease did not develop in fish held at 9°C, although the organism persisted in these fish for 5 months. The disease developed in fish held at temperatures of 12°C-18°C. The disease developed more rapidly and was more severe in fish held at the higher temperatures.

Experiment 3. Effects of different water temperatures on clinically-affected fish.

MATERIALS AND METHODS

In order to study the course of disease in naturally-infected, clinically-affected fish held at six different temperatures, 60 rainbow trout, derived from group A in chapter 3, were exposed at the experimental

site from 30 April 1982 and transferred to the laboratory on 15 July 1982 when they were showing clinical signs of PKD. They were divided into six batches of 10 fish and held in small tanks under conditions described in chapter 2. The water supplies to these tanks were adjusted over a 7-day period to 7°C, 9°C, 12°C, 15°C, 18°C and 21°C from an ambient temperature at the farm of approximately 18.5°C. Fish were sampled on 3 September 1982 as shown in Table 5.6. In order to investigate whether fish held at 15°C or less until recovery would suffer a recrudescence of disease at a higher water temperature, the water temperatures of the water supplies to tanks previously set at 7°C, 9°C, 12°C and 15°C were gradually increased to 18°C from 4 to 11 September 1982 and held at this temperature until termination of the experiment on 12 November 1982.

Evidence of infection and its severity were assessed by degree of renal swelling and histological appearance of renal sections as described in chapter 2.

RESULTS

Of ten fish sampled at the farm on 15 July 1982, three had renal swelling of grade 1, five of grade 2 and two of grade 3. The numbers of fish with various grades of renal swelling sampled at the laboratory are summarized in Table 5.6. The histological findings are recorded in Table 5.7. One fish from both the 9°C and 12°C tanks died prior to 3 September 1982 with severe peritonitis secondary to perforation of the abdominal wall due to an acanthocephalan infestation. In the 18°C tank five fish died between 3 and 17 August 1982 suffering from ichthyophthiriasis and PKD. In the 21°C tank all fish died between 26 July and 3 August 1982. No cause could be determined except the combined effects of PKD and heat stress.

DISCUSSION

Despite the small numbers of fish in each group, certain findings are worthy of comment. All five fish in both the 7°C and 9°C tanks showed signs of healing 43 days after the water temperatures were attained, and these signs were advanced in four of the five fish in each group. Fish in the 15°C and 18°C tanks sampled at this time only showed early signs of healing and recovery. The results for the 12°C tank were intermediate between these two. It would appear, therefore, that water temperature is inversely related to the recovery rate of PKD. This supports the conclusion of Ferguson (1981) that falling water temperature speeds the recovery of fish from PKD. It is not clear how the lower temperatures exert their effect. It may be by enhancing the killing of PKX cells and/or by decreasing their multiplication rate. In this respect, it is interesting to note that only one fish in each of the 7°C and 9°C tanks had detectable PKX cells when sampled while three in the 18°C tank had detectable PKX cells. Whatever the effect, it presumably counteracts the tendency of lower water temperatures to decrease the effectiveness of the host's immune response (Avtalion et al., 1973). It was shown that fish which had recovered in the 7°C, 9°C and 12°C tanks did not suffer a recrud-escence of disease when they were held at 18°C for 62 days. No PKX cells were detected in the kidneys of these fish at the end of this period. This differs from results in the previous experiment where sub-clinically infected fish held at 9°C for 180 days and then moved to a tank at 16°C developed gross pathological changes 85 days after moving. It may be,therefore, that the presence of the organism alone does not initiate a protective response, but that development of clinical disease induces some resistance in the fish. Three of five fish in the 15°C tank warmed to 18°C still had detectable PKX cells in their kidneys after

62 days. This agrees with the field findings of Schlotfeldt (1983a,b) and Smith et al. (1984) that infection is prolonged in fish held at about 15°C.

The findings that all fish held at 21°C died correlated with field reports that PKD-affected fish are particularly susceptible to heat stress despite adequate water flow and aeration.

STUDY 3. INVESTIGATION OF THE EFFECTS OF AGE AND PREVIOUS INFECTION ON THE SUSCEPTIBILITY OF RAINBOW TROUT TO PKD

Introduction

Ferguson and Ball (1979) postulated from their field observations and laboratory experiments with rainbow trout that infection of 0+ fish with PKD increased the resistance of the fish to disease as 1+ fish. They did not consider that age per se was a factor in this resistance to disease.

Results from Study 2 demonstrated that the presence of the PKX organism in fish for prolonged periods in the absence of clinical disease did not induce resistance to the disease. However, there was some evidence that, following the development of clinical signs, a degree of resistance was induced in surviving fish.

The four experiments described in this section were designed to investigate the effects of age and previous exposure on the susceptibility of rainbow trout to infection with PKD.

Experiment 1. Effect of age on the susceptibility of previously unexposed rainbow trout to PKD.

MATERIALS AND METHODS

Twenty yearling rainbow trout, average weight 700 g, and 20 fingerling rainbow trout, average weight 5 g, from farm A, were transported to the

experimental site on 10 June 1982. The yearlings were held in a 6 m tank, the fingerlings in a 1 m^3 cage. Both groups were managed as described in chapter 2. Fish were sampled as shown in Table 5.8. The degree of infection was assessed by grade of kidney swelling and kidney histopathology as described in chapter 2, and for fish sampled on 31 August 1982 by haematocrit values measured as described in chapter 4.

RESULTS

The degree of renal swelling and renal histopathological lesions found in fish at each sampling are detailed in Tables 5.8 and 5.9. The average PCV in yearling fish sampled on 31 August 1982 was 0.23, range 0.10-0.40, and in fingerling fish was 0.24, range 0.11-0.42. Yearling fish from each sampling contained intestinal or renal lesions of nephrocalcinosis.

DISCUSSION

Although only five yearling fish were sampled at the end of the experimental period, advanced histopathological lesions of PKD comparable to those seen in the fingerlings, were present. In addition, the haematocrit values showed individuals in both groups with severe anaemia and average values below accepted average values (Barnhart, 1969; McCarthy *et al.*, 1975). These findings support the conclusion of Ferguson and Ball (1979) that there is no age resistance to PKD and that, like fingerling fish, yearling fish are susceptible to PKD in their first summer on infected water.

Experiment 2. Effect of previous exposure to PKD on susceptibility to subsequent infection.

MATERIALS AND METHODS

One hundred and five 1+ rainbow trout which had survived clinical PKD the previous summer were used in this experiment. They were derived from survivors of group A fish (described in chapter 3) which had been moved as 80 mm fingerlings from farm A to the experimental site on 30 April 1982 and held in a 6 m concrete tank. They were managed as described in chapter 2. Yearling fish were sampled at intervals between 10 March 1983 and 2 March 1984. Infection was assessed by gross and histopathological examination of renal samples as described in chapter 2. Results were compared with those from 0+ rainbow trout in group A sampled in 1982 and group B fish sampled in 1983. These were described in detail in chapter 3.

RESULTS

Of the 105 yearling fish, one fish, sampled on 15 August 1983, showed renal swelling (grade 1). None of 40 kidney specimens from fish sampled between 10 March and 2 June 1983 contained evidence of PKD, nor did those of 25 fish sampled between 2 December 1983 and 2 March 1984. Histological findings in re-infected fish are recorded in Table 5.10. Lesions were circumscribed and involved small areas of tissue.

Between 30 June and 19 September 1983, of 25 1+ fish sampled 12 were PKX-positive and none had renal swelling of grades 2 to 4. In comparison, of 80 0+ fish from group A sampled between 1 July and 23 September 1983, 69 were PKX-positive, 34 had renal swelling of grades 2 to 4 and 23 were showing signs of recovery. Of 56 group B fish sampled between 30 June and 1 September 1983, all 51 were PKX-positive, 31 had renal swelling of grades 2 to 4 , and five showed signs of recovery.

DISCUSSION

The results showed that a smaller proportion of 1+ rainbow trout exposed for a second summer to PKD became detectably infected with PKX cells than 0+ rainbow trout exposed in their first summer. Lesions in the 105 fish tested were minimal and none showed clinical signs of PKD. It was shown in the previous experiment that 1+ rainbow trout exposed to PKD for the first time were fully susceptible to the disease. It seems likely, therefore, that the difference reflects a development of resistance in fish previously exposed to PKD. The resistance does not seem to prevent infection of the fish but, rather, limits the extent of lesions. This is in contrast to the findings of Hedrick et al. (1985b) in 30 steelhead trout surviving clinical disease. On re-challenge, these fish did not develop clinical signs or contain detectable PKX cells.

Experiment 3. Effect of different severities of PKD infection on susceptibility to subsequent disease.

MATERIALS AND METHODS

In order to study the susceptibility of rainbow trout to natural infection following recovery from different degrees of PKD infection, three groups of fish, designated A, B and C, which had survived PKD infection previously, were used. A fourth control group, D, comprised previously uninfected fish. Details of these groups are given in Table 5.11.

All groups were held and maintained in 1 m^3 cages placed in a 6 m tank as described in chapter 2.

Groups A and B were sampled on 30 July 1984, groups C and D on 6 June, and 6, 23 and 30 July 1984. Severity of infection was assessed by histological examination of renal tissue and by grading kidney swelling as described in chapter 2.

RESULTS

The grades of renal swelling and renal histopathology in fish at each sampling are detailed in Tables 5.12 and 5.13. No fish in group A exhibited renal swelling greater than grade 1, whereas fish in groups B and C developed renal swelling up to grade 3, and fish in group D up to grade 4. Clinical signs of PKD were seen in fish from groups B, C and D, but not in fish from group A.

DISCUSSION

In previous experiments described in Study 2 and Study 3, there was evidence that fish infected but not clinically-affected with PKD did not develop resistance to the disease, while those that became clinically-affected did. In this experiment, control fish in group D, thought to have been previously uninfected, and infected fish in groups B and C, which had not previously suffered clinical disease, developed disease with renal swelling up to grade 3 or 4 and extensive histopathological lesions of PKD. This confirmed results from Study 2 that sub-clinical infection with the PKX organism does not necessarily induce resistance to disease. The results suggested that infection causing greater than grade 1 renal swelling was required to induce resistance. Unfortunately, the number of fish available in group A which had previously developed renal swelling up to grade 2 was too small to draw firm conclusions about their susceptibility to disease. However, it was interesting to note that none of these fish had developed clinical signs by the end of July when fish in other groups had.

Experiment 4. Susceptbility to PKD of fry derived from broodstock which had survived clinical PKD.

MATERIALS AND METHODS

In order to study whether fry from broodstock which had survived clinical PKD were susceptible to the disease, eggs were stripped on 5 January 1984 from group A rainbow trout (described in chapter 3) which had survived two summers at the experimental site. The eggs were fertilized and then placed without disinfection in hatchery troughs supplied with flowing dechlorinated tap water at 10°C. Diseased eggs were removed every 7 days. Hatching occurred from 7 February 1984 and first feeding began on 2 March 1984. Thirty fry, average length 40 mm, were transferred to the experimental site on 18 May 1984 and held in a 115 l circular plastic tank supplied with river water. They were fed and maintained as described in chapter 2 and sampled on 12 and 23 July 1984.

Control rainbow trout fingerlings, average length 70 mm, derived from uninfected broodstock at farm C, were introduced to the field site on 9 May 1984 and maintained in a 6 m concrete tank as described in chapter 2. These fish were sampled on 5 and 30 July 1984.

Fry derived from group A fish were also held at the laboratory throughout the experimental period at 16°C and were sampled in July and September.

Infection was assessed from the degree of renal swelling and histopathological changes.

RESULTS

Fish in both groups at the experimental site developed PKD. The grades of renal swelling seen in the fish sampled are detailed in Table 5.14. Ten test fry died between 12 and 23 July 1984. No control fish

died.

None of the fry sampled at the laboratory showed gross or histopathological signs of PKD.

DISCUSSION

The results showed that fry from broodstock surviving PKD during two summers on infected water were susceptible to infection as were fingerling rainbow trout derived from previously uninfected broodstock. This suggested that no lasting immunity to PKD is transmitted from broodstock to offspring.

Although Ellis *et al*. (1982) suggested that transfer of PKD with eggs might occur, these was no evidence of this in the present study. None of the fry held in uninfected water at the laboratory developed PKD. However, eggs were stripped from fish during January. It may have been that the organism was not present at this time.

STUDY 4. INVESTIGATION OF THE SUSCEPTIBILITY OF BROWN TROUT AND ATLANTIC SALMON TO PKD

Introduction

As described in chapter 1, PKD in the United Kingdom has been observed in other salmonids besides rainbow trout, namely brown trout, char and Atlantic salmon. Although some occurrences in both brown trout and Atlantic salmon have been severe (Ellis *et al*., 1982, 1985), field observations of PKD in brown trout on farms producing both brown and rainbow trout have suggested that the disease is less severe in brown trout. Severe outbreaks of PKD in rainbow trout at a farm on the west coast of Scotland were reported by Ferguson and Needham (1978). Atlantic salmon held under similar conditions at the farm were unaffected.

The experiment in this study was designed to investigate the susceptibility of brown trout and Atlantic salmon to natural infection at the experimental site.

Materials and Methods

Fifty brown trout fingerlings hatched at farm D and 50 salmon parr, derived from Uist eggs from farm F, were transferred in May to the experimental site where they were held and maintained in separate 1 m^3 cages placed in a 6 m diameter circular tank.

Brown trout and Atlantic salmon were sampled as shown in Table 5.15. Degree of infection was assessed by grade of kidney swelling and by histological examination of kidney sections as described in chapter 2. Kidney sections were also stained by Giemsa's method (Table 3.2). The findings were compared with those from group A rainbow trout taken at similar times after introduction to the field site and described in detail in chapter 3.

Results

The number of fish sampled, the grades of kidney swelling encountered and the histopathological lesions seen in kidney sections are detailed in Tables 5.15 and 5.16. No clinical signs were seen in sampled salmon or brown trout. However, fish of both species developed kidney swelling up to grade 2. Renal histopathological changes included haemopoietic hyperplasia, vascular aggregations, diffuse inflammation, and glomerulus and tubule destruction. In recovering brown trout, eosinophilic granule cells were numerous in areas of organization.

In H & E stained sections, renal tubules in brown trout sampled 88 days after introduction to the field site and in Atlantic salmon sampled

84 days after introduction contained intraluminal cells with similar staining characteristics to PKX cells but of more variable shape. These cells in Giemsa-stained sections were seen to contain two spherical polar bodies.

Discussion

Infection and development of renal swelling occurred in both brown trout and Atlantic salmon at approximately the same time after introduction to the field site as in rainbow trout of group A. However, unlike the rainbow trout, neither the brown trout nor the Atlantic salmon developed renal swelling greater than grade 2, and neither group showed clinical signs or mortalities. Although the holding conditions at the farm were similar for all three species, the stocking densities of the brown trout and Atlantic salmon were less than for the rainbow trout. This may have influenced the results. Despite the limited number of samples from brown trout and salmon, the results suggested that not all fish became detectably infected and that recovery was more rapid in both brown trout and salmon than in rainbow trout. It may, therefore, be that both the brown trout and Atlantic salmon strains in this experiment were less susceptible to PKD than the rainbow trout.

It is interesting to note that no crystals were found in either the brown trout or salmon renal sections despite intra-vascular aggregations and diffuse inflammatory changes. At present, it is not certain if the intra-tubular cells, possibly representing immature myxosporidan spores, were a stage of PKX cell development. However, they were morphologically similar to those described by Kent (1985) and Kent and Hedrick (1985a,b, c) in steelhead trout.

GENERAL DISCUSSION

Prior to this work, environmental factors were suspected of influencing the initiation, course and development of PKD. Results from Study 1 demonstrated that at the experimental site PKD was a seasonal disease with fish only becoming infected between April and October. Seasonal variation has been demonstrated for other protozoan infections of fish, with pathogens having absolute temperature ranges in which they can survive and optimum ranges in which they can cause disease (Reichenbach-Klinke,1981). For example, Chilodonella infestation in cultured carp is a particular problem in Russia during the winter months whereas the organism dies during the summer. Conversely, ichthyophthiriasis is a greater problem when water temperatures are rising, allowing a more rapid reproductive cycle (Dogiel, Petrushevski and Polyanski, 1958).

It was not clear whether the infective stage of the PKX cell only appeared as temperatures rose in the spring, or whether it was constantly present, but fish were intractable to infection at the lower temperatures in the winter months. It was shown that fish held at 9°C were not intractable to experimental inoculation, but it was still not known whether they would be susceptible to natural infection at this or lower temperatures.

At 9°C it was shown that fish could remain infected for 5 months without developing clinical disease. At temperatures of 12°C-18°C clinical disease developed in fish infected for the first time. It appeared to develop more rapidly and be more severe at the higher temperatures. There was a marked increase in PKX organisms seen at these temperatures suggesting that multiplication was occurring. Such an increase was not seen at 9°C. The results of the field experiments support the observations by Ferguson and Ball (1979) and Ellis et al.

(1982) that severity of the disease increased as water temperatures rose while decrease of severity occurred as temperatures declined. Results in Study 2 showed that at lower temperatures healing and recovery of clinically-affected fish was enhanced.

The finding that the PKX organism would persist in sub-clinically infected fish at 9°C for 5 months raised the question of whether it could overwinter in some fish on the farm. Studies at the experimental site failed to show this.

With regard to protection against disease, fish that had been infected with the organism but had not become clinically affected or had only developed minimal renal swelling were shown not to be resistant to the disease. This may have been due to a decrease in the effectiveness of the host's immune response at lower water temperatures, as has been demonstrated by Avtalion et al. (1973) and reviewed in detail by Avtalion (1981).

Fish that had survived PKD in their first summer on infected water appeared to develop some resistance to the disease. This resistance did not prevent re-infection but limited the development of lesions. It seemed that initial infection had to induce greater than grade 1 swelling of the kidneys for this resistance to develop.

No evidence was found for age resistance and yearling fish like fingerlings were shown to be susceptible to disease in their first summer on infected water.

Fry from broodstock which had survived clinical PKD were not resistant to disease. It may be that the protection of the broodstock was not due to antibodies alone, but to a combination of antibodies and cell-mediated immunity. Also, any antibodies produced may not have been protective. However, at present, maternal antibody transfer to ova, although proven in the yolk sac in chickens (Good and Papermaster, 1964),

has not been proven in fish. Negative evidence was provided by the study of Voss, Fryer and Banowetz (1978) in which they showed the presence of a lectin in chinook salmon ova with properties of haemagglutination and bacterial growth inhibition but not immunoglobulins assayed by precipitin tests. However, the mechanism for immunoglobulin transfer by a membrane transport system probably does exist (Groberg, Voss and Fryer, 1979). A further possibility is that, by the time fry were exposed to infection at about 12 weeks post-hatch, any transferred maternal antibody had declined to a non-protective level.

With regard to species susceptibility, both the brown trout and Atlantic salmon in this study became infected and developed pathology similar to, but not as extensive as, that in rainbow trout. Vascular pathology, in particular, was not such a marked feature. It may be that these species are less susceptible to PKD than rainbow trout. Such interspecific differences occur in other diseases, for example, in furunculosis caused by Aeromonas salmonicida. This condition results in serious losses in brook trout, Atlantic salmon and brown trout (McCarthy, 1978), whereas certain strains of rainbow trout appear to have an innate resistance, perhaps based on a natural toxin neutralization factor in the serum (Cipriano, 1983). Although severe losses due to PKD have occurred in Atlantic salmon (Ellis et al., 1985), the strain used in the present study appeared less susceptible than the rainbow trout. This perhaps confirms the suggestion of Ellis et al. (1982) that certain strains of Atlantic salmon are more resistant than others. Such intraspecific differences were noted by Schafer (1968) in resistance to disease caused by Ceratomyxa shasta. In this case, cultured strains of rainbow trout were noticeably less resistant than feral rainbow trout and other salmonids.

The finding of intraluminal cells similar to myxosporidan spores in

both brown trout and Atlantic salmon with PKD may be of significance in the natural transmission of PKD. This aspect will be discussed further in chapter 7. The implications for management and control of PKD arising from the studies investigating the infective period at the experimental site and the effects of water temperature on the course and severity of the disease will also be considered in more detail in chapter 7.

Table 5.1. The time course and infective period of PKD at the experimental site: experimental and sampling periods for fish in groups A-D held at the experimental site.

Experimental group	Fish at experimental site: from	to	Sampling period in days after introduction to the experimental site	No. of fish examined during sampling period	No. of days after introduction to the experimental site until first PKX positive renal samples
A	30.4.82	25.3.83	5 - 330	175	36
B	10.3.83	1.9.83	16 - 176	91	85
C batch i	10.6.82	31.8.82	28 - 83	23	42
" ii	24.6.82	23.9.82	29 - 92	11	54
" iii	8.7.82	23.9.82	40 - 78	21	40
" iv	22.7.82	9.11.82	26 - 110	13	26
" v	12.8.82	9.11.82	4 - 89	18	19
" vi	2.9.82	2.11.82	61	13	61
D batch i	6.10.83	30.7.84	29 - 298	75	* 91 and 274
" ii	4.11.83	30.7.84	62 - 269	55	245
" iii	2.12.83	6.8.84	66 - 305	70	263
" iv	5.1.84	30.7.84	60 - 207	50	183
" v	3.2.84	30.7.84	87 - 178	50	154

* no positive samples from 92-273 days after introduction to experimental site

Table 5.2. Time course and infective period of PKD at the experimental site: experimental and sampling periods for fish in groups A-D transferred from the experimental site to the laboratory holding facilities.

Experimental group	Fish transferred from the experimental site and held at the laboratory	Sampling period in days after introduction to experimental site	No. of fish examined during sampling period	No. of days after introduction to the experimental site until first PKX positive renal samples
A batch i	11.5.82 — 9.7.82	71	4	71
" ii	19.5.82 — 20.9.82	51 - 144	8	71
" iii	27.5.82 — 18.8.82	74 - 111	10	74
" iv	3.6.82 — 12.8.82	53 - 105	5	53
" v	10.6.82 — 20.9.82	74 - 144	7	74
" vi	17.6.82 — 20.9.82	74 - 144	10	74
B batch i	25.3.83 — 19.5.83	29 - 70	8	no PKX positive samples
" ii	11.4.83 — 3.6.83	70 - 85	7	" " " "
" iii	20.4.83 — 20.6.83	70 - 102	14	" " " "
" iv	4.5.83 — 13.7.83	70 - 125	19	102
" v	20.5.83 — 20.6.83	85 - 102	10	102
" vi	26.5.83 — 20.6.83	102	5	102
C	not applicable	not applicable	not applicable	not applicable
D batch ia	7.11.83 4.1.84	46 - 90 ,	12	55
" ib	6.2.84 6.4.84	183	10	no PKX positive samples
" ii	5.12.83 11.1.84	61 - 68	7	" " " "
" iii	5.1.84 9.3.84	77 - 98	25	" " " "
" iv	6.2.84 6.4.84	92	10	" " " "
" v	5.3.84 30.4.84	90	5	" " " "

Table 5.3. Study 2 experiment 1. The effects of different water temperatures on the susceptibility of rainbow trout to PKD when inoculated intraperitoneally with an infected kidney suspension: renal pathology.

Sample date, days after inoculation	Tank * designation	No. of fish in sample	No. of fish with kidney swelling	Number of renal specimens at each sampling containing: PKX cells	haemo-poietic hyper-plasia	diffuse inflamm-ation	glomerular and tubule destruction	vascular aggreg-ations	crystals
49	A	5	0	0	1	0	0	0	0
	B	5	0	2	1	0	0	0	0
	C	5	0	4	5	0	0	0	0
85	A	10	0	2	9	0	0	0	0
	B	10	0	2	5	0	1	0	0
	C	10	3	7	8	5	5	5	0
	Totals	45	3	17	29	5	6	5	0

* Water in tank A maintained at 9°C
Water in tank B raised from 9°C to 16°C 7 days after inoculation
Water in tank C maintained at 16°C

Table 5.4. Study 2 experiment 2. The effects of different water temperatures on the course of PKD in sub-clinically infected rainbow trout: sampling schedule.

Sample date	Days after initial exposure	Number of fish in sample from tanks at: 9°C	12°C	15°C	18°C
8.7.83	43	10	10	10	10
20.7.83	55	10	10	10	10
25.7.83	60	0	0	0	4
28.7.83	63	0	0	0	2
2.8.83	68	5	5	5	5
8.8.83	74	0	0	0	13
17.8.83	83	10	10	10	-
31.8.83	97	5	5	5	-
14.9.83	111	5	5	5	-
29.9.83	126	5	5	5	-
13.10.83	140	5	5	2	-
2.11.83	160	5	5	-	-
16.11.83	174	5	5	-	-
30.11.83	188	5	5	-	-
15.12.83	203	5	5	-	-
6.1.84	225	5	5	-	-
Totals	-	80	80	52	44

- no fish surviving

Table 5.5a. Study 2 experiment 2. The effects of different water temperatures on the course of PKD in subclinically infected rainbow trout: renal histopathology in fish held at 9°C.

Days after initial exposure	No. of fish sampled	Number of renal specimens at each sampling showing: PKX cells	haemo-poietic hyper-plasia	diffuse inflammation	glomerular and tubule destruction	vascular aggreg-ations	crystals	organizing diffuse inflammation	increased lipofuscin
43	10	9	8	1	0	0	0	0	0
55	10	8	8	0	0	0	0	0	0
68	5	5	5	0	0	1	0	0	0
83	5	5	5	0	0	0	0	0	0
97	5	5	5	1	0	0	1	0	0
111	5	5	4	4	1	0	0	0	0
126	5	4	3	4	0	2	0	0	1
140	5	2	2	0	0	0	0	0	0
160	5	2	1	1	0	0	0	0	2
174	5	0	0	0	0	0	0	0	1
188	5	0	0	0	0	0	0	0	0
203	5	0	0	0	0	0	0	0	1
225	5	0	0	0	0	0	0	0	0
Totals	75	45	41	11	1	3	1	0	5

Table 5.5b. Study 2 experiment 2. The effects of different water temperatures on the course of PKD in subclinically infected rainbow trout: renal histopathology in fish held at 12°C.

Days after initial exposure	No. of fish sampled	Number of renal specimens at each sampling showing: PKX cells	haemo-poietic hyper-plasia	diffuse inflammation	glomerular and tubule destruction	vascular aggreg-ations	crystals	organizing diffuse inflammation	increased lipofuscin
43	10	10	8	0	0	0	0	0	0
55	10	10	9	0	0	0	0	0	0
68	5	5	4	4	1	3	1	0	0
83	5	5	5	5	5	5	3	0	0
97	5	5	4	4	3	4	0	0	0
111	5	5	5	4	4	3	1	0	0
126	5	5	3	4	5	1	1	1	1
140	5	5	3	4	4	0	0	0	0
160	5	4	3	3	2	1	2	1	1
174	5	3	3	3	3	1	1	1	2
188	5	2	1	1	1	0	0	0	1
203	5	0	0	0	0	0	0	0	2
225	5	3	3	1	2	1	1	2	0
Totals	75	62	51	33	30	19	10	5	7

Table 5.5c. Study 2 experiment 2. The effects of different water temperatures on the course of PKD in subclinically infected rainbow trout: renal histopathology in fish held at 15°C and 18°C.

Tank designation	Days after initial exposure	No. of fish sampled	Number of renal specimens at each sampling showing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations	crystals	organizing diffuse inflammation	increased lipofuscin
15°C	43	10	10	10	1	0	0	0	0	0
	55	10	10	10	8	2	4	0	0	0
	68	5	5	5	5	2	4	2	0	0
	83	5	5	5	5	5	5	5	0	0
	97	5	5	3	5	3	3	3	0	0
	111	5	4	3	4	4	2	4	0	0
	126	5	5	5	3	3	1	2	2	3
	140	2	2	2	2	2	0	0	1	0
Totals		47	46	43	33	21	19	16	3	3
18°C	43	10	10	10	1	0	1	0	0	0
	55	10	10	10	10	8	7	0	0	0
	60	4	4	3	4	4	0	0	0	0
	63	2	2	2	2	0	0	0	0	0
	68	5	5	3	5	3	3	1	0	0
	74	13	13	13	13	13	13	13	0	0
Totals		44	44	41	35	28	24	14	0	0

Table 5.6. Study 2 experiment 3. The effects of different water temperatures on the course of PKD in clinically-affected rainbow trout: gross renal pathology.

Sample date	Tank designation	No. of fish in sample	Number of fish at each sampling with renal swelling of grade: 0	1	2	3	4	5
3.9.82	7°C	5	3	0	0	0	0	2
	9°C	5	4	0	0	0	0	1
	12°C	5	2	1	1	0	0	1
	15°C	5	2	1	2	0	0	0
	18°C	5	0	2	1	1	1	0
12.11.82	* 7°C	5	5	0	0	0	0	0
	* 9°C	4	4	0	0	0	0	0
	* 12°C	4	4	0	0	0	0	0
	* 15°C	5	3	0	1	0	0	1

* temperature of the tank water prior to raising it to 18°C between 4th and 11th September.

Table 5.7. Study 2 experiment 3. The effects of different water temperatures on the course of PKD in clinically-affected rainbow trout: principal renal histopathological changes.

Sample date	Tank designation	No. of fish in each sample	Number of renal specimens at each sampling showing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations	crystals	organizing diffuse inflammation	increased lipofuscin	Total no. of fish showing histopathological lesions
3.9.82	7°C	5	1	1	1	1	1	1	5	5	5
	9°C	5	1	2	1	1	1	2	5	5	5
	12°C	5	2	2	2	2	1	1	2	3	4
	15°C	5	2	1	2	2	1	2	2	4	5
	18°C	5	3	3	3	3	2	2	2	3	5
12.11.82	7°C	5	0	0	0	0	0	0	0	0	0
	9°C	4	0	2	0	0	0	0	0	2	2
	12°C	4	0	2	0	0	0	0	1	2	2
	15°C	5	3	3	1	0	2	1	2	2	4

Table 5.8. Study 3 experiment 1. Effect of age on susceptibility to PKD: gross renal pathology in yearling and fingerling fish following primary infection.

Fish group	Sample date	No. of fish in sample	Number of fish at each sampling with kidney swelling of grade: 0	1	2	3	4	5
Yearling	10.6.82	5	5	0	0	0	0	0
	17.6.82	5	5	0	0	0	0	0
	5.7.82	2	2	0	0	0	0	0
	9.8.82	3	2	0	1	0	0	0
	31.8.82	5	2	1	1	1	0	0
Fingerling	10.6.82	5	5	0	0	0	0	0
	31.8.82	15	0	3	4	4	4	0

Table 5.9. Study 3 experiment 1. Effect of age on susceptibility to PKD: principal renal histopathological changes in yearling and fingerling fish following primary infection.

Fish group	Sample date	No. of fish in sample	Number of renal specimens at each sampling containing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregation	crystals
Yearling	10.6.82	5	0	0	0	0	0	0
	17.6.82	5	0	0	0	0	0	0
	5.7.82	2	0	0	0	0	0	0
	9.8.82	3	3	2	0	1	1	0
	31.8.82	5	4	4	4	2	4	4
Fingerling	10.6.82	5	0	0	0	0	0	0
	31.8.82	15	15	10	9	6	1	1

Table 5.10. Study 3 experiment 2. Effect of previous exposure to PKD on susceptibility to subsequent infection: principal renal histopathological changes in re-infected yearling rainbow trout.

Sample date	No. of fish in sample	Number of renal specimens at each sampling containing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations	crystals	increased lipofuscin
30.6.83	5	1	0	0	0	0	0	0
4.8.83	10	9	8	5	0	8	2	0
15.8.83	5	2	2	2	0	0	1	0
19.9.83	5	0	0	0	0	0	0	0
11.10.83	10	2	1	1	0	0	1	0
4.11.83	5	2	0	1	1	0	1	0
Totals	40	16	11	9	1	8	5	0

Table 5.11. Study 3 experiment 3. Effect of severity of previous PKD infection on susceptibility to subsequent disease: groups of fish used.

Group	History prior to re-exposure at the field site	Degree of infection in majority of fish sampled	Previous designation
A	1. Moved to experimental site 26.5.83 as 50-70 mm uninfected fish. 2. Moved to laboratory 23.6.83. 3. Held at 12°C until 11.5.84. 4. Sampled up to 32 weeks after initial exposure. 5. Re-introduced to field site 11.5.84.	Grade 2 renal swelling	Study 2, experiment 2, 12°C group
B	1. Moved to experimental site 26.5.83 as 50-70 mm uninfected fish. 2. Moved to laboratory 23.6.83. 3. Held at 9°C until 11.5.84. 4. Sampled up to 32 weeks after initial exposure. 5. Re-introduced to field site 11.5.84.	Grade 1 renal swelling	Study 2, experiment 2, 9°C group
C	1. Moved to experimental site 6.10.83 as 75 mm uninfected fish. 2. Held at site until 30.7.84. 3. Sampled up to 43 weeks after introduction to site.	PKX-positive, no renal swelling during 1983	Study 1, batch Di
D	1. Moved to experimental site 3.2.84 as 75 mm uninfected fish. 2. Held at site until 30.7.84. 3. Sampled up to 25 weeks after introduction to site.	PKX-negative prior to start of this experiment	Study 1, batch Dv

Table 5.12. Study 3 experiment 3. Effect of severity of previous PKD infection on susceptibility to subsequent disease: gross renal pathology in re-infected and control fish.

Fish group	Severity of previous infection/ maximum grade of renal swelling	Sample date	No. of fish in sample	Number of fish at each sampling with renal swelling of grades: 0	1	2	3	4	5
A	2	30.7.84	5	4	1	0	0	0	0
B	1	30.7.84	8	0	1	1	6	0	0
C	0	6.6.84	5	5	0	0	0	0	0
		6.7.84	5	3	2	0	0	0	0
		23.7.84	10	6	2	2	0	0	0
		30.7.84	20	4	4	4	8	0	0
D	uninfected	6.6.84	5	5	0	0	0	0	0
		6.7.84	5	0	5	0	0	0	0
		23.7.84	10	0	1	6	3	0	0
		30.7.84	20	1	7	7	3	2	0

Table 5.13. Study 3 experiment 3. Effect of severity of previous PKD infection on susceptibility to subsequent disease: principal renal histopathological changes in re-infected and control fish.

Fish group	Severity of previous infection/ maximum grade of renal swelling	Sample date	Number of renal specimens at each sampling containing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations	crystals
A	2	30.7.84	3	3	2	0	2	0
B	1	30.7.84	8	8	8	7	7	4
C	0	6.6.84	0	0	0	0	0	0
		6.7.84	5	5	3	2	3	0
D	uninfected	6.6.84	0	0	0	0	0	0
		6.7.84	5	5	4	4	4	0

Table 5.14. Study 3 experiment 4. Susceptibility to PKD of fry from previously infected broodstock: gross renal pathology.

Fish group	Sample date	No. of of fish sampled	Number of fish at each sampling with renal swelling of grades: 0	1	2	3	4	5
Rainbow trout fry from previously infected broodstock	12.7.84	5	2	3	0	0	0	0
	23.7.84	15	0	1	3	8	3	0
Control rainbow trout fingerlings from uninfected broodstock	5.7.84	5	0	5	0	0	0	0
	30.7.84	10	0	0	3	4	3	0

Table 5.15. Study 4. Susceptibility of naturally-infected brown trout, Atlantic salmon and rainbow trout to PKD: gross renal pathology.

Fish species	No. of days after introduction to experimental site	No. of fish in sample	Number of fish at each sampling with renal swelling of grades: 0	1	2	3	4	5
	0	20	20	0	0	0	0	0
Brown	49	10	10	0	0	0	0	0
trout	71	5	0	3	2	0	0	0
	88	2	1	0	0	0	0	1
	116	5	4	0	0	0	0	1
	32	5	5	0	0	0	0	0
Atlantic	59	5	2	3	0	0	0	0
salmon	84	5	3	0	2	0	0	0
	100	3	3	0	0	0	0	0
	35	10	10	0	0	0	0	0
	48	10	10	0	0	0	0	0
Rainbow	62	10	10	0	0	0	0	0
trout	69	10	3	3	3	1	0	0
	83	10	0	0	6	4	0	0
	101	5	0	0	0	2	0	3
	115	5	0	0	0	0	1	4

Table 5.16. Study 4. Susceptibility of naturally-infected brown trout, Atlantic salmon and rainbow trout to PKD: principal renal histopathological changes.

Fish species	No. of days after introduction to experimental site	No. of fish in sample	Number of renal specimens at each sampling containing: PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations	crystals	increased lipofuscin
Brown trout	0	20	0	0	0	0	0	0	0
	49	10	8	6	2	0	0	0	0
	71	5	5	4	5	5	4	0	0
	88	2	0	2	0	0	0	0	2
	116	5	0	0	0	0	0	0	0
Atlantic salmon	32	5	0	0	0	0	0	0	0
	59	5	3	3	2	2	0	0	0
	84	5	2	0	2	2	2	0	0
	100	3	1	1	0	0	0	0	0
Rainbow trout	35	10	1	0	0	0	0	0	0
	48	10	8	4	3	1	2	0	0
	62	10	10	10	4	3	9	0	0
	69	10	10	8	9	7	8	1	0
	83	10	10	9	10	8	8	6	0
	101	5	5	1	2	5	2	5	1
	115	5	5	5	1	3	2	2	4

Fig. 5.1. Seven day rolling average maximum and minimum water temperatures at the experimental site in 1983.

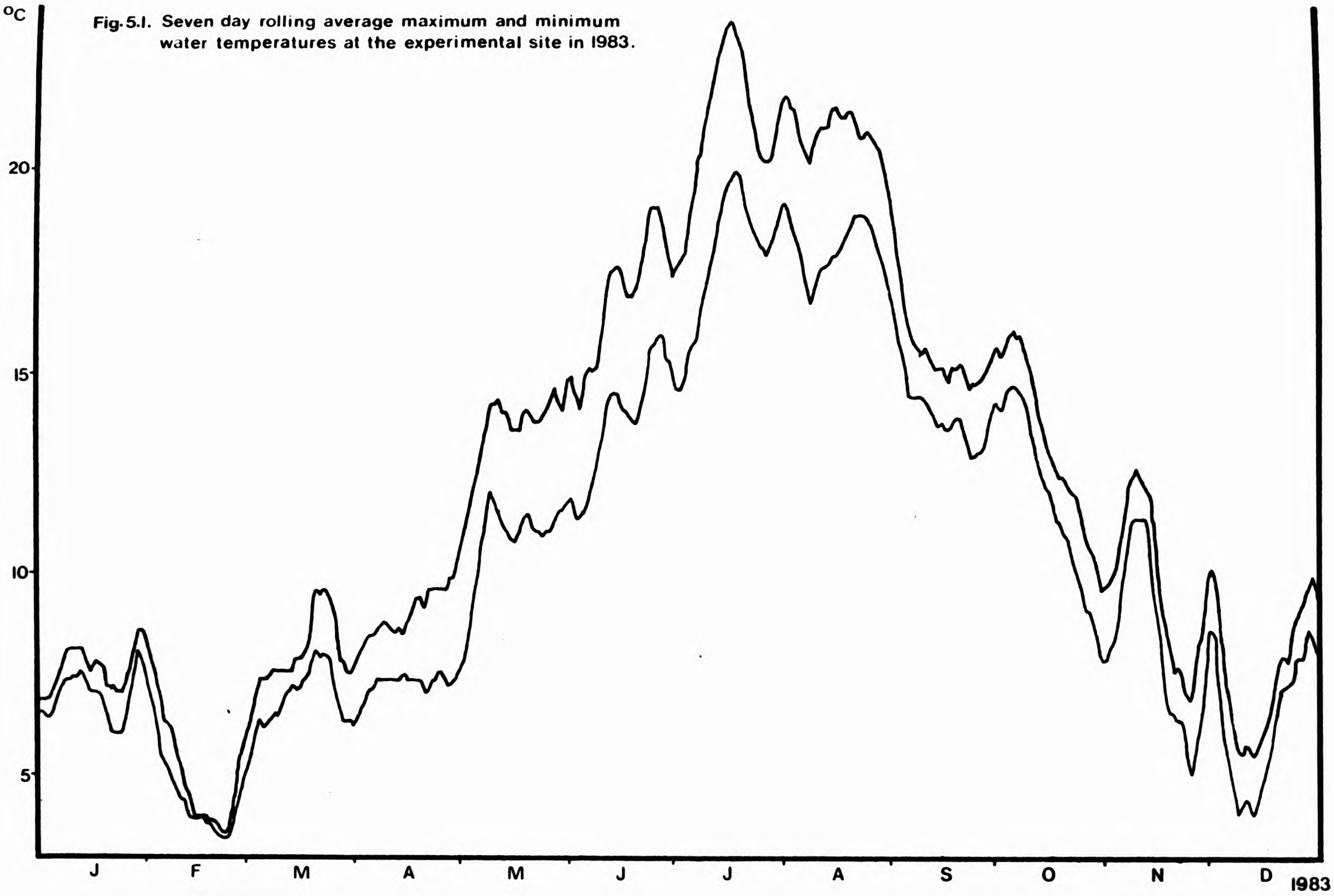
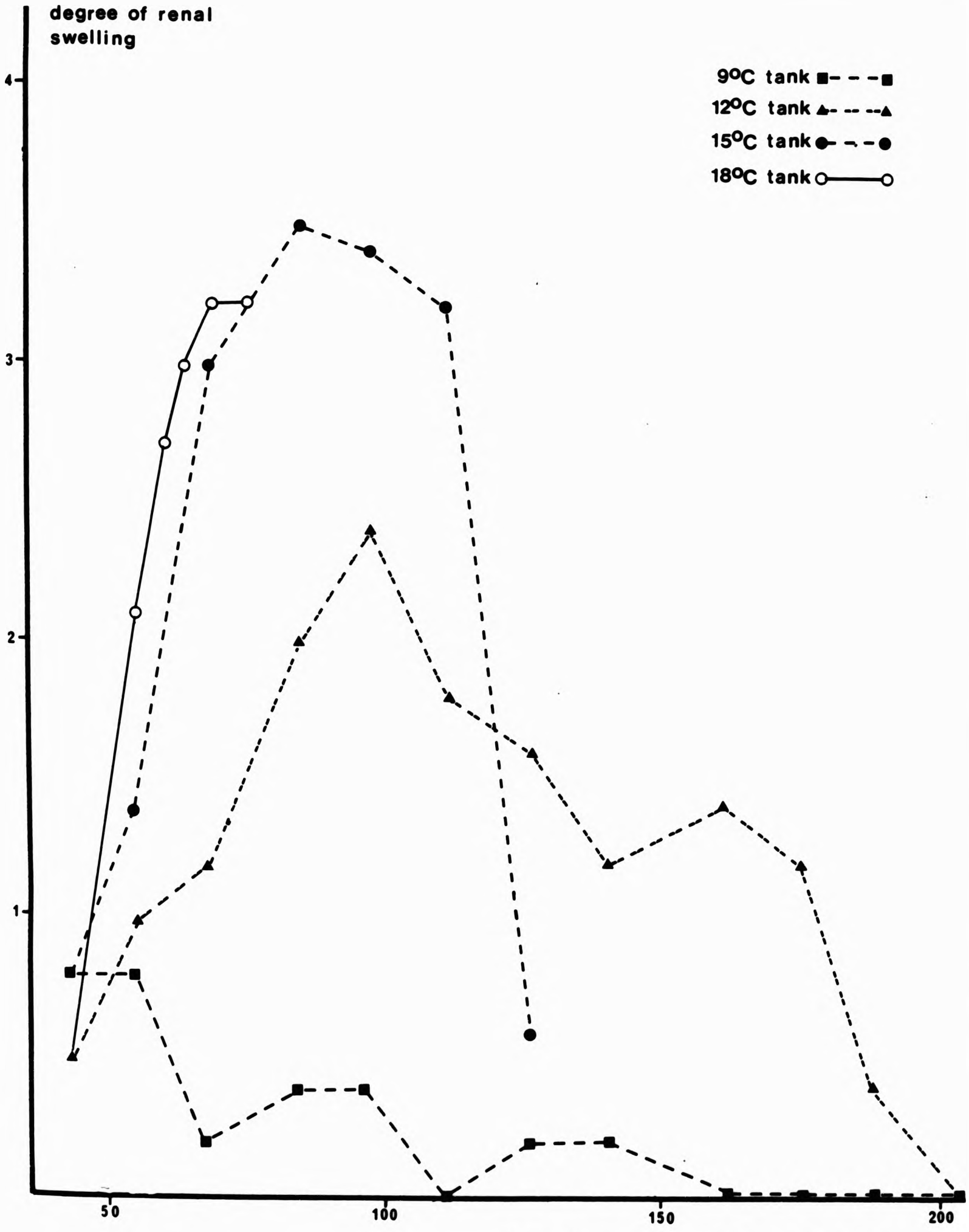


Fig.5.2. Average degree of renal swelling at each sampling in fish held in water at 9°C, 12°C, 15°C and 18°C during the investigation of the effect of water temperature on the development of PKD. Experiment 2, Study 2, Chapter 5.



Chapter 6

The experimental transmission of PKD

INTRODUCTION

It was shown in chapters 3, 4 and 5 that rainbow trout could be naturally infected with PKD by introducing them to the experimental site between April and October. However, it was not possible to infect fish by this method during the winter months.

Previous attempts to transmit PKD to rainbow trout were described in chapter 1. Holding uninfected fish in tanks supplied with water from tanks containing infected fish; holding them in water containing sediment from PKD-infected ponds; or holding them in water containing cultured infected kidney, homogenates of fresh, infected kidney tissue or faeces from PKD-infected fish, all failed to infect fish with PKD (Ferguson and Ball, 1979; D'Silva *et al*., 1984). However, intraperitoneal injection of a homogenate of PKD-infected kidney tissue in PBS was successful in establishing a kidney infection (D'Silva *et al*., 1984).

The series of transmission experiments described in this chapter were undertaken coincidentally with those of D'Silva *et al*. (1984) to try and experimentally reproduce the disease under laboratory conditions so that it could be studied throughout the year. It was not known what the infective stage of the organism was or how or whether it was released from infected fish. The first two experiments were designed, therefore, to investigate whether uninfected rainbow trout could be infected by cohabitation with (a) infected rainbow trout and (b) infected Atlantic salmon. Unfortunately, several attempts to set up a similar experiment with infected brown trout failed because the brown trout died from other causes. In subsequent experiments attempts were made to reproduce PKD by inoculating uninfected rainbow and brown trout with infected kidney

suspensions by different routes. Each experiment is described and discussed separately then the results are considered together in the General Discussion at the end of the chapter.

ATTEMPTS TO TRANSMIT INFECTION BY DIRECT CONTACT WITH INFECTED FISH

Experiment 1. Attempt to transmit infection from infected to uninfected rainbow trout.

MATERIALS AND METHODS

Ten rainbow trout, average length 107 mm, derived from group A (chapter 3), were held at the experimental site for 6 weeks in May and June, then transferred to the laboratory and maintained in a small tank supplied with dechlorinated, aerated tap water at 16°C as described in chapter 2. Ten fish from the same source as group A with no previous exposure to PKD were placed in the same tank as the infected fish after their adipose fins had been removed.

At the experimental site 10 fish in group A were sampled on the day that the test fish were transferred.

At the laboratory, both groups of fish were sampled 25-95 days after mixing. Degree of renal swelling was noted and tissue samples were processed for histological examination as described in chapter 2. Organs examined included brain, caeca, gills, heart, kidney, large and small intestine, liver, pancreas, spleen and stomach.

RESULTS

Eight of 10 fish, sampled at the experimental site on the day that infected fish were transferred, contained renal PKX cells.

At the laboratory, three fish died from ichthyophthiriasis 61-88 days after mixing. Of nine surviving fish previously exposed at the experi-

mental site, all contained PKX cells, three developed grade 2 renal swelling and two developed grade 1 renal swelling. Two of four sampled at 95 days contained renal lesions of haemopoietic hyperplasia, diffuse inflammation, glomerulus and tubule destruction, vascular lesions and crystals. Several fish in this group contained intestinal Pomphorynchus acanthocephalans.

Of eight surviving initially uninfected fish, four of which were sampled 95 days after mixing with infected fish, none showed evidence of PKD infection.

DISCUSSION

No transmission of PKD was demonstrated between infected and non-infected 0+ rainbow trout despite close contact of fish and their excretory products. Some fish were in contact for 95 days, a period previously shown to be sufficient for PKD to develop at 16°C in the laboratory (chapter 5), and in the summer months at the experimental site (chapters 3 and 5). These results support findings of other workers that water holding infected rainbow trout or faeces from infected fish has not been shown to be infective to other fish (Ferguson and Ball, 1979; D'Silva et al., 1984).

Experiment 2. Attempt to transmit infection from infected Atlantic salmon to uninfected rainbow trout.

MATERIALS AND METHODS

Twenty 75 mm fingerling rainbow trout from farm C, previously unexposed to PKD, and 10 salmon parr from farm F, held at the experimental site for 9 weeks from May, were placed in a medium tank supplied with dechlorinated aerated tap water at 16°C. These fish, managed as described

in chapter 2, were sampled after 34 and 56 days. Kidney tissue was prepared for histological examination as described in chapter 2.

Five parr from the same batch of salmon were sampled at the experimental site prior to the start of the experiment.

RESULTS

Of the five parr held and sampled at the experimental site two showed no evidence of PKD, while three had swollen kidneys with renal tissue containing lesions associated with PKD and many PKX cells as well as occasional intratubular parasitic cells.

At the laboratory, both salmon parr and rainbow trout showed signs of white spot after 32 days, with several dying 32-34 days after the start of the experiment. Treatment with malachite green and formalin was performed on days 34, 35 and 36 as described in chapter 2. Of seven salmon sampled after 34 days, none showed swollen kidneys or histological lesions of PKD. However, four did contain possible PKX cells within renal tubules. None of the rainbow trout sampled after 34 days (five fish), and after 56 days (12 fish) showed histological lesions of PKD, although several had swollen kidneys due to haemopoietic hyperplasia.

DISCUSSION

The results suggest that there was no detectable fish-to-fish transmission between infected salmon parr and uninfected fingerling rainbow trout under the conditions described. Although ichthyophthiriasis necessitated chemotherapy, there was opportunity for transmission of PKD prior to treatment. The swollen kidneys of some rainbow trout could have reflected the host's response to the ectoparasite, as a neutrophilia has been described in ichthyophthiriasis in mirror carp (Hines and Spira,

1973).

The significance of the intratubular cells in the salmon parr kidneys was not known. However, they resembled both the Myxosporidan trophozoites and developing spores in steelhead trout described by Kent and Hedrick (1985c) and the intraluminal cells described in study 4 chapter 5 in both brown trout and Atlantic salmon.

ATTEMPTS TO TRANSMIT INFECTION BY INOCULATION OF FISH WITH INFECTED KIDNEY SUSPENSIONS BY DIFFERENT ROUTES

General Materials and Methods

Details of the materials and methods are described in chapter 2.

PKD-free 0+ rainbow trout used in these experiments came from farms A and C or were hatched from eggs at the laboratory. PKD-free brown trout were from farm D.

Fish in each experiment were held at the laboratory in small tanks suppliedwith dechlorinated, aerated tap water at 16°C.

Seven routes of inoculation were investigated. These were intraperitoneal by injection, intraperitoneal via the cloaca, *per os*, into the stomach, via the gills, onto the skin by dipping, and subcutaneous by injection.

Uninfected kidney tissue for inoculation was derived from PKX-free 0+ rainbow trout from farm A.

PKX-positive kidney tissue for inoculation was derived from 0+ rainbow trout naturally infected at the experimental site during May and June and in some cases subsequently held at the laboratory to develop clinical disease. Kidneys used showed grades 1-4 swelling except in experiment 6 where kidneys showed grade 5 swelling. All kidneys were shown on histological examination to contain lesions associated with PKD and PKX cells.

No estimate was made of the number of PKX cells inoculated.

Kidneys were removed using sterile techniques and, except in experiment 10, where they were cut into 2 mm^3 pieces, were disrupted and suspended in sterile PBS. They were inoculated within 1 hr of the death of the donor fish.

Fish for inoculation intraperitoneally, by stomach tube, via the gills, by dipping and subcutaneously were anaesthetized prior to inoculation.

Infection in inoculated fish was assessed from the gross and histopathological appearance of the kidney. Gills, livers, intestines and spleens were examined histologically in the control fish in experiments 4 and 7 and striated muscle from the injected site was examined in subcutaneously inoculated fish in experiment 14.

Experiments 3 and 4. Attempts to reproduce disease in rainbow trout by intraperitoneal injection of coarse infected kidney suspensions.

MATERIALS AND METHODS

Details of the groups of fish and inocula used are given in Table 6.1. Fish in experiment 3 were sampled 28 days after injection. PCV was measured as described in chapter 4 in addition to the examination of the kidneys. Fish in experiment 4 inoculated with infected kidney suspensions were sampled 10-51 days after injection. Fish inoculated with uninfected kidney suspensions or PBS were sampled 40 days after injection.

RESULTS

None of the fish injected with PBS or uninfected kidney suspensions showed gross or histopathological lesions of PKD.

In experiment 3, four of 10 inoculated fish died with abdominal distension and peritonitis. The six remaining fish sampled 28 days after

injection all had swollen kidneys of grade 1 or 2 and histological lesions of PKD with PKX cells. Three had distended abdomens, gill pallor and small quantities of clear ascitic fluid. Three had signs of peritonitis. PCV values ranged from 0.06 to 0.28.

In experiment 4, PKX cells were recognisable in kidney samples from nine of 14 fish examined 24-51 days after inoculation with infected kidney material. Two of these fish had kidneys with grade 1 swelling and three had grade 2 swelling. Haemopoietic hyperplasia was present and diffuse inflammatory lesions with fluid-filled spaces were occasionally seen. Vascular aggregations and crystals were found in samples taken from 35 days after injection.

DISCUSSION

Results demonstrated that intraperitoneal injection of PKD-free rainbow trout with PBS or uninfected kidney tissue in PBS did not produce histological changes associated with PKD. Intraperitoneal injection of infected kidney material was successful in establishing a detectable kidney infection and inducing histological lesions of PKD in 15 of 30 fish. Subsequent experiments by D'Silva *et al*. (1984) also showed that PKD could be transmitted to previously uninfected rainbow trout by intraperitoneal injection of renal tissue containing PKX cells.

A complication of the intraperitoneal inoculation was peritonitis. Care was taken in subsequent experiments to minimize contamination of the suspension to be injected.

Experiments 5 and 6. Attempts to reproduce disease in rainbow trout by intraperitoneal injection of filtered infected kidney suspensions.

MATERIALS AND METHODS

Details of the groups of fish and inocula used are given in Table 6.2. Infected kidneys used in experiment 6 contained fibrous nodules which were broken up by drawing and expressing the suspension through a 10 ml plastic syringe.

Filters were prepared from polyester precision mesh (Simon Textile Co., Salford) to fit Swinnex-Millipore 47 mm holders (Millipore, Molsheim, France), using material with pore diameter sizes of 60 µm, 30 µm, 20 µm and 10µm. A 0.8 µm Millex AA disposable filter (Millipore) was also used. Filters were autoclaved before use.

In experiment 6, some of the suspension passed through a 60 µm then a 20 µm filter was homogenized in an all-glass Uni-form homogenizer, internal clearance 0.152-0.254 mm (Jencons [Scientific] Ltd., Leighton Buzzard, Beds.). A wet preparation of the homogenate was viewed by interference contrast microscopy.

In experiment 5, two fish from each group were sampled 29 days after injection. The remaining fish were sampled 48 days after injection.

In experiment 6, all fish were sampled 55 days after injection.

RESULTS

A wet preparation of the homogenized 20 µm filtrate viewed by interference contrast microscopy contained many intact cells but no clumps of cells and no visible large PKX cells.

Results of experiment 5 are shown in Table 6.3. Of the fish sampled 29 days after inoculation, those receiving the 60 µm filtrate had renal PKX cells without detectable histopathological lesions. Forty-eight

days after inoculation, in group 1, two of five fish with renal PKX cells had grade 1 kidney swelling and haemopoietic hyperplasia, with vascular lesions in one; in group 2, one PKX-positive fish had grade 1 renal swelling, while six showed haemopoietic hyperplasia and three, diffuse inflammation; in group 3, haemopoietic hyperplasia was seen in the infected fish, while in group 4, no lesions were detectable.

Results of experiment 6 are recorded in Table 6.4. Only fish in groups 1-4 developed a detectable renal infection with PKX cells and histopathological lesions.

DISCUSSION

In experiments 3 and 4, peritonitis was found to be a complication of intraperitoneal inoculation of coarse kidney suspensions. In experiments 5 and 6, using suspensions from which large aggregates of kidney cells had been removed by filtration, peritonitis was found not to occur. Unfortunately, using filtered suspensions reduced the detectable renal infection rate. However, this may have been due solely to dilution of the infective agent during preparation of the filtrates.

The results demonstrated that infected kidney suspensions, passed through filters with 60 to 10 μm pore sizes, and injected intraperitoneally, established detectable renal infections, with PKX cells and related histopathological lesions. Suspensions passed through a filter with a 0.8 μm pore size did not produce a detectable infection. This suggested that in these experiments the infective agent was greater than 0.8 μm in diameter and, therefore, that PKD does not have a viral aetiology.

Homogenization of the 20 μm filtrate to disrupt large PKX cells and release their intracellular inclusions prior to filtration was not shown to increase the infectivity of the suspension. In experiment 6, fewer

days after inoculation, in group 1, two of five fish with renal PKX cells had grade 1 kidney swelling and haemopoietic hyperplasia, with vascular lesions in one; in group 2, one PKX-positive fish had grade 1 renal swelling, while six showed haemopoietic hyperplasia and three, diffuse inflammation; in group 3, haemopoietic hyperplasia was seen in the infected fish, while in group 4, no lesions were detectable.

Results of experiment 6 are recorded in Table 6.4. Only fish in groups 1-4 developed a detectable renal infection with PKX cells and histopathological lesions.

DISCUSSION

In experiments 3 and 4, peritonitis was found to be a complication of intraperitoneal inoculation of coarse kidney suspensions. In experiments 5 and 6, using suspensions from which large aggregates of kidney cells had been removed by filtration, peritonitis was found not to occur. Unfortunately, using filtered suspensions reduced the detectable renal infection rate. However, this may have been due solely to dilution of the infective agent during preparation of the filtrates.

The results demonstrated that infected kidney suspensions, passed through filters with 60 to 10 μm pore sizes, and injected intraperitoneally, established detectable renal infections, with PKX cells and related histopathological lesions. Suspensions passed through a filter with a 0.8 μm pore size did not produce a detectable infection. This suggested that in these experiments the infective agent was greater than 0.8 μm in diameter and, therefore, that PKD does not have a viral aetiology.

Homogenization of the 20 μm filtrate to disrupt large PKX cells and release their intracellular inclusions prior to filtration was not shown to increase the infectivity of the suspension. In experiment 6, fewer

days after inoculation, in group 1, two of five fish with renal PKX cells had grade 1 kidney swelling and haemopoietic hyperplasia, with vascular lesions in one; in group 2, one PKX-positive fish had grade 1 renal swelling, while six showed haemopoietic hyperplasia and three, diffuse inflammation; in group 3, haemopoietic hyperplasia was seen in the infected fish, while in group 4, no lesions were detectable.

Results of experiment 6 are recorded in Table 6.4. Only fish in groups 1-4 developed a detectable renal infection with PKX cells and histopathological lesions.

DISCUSSION

In experiments 3 and 4, peritonitis was found to be a complication of intraperitoneal inoculation of coarse kidney suspensions. In experiments 5 and 6, using suspensions from which large aggregates of kidney cells had been removed by filtration, peritonitis was found not to occur. Unfortunately, using filtered suspensions reduced the detectable renal infection rate. However, this may have been due solely to dilution of the infective agent during preparation of the filtrates.

The results demonstrated that infected kidney suspensions, passed through filters with 60 to 10 μm pore sizes, and injected intraperitoneally, established detectable renal infections, with PKX cells and related histopathological lesions. Suspensions passed through a filter with a 0.8 μm pore size did not produce a detectable infection. This suggested that in these experiments the infective agent was greater than 0.8 μm in diameter and, therefore, that PKD does not have a viral aetiology.

Homogenization of the 20 μm filtrate to disrupt large PKX cells and release their intracellular inclusions prior to filtration was not shown to increase the infectivity of the suspension. In experiment 6, fewer

fish in group 4 (injected with the homogenized 10 μm filtered suspension) developed detectable renal infection than in group 3 (injected with the 10 μm filtered suspension without homogenization) (Table 6.4).

Experiments 7 and 8. Attempts to reproduce disease in brown trout by intraperitoneal injection of coarse infected kidney suspensions from rainbow trout.

MATERIALS AND METHODS

Details of the groups of brown trout and inocula used are given in Table 6.5. Fish in experiment 7 group 1 were sampled 7, 14, 26, 31 and 40 days after injection, as shown in Table 6.6, and fish in groups 2 and 3 were sampled 40 days after injection. Fish in experiment 8 group 1 were sampled 48, 63 and 105 days after injection, and in group 2, 48 and 105 days after injection.

RESULTS

In experiment 7, none of the control fish (groups 2 and 3) developed gross or histopathological signs of PKD. Gross and histopathological lesions seen in brown trout in group 1 (injected with an infected kidney suspension) are detailed in Table 6.6. Three fish in this group died with ichthyophthiriasis 31-38 days after injection and were not sampled.

In experiment 8, two rainbow trout and two brown trout sampled 48 days after injection contained renal PKX cells and histopathological lesions of PKD. One of three rainbow trout sampled 105 days after injection had severe lesions of PKD. None of the 13 brown trout sampled 63 and 105 days after inoculation contained detectable PKX cells or lesions, but both fish sampled 63 days after injection and six of 11 sampled 105 days after injection contained cells similar to Myxosporidan trophozoites and

immature spores within some renal tubules.

DISCUSSION

Results from both experiments demonstrated that PKD-infected renal tissue from rainbow trout, when injected intraperitoneally into brown trout, can establish a renal infection. Lesions in brown trout were similar to those seen in the field following natural infection with PKD (chapter 5). This suggests that the disease in brown trout and rainbow trout may be caused by the same organism.

The significance of the cells similar in appearance to myxosporidan trophozoites and immature spores in the renal tubules of infected brown trout will be considered further in chapter 7.

Experiment 9. Attempt to reproduce disease in rainbow trout and brown trout by intraperitoneal inoculation via the cloaca of a coarse infected kidney suspension.

MATERIALS AND METHODS

Details of the groups of fish and inocula used are given in Table 6.7. Inoculation was carried out using an epidural canula (Ref. 100/380/010, Portex Ltd., Hythe, Kent) inserted into the peritoneal cavity via the cloaca. Fish were sampled 44 and 77 days after inoculation.

RESULTS

One brown trout (group 2) showed histological evidence of PKD 44 days after inoculation and one fish from each group showed histological evidence 77 days after inoculation.

DISCUSSION

Results from experiments 3, 4, 6, 7 and 8 in which rainbow and brown trout were injected intraperitoneally with coarse infected kidney suspensions showed 28 of 35 rainbow trout with evidence of PKD 24-105 days after inoculation and 15 of 30 brown trout with evidence of PKD 14-105 days after inoculation. However, in this experiment using intraperitoneal inoculation via the cloaca a smaller proportion of fish (one of 10 rainbow trout and two of 10 brown trout) became detectably infected 44-77 days after inoculation. The reason for this difference was not known, although some fish may not have been inoculated into the peritoneal cavity despite every effort to ensure this. The infective dose may have been lower and the fish may have been more resistant to infection, Nevertheless, the results did demonstrate that fish could become infected by this route.

Experiments 10 and 11. Attempts to reproduce disease in rainbow trout by feeding infected kidney material.

MATERIALS AND METHODS

Details of the groups of fish and inocula used are given in Table 6.8. In experiment 10, the 20 experimental fish were starved for approximately 6 hr before 1 g of kidney, cut into 2 mm^3 pieces, was put into the holding tank. The water flow was halted until all the pieces had been eaten. The procedure was repeated 7 days later with naturally-infected donor fish kidneys from the same infected stocks. Five fish were sampled 48 days after the initial feed and 15 fish 63 days after.

In experiment 11, the 10 experimental fish were anaesthetized and given a coarse kidney suspension into the stomach via an epidural canula (Ref. 100/380/010, Portex Ltd., Hythe, Kent) attached to a 2 ml syringe.

A similar procedure was repeated 8 days later. Fish were sampled 41, 71, 92 and 104 days after the initial inoculation.

RESULTS

None of the 30 fish exhibited kidney swelling or histopathological lesions of PKD.

DISCUSSION

It was not found possible to establish a detectable kidney infection with PKX cells in rainbow trout by feeding affected kidney from naturally-infected fish. These findings agree with those of D'Silva et al. (1984). This will be discussed further at the end of the chapter.

Experiment 12. Attempt to reproduce disease in rainbow trout by inoculation of a coarse infected kidney suspension via the gills.

MATERIALS AND METHODS

Ten uninfected rainbow trout fingerlings, average length 80 mm, from farm A, were inoculated by flushing 2 ml aliquots of a coarse suspension of infected kidney material over the gills, twice for each set of gill arches. The inoculum comprised approximately 1 g of kidney tissue from two naturally-infected rainbow trout in 10 ml sterile PBS.

Fish were sampled 41, 71, 92 and 104 days after inoculation.

RESULTS

None of the 10 fish exhibited kidney swelling or histological signs of PKD.

DISCUSSION

It was not found possible to establish a detectable kidney infection with PKD by flushing the gills of rainbow trout with infected kidney suspensions.

Experiment 13. Attempt to reproduce disease in rainbow trout by dipping fish in a coarse infected kidney suspension.

MATERIALS AND METHODS

Ten uninfected rainbow trout fingerlings, average length 80 mm, from farm A, were immersed tail first up to a level just posterior to the cloaca for approximately 30 sec in a coarse infected kidney suspension. This suspension comprised approximately 1 g of kidney tissue from two naturally-infected rainbow trout in 10 ml sterile PBS.

Fish were sampled 41, 71, 92 and 104 days after immersion.

RESULTS

None of the 10 fish exhibited kidney swelling or histological signs of PKD.

DISCUSSION

It was not found possible to establish a detectable kidney infection with PKD by dipping fish in an infected kidney suspension. The skin was thought to be intact in these fish. In order to study whether initial skin damage was required, in the following experiment an attempt was made to establish infection by subcutaneous inoculation.

Experiment 14. Attempt to reproduce disease in rainbow trout by subcutaneous injection of a coarse infected kidney suspension.

MATERIALS AND METHODS

Groups of fish and inocula used are shown in Table 6.9. The 10 rainbow trout inoculated subcutaneously were injected dorsal to the lateral line and anterior to the dorsal fin using a 25 gauge 5/8 inch needle. The 10 control fish inoculated intraperitoneally were injected using the same homogenate and needle size.

Fish were sampled 52 and 58 days after injection. Sections of striated muscle from both groups of fish were taken from the region of the injection site.

RESULTS

None of the fish had obviously swollen kidneys. Eight of 10 control fish inoculated intraperitoneally contained renal PKX cells and evidence of haemopoietic hyperplasia. Five of these also had areas of diffuse inflammation. Muscle sections showed no evidence of inflammation or PKX cells.

Seven of 10 fishinoculated subcutaneously contained renal PKX cells and evidence of haemopoietic hyperplasia. One also had areas of diffuse inflammation. Muscle sections from four fish contained inflammatory lesions between muscle fibres and three of these contained PKX cells.

DISCUSSION

Results from experiment 13 suggested that fish could not be infected via intact skin with an infected kidney suspension. However, results from this experiment showed that if the suspension is introduced below the epidermis and dermis a renal infection with PKD can be established.

GENERAL DISCUSSION

Little is known of the life cycle or mode of transmission of the aetiological agent of PKD. With regard to invasion by the infective agent, Ghittino *et al*. (1977) considered ingestion the most plausible mode of entry and Klontz and Chacko (Anon, 1983) postulated that infection occurred by eating contaminated seagull faeces. The oral route of infection is implicated in several other protozoan diseases, for example, whirling disease caused by the Myxosporean protozoan *Myxosoma cerebralis*. In this disease, alkaline conditions in the intestinal tract distal to the stomach have been shown to induce *M. cerebralis* spores to release their polar filaments prior to sporoplasm release (Uspenskaya, 1957). Experimental transmission of a *Parvicapsula* sp. infection to coho salmon has been successful following stomach intubation of spores (Johnstone, 1984).

Ghittino *et al*. (1977) also considered the gills, with their large surface area, as a possible route of infection. They suggested that once the parasite had entered the fish it travelled by the lymphatic and blood systems to the kidney and other areas of haemopoietic activity. Ferguson and Needham (1978) found the organism in the capillaries of the intestine and gill supporting this hypothesis.

The skin represents another route of entry but may require damage before penetration of the parasite can occur. Several blood-borne parasitic infections occur in this way either directly or indirectly through the agency of a vector. The cercariae of the digenean blood flukes, *Sanguinicola davisi* and *S. klamathensis*, released from the intermediate snail hosts (*Oxytrema* sp. and *Fluminicola* sp., respectively) actively penetrate the skin around the pelvic and pectoral fins prior to entering the circulation (Wales, 1958; Evans and Heckmann, 1973). *Trypanosoma*

and Cryptobia infections of many fish species are introduced through the skin by vectors. Experimental infection was described by Robertson (1912) where the leeches, Hemiclepsis marginata and Piscicola geometra, caused the spread of both types of protozoan infection when penetrating the skin to suck blood. In PKD it is thought unlikely that a vector is necessary for transmission. Alderman and Rodgers (1984) demonstrated that water from a PKD-infected river when passed through precision mesh filters down to a pore size of 60 µm still caused infection of 0+ rainbow trout with PKD.

The main purpose of the transmission experiments described in this chapter was to try and reproduce the disease under laboratory conditions so that it could be studied throughout the year. Using natural transmission in the field it had been found that fish could only be infected between April and October. Initially, attempts were made to transmit the infection by cohabiting infected rainbow trout and Atlantic salmon with uninfected rainbow trout. Attempts to set up a third experiment using infected brown trout failed because the brown trout died from other causes. None of the 25 initially uninfected rainbow trout sampled had detectable renal infection with PKX cells after cohabitation with infected fish. Several reasons were considered for this failure to detect direct transmission. Firstly, it could be argued that if transmission was inefficient the number of fish sampled was too small to detect it. Secondly, it may be that, under the experimental conditions imposed, infected fish were not releasing infective forms of the parasite. This may have been because:

1. Some trigger such as decreasing or fluctuating temperatures is required to induce such release.

2. Fish may have to die and decompose before the parasite is released.

This is comparable to the situation in whirling disease (reviewed in detail by Halliday, 1976) caused by M. cerebralis. In the life cycle of this parasite, although spores may be released in the faeces of infected fish while they are alive (Uspenskaya, 1957) and an avian intermediate host has been implicated in transmission of the disease by finding M. cerebralis spores in the hindgut of the kingfisher (Schäperclaus, 1954), it is generally accepted that the major release of spores occurs when the host fish dies and infected cartilage and bone disintegrate.

3. Fish were releasing non-infective forms of the parasite which required a period of maturation in the environment and/or in one or more intermediate hosts before being infective to rainbow trout. Again, in whirling disease the spores released from dead fish are possibly not directly infective to other fish but require several months of "aging", a process perhaps involving the spores being eaten by an oligochaete Tubifex sp. prior to becoming infective (Markiw and Wolf, 1983).

In two other protozoan diseases affecting salmonids, namely ichthyophthiriasis and amoebiasis, a period of maturation is required for the parasite to become infective. In ichthyophthiriasis, MacLennan (1935) found that the trophozoite stage of the parasite, having left the host fish epithelium, encysted and underwent protoplasmic division into ciliated cells, often numbering several hundred, which then broke out of the cyst and actively sought a new host. In infection with Schizamoeba salmonis, Davis (1926) described the trophozoite stage developing into a cyst prior to release from the intestine of the fish and subsequent ingestion by another fish.

4. Rainbow trout may be non-productive, dead end hosts. Klontz and Chacko (Anon, 1983) considered that the aetiological agent of PKD was in the gastro-intestinal tract of piscivorous birds with fish which ingested

infected bird faeces subsequently developing PKD without releasing any further stages of the parasite. They cited epidemiological evidence to support this hypothesis. At the Hagerman State Hatchery in Idaho, rainbow trout in raceways protected by anti-bird wires remained uninfected whereas those in raceways progressively further away from the anti-bird wires were progressively more heavily infected with PKD. Certainly, in chapter 3 few PKX cells and only one possible spore were seen in renal tubules in heavily infected 0+ rainbow trout. Bradbury (1981) failed to isolate PKX cells or spores from PBS washings from ureters of infected rainbow trout. However, the finding of cells,similar to the trophozoites and developing spores described in 0+ steelhead trout (Kent and Hedrick, 1985a,b,c), in renal tubules of both Atlantic salmon and brown trout in the transmission experiments described in this chapter was interesting in that the cells may represent a further stage in the development of the PKX cell not seen in rainbow trout.

The third reason considered for failure to detect direct transmission was that, if infective forms of the organism were present, the conditions of the experiment may have been unsuitable for allowing infection of the host, despite previous results (chapter 5) demonstrating that the conditions were suitable for the progress of clinical disease once fish were infected.

In subsequent experiments, the results of which are summarized in Table 6.10, it was demonstrated that by using infected kidney suspensions from naturally-infected rainbow trout it was possible to establish a renal infection in rainbow trout and brown trout after intraperitoneal inoculation and in rainbow trout after subcutaneous injection. Coarse suspensions gave the best results. However, the filtration method diluted the infective dose and this may have been the reason for the decreased take.

Nevertheless, filtration did appear to decrease the problem of peritonitis due to experimental technique. The results suggested that the PKX cell was involved in the aetiology of PKD and that the method of producing the suspension did not kill all PKX cells.

The experimentally-induced disease differed from that after natural infection. None of the fish that developed renal infection with PKD (total 92) developed renal swelling greater than grade 2 despite large numbers of PKX cells being present in the kidneys of some injected fish. There was less renal haemopoietic hyperplasia and diffuse inflammation than in field conditions.

Other routes of inoculation (summarized in Table 6.10) using infected kidney suspensions were not successful. Although the numbers of fish used were small, using similar numbers a proportion of the fish did succumb to disease following intraperitoneal inoculation. The number of PKX organisms inoculated varied in each experiment and this may have influenced the results. The finding that fish could not be infected by other routes does not imply that these are not the natural routes of infection in the field. It is unlikely that the PKX cell is the infective form of the organism. The infective form may well invade the fish via the skin, gills or intestinal tract.

With regard to the original purpose of these transmission experiments, therefore, it was concluded that PKD could be maintained in rainbow trout, and possibly brown trout, by intraperitoneal implants of renal tissue containing PKX cells and in rainbow trout by subcutaneous injection of PKX-positive renal tissue. The resulting disease is similar to but less severe than that seen after natural infection.

Table 6.1. Experiments 3 and 4. Groups of fish injected intraperitoneally with coarse suspensions of kidney tissue from uninfected or naturally-infected 0+ rainbow trout or with PBS.

Experiment number	Group number	Source of PKD-free fish inoculated	Average length (mm)	No. of fish inoculated	Inoculum	Amount inoculated (ml)
3	1	Farm A	80	5	1 g kidney from 2 infected rainbow trout in 5 ml sterile PBS	0.2
3	2	Farm A	80	5	1 g kidney from 2 infected rainbow trout in 5 ml sterile PBS	0.5
4	1	Farm A	50	20	0.5 g kidney from 2 infected rainbow trout in 5 ml sterile PBS	0.1 to 0.2
4	2	Farm C	115	10	0.5 g kidney from 2 uninfected rainbow trout in 5 ml sterile PBS	0.2
4	3	Farm C	115	10	PBS	0.2

Table 6.2. Experiments 5 and 6. Groups of fish from farm C injected intraperitoneally with filtered suspensions of kidney tissue from naturally-infected 0+ rainbow trout.

Experiment number	Group number	Average length (mm)	Number of fish inoculated	Inoculum	Amount (ml) inoculated
5	1	60	15	1 g kidney from 2 rainbow trout/10 ml sterile PBS passed through 60 µm filter + 5 ml sterile PBS	0.3
5	2	60	15	10 ml 60 µm diluted filtrate passed through 30 µm filter + 5 ml sterile PBS	0.3
5	3	60	15	10 ml 30 µm diluted filtrate passed through 20 µm filter + 5 ml sterile PBS	0.3
5	4	60	15	10 ml 20 µm diluted filtrate passed through 10 µm filter + 5 ml sterile PBS	0.3
6	1	82	10	4.5 g kidney from 5 rainbow trout/25 ml sterile PBS in coarse suspension	0.2
6	2	82	10	Coarse suspension passed through 60 µm then 20 µm filters	0.2
6	3	82	10	Coarse suspension passed through 60 µm, 20 µm then 10 µm filters	0.2
6	4	82	10	Homogenized 20 µm filtrate passed through 10 µm filter	0.2
6	5	82	10	Coarse suspension passed through 60 µm, 20 µm, 10 µm then 0.8 µm filters	0.2
6	6	82	10	Homogenized 20 µm filtrate passed through 10 µm then 0.8 µm filters	0.2

Table 6.3. Experiment 5. Numbers of fish in each group with detectable renal PKX cells at each sampling.

Group number	1	2	3	4
Filtrate injected	60 µm	30 µm	20 µm	10 µm
Number of fish inoculated	15	15	15	15
*Number of fish dying from causes unrelated to PKD	0	1	10	9
Number of fish sampled 29 days after inoculation	2	2	2	2
Number of fish positive for PKX cells 29 days after inoculation	2	0	0	0
Number of fish sampled 48 days after inoculation	13	12	3	4
Number of fish positive for PKX cells 48 days after inoculation	5	7	3	1

* Fish in groups 3 and 4 died from failure of air and water supplies

Table 6.4. Experiment 6. Numbers of fish in each group with detectable renal PKX cells and associated histopathological lesions 55 days after injection.

Group number	1	2	3	4	5	6
Suspension injected	coarse	20 µm	10 µm	homogenized 10 µm	0.8 µm	homogenized 0.8 µm
Number of fish inoculated	10	10	10	10	10	10
Number of fish sampled 55 days after inoculation	10	10	10	10	10	10
Number of fish positive for PKX cells	10	4	6	3	0	0
Number of fish PKX positive with haemopoietic hyperplasia	8	4	6	3	0	0
Number of fish PKX positive with diffuse inflammation	1	0	1	0	0	0

Table 6.5. Experiments 7 and 8. Groups of rainbow trout and brown trout injected intraperitoneally with coarse suspensions of kidney tissue from uninfected or naturally-infected 0+ rainbow trout or with PBS.

Experiment number	Group number	Source of PKD-free fish for inoculation	Species inoculated	Number of fish inoculated	Inoculum	Amount (ml) inoculated
7	1	Farm D	Brown trout	20	0.23 g kidney from 2 infected rainbow trout/5 ml sterile PBS	0.1-0.15
7	2	Farm D	Brown trout	10	0.5 g kidney from 2 uninfected rainbow trout/5 ml sterile PBS	0.2
7	3	Farm D	Brown trout	10	Sterile PBS	0.2
8	1	Farm D	Brown trout	15	0.4 g kidney from 2 infected rainbow trout/5 ml sterile PBS	0.2
8	2	Farm C	Rainbow trout	5	0.4 g kidney from 2 infected rainbow trout/5 ml sterile PBS	0.2

Table 6.6. Experiment 7. Gross and histopathological lesions of PKD in brown trout in group 1 after injection with infected kidney tissue from rainbow trout.

Number of days after inoculation	Number of fish sampled	Number of fish with renal swelling of grades:			Number of fish with kidneys containing:				
		0	1	2	PKX cells	haemopoietic hyperplasia	diffuse inflammation	glomerular and tubule destruction	vascular aggregations
7	2	2	0	0	0	0	0	0	0
14	2	2	0	0	2	0	0	0	0
26	2	0	2	0	2	2	1	1	0
31	4	0	2	2	2	2	2	0	1
40	7	0	4	3	7	1	5	5	1
Total	17	4	8	5	13	5	8	6	2

Table 6.7. Experiment 9. Groups of rainbow trout and brown trout inoculated intraperitoneally via the cloaca with coarse suspensions of kidney tissue from naturally-infected 0+ rainbow trout.

Group number	Source of PKD-free fish for inoculation	Species inoculated	Average length (mm)	Number of fish inoculated	Inoculum	Amount (ml) inoculated
1	Farm A	Rainbow trout	85	10	0.5 g kidney from 2 infected rainbow trout/5 ml sterile PBS	0.2
2	Farm D	Brown trout	80	10	0.5 g kidney from 2 infected rainbow trout/5 ml sterile PBS	0.2

Table 6.8. Experiments 10 and 11. Groups of rainbow trout fed by mouth or via stomach tube with kidney material from naturally-infected 0+ rainbow trout.

Experiment number	Source of PKD-free fish for inoculation	Average length (mm)	Number of fish inoculated	Inoculation number	Inoculum	Amount inoculated
10	Laboratory-hatched	55	20	1	1 g kidney from 5 infected rainbow trout in 2 mm^3 pieces/ 15 ml sterile PBS	1 g fed to 20 fish
				2	As for first inoculum	1 g fed to 20 fish
11	Farm A	80	10	1	1 g kidney from 2 infected rainbow trout/10 ml sterile PBS as a coarse suspension	0.5 ml by stomach tube
				2	As for first inoculum	0.5 ml by stomach tube

Table 6.9. Experiment 14. Groups of rainbow trout injected subcutaneously or intraperitoneally with a coarse kidney suspension from naturally-infected 0+ rainbow trout.

Group number	Source of PKD-free fish for inoculation	Average length (mm)	Number of fish inoculated	Route of inoculation	Inoculum	Amount (ml) inoculated
1	Laboratory-hatched	80	10	Subcutaneous	0.75 g kidney from 3 infected rainbow trout as a coarse suspension in 10 ml sterile PBS	0.15
2	Laboratory-hatched	80	10	Intra-peritoneal	0.75 g kidney from 3 infected rainbow trout as a coarse suspension in 10 ml sterile PBS	0.15

Table 6.10. Experiments 3 to 14. Summary of results.

Experiment number	Inoculation procedure	Species of fish inoculated	Number of fish inoculated with kidney material from naturally-infected rainbow trout	Number of fish developing renal infection with PKD
3	Intraperitoneal injection	RT	10	6
4	" "	RT	20	9
5	" "	RT	60	18
6	" "	RT	60	23
7	" "	BT	20	13
8	" "	(i) BT	15	2
		(ii) RT	5	3
9	Intraperitoneal via cloaca	(i) RT	10	1
		(ii) BT	10	2
10	Per os	RT	20	0
11	Into stomach	RT	10	0
12	Via gills	RT	10	0
13	Onto skin	RT	10	0
14	(i) Subcutaneous injection	RT	10	7
	(ii) Intraperitoneal injection	RT	10	8
		Totals	280	92

RT = rainbow trout; BT = brown trout

Chapter 7

General Discussion

Prior to work described in this dissertation, PKD was emerging as an economically important disease to the fish farming industry in the U.K. yet, as described in chapter 1, many aspects of the disease were poorly understood. The Ministry of Agriculture, Fisheries and Food, Fish Diseases Laboratory (FDL) at Weymouth was surveying the extent of the problem and investigating the aetiology of the disease. The disease was prevalent in the south of England with the Rivers Avon and Test particularly affected. An experimental site was available on the River Avon where PKD was occurring annually. It was decided, therefore, that the field work described in this dissertation should be conducted at FDL, although the project was supervised from the University of Stirling, Institute of Aquaculture, a renowned centre for the study of fish diseases.

Certain lines of investigation into PKD were already progressing at FDL and these were to remain outside the scope of the project described here. These included the chemotherapy of PKD and the isolation of the infective agent from river water or infected fish tissues. The present research programme was initiated to investigate aspects of the disease which it was thought would aid development of a rational approach to the control of the disease. Four lines of investigation were pursued:

1. A study of the sequential pathology of the disease from initial infection to death or recovery of the fish.
2. A study of the haematology throughout the course of infection.
3. A study of the epidemiology, including the time course and infective period of PKD at the experimental site and the effects of water temperature, age, previous exposure to infection, and species on the development of the disease.

4. A study of experimental transmission of the disease.

It was hoped that these studies would help elucidate the pathogenesis of the disease and the development of resistance to it.

Previous work on the pathology of the disease had been confined to studies in clinically-affected fish (Ferguson and Adair, 1977; Ghittino et al., 1977; Ferguson and Needham, 1978; Roberts, 1978). Haematological studies were limited to packed cell volume measurements by Ferguson and Needham (1978) and differential cell counts made from two blood smears by Ghittino et al. (1977). However, during the period of research described here a more detailed investigation of various haematological aspects of the disease was conducted by Hoffmann and Lommel (1984). Certain of their conclusions were inconsistent with those of other authors. Comparison was only made between severely affected fish and uninfected fish, with no attempt to trace the development of changes in blood values during a disease outbreak.

With regard to the epidemiology of PKD, from previous work the age of fish at first exposure and water temperature had been identified as factors of possible importance, although how they affected the incidence of the disease was not understood. The infective period had not been studied in detail and had not been correlated with environmental changes. The role of fish species, other than rainbow trout, in the maintenance and spread of PKD was not known. Opinions differed as to the effects of water quality on the development and severity of PKD. Initially, it was thought to be a disease found only in soft, acid water classed as oligotrophic (Ferguson and Needham, 1978). However, subsequently serious outbreaks were described in hard, alkaline water classed as eutrophic (Scott,1979).

Resistance to PKD was investigated by considering the effects of

previous exposure to the disease, and age and species susceptibility. Specific resistance to PKD in 1+ rainbow trout surviving infection the previous summer had been postulated by Ferguson and Ball (1979), but it was not known what type of resistance it was, whether it resulted from infection alone or required the development of clinical disease, whether it prevented re-infection and whether it allowed the development of carrier fish. Genetic variation between strains of brown trout and Atlantic salmon was considered by Ellis et al. (1982, 1985) as a possible explanation of differing susceptibility to PKD, and interspecies differences were noted in rainbow trout and Atlantic salmon parr by Ferguson and Needham (1978). No attempts to quantify these differences had been made.

It was felt that success in carrying out future studies on the pathogenicity of the causative agent of PKD and immunity to infection would be aided by the ability to reproduce the disease throughout the year by experimentally inoculating fish. Previous attempts to experimentally transmit the disease had met with little success (Ferguson and Ball, 1979), although experiments by D'Silva et al. (1984) carried out concurrently with work described here demonstrated that intraperitoneal injection of infected kidney suspensions from rainbow trout could be used to induce a kidney infection with PKX cells in previously uninfected rainbow trout.

PATHOGENESIS

The route of entry of the causative agent of PKD is not known. Possible natural routes of infection include the alimentary tract, the gills, the skin and the urino-genital tract via the cloaca. The infective form of the organism had not yet been identified so it was not possible to investigate routes of infection experimentally. However, using the PKX cell (the form of the organism associated with all cases of PKD) in

kidney suspensions it was not found possible to infect fish orally, via the gills or across intact skin. These organisms would only cause disease if inoculated subcutaneously or intraperitoneally. In experiments described in chapters 5 and 6 bodies were seen in both Atlantic salmon and brown trout kidneys which histologically resembled immature myxosporidan spores described by Kent and Hedrick (1985a,b,c) in steelhead trout with PKD. Similar intraluminal spore-forms have now been detected in renal tubules of Atlantic salmon, brown trout and grayling thought to have recovered from PKD. The possible involvement of myxosporidans in the aetiology of PKD is currently under study. If a myxosporidan rather than haplosporidan aetiology is shown, then by analogy with other myxosporidans the digestive tract represents a probable route of entry (Hofer, 1906; Kudo, 1977; Mitchell, 1977; Johnstone, 1984).

Although the route of entry of the organism into the host is not known, histological evidence suggested that in infected fish the organism arrived via the circulation in the organs responsible for filtering foreign material, possibly in close association with macrophages. There was little evidence of progression into the renal tubules as suggested by Ferguson and Needham (1978). It was noticeable that the organism was found first in the kidney and spleen and attained higher numbers here than in any other tissue examined, suggesting some predilection for these sites. This may have been because macrophages tend to migrate to these tissues after phagocytosing foreign material and may, therefore, have carried organisms to these sites. Equally, however, PKX cells could have settled preferentially in these tissues and have subsequently been phagocytosed by macrophages. Ferguson (1984) described bacterial phagocytosis by macrophages in close association with renal peritubular capillary endothelial cells.

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Previous studies had noted external signs of abdominal distension, anaemia and exophthalmos associated with PKD and had linked them with possible renal and circulatory failure (Ghittino et al., 1977), but no attempt had been reported to relate sequential gross and histo-pathology to clinical signs and to assess the pathogenesis of the disease. Results in chapter 3 showed infection with PKX cells preceded the first detectable tissue changes by about two weeks. Haemopoietic hyperplasia was the most constant initial tissue change. Diffuse inflammation, glomerulus and tubule destruction, and vascular lesions then developed and as their severity increased gross pathology and clinical signs occurred. Anaemia was found once vascular pathology, including haemoglobin crystal formation, was pronounced. The chronological relationship of these developments was summarized in Fig.3.44.

Results of electron microscopical studies suggested that the initial generalized haemopoietic hyperplasia involved both erythrocyte and leucocyte precursors. However, the two aspects of renal pathology thought to be most significant in the pathogenesis were vascular lesions and the chronic inflammation centred on areas of tissue containing PKX cells. In rainbow trout, vascular changes are a major part of the pathological picture and could help explain the development of renal failure and anaemia. Chronic inflammation involving macrophages, lymphocytes and occasionally fibroblasts, is the lesion described in previous reports of PKD (de Kinkelin and Gérard, 1977a; Ghittino et al., 1977; Ferguson and Needham, 1978) and results in the renal enlargement which is a feature of the disease. Both haemopoietic tissue and excretory elements appear destroyed by the reaction, probably contributing to both anaemia and loss of fluid balance. Although this type of chronic inflammatory response was found in other organs, it was not as severe as that observed

in the kidney and spleen. This suggested that the function of these other organs may not have been as seriously impaired.

During the recovery phase it was found that healing of lesions appeared to progress rapidly where there was an apparent destruction of PKX cells, but more slowly where fibrosis was a prominent element of the reaction. The histological and ultra-structural findings suggested that PKX cells were undergoing necrosis. This may have been due to an acquired host response involving humoral and/or cellular immunity. This is discussed further under "resistance to PKD".

Batches of previously infected recovering fish tested between October and March (chapter 3 and unpublished data) showed no evidence of PKX organisms or healing nodules by March, suggesting that resorption of nodules occurs and that fish clear the infection.

Ferguson and Needham (1978) suggested that the kidney may be a route of exit for PKX cells. They based this hypothesis on histological evidence of PKX cells in some renal tubule lumens. In the present study few organisms were found in this site and none were demonstrated in ureteral sections in rainbow trout. Furthermore, only one possible intra-tubular spore-form was seen in this species. It may be that organisms are released by another route or in an, as yet, unrecognised form. Extensive studies of other tissues failed to demonstrate this. Alternatively, it may be that rainbow trout are aberrant hosts for the PKX organism and have a non-productive infection.

In rainbow trout, several factors, besides fish species, were found to affect the pathogenesis of PKD. Temperatures of 15°C and 18°C apparently enhanced parasite multiplication in subclinically-affected fish compared to that at 9°C and 12°C. At 9°C development of clinical disease was suppressed. In clinically-affected fish, recovery rate was increased

as temperatures decreased from 18°C to 7°C. Field observations suggested that stress would kill anaemic fish with PKD. These fish might otherwise have recovered. A previous exposure to PKD resulting in a renal swelling of at least grade 2 did not prevent re-infection but did apparently limit lesions and prevented clinical disease.

The haematological study during the development of PKD was a necessary adjunct to that of the pathology. In some instances the appearance of lesions and changes in blood picture correlated precisely, for example, the appearance of intravascular aggregations and vascular occlusions with the decrease in thrombocyte numbers. However, in many instances fish with swollen kidneys and advanced histopathological lesions associated with PKD presented blood pictures within the normal ranges for rainbow trout. This suggested that diseased fish did not develop an anaemia, but a haemodilution secondary to progressive failure of fluid balance control. However, it was found that several fish with significant decreases in erythrocyte values but normal leucocyte numbers had serum or plasma protein concentrations and urea levels within normal ranges. A haemodilution effect would have lowered these values. It seemed, therefore, that a true anaemia did develop in a proportion of severely diseased fish. In a smaller proportion of fish oedematous changes also occurred, resulting in decreases of all blood values. These findings could be related to the field observation that many fish die during a PKD outbreak when stressed, whereas only a few die if managed carefully. Anaemic fish are unable to adapt quickly to stressful situations but should survive if managed correctly. However, if anaemic fish also develop fluid imbalance, they are likely to die whatever the management conditions.

Anaemias can be divided into three broad categories, those arising from blood loss, those caused by erythrocyte destruction in excess of any

compensatory increase in erythropoiesis, and those resulting from decreased erythrocyte production. Roberts (1978) considered the anaemia associated with PKD to be hypoplastic, whereas Hoffmann and Lommel (1984) considered it to be haemolytic. Using the findings from the present study, the evidence for the anaemia associated with PKD belonging to one or more of the three broad categories is evaluated.

The first category arises from internal or external haemorrhage and is seen in salmonids, especially in viral haemorrhagic septicaemia. In PKD there is no consistent evidence of external blood loss. In this study, ecchymoses were seen in one oedematous fish. Evidence of internal bleeding was limited to the finding of blood cells in ascitic fluid samples from severely affected fish, and petechiation in some internal organs (chapter 3). However, on histological examination this petechiation was seen to be associated with obstructed blood vessels rather than with areas of haemorrhage. It seems unlikely that blood loss contributes significantly to the development of anaemia in PKD.

The second category of anaemia includes the haemolytic anaemias, and is exemplified by vibriosis caused by Vibrio anguillarum in many species of fish including salmonids. In this disease haemolysins, released by the causative organism, result in haemolysis, with subsequent deposition of large amounts of iron, as haemosiderin and ferritin, in splenic melano-macrophage centres.

In man, signs of haemolytic anaemia relate to excess erythrocyte destruction and increased erythropoiesis. Most human haemolytic anaemias involve extravascular haemolysis. However, intravascular haemolysis does occur in some anaemias, for example, associated with some malarial infections and clostridial septicaemias, and in certain microangiopathic conditions. In these cases, the signs may include haemoglobinaemia; the

absence of haptoglobin, resulting from the binding of free haemoglobin in the circulation and removal by hepatocytes; haemoglobinuria, resulting from the binding capacity of circulating haptoglobin being exceeded and free haemoglobin being filtered by renal glomeruli in excess of the amount which can be re-absorbed in the proximal tubules; the deposition of iron, extracted from resorbed haemoglobin, within tubule epithelial cells as ferritin and haemosiderin; haemosiderinuria, resulting from sloughing of tubular epithelial cells containing haemosiderin; methaemalbuminaemia, resulting from oxidized haemoglobin binding to albumin; and decreased haemopexin, resulting from haem, released from haemoglobin, binding to haemopexin to be processed in the liver. Further signs of excess erythrocyte destruction include (a) decreased erythrocyte survival times; (b) increased haem catabolism, resulting in increased serum unconjugated bilirubin, increased endogenous carbon monoxide production, an increased rate of bilirubin production and an increased rate of urobilinogen excretion; and (c) increased serum lactate dehydrogenase activity. In man, increased erythropoiesis is seen in the blood picture where reticulocytosis with polychromatophilia, macrocytosis, erythroblastosis, leucocytosis and thrombocytosis occur; in the bone marrow where there is erythroid hyperplasia; in increased plasma and erythrocyte iron turnover; and in increased erythrocyte creatine levels and erythrocyte uroporphyrin 1 synthetase, hexokinase and glutamate oxalate transaminase activities.

In the present study, evidence of increased haemolysis included significant amounts of Perls'-positive material in the spleens of some fish. The PCV values of these fish ranged from 0.23 down to 0.09. However, an increase in Perls'-positive material in spleens of fish with decreased PCV values was an inconstant finding and suggested that factors other than haemolysis were involved. Intravascular fusiform crystals

(chapter 3) were thought to indicate a haemoglobinaemia, following haemolysis, and to explain the maintenance or increase of haemoglobin levels in some fish where other parameters indicated anaemia. The histological appearance of vascular lesions and obstruction suggested that intravascular haemolysis following haemostasis could be occurring. These vascular lesions may be analogous to those of human microangiopathy. In this condition, haemolysis may occur by mechanical damage to erythrocytes forced against fibrin strands. The fibrin component of the vascular lesions in PKD was not prominent and an inconstant finding. Also, no cells equivalent to schistocytes were seen in blood smears from PKD-infected fish. However, fish erythrocytes may be more fragile than human erythrocytes and, unlike human erythrocytes, being nucleated, may not have the ability to reseal their outer membrane after damage.

Neither hypochromia nor poikilocytosis were frequent findings in the present study, although they were in others (Hoffmann and Lommel, 1984). Although hypochromia may be found in anaemias associated with chronic infections in man, it is not a finding in haemolytic anaemias. In contrast, polychromasia, often present in this study, is a common occurrence. Hypochromasia and poikilocytosis are more often seen in hypoplastic anaemias especially where iron is deficient. In haemolytic anaemias sufficient iron is generally released for recycling and accelerated erythropoiesis (Roberts, 1978).

No haemoglobinuria was noted during the experimental period, and no Perls'-positive material was seen on histological examination in renal tubule epithelial cells. This suggested that if haemolysis was occurring it did not exceed the haemoglobin binding capacity of haptoglobin. There was no evidence of significantly increased serum bilirubin levels, which might be expected if intravascular haemolysis was occurring. How-

ever, this parameter varies and may be within normal limits in human patients with haemolytic anaemia.

Evidence of increased erythropoiesis in PKD in the present study was seen histologically in the increase in haemopoietic tissue in the kidney, especially during the earlier part of the infection, and in the centres of secondary haemopoiesis in the liver later in the course of the disease. However, the numbers of immature red cells were not consistently elevated in anaemic fish, and in severely oedematous fish the numbers of aberrant cells and misshapen immature cells increased, suggesting a hypoplasia rather than accelerated erythropoiesis.

The third category of anaemia, arising from decreased erythrocyte production, has a diverse causation including nutritional deficiencies such as cyanocobalamin and iron deficiencies in man; replacement of haemopoietic tissue in the spleen and kidney as in granulomatous conditions such as bacterial kidney disease, ichthyophoniasis nocardiosis and tuberculosis in fish; destruction of haemopoietic tissue as in infectious haematopoietic necrosis (IHN) in fish; and damage to the excretory part of the kidney responsible for erythropoietin production as in some infectious or, more rarely, in neoplastic conditions.

In man, hypoplastic anaemia is often associated with chronic renal failure (CRF) resulting in a majority of cases from chronic pyelonephritis and chronic glomerulonephritis (Cappell and Anderson, 1971). The anaemia in PKD had many features in common with this type of anaemia. In man, the anaemia is usually normocytic and normochromic, although there may be a slight increase in reticulocytes. Polychromatophilia is unusual. Leucocyte numbers often remain within normal limits, although a neutrophilia may occur. Platelet numbers may be elevated, although platelet function may be severely impaired (Anon, 1975). The bone marrow may be hypercellular but erythropoiesis fails to counteract the anaemia. The

erythrocyte survival time is said to be decreased, especially in the later stages of the disease (Cappell and Anderson, 1971; Anon, 1975; Wintrobe, 1981). Values in osmotic fragility tests remain within normal limits as do serum bilirubin values. A decrease in blood haemoglobin concentration correlates with an increase in blood urea nitrogen (Wintrobe, 1981). The major factor in the development of anaemia in uraemia appears to be decreased erythropoietin secretion (Anon, 1975). This hormone is mainly secreted by the juxtaglomerular apparatus of the kidney nephrons. Exaggerations of the anaemic condition may occur due to haemodilution as a result of impaired fluid and electrolyte balance.

In PKD, the present study demonstrated the anaemia to be normochromic and normocytic as in CRF in man. Unlike the anaemia associated with CRF, polychromatophilia was common. However, it also occurred in uninfected fish and could not, therefore, be said to be a particular feature of the anaemia in PKD. Leucocyte numbers showed wide variation but were often within accepted normal limits. Neutrophilia was present in groups of fish with anaemia. Haemopoietic tissue was present and hypercellular even in severely affected fish. Serum bilirubin levels were within normal limits. Erythrocyte survival times were not examined. However, the increased amounts of Perls'-positive material seen in spleens from some anaemic fish may have represented increased erythrocyte destruction similar to that found in the later stages of human CRF. The marked decreases in PCV values in some fish described in chapter 4 may have been due to the hydraemia of fluid imbalance. Histologically, tubule and glomerulus destruction appeared progressive in PKD with large areas of the excretory kidney involved in the inflammatory reaction around PKX cells (chapter 3). It seemed possible, therefore, that a large proportion of nephrons became damaged with a resultant effect on erythropoietin prod-

uction. Unlike CRF in man, blood urea levels were not consistently elevated in fish with PKD. Some had raised levels and slight anaemia while others had normal levels but severe anaemia. However, in rainbow trout, the gills rather than the kidney are the main excretory organ of urea, and raised blood urea levels are, therefore, more likely to reflect deficient gill function rather than kidney function.

In conclusion, the anaemia of PKD shared features with both those anaemias resulting from haemolysis and those resulting from hypoplasia. As mentioned previously, Roberts (1978) considered the anaemia to be hypoplastic and Hoffmann and Lommel (1984) considered it to be haemolytic. It seems likely that, as with many anaemias, in PKD it has a multifactorial causation. Results of the work described here suggested that it shared more features in common with hypoplastic anaemias than with haemolytic anaemias. Further evidence for this might be derived from faecal urobilinogen levels, erythrocyte survival times using labelled erythrocytes and labelled iron, haemoglobinaemia and haemoglobinuria evaluations and erythrocyte osmotic fragility tests.

Studies of both the pathology and haematology in infected fish at different stages of the disease demonstrated that renal swelling and the identification of PKX cells in kidney impression smears were valuable in the rapid diagnosis of PKD but that severity of infection was more accurately assessed if the haematocrit value was also taken into consideration.

EPIDEMIOLOGY

Infective period and the effects of water temperature

At the experimental site in 1983 0+ rainbow trout were infected from the beginning of May to the end of September (chapter 5). Clinical

disease occurred from mid-July to September. In 1982 fish introduced after mid-July, although infected, did not develop clinical disease. This seasonality was also evident in 1984 and 1985, and field reports from several infected farms in the south of England suggested a similar pattern. The water temperatures recorded at the experimental site and the results of experiments in chapter 5 investigating the effects of water temperature on subclinically and clinically-affected fish suggest that the infective period was determined by the spring rise and autumn fall in water temperatures. It is interesting to note that persistent PKD throughout the year was recorded where water temperatures were maintained between 15-18°C (Schlotfeldt, 1983a,b). As discussed in chapter 5, several parasitic diseases show seasonality associated with rising and falling water temperatures. Members of the Myxosporida are no exception. The temperature range for occurrences of disease caused by Ceratomyxa shasta, a salmonid myxosporidan only found in the north-west of the U.S.A., was recorded by Schafer (1968) and was similar to that of PKD at the Hagerman hatchery described by Smith et al. (1984). Outbreaks occurred from May to November but not at temperatures below 10°C. Infections with the myxozoan Parvicapsula decreased in coho salmon from November to March as temperatures decreased from 10°C to 6°C (Johnstone, 1984).

The results of study 2 chapter 5 showed that at water temperatures between 9-12°C development of clinical PKD in subclinically-infected fish was prevented or delayed and between 7-12°C recovery from clinical PKD was hastened. In fish infected with C. shasta clinical disease did not develop at temperatures below 10°C (Schafer, 1968). In PKD, multiplication of PKX cells appeared to occur in the kidney; the rate of multiplication seemed to be temperature-dependent. The importance of these results in relation to the control of PKD will be considered later.

Host specificity and the spread and maintenance of PKD

At the experimental site, Atlantic salmon, brown trout and rainbow trout became infected with PKX cells (chapter 5). In addition, brown trout inoculated with infected kidney tissue from rainbow trout developed internal evidence of PKD (chapter 6). These results suggested that PKD in the three species may have a similar aetiology. Only rainbow trout developed clinical PKD. Lesions in brown trout and Atlantic salmon were similar to those seen in rainbow trout including haemopoietic hyperplasia, diffuse inflammation and glomerulus and tubule destruction. These changes were not as severe as in rainbow trout with grades 3 or 4 renal swelling. Vascular pathology was limited and no crystals were seen. Furthermore, no losses occurred in the brown trout and Atlantic salmon groups. While it may have been that too few brown trout and Atlantic salmon were examined to find severe lesions, nevertheless, the proportion of rainbow trout severely affected suggested that there was a difference in susceptibility to PKD between the three species. This may reflect a difference in the degree of adaptation of the causative agent of PKD to its host. Nevertheless, clinical disease has been reported in some strains of both Atlantic salmon and brown trout under farm conditions (Ellis et al., 1982, 1985). Other strains were found less affected. This suggests genetic variation exists between species and strains in their susceptibility to PKD.

In the present study intraluminal spore-forms, possibly Myxosporidan, were found in PKD-infected Atlantic salmon and brown trout (chapter 5). Similar forms were found in brown trout inoculated intraperitoneally with a coarse suspension of rainbow trout kidney containing PKX cells (chapter 6). On histological examination, neither uninjected brown trout nor the rainbow trout kidney used for inoculation contained detectable Myxosporidan-

like spores. It was felt that the spores could be a further stage of the PKX cell, similar to those described by Kent and Hedrick (1985a,b,c). Throughout the period of study immature spores were detected in only one rainbow trout. This may again indicate that the host/parasite relationship was not so well adapted in the rainbow trout as in the other species.

In the transmission experiments (chapter 6), no detectable transmission occurred between infected rainbow trout or infected Atlantic salmon and uninfected rainbow trout held in a shared water supply. Histopathological findings (chapter 3) suggested that few PKX cells or spores were present in renal tubules or ureters of infected rainbow trout throughout the course of the disease. These results may indicate that PKX cells do not reach maturity in or, as suggested earlier, are not released from, infected rainbow trout. These possibilities are important when considering the spread and maintenance of PKD.

As described in chapter 1, PKD is widespread throughout the United Kingdom and has been shown to occur annually at many sites. Although PKD-infected rainbow trout are known to have been moved to farms on at least 13 rivers, there is no proof that these rivers have become permanently infected by introducing infected stock. If rainbow trout are non-productive hosts for the PKX cell, then those species where intraluminal spore-forms are encountered regularly may represent the natural reservoir of infection. Apart from rainbow trout, other salmonids found suffering from PKD include Atlantic salmon, brook trout, brown trout, char, grayling, two species of Pacific salmon and steelhead trout. Eighteen species of coarse fish from rivers supplying PKD-positive sites have been examined and the pike was found to be infected (Seagrave et al., 1981; Bucke, 1984). Spores similar to those described by Kent and Hedrick (1985a,b,c) have only been recognised regularly in Atlantic salmon, brown trout,

grayling and steelhead trout. It seems possible, therefore, that in the U.K. wild salmonids are the source of infection for rainbow trout. PKD has been diagnosed in wild brown trout from a river supplying a salmon hatchery where the disease occurred annually (Wootten and McVicar, 1982). Grayling and brown trout are indigenous in most of the rivers known to supply PKD-infected farms. At least four rivers supplying infected farms are known to be regularly stocked with brown trout from infected farms. It may be that movement of infected rainbow trout from one site to another is not important in the spread of PKD, but that movement of other infected salmonids, especially brown trout, is.

Results of field studies in chapter 5 showed that farmed rainbow trout subclinically-infected at the end of summer or surviving clinical PKD had no detectable PKX cell infection the following spring. This implied that they did not become PKD carriers. Nevertheless, it was shown that PKX cells were able to survive in rainbow trout kept at 9°C for many months (chapter 5). Carriage overwinter, therefore, remained a theoretical possibility. The temperature of most rivers in the U.K. dips below 9°C during the winter months. Further experimentation would help establish whether PKX cells could persist in fish held at these lower temperatures.

As wild salmonids are a potential natural reservoir of infection, it is interesting to consider whether they maintain the causative agent over-winter. No work has yet been reported on PKX cell or spore survival in wild salmonids. However, it is interesting to note that a Sphaerospora species, a Myxosporidan, has been found in the kidneys of 104 out of 271 brown trout sampled in Bavaria in an area where PKD is endemic (Fischer-Scherl, El-Matbouli and Hoffmann, 1986). The samples were collected from April to June and from August to November. Although the authors do not consider that there is a link between the two infections, the spore-forms that they describe have similarities to those seen in the present study

and those described by Kent and Hedrick (1985a,b,c). The spores seen in samples collected from April to June may represent residual infections from the previous year.

During the study of the infective period at the experimental site (chapter 5) evidence suggested that infective organisms were in the water from the beginning of May and that their numbers increased during June and July. If the causative agent is found not to overwinter in wild or farmed fish, then an intermediate host may be involved which releases organisms as spring water temperatures increase. Although protozoa of the order Myxosporida generally have direct life cycles (Mitchell, 1977), recent findings suggest that in whirling disease the spores of M. cerebralis become infective only after a period within an aquatic tubificid oligochaete (Markiw and Wolf, 1983). However, in the present study, no intermediate host was identified for PKD.

Resistance to PKD

Little is known about resistance to PKD. Studies have so far been hampered by the inability to culture and purify the causative organism.

Previously unexposed brown trout and Atlantic salmon were apparently less susceptible to clinical PKD than previously unexposed rainbow trout (chapter 5). This suggested that interspecies variation in innate resistance existed. There was some evidence for the development of an acquired resistance in rainbow trout following challenge with the causative organism of PKD (chapter 5). 0+ fish which had developed kidney swelling of grade 2 and above, and those surviving clinical disease did not develop clinical disease when rechallenged as 1+ fish, despite some fish becoming re-infected. Hedrick et al. (1985a,b) found a similar resistance in re-challenged 1+ steelhead trout. No evidence of re-infection was detected

in these fish. Previously unexposed 1+ fish were fully susceptible to the disease as were 1+ fish which had previously been subclinically infected as 0+ fish. It seemed, therefore, that for resistance to occur it was necessary for fish not only to become infected but also to develop at least grade 2 renal swelling. The histological findings (chapter 3) suggested that cell types involved in both cellular and humoral immunity were increased as infection progressed. However, in the 0+ rainbow trout it was only when the disease process was advanced that evidence of PKX cell necrosis was found. There was no evidence of transmission of resistance from broodstock to fry. Fry from broodstock surviving clinical PKD were fully susceptible to the disease.

Antigen purification would greatly enhance further research in this field.

CONTROL OF PKD

Control measures previously used against PKD may be divided into those aimed at delaying the onset of clinical disease and those directed at decreasing mortalities once PKD has occurred. The results of chapters 3, 4 and 5 provide a rationale for these control measures and indicate other measures which might be of use.

With regard to delaying the onset of disease, some success in managing PKD in Northern Ireland was achieved by transferring fingerling rainbow trout from spring or borehole-fed hatcheries to river-fed on-growing facilities in July rather than May (Ferguson and Ball, 1979). This control measure, seen in the light of results in chapter 5, may have been working in two ways. Firstly, by putting fish out in July, PKD may not have had sufficient time to develop to clinical disease before water temperatures started their autumn fall and so curtailed further progress

of the disease. Secondly, fish exposed in July may have developed PKD to the stage where they had grade 2 renal swelling before recovery occurred. These fish would then have gained a resistance, as shown in chapter 5, which would have prevented clinical disease in the following year. However, this management practice proved unreliable in the south of England (Bucke et al., 1981). The results in chapter 5 suggest that, for the procedure to be successful, the date of initial exposure is critical but cannot be judged precisely because of the yearly variation in water temperatures. If fish are put out too early they have time to develop clinical disease; if put out too late they do not develop the disease to a degree which results in a protective response. Furthermore, this method of control would be impracticable for many farms, since fry supplies peak during March and fry suppliers need to have moved their fish onto on-growing sites by June, since spring water sources will be decreasing by that time. In the south of England farms producing fish for the table need to sell most of a year's production by December so that restocking can begin the following spring. This maximizes growth potential and allows stock to reach marketable size by the end of the same year.

The results in chapter 5 suggest that, if borehole or spring water is available, a regime may be devised for decreasing mortalities in the fishes' first summer and for producing fish resistant to PKD in their second summer. For each farm the development of PKD would have to be monitored from kidney smears and grading of kidney swelling. These findings would have to be correlated with daily water temperatures. It would then be possible to predict when grades 2 to 3 renal swelling would be likely to occur. At this stage, the water supply to tanks holding fry/fingerlings for growing on the following summer would be diluted with

the spring or borehole water, which has a temperature of 9-10°C. By reducing the combined water temperature to about 12°C it would be possible to delay further development of the disease until the river water temperatures declined in the autumn. Such fish should be resistant to clinical disease the following year. Farms using large volumes of water would be unable to treat all stock in this way. The amount of borehole/spring water required on a given farm can be calculated from (a) the number and size of fish being held, (b) the temperature of the river supply, and (c) the temperature of the borehole/spring water. For example, the daily water requirement for 100,000 fingerlings at 10g each is approximately 500,000 l (subject to available oxygen in the water). If the river inflow were at an average temperature of 20°C and the borehole/spring water at 10°C, the water supplies would have to be mixed in the ratio 20:80 to give a temperature of 12°C, i.e. 100,000 l river water: 400,000 l borehole/spring water per day. Similar calculations would have to be made for each farm depending on the variables noted above.

With regard to decreasing mortalities once PKD has occurred, measures used include reducing feeding to a maintenance level, avoiding handling of fish where possible, lowering stocking densities, treating secondary infections prior to clinical PKD developing and maintaining the quality of the water supply especially with regard to its oxygen content. The effectiveness of such measures may be explained in the light of the observations in chapters 3 and 4. As PKD developed, the functions of the kidney became impaired, especially that of haemopoiesis. This resulted in a severe, progressive anaemia. Anaemic fish may be expected to be susceptible to decreased oxygen availability and increased oxygen demand. Therefore, measures protecting the oxygen supply and avoiding stress should reduce the problems of anaemia. This may also have the

advantage of decreasing stimulation of the pituitary-interrenal axis, reviewed by Donaldson (1981), and so reduce production of corticosteroids. These hormones are important in the modification of cellular and humoral responses to antigens and generally decrease the effectiveness of such responses in fish (Ellis, 1981), This has been shown experimentally by Anderson, Roberson and Dixon (1982) where rainbow trout injected with a corticosteroid and then challenged with a bacterin derived from Yersinia ruckeri failed to produce antibody titres similar to those in uninjected fish.

Other measures which may be considered include treatment of the water supply and treatment of the fish. It is not known if physical and chemical methods of water treatment, such as ultraviolet light irradiation, chlorination and sand filtration, would be successful in destroying or removing the infective stage of the PKX cell. However, the incidence of the disease caused by C. shasta was reduced when water supplies were treated with combinations of ultraviolet light irradiation and chlorination (Sanders, Fryer, Leith and Moore, 1972), and when water was filtered through 25 µm filters of silica sand prior to ultraviolet light irradiation (Bower and Margolis, 1985). The practicality of such treatments on fish farms was not assessed. However, such methods may merit investigation with regard to controlling PKD.

As described in chapter 2, ichthyophthiriasis was a recurrent problem in fish exposed to PKD at the field site and then returned to the laboratory tanks. When the condition occurred it was treated with a combination of malachite green and formalin. It was noted in chapter 5 study 2 that a decrease in PKX cell numbers coincided with the initiation of such a combined treatment. Preliminary studies have since shown that, although formalin apparently exacerbates PKD, malachite green is effective

in treating PKD-infected, laboratory-held rainbow trout. Further detailed laboratory and field evaluation of the use of malachite green in the therapy of PKD is in progress.

CONCLUSION

It has become evident during research for this dissertation that certain aspects of PKD merit further detailed investigation. Studies of the pathology and haematology of PKD have elucidated the pathogenesis, but the pathogenicity of the PKX cell is still unclear. Isolation and culture of the organism is necessary before this can be studied. This is also required before passive and active protection tests can be undertaken and other aspects of the immunology investigated. Now that laboratory maintenance of the disease has become possible (chapter 6), isolation and culture can be attempted throughout the year.

Studies in chapters 5 and 6 have shown that wild salmonids, such as brown trout and grayling, may be more important in the spread and maintenance of the disease than rainbow trout, and further investigation of their possible role as a natural reservoir of infection is indicated. This would involve studying their disease status in rivers supplying infected farms and using them, rather than rainbow trout, in transmission experiments.

Understanding of the disease process and the ways in which water temperature affects PKD has increased during the research period and has led to the formulation of a practical control measure for managing the disease. However, the line of investigation which perhaps provides the best opportunity for developing a treatment against the disease relates to the use of malachite green. If this dye proves to be therapeutic, and problems related to its toxicity are overcome, then the strategic use of

this chemical, combined with knowledge of the disease, such as when it occurs and how quickly it occurs, should prevent many of the losses caused by PKD.

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Appendix 1

Solutions, reagents and stains

(a) Formulation of 10% neutral buffered formalin.

40% formaldehyde	100 ml
Sodium dihydrogen orthophosphate	40 g
Disodium hydrogen orthophosphate (anhydrous)	6.5 g
Distilled water	900 ml

Chemicals supplied by BDH Chemicals Ltd., Poole, Dorset.

(b) Formulation of Gill's Haematoxylin No.3.

	% by weight
Ethylene glycol	30.0
Aluminium sulphate	4.2
Haematoxylin	0.6
Citric acid	0.14
Sodium iodate	0.06
Distilled water	65.0

(c) Formulation of phosphate buffered saline (PBS).

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen orthophosphate	0.2 g
Distilled water	1 litre

(d) Formulation of modified Dacie's fluid.

40% formaldehyde	10 ml
Trisodium citrate	31.3 g
Brilliant cresyl blue	1.0 g
Distilled water	1 litre

(e) Formulation of Drabkin's solution (following Dacie and Lewis, 1975).

Potassium ferricyanide	20 mg
Potassium cyanide	50 mg
Distilled water	1 litre

The solution is kept in a brown glass bottle in the dark at room temperature.

Appendix 2

Staining procedures

(a) Schedule for staining sections with haematoxylin and eosin.

1. Dewax section in CNP 30 or xylene for 2 min.
2. Second bath of CNP 30 or xylene for 2 min.
3. 100% alcohol for 2 min.
4. 100% alcohol for 2 min.
5. Wash in running tap water until all alcohol is removed. Shake off excess water.
6. Gill's haematoxylin No.3 undiluted for 3 min.
7. Wash in running tap water for 5 min until bluing occurs,
8. Differentiate with 1% acid alcohol (1% concentrated hydrochloric acid in 70% alcohol) for about 10 sec.
9. Wash in running tap water again for 5 min until blue again.
10. Check differentiation has occurred to required degree. If not, repeat stages (8) and (9).
11. Eosin (1% aqueous) for 3 min.
12. Wash quickly with tap water.
13. Dehydrate again through alcohols, 70% 100% 100% 50/50 100% alcohol/CNP 30 or xylene. 1-2 min each bath.
14. Back into CNP 30 or xylene 2 min.
15. Second bath of CNP 30 or xylene 2 min.
16. Mount in DPX (BDH Chemicals Ltd., Poole) under a coverslip.
17. Place on hot plate to dry for about 20 min.

(b) Preparation and staining procedure for kidney impression smears.

Kidney tissue for examination is excised from the sample fish by dissection as described in chapter 2. The cut edge of the tissue is touched lightly against absorbent paper to remove excess blood and then is pressed lightly against a chemically clean microscope slide. Several impressions can be made from one piece of kidney on one slide. The smear is air dried and fixed in 100% methanol for 5-10 min.

Staining schedule (After Disbrey and Rack, 1970)

May-Grünwald stock solution:

3 g May-Grünwald stain powder dissolved in 1,000 ml acetone-free methanol, by heating to 50°C. Allow to cool, shaking intermittently.

Working solution:

2 parts stock solution

1 part distilled water buffered to pH 6.8

Giemsa stock solution:

7.5 g Giemsa stain powder dissolved in 500 ml glycerol at 56°C for 1-2 hr. 500 ml methanol added. Leave for 7 days and then filter.

Working solution:

1 part Giemsa stock solution

9 parts distilled water buffered to pH 6.8

The two working solutions should be freshly prepared.

After fixing the smear in methanol for 5-10 min, the staining procedure is as follows:

1. May-Grünwald working solution 5-10 min.
2. Giemsa working solution 10-20 min.
3. Rinse in several changes of distilled water buffered to pH 6.8.
4. Blot dry.
5. The smear may be examined directly under oil or rinsed in xylene and mounted in a neutral mounting medium such as DPX.