STUDIES ON THE IDENTITY OF THE RODLET CELL IN TELEOST FISH

By

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ABSTRACT

The nature of the rodlet cell of teleost fish has been the subject of speculation since its first published description in 1892. Research since then has described the rodlet cell morphologically and/or histochemically, both at the light and electron microscopic levels. However, whether the rodlet cell is a "parasite" or a normal cell has been a subject of debate, and there has been no consensus on its affinity or function.

While the detailed morphology of the rodlet cell is known, no previous study has considered the ecological parameters of the fish with respect to the presence of rodlet cells. In this investigation, the available literature on rodlet cells was examined, and the 114 teleost species reported to contain rodlet cells classified for geographic range, habitat and food choices. There appeared to be no geographical or habitat pecularities in this group, but a comparison of the food choices of these 114 species with a known, diverse, geographically circumscribed population of 326 species (Wheeler, 1969) indicated that the former had proportionally 60 times more species which were listed as omnivorous, 6 times as many which fed on bottom detritus and 3 times as many which were herbivorous than the latter. If the rodlet cell represented a parasitic organism, such information could have implications for its mode of dissemination.

This investigation has been the first to employ modern molecular biological techniques to explore the nature of the rodlet cell. The DNA

of the rodlet cell nucleus has been compared qualitatively, using hybridization in situ, and quantitatively, using two methods of microdensitometry, with cells known to contain the fish genome but no rodlets. Because the DNA of rodlet cell nuclei hybridizes to the same extent to genomic DNA of the fish as does the DNA of the fish's own cells, and because there appears to be no statistically significant difference in the quantity of DNA in rodlet cell nuclei compared to fish cells of the same species, the rodlet cell itself has been concluded to be of teleost origin.

However, earlier observations in this laboratory had shown a light microscopic "RNA-type" histochemical reaction in the rodlet cores, after both acridine orange and methyl green-pyronin staining methods; furthermore, the cores were Feulgen-negative. The procedures differentiate DNA from RNA in most situations. The nuclease-gold technique of Bendayan (1981) was used in electron microscopic histochemistry to confirm the presence of nucleic acid(s). The rodlets were found to contain DNA, but no RNA. Further studies using S1 nuclease, which is specific for single-stranded DNA, indicated that the DNA of the rodlet labels also with S1-gold. The DNA of the rodlet, in all cases, is confined to the periphery of the rodlet core, and the DNA appears to be spirally distributed and of differing concentration along the length of the core. Because the rodlet core failed to react to the Feulgen procedure and failed to hybridize to genomic DNA, it must contain non-genomic DNA which is in a different conformation from the DNA found in the nucleus of the rodlet cell. Arguments are presented which consider the possible origins of rodlet DNA, and conclude that the rodlet DNA is exogenous, thus supporting the hypothesis that the rodlet, at least, is a "parasite".

This study represents the first time that the rodlet cell and the rodlet itself have been regarded as separate entities. It is postulated that the rodlet-containing cell itself is a teleost cell and the rodlet an exogenous DNA-containing structure. The phylogenetic affinity of the rodlet is unknown, as is the precise nature of the rodlet DNA; speculation includes both single-stranded and Z-DNA. Directions for future research are considered.

ACKNOWLEDGEMENTS

In a study such as this one, where the techniques used to address the problem come from several fields of biology, I have been fortunate to have had superb cooperation from people with expertise in many areas. It is a pleasure to acknowledge their help.

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INTRODUCTION TO THE PROBLEM

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INTRODUCTION TO THE PROBLEM

The rodlet cell of teleost fish has been discovered independently several times since its first description from various tissues and epithelia by Thélohan (1892a, b). Thélohan (1892a) found the cells to be:

> ordinarily ovoid, sometimes a bit irregular; they have a thick enveloping membrane. . . at one of the extremities one sees a nucleus; the rest of the cavity is filled with small, very narrow rodlets which seem to converge toward a point situated most often opposite the nucleus. . . . [The cells] are never intracellular. Their dimensions are not the same in different fish [translation by L. Barber].

Such a description is still valid today, unlike some others which have succeeded it.

Thélohan described the rodlet cells from kidney and intestinal epithelium, ovarian connective tissue, gill epithelium and subcutaneous connective tissue from both freshwater and marine teleosts. He found the rodlet cells ranged in size from 6-9 µm X 4-6 µm in stickleback to 15 X 10-12 µm in the tench. Thélohan commented, "Their parasitic nature seems to me to be almost beyond doubt, but they present such particular characteristics that I have not been able to discover in them [any] affinities with any known form of parasite" (translation by L. Barber). In a more detailed paper, Thélohan (1892b) presented a description of both rodlet cells and the rodlets themselves, but he did not assign a name to the cells, even though he considered them sporozoans.

Laguesse (1895), who was aware of Thélohan's studies, described rodlet cells in the pancreatic ducts of the hepatopancreas of Crenilabrus crenilabrus, concurred with Thélohan's assessment of the rodlet cell as a parasitic sporozoan, named it Rhabdospora thélohani at the suggestion of Henneguy and suggested it was probably a coccidian. In this, he was seconded by Labbé (1896) who included a description and figure of R. thélohani in a treatise on coccidians; in his figure, each rodlet is pictured incorrectly with a tiny nuclear body at the basal end of the rodlet.

Until 1906, no authors suggested that the rodlet cell was other than a parasite. However, in that year, Plehn (1906a,.b) described a cell of the same morphology from the endothelium of the bulbus arteriosus, intestine and kidney duct of the barbel, carp, goby, and trout (scientific names not given); she considered the cell was a unicellular gland, probably endocrine. In a sharp exchange of letters, she defended her opinion against Laguesse (Laguesse, 1906; Plehn, 1906b), but later included a description of the rodlet cell from the bulbus arteriosus of the carp in a volume on fish pathology, in a section devoted to protozoan parasites (Plehn, 1924). Initially stating, "They certainly appeared to be parasites", at the end of the section, having described their appearance and indicated some of the sites of somatic distribution, she concludes, "They must have a mighty task to fulfil, which for the present, is unknown" (translation by L. Barber). The ambivalence is characteristic of the position taken by many subsequent investigators.

The rodlet cell was discovered again by Smallwood and Smallwood (1931) in the intestine of Cyprinus carpio from Lake Oneida (New York,

USA), where it was classed unequivocally as a stage in development of the goblet cell. Duthie (1939) discovered the rodlet cell in the mesenteries of fish in which he was describing the blood cells; he considered it was an end stage in granulocyte development, and that the elongate cytoplasmic rodlets were specific granules coalescing and being expelled. Catton (1948, 1951) observed rodlet cells in the same loci and concurred with Duthie, as, later, did Stolk (1956, 1957) and Weisel (1962, 1973).

In 1949, Al-Hussaini (1949a, b) mentioned "pear-shaped" cells which he had discovered in the digestive tract of several teleosts; he thought, as had Smallwood and Smallwood (1931), that they were a type of mucus-producing cell. The term "pear-shaped" cell has persisted in the literature (e.g., Bishop and Odense, 1966; Iwai, 1968; Hirji and Courtney, 1979), although it has essentially been superseded by the term "rodlet cell" first used by Bullock (1963).

Dawe et al. (1964), investigating hepatic neoplasms in Catostomus commersoni, found rodlet cells associated with the neoplastic epithelium; the authors called them "protozoa" and considered they lay within the neoplastic epithelial cells that were forming ductlike structures. However, close examination of the figures accompanying the paper does not support unequivocally an intracellular position for the cells. Hoffman (in Dawe et al., 1964) expressed the opinion that the "protozoa" were Haplosporidian parasites, although he later considered the cells as normal components of the fish (Hoffmann, personal communication). Dawe et al. (1964) also examined hepatic neoplasms in the brown bullhead, Ictalurus nebulosus, but found no rodlet cells in that species.

Wilson and Westerman (1967) discovered and described "foliaceous

cells" from the olfactory epithelium of Carassius auratus which are morphologically identical to rodlet cells. These authors suggested the cells might be either sensory cells or a type of mucus-producing cell. Ojha and Kapoor (1973) in a study of the olfactory epithelium in a cyprinid, Labeo rohita, figured a cell morphologically identical to the rodlet cell, and called it a "goblet cell"; ironically, they cited Wilson and Westerman (1967) but made no comment on the latter's interpretation of the "foliaceous cell".

Fearnhead and Fabian (1971) found a cell they termed the "rod cell" in the gills of Monodactylus argenteus, an euryhaline, estuarine perciform from South Africa. These authors proposed an osmoregulatory function for the "rod cell", since they noted its disappearance from fish that were experimentally transferred to fresh water. They had found no previous reference to such a cell, and in their opinion, the "rod cell" was probably a functional unit of the gill, possibly specific to M. argenteus. While they considered the possibility that the cell might be a parasite because of the "features not normally found in metazoan cells", they concluded that if indeed it were a parasite, with such a heavy infestation as the fish displayed, the fish should show behavioural changes such as increased respiration, rubbing of the opercula on tank walls or loss of condition, none of which occurred. Morphologically, the "rod cells" of Fearnhead and Fabian (1971) are comparable to rodlet cells of other species. Mattey et al. (1979, 1980), in Britain, examined the gills and pseudobranch of the euryhaline serranid Dicentrarchus labrax from the Plymouth estuary. They noted that the numbers of rodlet cells declined when the fish were moved to water of reduced salinity, and proposed that rodlet cells had an osmoregulatory function, supporting

the conclusions of Fearnhead and Fabian (1971).

Thus, examination of the literature raises several obvious questions about the nature of the rodlet cell and its somatic and ecological distribution:

1) What is the rodlet cell: is it a parasite or a normal tissue component? If the rodlet cell is a parasite, what is its affinity? If it is a normal cell, what is its function?

If the rodlet cell is a parasite, what can be said about its affinities? Some authors consider the rodlet cell as a "parasite" (e.g., Thélohan, 1892a, b; Laguesse, 1895, 1906; Dawe et al., 1964, 1976; Mourier, 1970; Bannister, 1965, 1966; Barber and Westermann, 1975; Barber et al., 1979; Mayberry et al., 1979; Groman, 1982). Attempts to classify the rodlet cell have been unsuccessful; its inclusion in the Sporozoa as a "coccidian" (Thélohan, 1892b; Laguesse, 1895; Labbé, 1897) or an "apicomplexan" (Mayberry et al., 1979) have been rejected by others (Mourier, 1970; Paterson and Desser, 1981) on the basis of electron microscopic evidence. It seems quite clear that rodlet cells, whatever their nature, are not coccidian or apicomplexan parasites. Other investigators have carefully described the rodlet cell in whatever location it happens to occur; e.g., the intestinal epithelium (Bullock, 1963; Bishop and Odense, 1966; Hirji and Courtney, 1979) or the endothelium of the bulbus arteriosus (Flood et al., 1975), but have avoided offering an opinion as to its most likely function or identity.

If the rodlet cell is a normal cell, what is its function? The most prevalent body of opinion considers the rodlet cell to be a normal teleost cell of unknown function. Morrison and Odense (1978) noted that

authors in this category have tended to associate the rodlet cell's function with the particular organ they were investigating. Consequently, seen in the digestive tract, the rodlet cell has been called a mucous (= goblet) cell (Bühler, 1930; Smallwood and Smallwood, 1931; Al-Hussaini, 1949a, b, 1964; Eberl-Rothe, 1953), in the gill as an osmoregulatory (Fearnhead and Fabian, 1971; Mattey et al., 1979, 1980) or mucus-producing (Leino, 1974; Hughes and Byczkowska-Smyk, 1974) cell, in blood cell studies as an end stage in granulocyte development (Duthie, 1939; Catton, 1948, 1951; Stolk, 1956, 1957; Weisel, 1962), in olfactory epithelium either as a type of sensory cell (Wilson and Westerman, 1967) or a mucous cell (Wilson and Westerman, 1967; Ojha and Kapoor, 1973; Kapoor and Ojha, 1974), and in the archinephric and collecting ducts of the kidney as a glandular (Plehn, 1906a) or "secretory" (Desser and Lester, 1975; Morrison and Odense, 1978) cell. Clearly, there is no consensus on the nature or function of the teleost rodlet cell.

Identical evidence has been used by the proponents of both the normal-cell and "parasite" hypotheses to support their positions. The sporadic appearance of rodlet cells in individual fish has been cited as evidence for a parasite (Bannister, 1965, 1966; Barber and Westermann, 1975; Barber et al., 1979), but has been considered a consequence of season (Leino, 1979b), or osmotic (Mattey et al., 1979) or functional (Bullock, 1963; Desser and Lester, 1975) change in a somatic cell. The site variability seen among individuals and species has also been given as evidence of a parasitic nature, but the proponents of the normal-cell hypothesis have regarded the distribution of sites as too broad, pointing

out that most parasites are very site-specific, and even species-specific (Leino, 1974; Desser and Lester, 1975). Neither group has established the taxonomy and life cycles of the "parasite" nor the function of the "normal cell".

2) Is the rodlet cell restricted to the Teleostei? If so, are there any groups lacking the rodlet cell, or do all teleosts show it? There is some doubt that rodlet cells are unequivocally restricted to Teleostei in that some authors have stated that they have been found in hylid frogs (Hirji and Courtney, 1979); Morrison and Odense (1978) examined entire specimens of the spring peeper, Hyla crucifer, and the red-spotted newt, Triturus vulgaris, without finding rodlet cells. Apparently the basis of the notion of rodlet cells in the amphibian came from a mistranslation of a statement by Laguesse (1906). In the original statement, Laguesse indicates Plehn (1906a) has misinterpreted a description he published previously: he had described a cell from the arterial valves of amphibians and says rodlet cells are not equivalent to it. "The constituent cells [of the arterial valves] are generally polyhedral and not ovoid; they do not have the characteristic rodlets" (translation by L. Barber). As well, Stolk (1956) reported cells he called "discharging coarse granulocytes" which he equated with Duthie's (1939) "coarse granulocytes" in a specimen of the elasmobranch Scyliorhinus canicula. Certainly Duthie's "coarse granulocytes" were rodlet cells; there is no pictorial evidence that Stolk was describing an equivalent cell in the dogfish. If it were indeed a rodlet cell, this report would be the first from any organism outside the Teleostei.

3) Are there any common denominators for the somatic sites in which

rodlet cells have been found? Although rodlet cells have been reported as wandering cells of loose connective tissue by some authors (e.g., Thélohan, 1892; Plehn, 1906; Mayberry et al., 1979), Barber et al. (1979) reported they appeared to be confined to epithelia of various kinds. Leino (1974) and Desser and Lester (1975) reported that rodlet cells formed junctional complexes, specifically tight junctions, with adjacent epithelial cells in the gill, but Dawe et al. (1964) and Groman (1982) considered they were intracellular parasites.

4) Is there evidence of common ecological parameters in the behaviour of the fish reported to contain rodlet cells? No investigator has analyzed the matter of ecological parameters which might bear on the distribution of rodlet cells, such as habitat preference, feeding patterns and geographical distribution.

In spite of the length of time the rodlet cell has been known, and in spite of the number of publications on its morphology, the problem of its identity remains unresolved.

The solutions to the above questions are of some interest because rodlet cells have been reported in large numbers in fish kept under aquaculture conditions, or in diseased fish (Dawe et al., 1964; Anderson et al., 1976). As well, this is the first study to attempt to correlate rodlet cell prevalence with ecological parameters of the species reported in the literature to contain rodlet cells. While the literature consists of morphological and histochemical observations, modern methods in molecular biology have not previously been applied to the problem of rodlet cell identity. This study represents the first application of quantitative and qualitative molecular biological techniques for measuring

DNA content, and the first use of the nuclease-gold electron microscopic histochemical technique of Bendayan (1981) to the question of the nucleic acids present in rodlet cells.

THE AIM OF THE INVESTIGATION

The approaches to the problems outlined above take two major forms:

Evidence from Previous Studies

Ecology:

To investigate the question of whether rodlet cells show any common patterns of somatic occurrence and ecological incidence in teleost species, a list of all teleosts reported to contain rodlet cells was compiled, and a survey of their habitat preferences, food choices, geographical distribution and association with other teleosts was undertaken as far as available information on the species exists. In addition, the somatic sites where rodlet cells have been reported were tabulated, and compared to material taken from S. atromaculatus, Catostomus commersoni and Cyprinus carpio during the course of these investigations.

The Molecular Biological Approach

Isolation Experiments:

In an attempt to concentrate the rodlet cells in a mixed cell population, various centrifugal methods were tried, unsuccessfully (see Appendix I). Fortunately, rodlet cells are found in profusion in the gills of the northern creek chub, Semotilus atromaculatus, and so other

methods have been used to give the information ordinarily obtained from isolated cells.

Quantitative DNA Experiments:

If the rodlet cell is a parasite, then its nuclear DNA should be different from that of cells belonging to the host fish. To investigate this question, the DNA of rodlet cell nuclei was examined using microdensitometry after Feulgen staining.

Qualitative DNA Experiments:

If the rodlet cell is a parasite, its nuclear DNA will show sequences qualitatively different from those of the cells of the fish. To investigate this question, DNA-DNA hybridization in situ was carried out.

Nuclease-Gold Experiments:

Preliminary light and electron microscopic histochemical experiments (Barber et al., 1979) indicated that nucleic acid, probably RNA, was present in the cores of the rodlets. To clarify these observations, the nuclease-gold technique (Bendayan, 1981) was used in electron microscopic studies to establish the identity of the nucleic acid(s) in the rodlet cores.

CHAPTER II

MORPHOLOGY, HISTOCHEMISTRY, AND SOMATIC DISTRIBUTION OF RODLET CELLS

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MORPHOLOGY, HISTOCHEMISTRY, AND SOMATIC DISTRIBUTION OF RODLET CELLS

INTRODUCTION

Rodlet cells were first described in detail from the collecting ducts of kidney of a stickleback (identified as Pungitius pungitius, by Grünberg and Hager (1978), although not by Thélohan) as structures 10-12 µm X 5-8 µm, lying between epithelial cells, with the nucleus toward the periphery of the duct and the apical end at the lumen (Thélohan, 1892b). Thélohan also reported rodlet cells in large numbers in the intestinal epithelium, particularly the pyloric caecum, in a small perch (Perca fluviatilis), and also in the carp (Cyprinus carpio) intestinal epithelium, especially in the rectal mucosa.

Many of the descriptions of rodlet cells are incidental to more detailed morphological studies on organs (e.g., Laguesse, 1895; Al-Hussaini, 1949a, b; Fearnhead and Fabian, 1971), although a few morphological or histochemical studies concentrated upon rodlet cells have been published (e.g., Thélohan, 1892a, b; Plehn, 1906; Leino, 1974, 1982; Morrison and Odense, 1978; Barber et al., 1979; Mayberry et al., 1979). The work of Morrison and Odense (1978) represents the most recent survey of histochemical observations reported in the literature, but no one has, until now, examined the complete range of morphometric observations on rodlet cells.

THE QUESTION:

Are there common morphological and histochemical characteristics for rodlet cells in various species? Alternatively, have there been morphological details or histochemical reactions reported which are characteristic of rodlet cells in a group of teleosts, and which might give some indication of the phylogenetic origin of the cell?

While this chapter brings together work that has already been published, it also extends the histochemistry of rodlet cells and enlarges the number of species of teleosts which have been reported to contain rodlet cells. It is the first study ever to have brought the available morphological and histochemical information together. It also attempts to collate the reports on the sizes of rodlet cells in various teleosts, the somatic sites in which they have been found, and the opinion(s) the authors had as to their identity.

MATERIALS AND METHODS

Histological Studies

To extend the information on species containing rodlet cells including Antarctic species and those used for experiments in this study, a survey of somatic sites was carried out on a number of teleosts: the northern creek chub Semotilus atromaculatus and the white sucker Catostomus commersoni; Nototheniids were captured in the Southern Ocean by members of the British Antarctic Survey, and tissues excised and fixed in the field before shipment to Canada for study; Oryzias latipes was obtained from a commercial supplier (Carolina

Biological Supply Company); Rutilus rutilus was obtained as wild-caught specimens from the East Anglia Water Authority in Huntingdon, England.

Histochemistry

Information on the histochemical reactions of rodlet cells from various somatic sites was obtained by searching the rodlet cell literature. The ATP-ase reaction was performed on rodlet cells of Rutilus rutilus in order to demonstrate the presence of myosin, which is an ATP-ase. The ATP-ase method is found in Thompson (1966).

Tables 2-I and 2-II, which are compiled from information in the literature, record the sizes of rodlet cells in various teleost species, and somatic distribution of rodlet cells, respectively.

RESULTS AND DISCUSSION

MORPHOLOGY:

Light Microscopy

In all species, the rodlet cell is usually oval, elongate oval or slightly curved in shape, bounded by a thick refractile cell border and containing a basal nucleus. The cytoplasm contains several to numerous (3-50) (Bannister, 1966; Barber and Westermann, 1975; Al-Hussaini, 1964) elongate, arrow- or club-shaped rodlike structures up to 10 µm in length (Desser and Lester, 1975) depending upon the overall size of the cell. These "rodlets" (e.g., Bullock, 1963; Barber and Westermann, 1975; Mayberry et al., 1979) or "axial filaments" (Bannister, 1966) radiate from the apical end of the cell, extending approximately to the level of the nucleus. In the light microscope it is possible to discern

a very fine, central core in the rodlets, especially in teleost species having large rodlet cells; e.g., Catostomus commersoni (Barber and Westermann, 1975; Leino, 1982). Based upon the Brownian motion of recently discharged rodlets, detergent-treated to remove the peripheral substance, the core is stiff; the apical tip appears very fine as well. The rodlet core frequently displays staining characteristics differing from the rest of the rodlet substance (q.v.).

Table 2-I, a list of morphometric observations from various authors, shows that rodlet cells have different sizes, depending upon the species from which they come. The largest rodlet cells reported are 19-25 μm X 8-10 μm, in C. commersoni and Glyptocephalus cynoglossus (Desser and Lester, 1975; Barber and Westermann, 1975; Morrison and Odense, 1978), and the smallest reported appear to be 6.0 μm X 3.3 μm in Carassius auratus (Flood et al., 1975). Many of the papers reporting rodlet cells give no dimensions for the cells, or indicate their size as a fraction of the height of the other cells in the epithelium (e.g., Plehn, 1906).

The apex of the rodlet cell is at the free surface of the epithelium. Some authors report the presence of a small "stoma" (e.g., Bullock, 1963; Ciullo, 1975) connecting the cytoplasm of the rodlet cell to the lumen. Even the position of rodlet cells in the epithelium has been the subject of controversy. Dawe et al. (1964), in describing the light microscopic morphology of rodlet cells from hepatic neoplasms in C. commersoni, reported it as intracellular in the normal and neoplastic bile duct epithelium, but electron microscopy of rodlet cells in the same species shows that rodlet cells are intercellular. Groman (1982), using only light microscopy for tissues of Morone saxitilis, also was of the opinion

TABLE 2-I

REPORTED DIMENSIONS OF RODLET CELLS

SPECIES	LENGTH um	DIAMETER um	REFERENCE
Catostomus commersoni	25.4	8.5	Desser and Lester (1975)
" "	19.9	9.6	Barber and Westermann (1975)
" "	15	8	Dawe et al. (1964)
" "	13-16	6-9	Chaicharn and Bullock (1967)
Glyptocephalus cynoglossus	19	not given	Morrison and Odense (1978)
Ammocytes sp	16-18	8-10	Labbé (1896)
Tinca tinca	15	6-9	Thélohan (1892b)
Alburnus alburnus	15	10-12	Thélohan (1892b)
Phoxinus phoxinus	15-20	10	Bannister (1965,1966; Thélohan, 1892b)
Cyprinus carpio	12-15	10-12	Thélohan (1892b)
Semotilus atromaculatus	12-15	8-10	this study
Rutilis rubilio	12	8	Mayberry et al. (1979)
Leuciscus cephalus	12	7	Mayberry et al. (1979)
Alburnoides bipunctatus	12	6	Mayberry et al. (1979)
Scopnthalmus maximus	11.5	5	Anderson et al. (1976)
Puncitius punqitius	10-12	5-6	Thélohan (1892b)
Xiphophorus(Platypoecilus)variatus	8	4	Aronowitz et al. (1951)
Perca fluviatilis	6-9	4-6	Thélohan (1892b)
Rutilis rutilis	7.5	4.0	Zapata et al. (1978)
Brachydanio rerio	7.0	not given	Morrison and Odense (1978)
Carassius auratus	6.0	3.3	Flood et al. (1975)

that rodlet cells are intracellular, but most authors (e.g., Thélohan, 1892; Plehn, 1906; Leino, 1974; Barber et al., 1979) consider the rodlet cell lies between the epithelial cells bordering the free surface (Figs. 2-1, 2-2), and even in the light microscope is clearly seen to be intercellular. The rodlet cell border is variously called a capsule (e.g., Bannister, 1966; Morrison and Odense, 1978; Barber and Westermann, 1975), a fibrous border or layer (Mayberry et al., 1979; Leino, 1974) or a fibrous and granular encasing layer (Flood et al., 1975). The nucleus of the rodlet cell is basal, and has been reported as typically spherical or hemispheric. Horseshoe-shaped nuclei in rodlet cells have been reported only from salmonids (Plehn, 1924 (Fig. 115b); Bullock, 1963; Kimura, 1973; Modin, 1981). The nucleus is abluminal. If the rodlet cell is in a squamous epithelium or other confined space (Fig. 2-2), then the rodlet cell appears to lie horizontally, and the nucleus is positioned roughly parallel to the direction of the free surface.

Electron Microscopy

Numerous papers have been published on the fine structure of the rodlet cell (Bannister, 1966; Iwai, 1968; Mourier, 1970; Leino, 1974; Desser and Lester, 1975; Flood et al., 1975; Morrison and Odense, 1978; Barber et al., 1979; Mayberry et al., 1979; Mattey et al., 1979; Paterson and Desser, 1982). The rodlet cell (Fig. 2-3) is an oval cell enclosed in a unit membrane. Kimura (1973) and Desser and Lester (1975) report the presence of tight junctions at the cell apex; Leino (1974) reports that desmosomal junctions are present between rodlet cells and adjacent epithelial cells. Observations in this laboratory concur with both previous reports. Fig. 2-5 shows apparent tight junctions between

the apex of the rodlet cell of the gill of S. atromaculatus and the epithelial cells, and in the gill of C. commersoni, desmosomes have been seen. Immediately under the unit membrane lies a thickened layer 0.8-2.2 μm thick in the mature cell (Mourier, 1970; Desser and Lester, 1975; Flood et al., 1975; Mayberry et al., 1979). The layer consists of circularly disposed filaments 6.8-9.3 nm in diameter and up to 102.3 nm long (Flood et al., 1975; Desser and Lester, 1975; Mayberry et al., 1979), which may be actin on the basis of fibril size, and since the layer shows contractility in vivo (Leino, 1974; Barber et al., 1979). Mourier (1970), on the other hand, claims to have identified four fibrillar regions in the cell border: first, a fine, peripheral, evenly distributed layer he called a "feutrage de fond" (literally, a "bottom feltwork"); second, a band of 20 nm transverse fibres running parallel to the plasma membrane and recurring every 80 nm. These fibres, he reported, showed periodic transverse striae, and were of variable length. Third, fibres of size similar to the second group were seen bundled together at the junction between the vacuolar cytoplasm and the fibrillar border region; and, fourth, local concentrations of fibrils transversely disposed were seen near the cell membrane. None of the fibril groups reported by Mourier agrees with the 7-10 nm fibril dimensions reported by others, but Kimura (1973) reported the presence of 20 nm fibrils in the periphery of rodlet cells of Salmo gairdneri. Figs. 2-3 and 2-5 also show tubular structures associated with the apex of the cell, and the same electron density as the cell border, which appears somewhat thinned in the area. The tubular structures are approximately 80 nm in diameter; their origin and functions are unknown.

The nucleus of the rodlet cell is basal; it is hemispherical, spherical, horse-shoe or cup-shaped, and contains one or two small, central nucleoli; heterochromatin is present as a uniform band at the periphery of the nucleus, interrupted by nuclear pores. Isolated clumps of heterochromatin are also scattered through the nucleus.

The rodlets themselves, depending upon the teleost species, may be up to 10 μm in length, and individual rodlet cells may contain up to 50 rodlets (Bannister, 1966; Barber and Westermann, 1975). In general, the rodlet consists of two components: a narrow, elongate, slightly tapering electron-dense core running the entire length of the rodlet, and a surrounding coat which appears histochemically to be composed of a neutral to acidic glycoprotein (Leino, 1979, 1982). The coat is about half the thickness of the core material at the apical end of the rodlet, but broadens out into an oval, spherical or rhomboidal structure just above the level of the nucleus. In some species, and perhaps with some types of preparations, the rodlet core has shown a crystalline substructure (Desser and Lester, 1975); in other species, it appears to be uniformly electron-dense, and, along with the peripheral substance, amorphous. The rodlets are themselves membrane-bound, but the cores are not. The position of Morrison and Odense (1978) that the term "rodlet" applies only to the electron-dense core does not concur with earlier descriptions, and has not been adopted by subsequent investigators.

The rest of the cytoplasm contains large, electron-lucent cisternae of rough endoplasmic reticulum (RER) and moderately electron-dense bands of ribosome-containing material forming a meshwork which extends from

the inner side of the contractile layer to the membrane surrounding the rodlet peripheral substance. The vacuolar cisternae contain an electron-lucent or lightly flocculent substance; they are not empty as Desser and Lester (1975) reported. In observations on living rodlet cells, considerable fluid is discharged when the rodlets are expelled; it may be that the cytoplasmic meshwork and the fluid-filled vacuoles provide intracellular support for the rodlets.

When mitochondria have been reported in the rodlet cell, they have been described as having fewer and thicker cristae in mature rodlet cells than in young stages (Desser and Lester, 1975; Paterson and Desser, 1981) and have been generally found in the apex of the cell interspersed among the thin ends of the rodlets. In the species examined in this study, only early stages of rodlet cell development, having few rodlets, narrow cell borders and large Golgi complexes contain mitochondria scattered through the cell. Mature rodlet cells show either numerous whorled, membranous structures which may be degenerated mitochondria or smaller vacuoles, and tubular structures already described are associated with and perhaps replace some of the apical cell border (Fig. 2-3). Apical structures are expelled when the rodlet cell border contracts, along with the rodlets.

Sometimes a large Golgi complex is seen, especially in early stages; it lies to one side of the distal portion of the rodlet cell nucleus (Fig. 2-4); the Golgi complex may produce the peripheral substance of the rodlet, since it and the rough endoplasmic reticulum are known to be involved in adding carbohydrates to protein in other cells (Ham, 1980). Rodlet peripheral substance consists of glycoproteins. However, direct evidence of the association of the Golgi complex with the rodlet

peripheral substance is presently lacking.

Morphological studies show variations in the structure of the rodlet cell apex. In some species, a single, thick, finger-like projection covers the apical opening (Leino, 1974). This projection is approximately the same height as the microvilli of the adjacent intestinal epithelial cells. In other species, and in other tissues, the apical region may be smoothly oval (Mattey et al., 1979, in the pseudobranch of Dicentrarchus labrax), broadly tapering (Mayberry et al., 1979, in the stomach of Leuciscus cephalus), or with a few microvilli (Paterson and Desser, 1982, in the intestine of Notropis cornutus and a similar appearance in rodlet cells from the intestine of rainbow trout (Kimura, 1973)). Fig. 2-5 shows a rodlet cell apex with several projecting structures which may be part of the microridge patterns associated with the mucus-covered surfaces of teleost gills. The significance of the apparent variety or surface features in rodlet cells is not known.

In some species, the basal region is also of interest: there are sometimes broad extensions of the basal region into the surrounding tissue (Fig. 2-6). The "capsular" layer is thinner in this region, sometimes has been found to contain dense-core vesicles (Kimura, 1973) and the cell base is often vacuolated. In light microscopic preparations of tissues from Catostomus commersoni there was frequently a space at the cell base, and in electron microscopic preparations from the same species myelin figures were sometimes seen (Barber et al., 1979); both could represent preparation artefacts caused by fixation or dehydration, since these features have not been seen in all species.

DISTRIBUTION:

From the present studies, and from examining the precise orientation of rodlet cells in various sites, it would appear that rodlet cells are almost exclusively found in epithelia. The epithelia may be external: in the skin, fins or gill, or internal: in vascular endothelium, coelomic mesothelium, or intestinal or duct epithelia. The shapes of the cells comprising the epithelia appear not to be a determining factor in the presence of rodlet cells, although rodlet cells are not often found in stratified squamous epithelia. Assertions by some authors (e.g., Thélohan, 1892b; Plehn, 1906) that rodlet cells migrate freely through connective tissue are difficult to confirm, and serial sections would be required to establish unequivocally that the cells are not associated with a free surface, either external or vascular, which is absent from the section being examined.

Table 2-II is a list of sites from which rodlet cells have been reported since 1892. Since most of the reports of rodlet cells have been incidental to histological investigations of other systems; e.g., the digestive tract, Table 2-II may reflect more the trends of teleost histological research since 1892 than rodlet cell distribution.

Observations in this laboratory on C. commersoni and S. atromaculatus indicate that rodlet cells are widely distributed, but that there may be variations among species in terms of "usual" locations. Numerous reports mention large numbers of rodlet cells in the intestine (e.g., Leino, 1974; Desser and Lester, 1975; Mayberry et al., 1979); however, examination of the intestine of S. atromaculatus has shown that even individual fish that have many rodlet cells in the gill have none

TABLE 2-II

MAJOR SITES OF DISTRIBUTION OF RODLET CELLS

SITE	PROPOSED IDENTITY	REFERENCE
DIGESTIVE SYSTEM		
- Pharynx	parasite	9
- Esophagus	secretory or gland cell	47
-Intestine, Stomach, Pyloric Caeca	parasite	13, 27, 44, 48, 62, 76, 110, 111
	mucous or goblet cell	2, 3, 20, 31, 70, 71, 101, 113, 114, 115
	secretory or gland cell	31, 47, 54, 67, 89, 91, 92, 79, 98, 117
	granulocyte	5, 24, 25, 26, 37, 79, 101
	no opinion	1, 15, 21, 22, 27, 38, 60, 61, 80, 130
-Pancreatic Duct	parasite	44, 65, 66
-Liver, Gall Bladder, Bile Duct	parasite	4, 24, 31, 44
	secretory cell	45, 47
KIDNEY		
	parasite	4, 9, 10, 44, 52, 76, 83, 111, 116
	granulocyte	25, 37
	secretory cell	37, 45, 70, 91, 92
CONNECTIVE TISSUE		
	parasite	8, 9, 44, 110, 111
	secretory cell	37, 82, 91, 107

Table 2-II cont'd

SITE	PROPOSED IDENTITY	REFERENCE
CIRCULATORY SYSTEM		
	parasite	46, 61
	secretory cell	34, 37,45, 47, 91, 92, 93, 120, 129
	no opinion	40
GILL/PSEUDOBRANCH		
	parasite	10, 11, 44, 61, 76, 110, 111
	secretory/osmoregulatory cell	34, 39, 70, 74, 75, 67, 82
	mucous cell	56, 70, 89
	granulocyte	24, 25, 37, 79
	no opinion	115, 130
OLFACTORY EPITHELIUM		
	parasite	7, 8, 18, 44, 67, 86
	mucous or secretory cell	7, 59, 71, 86, 127

In addition to the above sites, rodlet cells have been reported from neural and meningeal tissue (8, 9), eye (9), and gonad (9, 91, 110, 111). It is not clear whether these reports represent situations in which the rodlet cell is associated with blood vessels in the tissue or not. A few authors (21, 130) have been vague about precisely which of the examined tissues contained rodlet cells.

in the intestinal epithelium. Similarly, S. atromaculatus usually has large numbers of rodlet cells in the gill (Fig. 2-7), in connective tissue of the fin, in the endothelium of blood vessels in most organs, and in kidney collecting tubules. However, observations on Antarctic Nototheniids have shown rodlet cells common in kidney tubules, but very rare in the gill epithelium. Morrison and Odense (1978) in their review of rodlet cell distribution and morphology, emphasizing marine teleosts, especially Gadus morhua, found few or no rodlet cells in the gills. However, Fearnhead and Fabian (1971) noted very large numbers of rodlet cells in the gills of Monodactylus argenteus, a marine/estuarine species, and Mattey et al. (1979) found a similar situation in seawater-adapted Dicentrarchus labrax, gill and pseudobranch. Groman (1982), in his studies on the striped bass Morone saxitilis, reported rodlet cells in gill lamellae, olfactory epithelium, intestinal mucosa, the pancreatic and bile ducts, mesenteries, kidney tubules, urinary bladder epithelium, and the lateral line. The fish he examined were hatchery-reared; the other marine species mentioned here were wild-caught. Such disparities suggest there may be species-specific variations in the somatic distribution of rodlet cells; there is no information on what conditions may influence their presence.

HISTOCHEMICAL OBSERVATIONS

Light Microscopy

Several histochemical studies have been carried out on light microscopic preparations of rodlet cells (Plehn, 1906; Bullock, 1963;

Barber and Westermann, 1975; Morrison and Odense, 1978; Leino, 1982). Most investigators have regarded the rodlet as a uniform cytoplasmic body, and have reported staining reactions for the entire structure. Plehn (1906) applied stains for nucleic acids (haematoxylins, safranin) and protein (bleu de Lyon, eosin, haemalum), pointing out that the rodlets did not stain well with most stains. Bullock (1963) and Morrison and Odense (1978) showed that neutral polysaccharides in the rodlets were stained by the periodic acid-Schiff procedure (PAS), but that rodlets failed to react to lipid stains (Baker's acid haematin, Sudan black B), to some polysaccharide stains (Rinehart/Abul-Haj colloidal iron procedure) to nucleic acid procedures (thionin, azure C, Mallory's phosphotungstic acid haematoxylin), or to procedures for basic proteins (Sakaguchi technique for arginine). Bullock (1963) and Ciullo (1975) also carried out procedures for alkaline phosphatase, which indicate the presence of lysosomes, with negative results. Grünberg and Hager (1978) found no reaction of rodlets to haemalum or Delafield's haematoxylin, thus confirming results reported by Plehn (1906); they did report "a typical xanthoprotein reaction" with Van Gieson's stain and staining with Weiger's resorcin-fuchsin. Ciullo (1975) reported positive staining for proteins (fast green FCF, green with Masson's trichrome), neutral polysaccharides (PAS) but not acidic polysaccharides (alcian blue) although he did obtain staining with aldehyde fuchsin which may also stain sulphated mucosubstances (Pearse, 1983), and obtained variable responses to the Baker acid haematin procedure for phospholipids, leading him to speculate upon "different species of rodlet cells".

A few investigators recognized that the rodlet could be seen to consist of two components, even in the light microscope, and divided

their histochemical observations accordingly into reactions for the peripheral substance or sheath, and the rodlet "core". Barber and Westermann (1975) found entire rodlets, peripheral substance and core from C. commersoni, stained with PAS, fast green FCF, orange G and eosin, and that the cores of rodlets were pink after methyl green/pyronin and red after acridine orange, both procedures which demonstrate RNA or depolymerized DNA. Morrison and Odense (1978) showed general reactions of rodlets to proteins (eosin, Van Gieson's stain, mercury bromphenol blue), neutral polysaccharides (PAS), but no reactions for glycogen (Best's procedure for glycogen), argentaffin substances (using azo coupling) or lactic dehydrogenase. There is some uncertainty in recording the observations of Morrison and Odense (1978), because of their interpretation, mentioned above, of the structure of the rodlet itself.

The most intensive histochemical study on rodlets and rodlet cores is that of Leino (1982), who clearly differentiated between the reactions of the rodlet peripheral substance and those of the rodlet core. He found that rodlet peripheral substance contains glycoproteins with mannose, galactose or fucose sidechains (1,2-glycol groups), since the PAS staining is almost blocked by acetylation and restored by saponification. Leino (1982) has been the only investigator to report the presence of acid polysaccharides in rodlets, using alcian blue at pH 2.5, although other authors have applied this stain to rodlet cells of the same (Barber and Westermann, 1975) or different (Bishop and Odense, 1966) species without observing a positive reaction. Rodlets failed to react to the same stain at pH 1.0. Both strong and mild methylation abolished the alcian blue staining, but Leino remarked only

that the rodlet peripheral substance probably contained sialomucins, even though the methylation procedures cited also demonstrate the presence of sulphate groups and mucins. He found no reaction of rodlets to lipid stains, in agreement with Morrison and Odense (1978), Bullock (1963) and Barber and Westermann (1975), even after glutaraldehyde fixation. Leino also found no staining for nucleic acids using methyl green/pyronin applied to glutaraldehyde-fixed, methacrylate-embedded sections, or reactions to gallocyanin procedures which stain acid groups, especially nucleic acids. He obtained a strong reaction for proteins with basic groups (mercury bromphenol blue) and cyclic sites in amino acids (coupled tetrazonium), but very little reaction for basic proteins alone (Biebrich scarlet, pH 9.5, fast green FCF at pH 8.0), and only at high pH (pH 7.0) did the peripheral substance of the rodlet react to the azure A in a combined azure A/eosin stain. Thiazine dyes, of which azure A is one, tend to stain normally acidophilic proteins at high pH (Morrison and Odense, 1978).

Leino found the rodlet cores unreactive to all procedures except those for proteins: mercury bromphenol blue, Biebrich scarlet, coupled tetrazonium, PAS/light green, azure A/eosin. The rodlet cores failed to show reactions for lipid, polysaccharides/glycoproteins, or nucleic acids.

Leino's observations are in general agreement with those of other investigators, but at odds in specific but rather important details: for example, in the matter of reactivity to nucleic acid and phospholipid procedures. The methyl green/pyronin stain used by Barber and Westermann (1975) was performed on touch preparations, and observations made on

entire, isolated rodlets, not on plastic-embedded, sectioned material. Leino did not use acridine orange and fluorescence microscopy. Both Morrison and Odense (1978) and Ciullo (1975) obtained aldehyde fuchsin reactivity in rodlets, indicating the presence of phospholipids (Pearse, 1983), although all authors agree that sudan black reactivity for lipids is absent.

While rodlet cells have been generally unreactive to procedures for demonstration of enzymes, the contractile border of the rodlet cell is strongly reactive for ATPase, indicating the presence of myosin (Fig. 2-8). This is the first enzyme that has been demonstrated in rodlet cells, and it substantiates the idea that the contraction of the cell border is mediated by actin and myosin, even though the actin fibres are not arranged as for either skeletal or smooth muscle.

Electron Microscopy

In the electron microscope, Leino (1979, 1982), using Epon-embedded thin sections, confirmed the presence of 1,2-glycoproteins in the rodlet peripheral substance by the use of periodic acid-silver methenamine. In this procedure, the rodlet cores are unreactive, although the control procedure, without periodic acid, shows uniform nonspecific deposition of silver grains over the rodlet. Digestion of thin sections with pepsin removed material from the cores of rodlets, leaving them unstained after uranyl acetate/lead citrate. Mattey et al. (1979) differentiated rodlets from mucous granules by incubating Epon-embedded thin sections in 1% H_2O_2 for 30 minutes and then staining in 15% phosphotungstic acid for 2 hours; mucous granules showed an electron-dense deposit, and rodlets were unstained. Periodic acid followed by

phosphotungstic acid in 10% chromic acid showed reactions similar to the periodic acid-silver methanamine reaction in that the rodlet peripheral substance and mucous granules were both stained, indicating the presence of 1,2-glycoproteins.

THE ANSWER

The published literature on the morphology and histochemistry of rodlet cells does not give compelling evidence for either a normal cell or a parasite origin. Rodlet cells appear to vary in size according to the species in which they are found, but many normal cell types; i.e. erythrocytes, do the same. While the rodlet cell apex appears to vary according to the epithelium in which it is situated, and the nuclei of rodlet cells in salmonids appear to have a common morphology, the rodlet cells show inconsistent somatic distribution from species to species. This calls into question any hypothetical normal functions which might be attributed to the cell in a particular locus.

There would appear to be no histochemical characteristics unique to a particular group of teleosts, or providing any indication of possible function or phylogeny. The histochemical observations on rodlets and rodlet cells show that, with very slight variations, all previous reports on rodlets indicate a neutral or slightly acid glycoprotein peripheral substance. There have been no enzyme reactions reported. The core is protein with different, usually slightly enhanced, staining reactions; it probably contains some kind of nucleic acid, probably RNA or depolymerized DNA. The latter observation has been made doubtful because the nucleic acid has not been identified histochemically in sectioned preparations (Leino, 1982). This matter is pursued further in Chapter 6 of the thesis.

PLATE I

Fig. 2-1: Section of archinephric duct of Notothenia rossii showing rodlet cell (rc) lying at the lumen of the tubule between two columnar epithelial cells (arrows). The basal nuclei of the epithelial cells are indicated (n). The nucleus below the rodlet cell (n_2) is probably that of a wandering leukocyte. Hematoxylin and eosin. X 1500

Fig. 2-2: Section of gill filament of Semotilus atromaculatus showing portions of two rodlet cells (rc) positioned obliquely in the gill epithelium. The rodlets tend to be oriented perpendicular to the free surface. A mucous cell (mu) is seen adjacent to one rodlet cell. Hematoxylin and eosin. X 1500

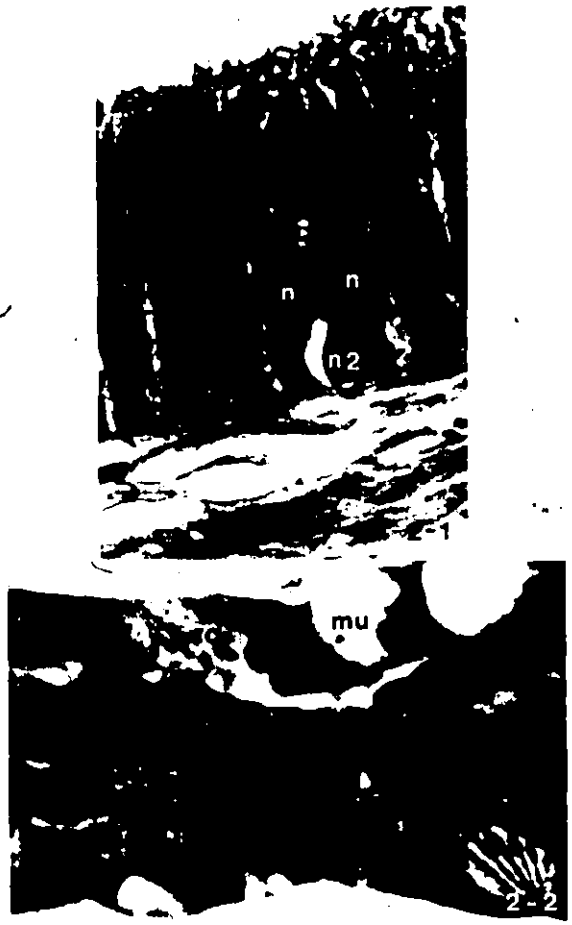


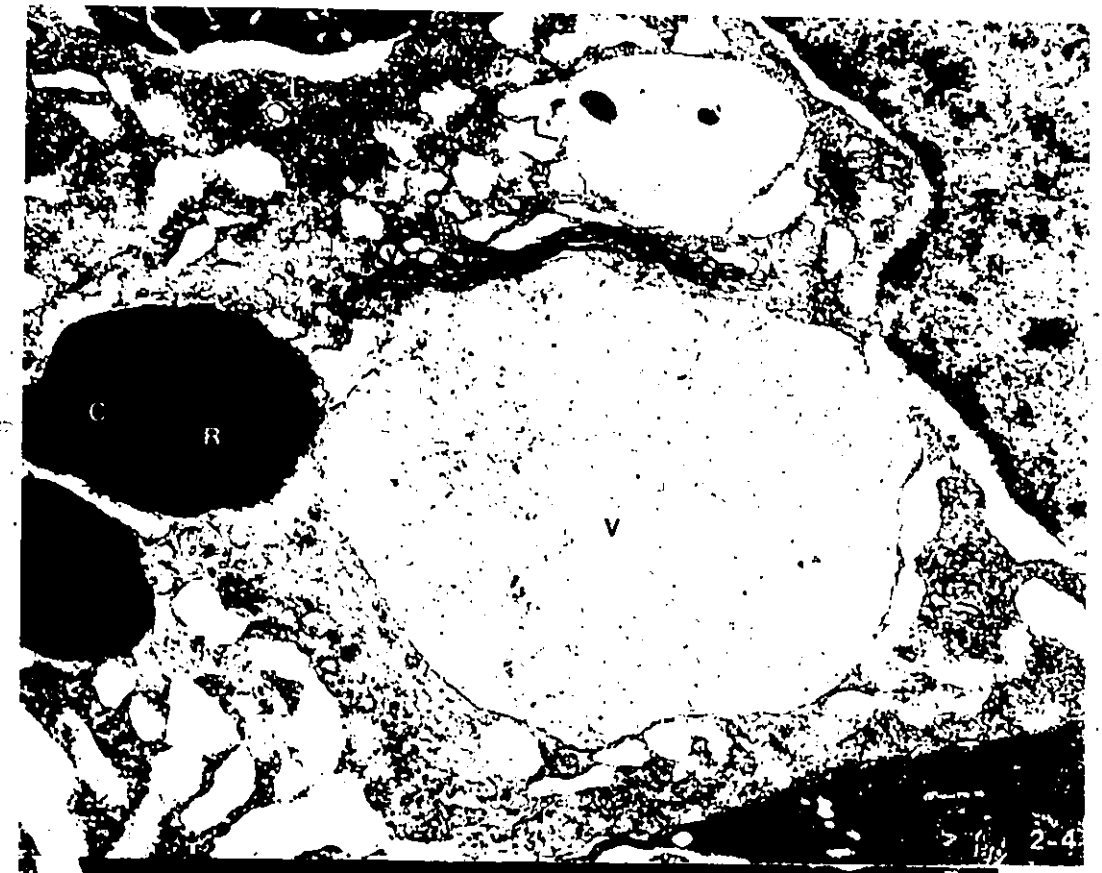
PLATE II

Fig. 2-3: Electron photomicrograph of a longitudinal section of a rodlet cell, showing the basal nucleus (N), wide cell border (B), and apical region (A). The rodlets (R) show an electron-dense core (C) and are seen lying in vacuolated cytoplasm. The thinned border at the cell base is indicated (arrow). The rodlets appear more electron-dense than is usually seen because of pre-treatment with RNAse A. Epon embedding; uranyl acetate and lead citrate stain. X 18,750

PLATE III

Fig. 2-4: Electron photomicrograph of young rodlet cell showing a thin cell border (B), two rodlets (R) with slightly more dense cores (C) and a large Golgi complex (GA) adjacent to a large vacuole (V) at the releasing face of the complex which lies adjacent to one rodlet and contains flocculent material. Smaller vacuoles (v) are also seen at the forming face of the Golgi complex; these contain electron lucent material. The nature of the large vacuolar structure lying between the forming face of the Golgi complex and the nucleus (N) is not known. X 29,900

Fig. 2-5: Apical region of rodlet cell from gill of Semotilus atromaculatus, showing rodlets (R), the cell border (B), and what appear to be microvillar or microridge structures at the cell apex (arrows) projecting from the free surface of the cell. The rodlet cells appear to be associated with adjacent epithelial cells by means of tight junctions (J). Elongated and whorled structures adjacent to the rodlets are thought to be the remains of mitochondria. The rodlet cell was previously treated with RNAse A. Uranyl acetate and lead citrate stains. X 30,000



2-4

2-5

PLATE IV

Fig. 2-6: Basal region of rodlet cell near the nucleus (N) showing extension of cell border (B) containing vacuoles (V). Unstained, unosmicated Epon section. X 42,000

Fig. 2-7: Section through gill filament of S. atromaculatus showing portions of 10 rodlet cells (rc) either contiguous or separated from each other by one or two cells. A mucous cell (M) is seen in the lower left corner. Epon section, unosmicated; uranyl acetate and lead citrate stains. X 7,700

2-7

PLATE V

Fig. 2-8: Rodlet cell (rc) from gill of Rutilus rutilus. The imprint preparation was treated to demonstrate ATPase; the border of the rodlet cell reacts very strongly (arrows), while other cells do not. The nucleus of the rodlet cell (n) is faint, and both ends of the rodlet cell show thinning of the cell border (<). No counterstain. X 1250

CHAPTER III

ECOLOGICAL PARAMETERS:
GEOGRAPHICAL DISTRIBUTION, HABITATS AND FOOD CHOICES
OF TELEOSTS REPORTED TO CONTAIN RODLET CELLS

CHAPTER III

ECOLOGICAL PARAMETERS: GEOGRAPHICAL DISTRIBUTION, HABITATS AND FOOD CHOICES OF TELEOSTS REPORTED TO CONTAIN RODLET CELLS

INTRODUCTION

While the morphology of the rodlet cell from several species of fish is well-documented in both light and electron microscopy, investigation of the ecological relationships of species known to contain rodlet cells has never been reported.

THE QUESTION

1) Is there any discernible phylogenetic pattern for the presence of rodlet cells?

2) Are there limiting factors of habitat, geographic range or food choices which may bear upon the incidence of rodlet cells in a species?

These questions were investigated by collating all the available reports of the incidence of rodlet cells with published information on the habitats, geographical distribution and food choices of the species.

MATERIALS AND METHODS

The information for these studies was collated from three types

of material:

1) Information on Species

The literature on rodlet cells since 1892 may be divided into two categories. The first is a group of published papers in which the authors have recognized the rodlet cell as a distinctive cell type, whatever they may have named it: "Stäbchendrüsenzell" (Plehn, 1906), "pear-shaped cell" (Al-Hussaini, 1949; Hirji and Courtney, 1979), "rod cell" (Fearnhead and Fabian, 1971), "apicomplexan parasite" (Mayberry et al., 1979) or Rhabdospora thélohani (Mayberry et al., 1979). A second literature source has been papers in which the rodlet cell has been figured, but identified as a stage of development of an already-recognized type of cell: Ojha and Kapoor (1973) and Hughes and Byczkowska-Smyk (1974) identified the rodlet cell as a stage in mucous cell development; Duthie (1939), and Catton (1948, 1951) considered it to be an end stage in blood granulocyte development. For both literature sources, the identification of the rodlet cell has been confirmed by examining line drawings or photographs.

For the phylogenetic survey of fish with and without rodlet cells, the classification and terminology used is that of Greenwood et al. (1969). Where possible, the modern names of species or genera have been given, but the names used in the original reports have also been included in square brackets (Tables 3-I and 3-II). Where rodlet cells have been reported for the first time from a species investigated in this laboratory, there is no reference number after the name in the tables; all other reports have a reference.

2) Information on Habitat and Food Choices

Information on habitat, geographical distribution and food choices was obtained from the following papers, atlases, and checklists: Axelrod et al. (1977); Burchett et al. (1983); CLOFNAM (1973): Grizzle and Rogers (1976); Lagler et al. (1977); Lee et al. (1980); Maitland (1977); Migdalski and Fichter (1976); Newdick (1979); Scott and Crossman (1973); Smith (1950); Targett (1981); Tarverdiyeva and Pinskaya (1980); Wheeler (1969, 1975). For some species, there appears to be no published information on habitat or food choices. Most authors have not indicated the degree of maturity of the individuals examined; unless indicated otherwise, the assumption has been that they are adult fish. Finally, unless the authors have stated that the individuals examined were hatchery-reared or aquarium specimens, the assumption has been that the species were caught in the wild.

Table 3-I is a survey of geographic range, and habitat and food choices for all the teleost species reported to contain rodlet cells. Table 3-II is a similar compilation for species in which rodlet cells have been sought, but not found. Table 3-III is a comparison of reported food choices of species from Table 3-I with those reported in Wheeler (1969) from Britain and Northwest Europe. This atlas catalogues a complete, geographically circumscribed fauna of both marine and freshwater species, and Table 3-I lists a geographically diverse mixture of freshwater and marine fish. It was felt that comparisons of teleosts in Table 3-I with a known teleost fauna (Wheeler, 1969) would make it possible to ascertain whether the habitats and food choices of species

with rodlet cells were characteristic of a general population of teleosts, or whether some obvious peculiarities exist in rodlet cell-containing species.

RESULTS AND DISCUSSION

Since 1892, approximately 114 species of teleosts from 43 families have been confirmed as containing rodlet cells in one or several body organs (Table 3-I).

Rodlet cells are found in evolutionarily diverse groups of teleosts, including the "primitive" Elopomorphi, as well as the "advanced" Percomorphi (Greenwood et al., 1969). The majority of fish reported in Table 3-I are found in the freshwater and marine environments of North America and Europe. Ostariophysi seem to have been particularly well represented; however, this should not be surprising since the group comprises a large proportion of the freshwater teleosts of North America. As well, in Europe, many freshwater teleosts are either ostariophysans or perciforms (Lagler, 1977).

The first reports of rodlet cells in the Antarctic teleosts Harpagifer bispinis, Parachaenichthys georgianus, Notothenia neglecta, N. larseni and N. (Trematomus) vicarius are presented in Table 3-I. The sole previous reports from this large, biogeographically isolated group are from the gill of Chaenocephalus aceratus, where the rodlet cell was not recognized as such (Hughes and Byczkowska-Smyk, 1974), and gills of N. rossii (Westermann et al., 1984). Rodlet cells were identified in the stomach of an Antarctic specimen of Borostomias antarcticus, a salmoniform lanternfish, found in deep, cold waters of latitudes higher

Glossary of Abbreviations used in Tables 3-I and 3-II

A - Adult

Alg - Algae

C - Crustaceans

F - Fish

I - Insects

IL - Insect Larvae

Inv - 'Invertebrates', not specified

J - Juvenile (after postlarval stage, but before sexual maturity

L - Larvae

M - Molluscs

Pl - Plankton (both Zooplankton and Phytoplankton)

Omn. - Omnivore

Sm - Small

Sq - Squid or other cephalopod

W - Worms

Y - Young (hatching to postlarval stage)

TABLE B-I

Teleost Species Reported to Contain Rodlet Cells: Distribution, Habitat, and Food Choices

(references are in brackets)

TAXONOMY	DISTRIBUTION	HABITAT	FOOD CHOICES
Superorder ELOPOMORPHI Anguilliformes			
Anguillidae			
Anguilla anguilla (vulgaris (46, 67) and fluviatilis (13)	Europe	Catadromous. Ylaarine,benthic A: Streams, estuaries, mud bottoms (28,73, 124)	I, C, F
A. rostrata (2)	North America	streams, estuaries, mud bottoms (124)	I, C, F
Congridae			
Conger oceanicus (82)	N. Atlantic	bottom, shallow water to 260 m (73, 124)	F, C, Sc
Superorder CLUPEOMORPHA Clupeiformes			
Clupeidae			
Clupea harengus harengus (82)	N. Atlantic	pelagic; at surface (78,124)	Y: Pl, C, F A: C, F, selected Inv.
Superorder OSTEOGLOSSOMORPHA Osteoglossiformes			
Osteoglossidae			
Osteoglossum bicirrhosum(45)	S. America, Amazon Basin	still, shallow, reedy backwaters; spawning near surface. Often large aquarium fish (6,124)	F,C
Superorder PROTACANTHOPTERYGII Salmoniformes			
Salmonidae			
Salmo trutta (45)	mid-basin, Damuce River	upper reaches of river, lakes. Very rare; survival doubtful (73, 124)	Y: Inv., F A: F, esp. Chondrostoma

Salmo gairdneri (21,45,61,80,82,91)	West Coast North America, Introd. elsewhere	oligotrophic streams, lakes Most reports from hatchery spms. (97)	L, C, F
S. trutta (21, 24)	Europe, Introd. to North America	fast streams, oligotrophic lakes, sea (68, 124)	Y: I, IL, C, F A: F, C
S. salar (21)	North America	Y: streams. A: pelagic, sea (124)	Y: any available food; not especially selective. A:C, F.
Salvelinus fontinalis (21, 82)	Eastern North America	clear, cold streams, lakes (68, 97)	Omnivore, incl. Alg., I, F.
S. namaycush (70)	North America	deep, cold lakes, rivers (68, 97)	Broad range: Pl to I, F to nice.
Thymallus thymallus (45)	N. of Pyrenees. N. England to USSR	large lakes; cool, swift unpolluted rivers. Often in shoals or pairs (124)	Omn., bottom Inv. terrestrial I and Occ. mammals.
Oncorhynchus kisutch (80)	Western North America	anadromous; inshore rivers to ocean (68, 124). Report from hatchery specimens.	Y: I, F, C, Sq A: F, C, Sq
O. tschawytscha (80)	Western North America	anadromous; Y: large rivers, lakes, A: N. Pacific. Migrates far upstream. (68, 97) Report from hatcheryspm.	Y: I, C, IL A: F, Sq, C
Esocidae			
Esox lucius (70)	Circumpolar, northern hemisphere, south to mid-USA	shallow lakes, quiet rivers, inshore near vegetation (124)	Y: Inv., esp. C,IL A: F, Amphibs.
Astronesthidae			
Borostomias antarcticus (76)	Polar Oceans North or South of 40°	mesopelagic or bathypelagic (23)	unknown
Superorder OSTARIOPHYSI:			
Cypriniformes			
Catostomidae			
Catostomus catostomus (123)	E. Siberia, Canada Northern USA	on bottom, stillwater, slow rivers (63)	Y: Pl, diatoms A: Bottom Inv.
C. commersoni (10,11,26,30,31,34,70)	Eastern Canada, Northern USA	Y: surface, pelagic A: bottom, warm shallow bays, lakes. (68, 124)	Y: surface Pl A: IL, I, C, M
Cyprinidae			
Abramis brama (45)	Ireland to Aral Sea. Arctic Ocean to Black/Caspian Seas	Y: midwater A: warm, stagnant water, slow rivers, lakes, estuaries Bottom dwellers.(73, 124)	Y: Pl, C A:Inv., esp. M, M, IL

Alburnoides bipunctatus (76)	Cont. Europe	swift water, both shoals and deep.(73, 124)	Zooplankton, I, C.
Alburnus alburnus (111)	Europe	shoaling surface feeder, rivers. (73, 123)	Pl, C, I, ...
Barbus barbus [fluviatilis] (45,91)	Europe	mid-river dweller, bottom-dweller; clear,deep, stony fast rivers, sand beds.'64)	IL, M, C, M, F, plants.
Brachydanio rerio (82)	Asia, esp. India	gravelly streams, (78, 124) Aquarium specimens.	Inv.
Carassius auratus (18, 40, 70, 76, 111,115, 127)	worldwide, mod. temperate zones	weedy ponds, lakes, rivers, often O_2 deficient (68, 124)	Omnivore; preference for phytoplankton.
Chondrostoma nasus (76)	Europe	mid-reaches, large,fast,deep rivers; breeds in gravelly streams. (73, 124)	Grazes Alg. from piles, also plant, animal bottom organisms.
Cyprinus carpio (1, 2, 36, 38, 62, 91 93, 101, 111, 116)	Introd.from Asia; widely found south of 50°	eutrophic ponds,lakes, slow rivers, over all bottoms, in all waters. Resist low O_2. (68,124)	Y: Omnivore; Bottom C, IL A: M, IL, C, Plants
Elopichthyes bambusa (113)	Amur River,USSR, Large rivers, China	Y: quiet backwaters for for first summer A: active, midwater fish (124)	Y: Inv. A: F
Erythroculter erythropterus (113)	Eastern USSR, China	large lakes, rivers	unknown
Labeo rohita (86)	S. Asia, India	large rivers in South, fast streams in North (78)	Omnivore: plants, I, IL, C, M
Leuciscus (Squalius) cephalus (76)	Europe	sometimes shoaling; running water, lakes; brackish water (124)	F, plants, chance Inv.
Notropis blennius (70)	Mississippi R., Southern USA	shoaling in lakes, slow rivers; over silt, sand, gravel (68,97)	Pl, Alg.
N. cornutus (89)	Great Lakes tributaries	clear, cool, weedless lakes (68, 97)	I, Alg., plants
N. wabratilis (70)	Tributaries of Ohio, Mississippi R.	clear, quiet, weedy pools (124)	unknown
Phoxinus phoxinus (8, 9, 111)	Europe	clean, flowing water, gravel bottoms (124)	Y: available food; usu. C, IL, Pl. A: Inv., C, plants
Pimephales notatus (70)	Central USA	wide var. of habitats; muddy streams, lakes (68, 97)	Generalized bottom feeder; detritus, Alg.
Ptychocheilus oregonense (120)	Western North America	lakes, slow rivers (68, 124)	Y:I, L, M, Pl A: also F, C.
Richardsonius balteatus (79)	Western North America	lakes, ponds, slow rivers, shoaling; spawns on gravel (68)	Omn: eso I, IL, Occ.Pl, So F
Rutilis rubilio (76)	S. Europe; Adriatic	standing water, slow rivers (73)	Inv., L, M
R. rutilis (1, 3, 24, 130)	Europe	all types of water; tolerates low O_2 conditions (73, 124)	Y: Omnivore: Pl, Inv.C,Alg. A:M, IL, Inv.
Semotilus atromaculatus	Eastern North America	small, clear streams, lake shores (68, 97)	Omnivore: I, C, F, Alg.
Squalius (Pachychilon) pictus (45, 75)	Europe	small shoals; upper layers of water (73)	M, F, C, I, Alg.
Tinca tinca (111)	Europe	still water, marshes, mud bottoms; tolerates low O_2 (123)	Y Alg., C, IL A:Bottom IL, C, M

Superorder CHARACIPHYSI			
Characiformes			
Distichodontidae			
Distichodus nilloticus (114)	East Africa	lakes, lower reaches of rivers (6, 124)	Plants, Inv.
D. rostratus (114)	East Africa	? as for nilloticus	? as for nilloticus
Characidae			
Hyphessobrycon callistus (107)	Amazon basin, Paraguay, S. Am.	shoaling, in standing and running water.(78,105) Aquarium spa.	Omnivore or carnivore I, inv.,plants.
Superorder PARACANTHOPTERYGII			
Gadiformes			
Gadidae			
Gadus morhua (15, 82)	North Atlantic	cold water;Y: at surface J:(over 2 mo.) demersal A: forage on bottom,but live above substrate (78, 124)	Y: small C J: C, [illegible] A:F, C, [illegible]
Lota lota (70)	Holarctic, N. of 40°	bottom of cold lakes, rivers (68, 97, 124)	Y: IL, C, F A: F, C, I
Melanogrammus aeglefinus (82)	North Atlantic	Y: pelagic J: (over 7 mo.) demersal A: sea bottom, 40-300m (123,124)	Y: C A: benthos, brittle stars, W, M
Pollachius virens (82)	North Atlantic	surface feeder, but caught on trawls, lines (123, 124)	Y: C, M, eggs J: C, F; A: F
Macrouridae			
Cetonurus globiceps (76)	Atlantic Western Pacific	benthopelagic; 1100-3000 m (50)	unknown
Chalinura leptolepis (76)	Pacific, Western North Atlantic	benthopelagic,680-4000 m (28)	unknown
Nezumia sclerolepis (76)	West Coast, North America	on bottom, above 1000 m (78, 124)	unknown
Ventrifossa atherodon (76)	IndoPacific	deep ocean (28)	unknown
Lophiiformes			
Lophiidae			
Lophius piscatorius (20, 117)	E. N. Atlantic, Mediterranean	lies partly buried, bottom to 1000 m (123, 124)	A: F, Occ. birds, C.

Atheriniformes Cyprinodontoidei			
Oryziidae			
Oryzias latipes	Japan, S. E. Asia	rice paddies, brackish water, quarium specimen. (78)	Omnivore, C. I.
Cyprinodontidae			
Fundulus heteroclitus (27)	East Coast, North America	creeks, tidal flats, in weeds, (68, 124)	Omnivore: Diatoms, C, eggs,Sm. F. grass.
Poeciliidae			
Gambusia affinis (22)	Southeastern USA	clear ponds, brooks, marshes, streams; Aquarium specimen (6,124).	IL, other Inv.
Poecilia reticulata (48, 82)	Central and N. South America	Fw. or brackish streams, pools; (68, 124) Aquarium specimen.	Alg..IL, C, eggs, F.
Xiphophorus helleri (70)	Central and N. South America	springs, streams, rivers swamps; Aquarium specimen (6,68,78,124).	Carnivore; Omn. in captivity
X. maculatus (107)	Central and N. South America	ponds, swamps, slow streams; aquarium specimen (6,68, 124)	Alg., live food
X. variatus (5)	Mexico	pools, ponds, lakes, slow streams,; tolerates low O_2	Alg., bottom detritus Pl
		(105) Aquarium specimen.	
Superorder ACANTHOPTERYGII Gasterosteiformes Gasterosteoidei			
Gasterosteidae			
Culaea (Eucalia)inconstans (52)	Great Lakes, N. to Gt. Slave Lake	cold, weedy streams, ponds, lakes to 55 a (68, 97)	C, eggs and larvae of F, M, W, Alg.
Gasterosteus aculeatus (48, 83)	Circumpolar, N. Hemisphere	In Fw: lakes,streams. M:nearshore shallows, grass (68, 97, 124)	Carnivore, esp. W, C.
Pungitius pungitius (45)	coastal N. Europe Asia, N. America, south to 40°N.	brackish, freshwater. weedy, swampy areas, small streams, ponds. Sometimes aquarium fish.(73,"124)	Inv., esp. C, IL
Channiformes Channoidei			
Channidae			
Ophicephalus [Channa?] punctatus (59)	Southeast Asia	bottom, swamps, ditches, often low O_2 (124)	Y: W,L A: F

Scorpaeniformes Scorpaenoidei			
Scorpaenidae			
Sebastes inermis (58)	Northwest Pacific	bottom, in rocky areas (73, 124)	C SaF?
Triglidae			
Aspitrigla (Trigla) cuculus (24, 25, 37)	Europe, coastal waters.	bottom-dweller, sand, mud gravel to 250 m. (73, 124)	C, Sq, Occ. F; usu. large, active prey
T. lucerna(corax and hirundo)(37)	Europe, coastal waters	sandy, muddy bottom to 150 m (28, 73, 124)	C, F, M, slow-moving prey
Hexagrammoidei			
Anoplopomatidae			
Anoplopoma fimbria (76)	West Coast USA, N. Pacific	Y: pelagic A: bottom-living on Cont. shelf above 300 m (73, 124)	Unspecialized feeders, F, C, M
Cottoidei			
Cottidae			
Cottus gobio (1, 3, 91)	Europe	moderate to slow streams, lakes; over hard bottoms, under obstacles (124)	Inv., C, I, Pl
Hemitripterus americanus (82)	Atlantic coast, Chesapeake Bay to Labrador	bottom-dwelling, clear water rocky grounds to 100 m (123)	C, F
Myxocephalus octodecemspinosus (82)	Atlantic coast, Northern USA	bottom-dwelling, nearshore(?) (68, 73, 78)	? prob. as for H. americanus
Superorder PERCOMORPHI Perciformes Percoidei			
Serranidae			
Dicentrarchus labrax (74)	Europe	Surf zone, rocky outcrops, many bottoms; anadromous (73, 124)	F, C, Sq
Morone saxatilis(44)	Atlantic Coast Nova Scotia to South Carolina	estuarine, ?anadromous(44)	?F
Centrarchidae			
Ambloplites rupestris (70)	North America	cool, clear lakes, near pilings, outcrops (68, 97)	Inv., C; IL, I

Micropterus dolomieui (70)	Eastern North America	clear fast streams, pools with gravel bottoms.(68, 97)	F, Amphibs, Inv., C, IL
Percidae			
Perca flavescens (70, 129)	North America	Lakes, slow rivers, under vegetation, > 10 a (68, 97, 124)	IL, C, F esp as adults
P. fluviatilis (25, 54, 111)	Europe	ponds, lakes, rivers, streams under, near obstacles (124)	Y: ZooPl, Inv., other fry A:IL, C, F
Stizostedion (Lucioperca) lucioperca (7)	Europe	murky water or shadows, large lakes, slow rivers, over hard bottoms (73, 124)	Y: C, IL A:F, very predatory
S. vitreum (69)	North America, Southern USA to Arctic Ocean	Y: pelagic moderately clear lakes, hard bottoms.	Y: Inv. A: F, I, C
Monodactylidae			
Monodactylus argenteus (39)	IndoPacific	pelagic, schools in estuaries, river mouths, harbours; prefers quiet waters (6, 102)	I
Sparidae			
Sparus aurata (35)	worldwide; tropical, subtropical oceans; Red Sea.	schooling; reefs, sandy to rocky bottoms cultured specimens (78, 124)	Omnivorous
Cichlidae			
Pseudotropheus sp.(45)	Lake Malawi, Africa	crevices in rock; often aquarium fish. (6, 105,124)	Can., often Alg.
Pterophyllum scalare (107)	Amazon Basin Guyana	weedy, slow streams, lakes; murky water Aquarium specimen (6,105,124).	Inv.
Labroidei			
Labridae			
Ctenolabrus rupestris (24,25)	Europe	Rocky, weed-covered shores; offshore in winter (124)	SnC, M, M
Labrus bergylta (37)	Europe	rocks, offshore reefs to 9m, among algae and weeds (78,124)	Mussels, other M, LgC, M
L. pimaculatus (mixtus) (37)	Eastern Atlantic, Mediterranean	over rocks, rough ground to 200 m (28)	C, M, F. Scavenger?
Symphodus(Crenilabrus) crenilabrus(65)	Europe	most spp. over rocky shores	? M, C, Inv.
Symphodus (Crenilabrus) melops (37)	Europe	rocky shores, pools;	Barnacles, M, C, M
Symphodus(C.) tinca (pavo) (37)	Eastern Atlantic Mediterranean	littoral, near rocks, eel-grass, 1-50 m (28) sedentary (124)	as for melops
Tautoglabrus adspersus (82)	Western N. Atlantic N.J. to Labrador	? as for mixtus (123)	?Scavenger

Notothenioidei			
Nototheniidae			
Notothenia larseni juvenile only	Southern Ocean, Atlantic sector	Y: pelagic J: demersal A: pelagic (105)	Pl, C (mysids, euphausiids), M, Salps
N. rossii (122)	Southern Ocean, Atlantic sector	Y: pelagic, offshore J: demersal over gravel, in weeds, (23,55) A: pelagic, offshore (23, 109)	Y: Pl, C, F, L J: C (mysids), F, Alg. A: C (euphausiids), other Inv.
N. (Trematomus) vicarius	Southern Ocean Atlantic sector	J: demersal, mud 90 - 240 m (23)	C, fish eggs.
Harpagiferidae			
Harpagifer bispinis	Southern Ocean, South Shetlands	under rocks near bottom, 10-20 m, or tide pools, weeds (109)	C (amphipods)
Bathydraconidae			
Parachaenichthys georgianus	Southern Ocean, South Georgia	inshore, weedy, to 100 m (108, 109)	F, C, Alg., Inv.
Channichthyidae			
Chaenocephalus aceratus (56)	Southern Ocean, South Georgia	over muddy bottoms, ambush predators (109, 124)	F, also C (mysids, amphipods) in some areas.
Agonoidei			
Agonidae			
Agonus cataphractus (63)	Europe	over sand to 30 m; usually shoaling (124)	M, C, Fish eggs, L
Scorpaenoidei			
Cottidae			
Cottus gobio (1)	Europe	fast streams, pools, lakes, over gravelly bottoms (123)	Summer: IL Winter: C, F
Acanthuroidei			
Siganidae			
Siganus argenteus (35)	Red Sea	seapelagic (35,78)	mainly plants
S. luridus (35)	Red Sea	associated with coral reefs (35)	mainly plants
S. rivulatus (35)	Red Sea	reefs or pelagic (35)	mainly plants

Scombroidei			
Scombridae			
Thunnus thynnus	Atlantic	migratory schools, mid-water column to surface (124)	Y: C (euphausiids, decapods) A: F, Sq
Stromatoidei			
Nomeidae			
Nomeus gronovii (60)	Caribbean	warm seas. Y: among tentacles of Physalia. A: virtually unknown (78, 124)	Pl, host. Host diet
Stromateidae			
Peprilus triacanthus (20)	North America, Atlantic coast south of 48°	cool water, small schools 1)deep, over mud, or 2) shallow over sand. Two spp? (78, 123)	SeF, Sq, C, ctenophores
Stromateus fiatola (20)	Africa, Atlantic coast	Y: pelagic A: hard and soft bottoms to edge of Cont. shelf, 12-80 m (123)	F, Sq, C, ctenophores
Belontiidae			
Trichopsus vittatus (G/H)	S.E. Asia, Malay Peninsula	weedy streams, ditches tolerates low O_2, air breather (105, 124). Aquarium spn.	Inv., some plants
Pleuronectiformes			
Pleuronectoidei			
Scophthalmidae			
Scophthalmus maximus (3)	Eastern N. Atlantic, Mediterranean	Y: pelagic A: on sand, shell gravel, fine gravel to 80 m. (124) Hatchery spns.	Y: C, esp. shrimp, F A: F
Pleuronectidae			
Glyptocephalus cynoglossus (82)	North Atlantic, Europe to North America	cool water (-1 to 10°C), muddy grounds, sands to 300m (123, 124)	Bottom Inv., e.g. C, M, W, starfish
Hippoglossoides platessoides (82)	North Atlantic, Europe to N. Am.	cool waters, fine sand, mud, 37-183 m (123, 124)	Sea urchins, C, F, M, starfish
Pseudopleuronectes americanus (82)	Atlantic coast, N. America	mud to hard bottoms to 37 m (123)	Bottom C, M, W
Soleidae			
Solea (vulgaris) solea (4)	European N. Atl., through Med. and W. African coast	shallows to 75 m., in sand mud, fine gravel. Y: pelagic (124) Hatchery spns.	Bottom M, SeC, M, brittle stars, F

TABLE 3-II

Species in which Rodlet Cells were Reported to be Absent: Habitat and Distribution.

TAXONOMY(REPORTS)	DISTRIBUTION	HABITAT (REFERENCE)	FOOD CHOICES
Superorder CLUPEOMORPHA			
Clupeiformes			
Clupeidae			
Alosa pseudoharengus (81)	N. Am. Atlantic coast, Gt. Lakes	pelagic, near surface of water;anadromous or landlocked (123)	Pl, F, C
Superorder PROTACANTHOPTERYGII			
Salmoniformes Myctophidoidei			
Myctophidae			
Electrona antarctica	Southern Ocean W. Antarctic region	mesopelagic; midwater column 700-1000m (123)	Pl
Gymnoscopelus braueri	Southern Ocean	mesopelagic; midwater column	Pl (copepods)
Superorder OSTARIOPHYSI			
Siluriformes			
Ictaluridae			
Ictalurus nebulosus(30)	Eastern N. Am. Introd. to Eur., West. N. Am., N.Z.	bottom-living; over mud, in weeds of ponds, slow rivers (123)	Inv.
Superorder PARACANTHOPTERYGII			
Gaciformes			
Muraenolepoidei			
Muraenolepididae			
Muraenolepis microps	South Atlantic, Southern Ocean	bottom-dwelling,inshore, 18-830 m (123)	Y: copepods

Superorder ACANTHOPTERYGII			
Perciformes			
Notothenioidei			
Nototheniidae			
Notothenia larseni fingerling only	Southern Ocean	Y: pelagic	Pl?
N. macrocephala	South Atlantic, Southern Ocean	A: bottom-dwelling	A: C, F, Alg.
Blennioidei			
Anarhichadidae			
Anarhichas lupus (61)	North Atlantic to Arctic Ocean	moderately deep water, 1-435 m. (28, 123)	echinoderms, C, M
Pleuronectiformes			
Pleuronectoidei			
Pleuronectes platessa (4)	European N. Atl. to W. Med.	shallow water, on sand, to 120 m. (123)	Inv: M, C, W, F

TABLE 3-III

Comparison of food choices between species with rodlet cells (Table 3-I) and a general, geographically circumscribed teleost population (Wheeler, 1969).

	General Population n=326		Population with rodlet cells n=109		Ratio of percentages rodlet cell pop'n: general population
FOOD CHOICES	# spp.	%	# spp.	%	
Crustacea	150	46.0	82	75.9	1.65:1
Fish	129	39.6	57	52.8	1.15:1
Plankton	63	19.3	19	17.6	0.91:1
Molluscs	62	19.0	28	25.9	1.36:1
Worms	50	15.3	17	15.7	1.03:1
Insects/Larvae	44	13.5	50	46.3	3.43:1
Detritus/Eggs	27	8.3	19	17.6	2.12:1
Plants/Algae	26	8.0	27	24.1	3.02:1
Cephalopods	21	6.4	10	9.3	1.44:1
Echinoderms	16	4.9	5	4.6	0.94:1
"Inverts"-Bottom	12	3.7	24	22.2	6.04:1
Omnivorous	1	0.3	20	18.5	60.37:1

than 40° (Mayberry et al., 1979).

For those teleosts in which rodlet cells are known, the general habitats range from tropical to polar, and from freshwater to marine. The species appear to be equally divided between freshwater and marine or euryhaline habitats: 49 are entirely marine, 55 freshwater, 11 may be considered euryhaline, and 7 are anadromous/catadromous. The majority of freshwater species (40 vs. 18) are stated, as adults, to prefer "still" water; i.e., lakes, ponds, or slow rivers, to the "swift" water of streams or fast rivers, although some species are found in both places. Thirty-eight species, both marine and freshwater, are commonly found over "soft" substrata of mud or sand, and 39 over "hard" bottoms of gravel or rock. The others are either pelagic, unknown, or not indicated in the literature.

It is clear, therefore, that rodlet cells are found in teleost fish from a broad range of habitats, and are not confined to those from a narrow segment of the environment.

Many of the species listed in Table 3-I appear to be bottom dwellers, or to spend extended periods on the bottom at some stages (40 species); more are pelagic through at least part of their life cycles (72 species). Table 3-I gives no information on relative numbers of rodlet cells in individuals of a species, since the numbers are individually so variable. Some groups have, however, been observed to have relatively few rodlet cells, generally. Salmonids, which are pelagic except when breeding in gravelly streams, have been rarely observed to have rodlet cells, and then only in the intestines of hatchery-reared specimens (Modin, 1981). The pelagic scombrids and clupeids have

occasionally been seen to have a few rodlet cells; however, Morrison and Odense (1978) found no rodlet cells in intestinal tissue from two specimens of Alosa pseudoharengus.

Of the 10 species listed in Table 3-II, where rodlet cells have been looked for and not found, only four are considered to be pelagic; the others are bottom-dwelling. For most of these 10 species, only one or two individuals, and from these generally only one or two tissues, were examined. In the light of the great variability in incidence of rodlet cells within different organs and among individuals, such negative evidence may mean only that the particular individuals or organs examined did not contain rodlet cells. To declare the absolute absence of rodlet cells from a species in such circumstances could be misleading. Table 3-II is a very small sample, considering the number of teleost species in the world; this group may not reflect the conditions generally prevailing when a species lacks rodlet cells.

The one large group of bottom dwellers which has been carefully examined, and from which rodlet cells have never been reliably reported, is the Siluriformes. Dawe et al. (1964) examined hepatic neoplasms of both Ictalurus nebulosus, a silurid, and Catostomus commersoni, an ostariophysan, both from the same environment; they reported large numbers of rodlet cells in the latter, but none in I. nebulosus. In addition, Grizzle and Rogers (1976), in their atlas of the anatomy and histology of the channel catfish, I. punctatus, show no illustrations of rodlet cells, and do not mention them in the text. Examination of gill tissues of I. nebulosus in this laboratory (Barber, unpublished) have shown none, although the gill is a common location for rodlet cells in

freshwater fish.

Although fish other than teleosts have been examined, e.g. Raja ocellata, a skate and Acipenser oxyrhynchus, the Atlantic sturgeon (Morrison and Odense, 1978) and Lampetra lamottenii and Petromyzon marinus, a brook and the sea lamprey, respectively (Barber, unpublished), rodlet cells have not been found. Stolk (1956) found a cell in Scyliorhinus catulus, an elasmobranch, which was said to be equivalent to the "coarse granulocyte" described by Duthie (1939) from the mesenteries of some teleosts. Duthie's Fig. 1 of the "coarse granulocyte" clearly represents a rodlet cell; Stolk's written description of the "coarse granulocyte" is consistent with the general morphology of the rodlet cell, but the accompanying line drawings show no cytoplasmic detail. Reports of rodlet cells in the paddlefish Polyodon spathula (Weisel, 1973) accompanied by a photograph indicate clearly that the cell described as a "rodlet cell" in this fish is not one. Inspection of the accompanying photographic figure would suggest that Weisel's "rodlet cell" is a large, rather elongate, irregularly-shaped, mucous-type of cell with spherical, PAS-positive cytoplasmic granules. Claims of rodlet cells in the sturgeon Acipenser ruthenus, a chondrostean, and in the holostean gar pike Lepisosteus osseus are unsupported by line drawings or photographs (Grünberg and Hager, 1978).

Table 3-III is a comparison between the food choices reflected in the species of Table 3-I and those of an atlas (Wheeler, 1969) for a geographically circumscribed population of both freshwater and marine teleosts found in Britain and Northwest Europe. The principal food choices of both groups are crustacea, fish, plankton, and molluscs. Of the species reported in Table 3-I, 82 are stated specifically to choose

"crustaceans" as food; indeed, some teleosts are selective in the types of crustaceans they prefer as food. Fifty-seven species are known to eat other fish: Burchett (1983) reported that Notothenia rossii at South Georgia preferentially consume fish as juveniles, even when euphausiids and amphipods may be more plentiful in the environment. Twenty-eight species choose molluscs, and some pleuronectids choose to eat mussels in preference to other molluscs (Wheeler, 1969).

Roughly equal proportions of plankton, echinoderm, worm, mollusc, fish and cephalopod eaters are seen in both lists (Table 3-III); this suggests that the teleosts listed in Table 3-I may be simply a representative group in spite of their geographical diversity. If this is true, then it emphasizes the disparities seen between the atlas and Table 3-I in the number of omnivorous and bottom-invertebrate-eating species. As well, Table 3-III shows two to three times as many insect/larvae, detritus-eating and herbivorous forms as does Wheeler (1969). The comparatively large number of omnivores seen in Table 3-I suggests a possible relationship between diet and the presence of rodlet cells in a species.

Evidence for seasonality in the presence of rodlet cells from some freshwater teleosts exists. Leino (1974) reported fewer rodlet cells in intestine and gill of Perca flavescens, Stizostedion vitreum, Esox lucius and Micropterus dolomieui during May-June and increased numbers in these organs in late summer (August-September). Observations in this laboratory on Catostomus commersoni (Ladd, unpublished) support this observation.

The ages of teleosts examined may be a factor also in the presence

of rodlet cells, although some observations are equivocal. Postlarvae or fingerlings of Notothenia larseni or N. rossii have not shown rodlet cells in tissues examined. However, cells have occasionally been seen in prehatching embryos of Oryzias latipes (Westermann and Jensen, unpublished) and in 14-day post-hatching Stizostedion vitreum (Leino, 1974). In all these species except O. latipes, the larvae and post-larvae are pelagic, while the juveniles and adults are demersal.

ANSWERS

1) Reliable reports of the incidence of rodlet cells are confined to the Teleostei. While it is not possible to say that some teleosts are not susceptible to the presence of rodlet cells, the Siluriformes have never been confirmed to contain them.

2) It is clear, from examining Tables 3-I and 3-III that a species is more apt to contain rodlet cells if it is demersal and omnivorous; pelagic species like scombrids and salmonids, while they may contain rodlet cells, apparently have fewer than demersal species. The information presented in this study also suggests there may be common denominators in habitat and/or food choices that influence susceptibility to rodlet cell presence. The broad geographical range of species with rodlet cells indicates that geographical considerations, even a longstanding barrier such as the Antarctic Convergence, do not limit the incidence of rodlet cells.

CHAPTER IV

MICRODENSITOMETRY EXPERIMENTS

CHAPTER IV

MICRODENSITOMETRY EXPERIMENTS

INTRODUCTION

The diagnostic features of the rodlet cell are the thick contractile capsule, basal nucleus and fine, elongate cytoplasmic rodlets. The nucleus may vary greatly in shape depending upon the teleost species from which the rodlet cells come, and whether the rodlets are present or have been expelled after contraction of the "capsule". However, apart from casual descriptions of position, heterochromatin distribution and general shape, the nucleus of the rodlet cell has been overlooked as a factor in determining the identity of the rodlet cell.

THE QUESTION

Is the nuclear DNA content of rodlet cells in a teleost species different from that of cells known to contain the genome of the species? If rodlet cells are derived from normal teleost cells, their nuclear DNA should have the same DNA content, or a simple multiple thereof, as cells known to belong to the fish. It is possible to quantify the amount of DNA in individual nuclei on a microscope slide using the Feulgen technique and microdensitometry, and different species of living organisms contain characteristic amounts of DNA in the nucleus. If rodlet cells are "parasites", then their nuclear DNA content might be expected to bear no

consistent relationship to the host DNA. In the present study, the hypothesis has been that erythrocytes of the species studied reflect the normal 2C DNA content of the tissues, and that other somatic cells are either 2C or a simple multiple of C. To test the hypothesis, the DNA contents of rodlet cells, erythrocytes and teleost cells larger than erythrocytes; e.g., leukocytes and epithelial cells, have been measured and compared statistically.

MATERIALS AND METHODS

Specimens

Touch preparations of gill or conus arteriosus were prepared from the white sucker, Catostomus commersoni, the carp, Cyprinus carpio, and the northern creek chub, Semotilus atromaculatus. They were air-dried, fixed in methanol, and dried for 24 hours or longer before being treated with the Feulgen procedure.

Histochemistry

Feulgen Procedure:

The slides were warmed in a 60°C oven, and hydrolyzed in 1N HCl for 11-15 minutes at 60°C. They were rinsed in cold 1N HCl, then in distilled water, and stained in freshly-prepared Schiff's reagent for 3 hours in the dark at room temperature. After staining, the slides were bleached in 3 changes of 0.25% sodium metabisulphite in dilute HCl and then washed for 15 minutes in running tap water. For counterstaining, the slides were immersed briefly in 0.01% fast green FCF in 95% ethanol, dehydrated through 100% ethanol, cleared in xylene, and mounted in DPX.

Immediately after mounting, the finished preparations were placed in a covered slide flat until examination, to reduce their exposure to ambient illumination.

Microdensitometers

The measurements of DNA content were carried out using both a Leitz MPV-3 microdensitometer, and a Vickers 85 scanning microdensitometer.

Using a Leitz MPV-3 Microdensitometer:

The Leitz MPV-3 microspectrophotometer measures the transmission of light through a specimen which has been circumscribed by a closely fitting optical diaphragm and illuminated by monochromatic light. The absorbance of the structure is calculated by comparing the light transmission values for the aperture with and without the specimen in the aperture. For the Feulgen procedure described above, a two-wavelength method (Patau, 1952) was used to calculate the amount of DNA present in the nuclei measured. Erythrocytes of the three species measured were assumed to represent the 2C condition, and the DNA content of rodlet cells were measured and compared to that calculated for the erythrocytes.

Using a Vickers M85 Scanning Microdensitometer:

With a scanning microdensitometer, a single wavelength of monochromatic light may be used to measure the absorbance of the aperture-enclosed nucleus. The monochromatic light is present as an operationally variable narrow beam that scans the aperture, taking readings 10,000 times/second and recording values for absorbances above a certain (operationally variable) threshold. The readings are integrated and appear as "units" on the machine. It is possible to take multiple

readings of a single specimen and to average these so that a value is presented of the absorbance with a very small distributional error. To calculate the relative absorbance, the background value obtained from the scan of the aperture without the specimen present is subtracted from the reading with the specimen in the aperture.

Sources of Error

Errors in the amount of material measured using the spectrophotometer fall into two general classes: systematic errors and preparation/biological artefacts.

1) Systematic errors may be of several different kinds, including distribution error, scattering errors (glare), stochastic errors, focusing error, and for the scanning microdensitometer, spot size error. Of these, the most important are the distribution and scattering errors. Stochastic errors, in which measurements are randomly displaced about a mean, may be compensated for by measuring large numbers of cells, and by measuring the same cell several times (in the scanning microdensitometer). If a specimen is out of focus, the microdensitometer readings will be lower because of a reduction in the apparent density of the specimen. Spot size error is related to the focus error, in that if the specimen is unfocused, the effective spot size is proportional to the decrease in effective optical density (Vickers manual). To avoid this error, careful focusing and choice of the smallest satisfactory spot size is an effective solution.

The distribution error results from local (in the case of cell nuclei, intranuclear) variations in optical density of the material being measured. For Feulgen procedures, the euchromatin and heterochromatin

areas show local density variations. In the Leitz MPV3 microdensitometer, the light transmission value measured represents an average value of light passing through the entire nucleus, as if the nucleus were of a single, uniform density. Visual inspection shows, of course, that this is not the case. The consequence of this distribution error is that measured values displayed by the machine will be lower than the actual DNA content of the nucleus. The two-wavelength method of Patau (1952) used in the determinations in these experiments largely compensates for this error by changing the apparent absorption characteristics of the nucleus in the two different monochromatic wavelengths. The scanning microdensitometer is designed precisely to reduce distributional error, and to simplify the very laborious calculations of DNA using the two-wavelength method. The monochromatic scanning spot measures the transmission of light through a very large number of individual, small areas within the nucleus itself, and then integrates all the readings into a consolidated value which is displayed. The instrument may be set so that light background areas are not included in the integrated measurement. Each small measurement is considered to represent an internally homogeneous region of the measuring area.

Scattering errors occur around the edges of the specimen to be measured, when the aperture surrounding the specimen does not meet the specimen edge precisely. For nuclei, there is always some light scattering since it is impossible to adjust the aperture to fit a nucleus precisely. As well, stray light from the environment may produce scattering errors; these are reduced in the Vickers M85 because the entire substage light source, the microscope objectives and the stage

are in lightproof enclosures. The best practical way to avoid scattering error in measurement is to position the aperture as closely around the specimen as possible; the scanning microdensitometer is much more flexible in this regard, since it has a rectangular mask which is variable for size and also for position angle in the field. Consequently, the mask could be fitted very closely around individual nuclei, with the principal scattering error occurring at the corners of the aperture. Setting the measurement threshold slightly above zero usually meant that the background was quite low. With the Leitz MPV3 microdensitometer, the circular and rectangular apertures were more adjustable, but they were situated only in the middle of the field, and the rectangular aperture angle could not be changed.

2) In addition to systematic errors, biological/preparation artefacts can also induce errors. For transmission/absorbance experiments, it is necessary that the chromophore used obey the Beer-Lambert Law, which states that the density of stain is directly proportional to the amount of material present, and that Transmission (T) = $\log_{10}(I_1/I_0)$. It applies strictly only when the light employed is monochromatic, all the light paths through the specimen are equal and the chromophore is homogeneously distributed. None of these conditions is precisely fulfilled in any biological preparation. In addition, when the material being measured is present in high concentration, interaction among chromophore molecules may change the absorption characteristics of the material. In some biological preparations, nuclei may be very dense, if they contain large amounts of heterochromatin, if they are senile and pycnotic, or if they are in a thick portion of an imprint where they

shrink upon drying after preparation. Such dense structures will tend to give readings lower than the actual amount of material present. This problem may be avoided by measuring cells at the periphery of the touch preparation, and avoiding areas where cells are piled on each other.

Other potential preparation and biological sources of error include differences in nuclear shape, morphological variability in shape among cells in a species, preparation loss of DNA, and for the rodlet cell, the effect of rodlet cell capsule thickness on hydrolysis and stain preparation. The latter possibility was tested using different hydrolysis times followed by Feulgen staining. For all hydrolysis times, the proportion of absorption was constant between rodlet cell and erythrocyte nuclei, and it has been assumed that rodlet cell and erythrocyte nuclei hydrolyze at the same rate, and that capsule thickness does not constitute a significant barrier to stain penetration. For the shape variations within nuclei of a species, some error is inevitable, but careful positioning of the aperture around the cell keeps the error to a minimum. The staining procedure was carefully controlled to minimize any preparation loss of DNA.

Experiments

Experiments 1 and 2 were carried out using the Leitz Microdensitometer; Experiment 3 used the Vickers M85 Scanning Microdensitometer.

Experiment 1: For the first experiment, all the preparations could not be made in one batch, and attempts were made to compensate for possible variations in staining by measuring at least five erythrocyte nuclei from each preparation and ascertaining that their measurements were the same as previous measurements for the species. For most slides,

15-30 erythrocytes and a comparable number of rodlet cell nuclei were measured for each specimen of Catostomus commersoni, Cyprinus carpio and Semotilus atromaculatus. Limited amounts of material from carp were available, and only 54 erythrocytes and 44 rodlet cells were measured, all from the same slide.

To determine the accuracy of measurements for the three species used in the experiments, erythrocytes from teleosts whose nuclear DNA values are also known, were studied. Erythrocytes of one Ictalurus nebulosus (brown bullhead) from Lake Erie, one hatchery-reared Salmo gairdneri (rainbow trout) and three Oryzias latipes (medaka) were measured, and their DNA contents compared with published values (Hinegardner and Rosen, 1972; Shapiro, 1976).

Experiment 2: In this experiment, gill touch preparations from sucker and chub and touch preparations of the conus arteriosus of carp were hydrolyzed and stained in the same way as in Experiment 1, but in a single batch to eliminate potential variation caused by differences in staining. Ten to twenty nuclei of erythrocytes, rodlet cells and cells with nuclei larger than erythrocytes; e.g., gill epithelial cells and blood leukocytes, were measured.

Experiment 3: The slides prepared for Experiment 2 were examined in the scanning microdensitometer, and measured according to methods outlined above. The scanning beam was set to approximately 525 nm, threshold value at 0.1 arbitrary units, beam width at 2 (of 5 widths), and band width 40 units. Attempts to use a smaller beam (width 1) resulted in malfunction of the microdensitometer. Erythrocytes were considered to represent the 2C genetic complement of the species examined, and rodlet cells and cells having nuclei approximately the same size as

the rodlet cell nuclei were measured to provide comparative DNA measurements. Each cell was scanned 5 times, and the average density of the background was subtracted from the average density of the specimen to obtain a value for the amount of DNA present.

The DNA content of cells of Cyprinus carpio is known to be 3.4 pg DNA/2C nucleus (Hinegardner and Rosen, 1972; Shapiro, 1976). This value was used to calculate the relative values of DNA for rodlet cells, erythrocytes and cells larger than erythrocytes.

Statistical Treatment

The nuclei of the cells of each species were compared within and between species using Student's "t" Test (Snedecor, 1961). Sample calculations are contained in Appendix II.

RESULTS

The results of measurements of DNA content of I. nebulosus, O. latipes and S. gairdneri are found in Table 4-I; the measurements from this study agree well with previously published values.

The results of Experiments 1, 2 and 3 are seen in Tables 4-II to 4-V.

Experiment 1

In this experiment, only erythrocytes and rodlet cells were measured. The results of the measurements are found in Table 4-II and the graphical distribution of DNA values for all three species in Fig. 4-1. Using this method, there were statistically highly significant differences ($p < 0.001$) between the DNA contents of erythrocyte and rodlet

FIGURE 4 - 1: Distribution of DNA in Erythrocytes and Rodlet Cells in some Teleosts.

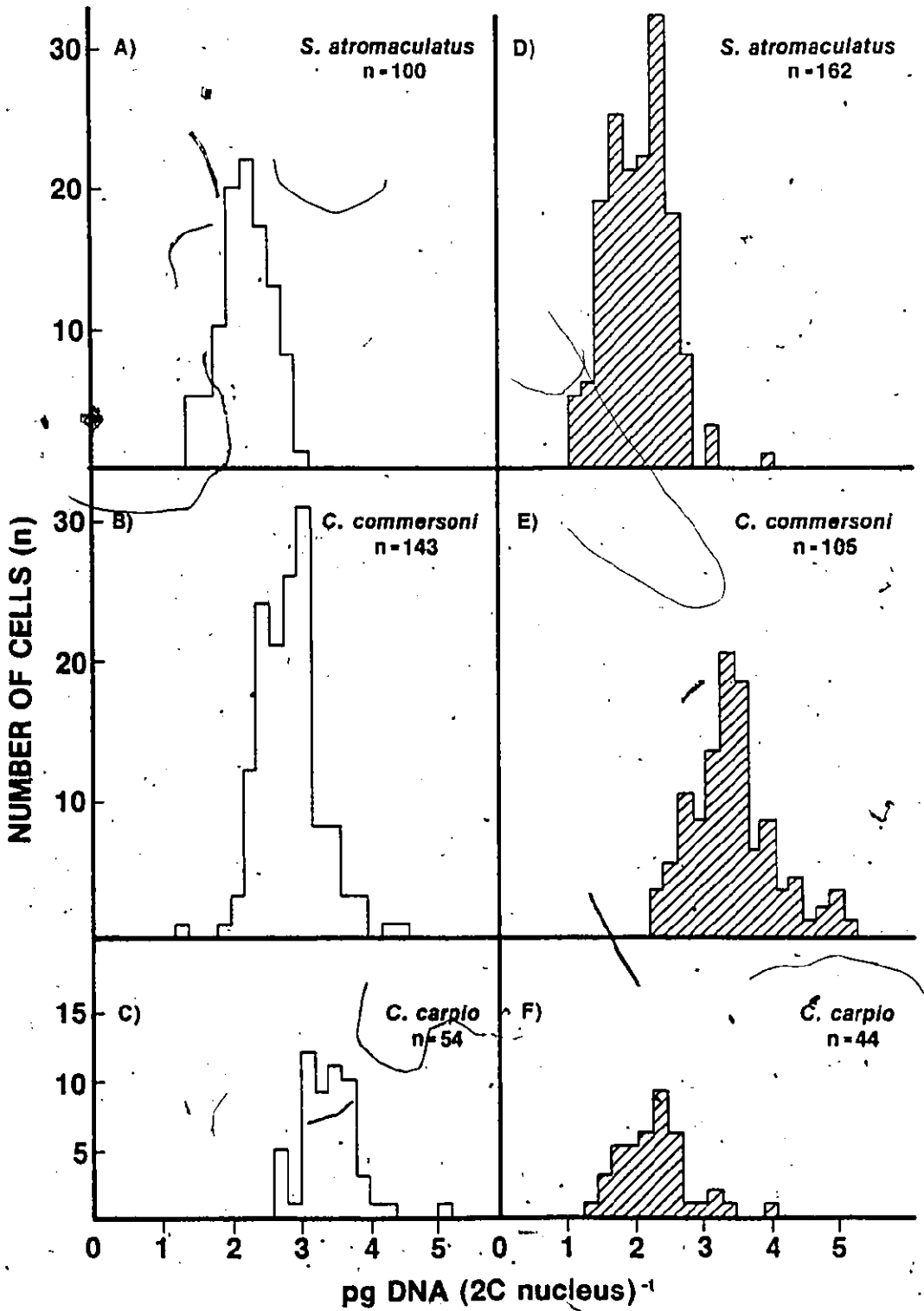


TABLE 4-I

COMPARISON OF NUCLEAR DNA CONTENT OF SOME TELEOST SPECIES WITH PREVIOUSLY PUBLISHED VALUES

SPECIES	DNA PG/NUCLEUS MEAN +/- S.E.M.	PREVIOUSLY PUBLISHED VALUES PG/2C NUCLEUS (MEAN)
I. nebulosus	2.19 +/-0.03 n=30	2.4#
O. latipes	2.15 +/-0.07 n=20	2.2#
S. gairdneri	5.50 +/-0.07 n=30	4.9 - 5.6*

#Based upon Hinegardner and Rosen (1972).
*From Shapiro (1976)

TABLE 4-II
EXPERIMENT 1

COMPARISON OF NUCLEAR DNA CONTENT IN ERYTHROCYTE AND RODLET CELL NUCLEI
LEITZ MPV3 MICRODENSITOMETER

CELLS	CHUB DNA (pg/nucleus +/-SEM) Range (pg)	SUCKER DNA (pg/nucleus +/-SEM) Range (pg)	CARP DNA (pg/nucleus +/-SEM) Range (pg)
Erythrocytes	2.30 +/-0.03 n=100	2.87 +/-0.04 n=143	3.4 +/-0.06 n=54
Range	1.43-2.92	1.38-4.48	2.62-5.02
Rodlet Cells	2.02 +/-0.04 n=162	3.38 +/-0.06 n=105	2.28+/-0.08 n=44
Range	0.97-3.89	2.29-4.99	1.39-3.96

STATISTICAL COMPARISONS BETWEEN AND WITHIN SPECIES

BETWEEN SPECIES

ERYTHROCYTES		
Chub vs. Sucker	t= +/-12.88	p<0.001
Chub vs. Carp	t= +/-17.29	p<0.001
Carp vs. Sucker	t= +/-4.98	p<0.001
RODLET CELLS		
Chub vs. Sucker	t= +/-21.66	p<0.001
Chub vs. Carp	t= +/-3.08	p<0.01
Carp vs. Sucker	t= +/-11.16	p<0.001

WITHIN SPECIES

CHUB		
Erythrocytes vs. Rodlet Cells	t= +/-5.92	p<0.001
SUCKER		
Erythrocytes vs. Rodlet Cells	t= +/-5.20	p<0.001
CARP		
Erythrocytes vs. Rodlet Cells	t= +/-13.64	p<0.001

TABLE 4-III

COMPARISON OF NUCLEAR DNA CONTENT USING TWO DIFFERENT MICRODENSITOMETERS

EXPERIMENT 2
LEITZ MPV-3 MICRODENSITOMETER

Cells	CHUB DNA (pg/nucleus±SEM) Range (pg)	SUCKER DNA (pg/nucleus ± SEM) Range (pg)	CARP DNA (pg/nucleus ± SEM) Range (pg)
Erythrocytes	2.06 ± 0.12 n = 10 1.64-3.01	3.66 ± 0.08 n = 12 3.45-4.10	3.4 ± 0.12 n = 18 2.49-4.24
Rodlet Cells	3.14 ± 0.49 n = 12 0.903-5.133	4.00 ± 0.19 n = 10 2.59-5.09	3.26 ± 0.45 n = 12 1.81-4.83
Larger Cells	2.64 ± 0.37 n = 10 1.84-4.31	4.43 ± 0.16 n = 14 3.76-5.11	3.32 ± 0.16 n = 15 2.58-4.76

EXPERIMENT 3
VICKERS SCANNING MICRODENSITOMETER

Cells	CHUB DNA (pg/nucleus±SEM) Range (pg)	SUCKER DNA (pg/nucleus ± SEM) Range (pg)	CARP DNA (pg/nucleus ± SEM) Range (pg)
Erythrocytes	2.45 ± 0.05 n = 20 2.10-2.96	5.23 ± 0.09 n = 20 4.76-5.59	3.4 ± 0.07 n = 22 2.92-4.08
Rodlet Cells	3.37 ± 0.18 n = 20 2.31-5.26	6.90 ± 0.29 n = 9 5.76-9.43	4.07 ± 0.28 n = 11 1.30-5.17
Larger Cells	3.06 ± 0.14 n = 19 2.19-4.58	7.71 ± 0.31 n = 18 5.60-9.79	4.11 ± 0.20 n = 26 2.74-6.09

TABLE 4-IV
EXPERIMENT 2
STATISTICAL COMPARISONS OF NUCLEAR DNA WITHIN AND BETWEEN SPECIES

LEITZ MPV3 MICRODENSITOMETER

BETWEEN SPECIES		
ERYTHROCYTES		
Chub vs. Sucker	t= +/-7.87	p<0.001
Chub vs. Carp	t= +/-7.22	p<0.001
Carp vs. Sucker	t= +/-1.30	n.s.
RODLET CELLS		
Chub vs. Sucker	t= +/-1.50	n.s.
Chub vs. Carp	t= +/-0.178	n.s.
Carp vs. Sucker	t= +/-1.40	n.s.
LARGER CELLS		
Chub vs. Sucker	t= +/-4.48	p<0.001
Chub vs. Carp	t= +/-3.73	p<0.01
Carp vs. Sucker	t= +/-4.72	p<0.001

WITHIN SPECIES		
CHUB		
Erythrocytes vs. Rodlet Cells	t= +/-1.94	n.s.
Erythrocytes vs. Larger Cells	t= +/-1.49	n.s.
Larger Cells vs. Rodlet Cells	t= +/-0.79	n.s.
SUCKER		
Erythrocytes vs. Rodlet Cells	t= +/-1.62	n.s.
Erythrocytes vs. Larger Cells	t= +/-4.27	p<0.001
Larger Cells vs. Rodlet Cells	t= +/-1.71	n.s.
CARP		
Erythocytes vs. Rodlet Cells	t= +/-0.35	n.s.
Erythrocytes vs. Larger Cells	t= +/-0.37	n.s.
Larger Cells vs. Rodlet Cells	t= +/-0.14	n.s.

TABLE 4-4
EXPERIMENT 3

STATISTICAL COMPARISONS OF NUCLEAR DNA WITHIN AND BETWEEN SPECIES
VICKERS SCANNING MICRODENSITOMETER

BETWEEN SPECIES		
ERYTHROCYTES		
Chub vs. Sucker	t= +/-41.7	p<0.001
Chub vs. Carp	t= +/-10.48	p<0.001
Carp vs. Sucker	t= +/-9.48	p<0.001
RODLET CELLS		
Chub vs. Sucker	t= +/-10.79	p<0.001
Chub vs. Carp	t= +/-2.16	p<0.05
Carp vs. Sucker	t= +/-6.85	p<0.001
LARGER CELLS		
Chub vs. Sucker	t= +/-10.27	p<0.001
Chub vs. Carp	t= +/-3.99	p<0.001
Carp vs. Sucker	t= +/-12.82	p<0.001
WITHIN SPECIES		
CHUB		
Erythrocytes vs. Rodlet cells	t= +/-4.81	p<0.001
Erythrocytes vs. Larger Cells	t= +/-4.22	p<0.001
Larger Cells vs. Rodlet Cells	t= +/-1.30	n.s.
SUCKER		
Erythrocytes vs. Rodlet Cells	t= +/-4.73	p<0.001
Erythrocytes vs. Larger Cells	t= +/-6.32	p<0.001
Larger Cells Vs. Rodlet Cells	t= +/-1.92	n.s.
CARP		
Erythrocytes vs. Rodlet Cells	t= +/-2.97	p<0.01
Erythrocytes vs. Larger Cells	t= +/-3.14	p<0.01
Larger Cells vs. Rodlet Cells	t= +/-0.132	n.s.

cell nuclei, not only within but also between species. For carp and chub, the erythrocytes showed significantly more DNA than the rodlet cells of the same species; in sucker, the converse is true, and the rodlet cells show significantly more DNA per nucleus than the erythrocytes. The statistically closest relationship is seen between the rodlet cells of carp and chub ($p < 0.01$).

Fig. 4-1 shows that the cell populations measured in Experiment 1 show an apparently normal distribution about the mean, and that the means of the erythrocyte and rodlet cell measurements appear not to overlap.

Experiment 2

In this experiment, the cells were measured on a Leitz Microdensitometer. Tables 4-III and 4-IV show that DNA contents of erythrocyte and larger cells assessed between species show highly significant differences ($p < 0.01$, $p < 0.001$) except for carp compared with sucker, but that there were no significant differences in the DNA content of rodlet cells from the three species. Within species, no statistically significant differences were found among any of the cell types for the chub and carp. For the sucker, the rodlet cells do not contain significantly more DNA than either the erythrocytes or larger cells, although the larger cells are measured as containing significantly more DNA than the erythrocytes ($p < 0.001$).

Experiment 3

The results of measurements of the same preparations as for Experiment 2, but using the scanning microdensitometer are found in Tables 4-III and 4-V. Using the scanning microdensitometer, it is possible

to perform several replicate measurements quickly upon a single nucleus. For the same number of cells as in Experiment 2, but with a larger number of actual measurements, comparisons of rodlet cells with erythrocytes and larger cells within the same species showed that rodlet cells show significantly different DNA values from the erythrocytes ($p < 0.01$, $p < 0.001$), but no significant differenoes from the larger cells of the same species. The carp showed the least significant difference between erythrocyte and rodlet cell DNA content. For carp and sucker, the t values of the differences between erythrocytes and larger cells of the same species were generally larger than for erythrocytes and rodlet cells ($t = \pm 4.22, 6.32, 3.14$ vs. $t = \pm 4.81, 4.73, 2.97$, respectively).

For both microdensitometers, and for all experiments, the DNA values of erythrocytes of S. atromaculatus, C. commersoni and C. carpio were highly significantly different from each other ($p < 0.001$), with one exception, which is carp vs. sucker in Experiment 2 where no significant difference was found between the erythrocytes of the species.

For both microdensitometers, and for all experiments, the smallest statistically significant differences were seen among the rodlet cells: in Experiment 1 the least significant difference was $p < 0.01$ for chub vs. carp rodlet cells; for Experiment 2, using the same instrument, there was no significant difference among rodlet cells of the three species. For Experiment 3, a range of significances was seen among rodlet cells between species, but these were smaller than were seen among either erythrocytes or larger cells in the same experiment.

Table 4-III shows the absolute DNA content of erythrocytes, larger cells and rodlet cells based on 3.4 pg/nucleus established for the carp (Hinegardner and Rosen, 1972). Clearly the values calculated for

the scanning microdensitometer did not agree with those for the Leitz microdensitometer, in that they were invariably larger. In addition, they failed to show changes which paralleled those of Experiments 1 and 2: in Experiment 3, rodlet cells showed a higher DNA content than the erythrocytes in the species; Experiment 1 showed the converse, and Experiment 2 was in between.

Experiments 2 and 3 (Tables 4-IV and 4-V) indicated consistently that no matter what type of microdensitometer was used to measure the cells, rodlet cells and the "larger cells" showed no significant difference in DNA content for any species studied.

DISCUSSION

Experiment 1 shows the advantage of measuring large numbers of cells, in that it is possible to construct a meaningful histogram (Fig. 4-1) which indicates that the erythrocytes and rodlet cells both have approximately normal distributions about their means, and that the means of the measured cells do not overlap. Clearly, for the populations of cells examined in Experiment 1 using the methods available, there is a significant quantitative difference between rodlet cell and erythrocyte DNA.

The experiment suffers from two disadvantages. The first is that with sufficiently large numbers of cells, it may be possible to show statistically significant differences between cell classes even when none really exist; as the number of degrees of freedom rise, the t value required to demonstrate statistically significant difference grows smaller. For example, the statistical calculations of Experiment 1 are

not borne out by Experiment 2, where no species shows a significant difference between rodlet cells and erythrocytes. Experiment 2 is based upon fewer cells; in both experiments, the measurements were carried out as carefully as possible. For Experiment 1, the chub and carp results could be explained on the basis of a combination of distributional and scattering errors, which would tend to give smaller apparent DNA values, but this does not account well for the sucker. What is not clear, if the "difference" perceived in the relative amounts of DNA in rodlet cells and erythrocytes in Experiment 1 is actually a measurement error, is why the bias is inconsistent: sucker rodlet cells show more DNA than the erythrocytes, but the reverse is true for chub and carp rodlet cells. However, that some real statistical differences may exist between the erythrocyte DNA and rodlet cell DNA values is confirmed by the scanning microdensitometric experiment where significant differences are also seen for all three species of fish, and by the histograms of Fig. 4-1.

The second disadvantage of Experiment 1 is that larger cells were not included in the experimental design. Human leukocytes have shown considerable variation in DNA content (Shapiro, 1976) and at the time Experiment 1 was performed, it was considered that the best comparison of DNA content lay between erythrocytes with a "known" 2C DNA complement and the rodlet cell, whose DNA values were totally unknown. As well, preliminary assessments of sucker leukocytes in the Leitz microdensitometer had shown a wide variation in DNA content, confirming the original assumption.

Experiments 1 and 2 present apparently contradictory evidence. In Experiment 1, where the rodlet cells in all species, based upon

statistical evidence, may be considered to have a quantitatively significantly different genome from 2C cells known to belong to the fish, in no case is the difference as large as 1C. This lends weight to the impression of variability, and consequently supports the hypothesis that the rodlet cell is a "parasite": Experiment 2, on the other hand, with fewer measured cells, shows no significant difference among the DNA contents of any of the measured cell types for chub and carp; only in the sucker is the difference significant ($p < 0.001$) for erythrocytes vs. larger cells. With less confidence, the results fail to support the hypothesis that the rodlet cell is a "parasite", based upon the apparent DNA content of the nucleus.

The results of the scanning microdensitometry of Experiment 3 support Experiment 1 when only rodlet cells and erythrocytes are compared (Tables 4-II and 4-V), but both Experiments 2 and 3 with their lack of significant difference between rodlet cells and larger fish cells cast doubt on whether this difference is really large enough to justify the hypothesis that the rodlet cell is a "parasite" unless the larger cells are also to be regarded as "parasites" (Tables 4-IV and 4-V). In addition, there is no apparent relationship between absolute amounts of DNA measured in the Leitz MPV3 and Vickers M85 (Table 4-III). The results of Experiments 2 and 3 also do not support those of Experiment 1 in that, for Experiment 2, only the carp rodlet cells show a smaller DNA content than the erythrocytes (Table 4-III) when measured with the Leitz microdensitometer. For all other species and either method of measurement, the rodlet cells show a greater average DNA content than the erythrocytes of the same species.

Part of the apparently close relationship of the rodlet cells among species measured with the Leitz MPV3 microdensitometer may reflect a consistent scattering error based upon their generally hemispheric nuclei and the intractability of the apertures for this instrument. The aperture of the scanning microdensitometer may be placed more closely around the nuclei of rodlet cells than can that of the Leitz MPV3. This reduces the scattering error, and the statistical results noted in Table 4-V may represent more nearly an accurate assessment of the actual differences in DNA content.

The differences in apparent DNA values for the same cells within species measured on different instruments is probably a distribution error caused by the "averaging" of light transmission of the Leitz microdensitometer, which tends to give lower values than the scanning microdensitometer. As well, the Leitz microdensitometer probably gives a larger scattering error: its optical system is not closed off from ambient light, although the readings were taken in subdued light. The inflexibility of the aperture arrangement frequently meant considerable areas of the measuring field were unfilled by the nucleus under consideration. The latter was, predictably, especially valid for the rodlet cells and larger cells in each species. Consequently, the scanning microdensitometer must be generally considered to give a more reliable measure of actual DNA content than the Leitz MPV3.

These experiments also call into question the reliability of microdensitometry in making fine distinctions between the DNA contents of cells in an organism. The range of DNA values of rodlet cells and larger cells for the various species encompasses the 2C value of the

erythrocytes, but more of them have an apparent value closer to 3C than 2C, especially in the sucker. Whether this is the result of gene amplification or measurement error due to the larger apertures required for the larger cells is not known. On the basis of such a limited study it is not possible to state whether the basis of the measurements, i.e., that erythrocytes contain a 2C genome, is accurate or not in the light of the apparent variations seen in other cells. The study does indicate that to compare DNA values in absolute terms requires consistent use of one type of microdensitometer, preferably supported by other analytical methods.

THE ANSWER

The fact that there was, consistently, no significant difference between rodlet cells of a species and the larger cells of the species, no matter which method of measurement was used, is substantial grounds for accepting the hypothesis the experiments were designed to test: namely, that the rodlet cell nuclear DNA content is not different from that of the cells known to belong to the fish. The hypothesis is not as clearly accepted when rodlet cells are compared to the erythrocytes within the species (compare Tables 4-IV and 4-V), although the "t" values for rodlet cells vs. erythrocytes are generally smaller than those between rodlet cells and larger cells of the species. One must conclude, therefore, on the basis of these experiments, that the DNA content of the nucleus of the rodlet cell is quantitatively similar to nuclei of a population of cells known to belong to the genome of the fish.

CHAPTER V

HYBRIDIZATION IN SITU

CHAPTER V

HYBRIDIZATION *IN SITU*

INTRODUCTION

The principle that denatured strands of DNA realign themselves *in vitro* precisely (Britten and Kohne, 1968) with complementary sequences, given enough time, has been used to determine evolutionary distance between organisms. The closer the species are to each other phylogenetically, the larger the number of genetic sequences they will have in common (Rice, 1972). As well, the more precisely paired two DNA sequences are, the more energy it takes to separate them; *i.e.*, the higher the temperature of denaturation.

In addition to reassociation experiments carried out on solutions of DNA *in vitro*, methods have also been developed to enable visual localization of reassociated DNA molecules on microscope slides, using nick-translated DNA and radioisotopically labeled nucleotide triphosphates (Rigby *et al.*, 1977; Brahic and Haase, 1978). Hybridization *in situ* is a rather imprecise method, unfortunately, since even with enhancement procedures only about 10% of the actual label can be demonstrated (Brahic and Haase, 1978). The first choice in these experiments was to isolate rodlet cells so their nuclear DNA could be extracted and hybridized *in vitro* to known types of DNA (Southern, 1975). The methods employed are outlined in Appendix I. To date no successful method has been devised for concentrating rodlet cells in numbers that would be useful

for the proposed experiments; it was felt that the time expended on the isolation experiments was better spent on methods which would give an answer to the question of qualitative similarity of rodlet cell nuclear DNA and that of somatic cells of the fish.

THE QUESTION

Do rodlet cell nuclei have qualitatively the same DNA as cells known to belong to the fish? In order to ascertain whether the nucleus of the rodlet cell contained DNA similar to that of known somatic cells in the same fish, hybridization _in situ_ studies were made.

MATERIALS AND METHODS

Northern creek chub (_Semotilus atromaculatus_) and white sucker (_Catostomus commersoni_), weighing approximately 60 g, were injected with 0.1 ml 10,000 IU/ml heparin 20 minutes before being killed by severing the spinal cord and dorsal aorta with a pair of scissors. Peripheral blood was collected in a siliconized, nuclease-free beaker, and DNA extracted (for details of protocol, see Appendix III). To reduce background label, microscope slides were prepared in the following manner: alcohol-rinsed slides were treated with 450 mM NaCl/45 mM Na citrate/0.02% w/v Ficoll, polyvinylpyrrolidone and BSA for 3 hours at 65°C, rinsed briefly in water and fixed for 20 minutes in freshly-prepared Carnoy's fluid (100% ethanol:glacial acetic acid, 3:1) (Brahic and Haase, 1978). The excised gills of the chub were touched to the prepared slides, and allowed to dry.

Air-dried preparations were fixed in Carnoy's fluid, treated with formamide, Proteinase K and dehydrated before the DNA was denatured in 0.07N NaOH for 3 minutes. The treated slides were dehydrated through 90% alcohol and allowed to air dry before the hybridization solution was applied.

DNA for nick-translation was obtained from several sources. Chub and sucker genomic DNA were prepared in the laboratory, and chub DNA was the principal tool used to investigate the rodlet cell nuclei of the respective species. Rainbow trout genomic DNA and cloned vitellogenin gene (a gift from T. T. Chen), a cloned rainbow trout oncogene (myc 1-8, a gift from R. A. Sonstegard) were also nick-translated and used for comparison purposes. Nick-translated chub genomic DNA was also applied to prepared frog, human and ciliate cells to confirm its specificity.

Details of the procedure for nick-translation are found in Appendix III. ^{125}I-cytidine triphosphate (New England Nuclear) was combined with cold adenine, thymidine and guanidine triphosphates as bases for the polymerization of the DNA after nicking with DNAse I. The nick-translated DNA was estimated using a scintillation counter for proportion of isotope uptake after being dissolved in hybridization buffer, then denatured by heating at 100°C for 10 minutes. Approximately 5×10^5 cpm (15-30 µl) of the nick-translated DNA was applied to each slide preparation, covered with a siliconized, nuclease-free coverslip, and incubated at 27-29°C for 65-72 hours on paper towels moistened with 300 mM NaCl/30 mM Na citrate in a shallow tray covered with plastic food wrap. After incubation, the coverslips were removed, and the slides washed for 24 hours in 3 changes of 600 mM NaCl/1 mM EDTA/10 mM Tris HCl

pH 7.4/50% formamide, then rinsed in 70% ethanol/300 mM sodium acetate, and dehydrated through 95% ethanol and air-dried.

Radioautography was performed using Kodak NTB-2 emulsion diluted 1:1 with 600 mM sodium acetate, to enhance the reaction of the emulsion to nuclear decay. The coated slides were air-dried, then stored with desiccant in light-proof boxes at 4°C for 1-3 weeks. The emulsion was developed in freshly-prepared Kodak D-19 diluted 1:2 with distilled water, rinsed 1 minute in 1% acetic acid, and fixed 4 minutes in Kodak acid hard fixer. Development and fixing were carried out at 20°C. For visualization, the completed preparations were stained either with Harris's hematoxylin and eosin or Scarba's red and fast green FCF (Humason, 1972).

In evaluating the genomic DNA preparations, an unlabeled cell was defined as one with fewer than 4 grains of silver per nucleus. This value had previously been adopted by Rossant (1982, personal communication) in her studies. In one experiment, three hundred of each of rodlet cells, erythrocytes and lymphocyte-sized nuclei (as defined below) were classed as labeled or unlabeled in each preparation. Because of the drastic pretreatment that slides undergo, cytoplasm of cells with nuclei approximately the size of rodlet cell nuclei (= lymphocyte-sized) is often not stained, and identification of these cell types is not possible. However, the thick "capsule" of the rodlet cell withstands complete destruction, and only rodlet cells which were clearly identifiable were included in the estimations.

To determine the specificity of the reaction of the chub genomic DNA after hybridization to chub cells and rodlet cells, the preparations were hybridized and washed at 45, 50, or 55°C in 30 mM Na citrate/300 mM

NaCl, then radioautographed and examined. The label over 100 each of rodlet cells and "other cells" (erythrocytes or leukocytes) was estimated and the number of silver grains counted precisely for 20 cells. As well, the background number of silver grains was estimated from counting 10 0.02 X 0.02 mm areas delineated by an ocular graticule over an area devoid of cells. To compare the background with the cell preparations, grains were counted for 10 areas of the same size over areas containing cells.

RESULTS

The results of the counting of label of the first experiments are seen in Table 5-I, and show that 56.3-73.6% of the rodlet cells are labeled with more than four silver grains, in the same preparations in which 71.0-99.7% of the nuclei of other cells were labeled. Grouping rodlet cells in which any number of grains were found over nuclei, and comparing them with similarly grouped somatic cells shows that for the control experiments, 87.0, 90.8 and 88.3% of rodlet cells showed some label, whereas 92.0, 99.7 and 98.7% of somatic cells were labeled under the same conditions. In the washing experiments, at 45°C, 97.0% of rodlet cells and 96.0% of somatic cells bore label; at 50°C, 84.0% and 92.0% of rodlet and somatic cells were labeled, respectively; at 55°C, 64.0% of rodlet cells and 68.0% of somatic cells showed some label.

The rodlet cells were identified on the basis of the capsule and clear, unlabeled, elongated, lightly stained cytoplasmic area (Figs. 5-1, 5-2). Sometimes the individual rodlets were also visible in spite of the pretreatment of the tissue. Erythrocytes were identifiable by nuclear shape and size (Fig. 5-2), although the cytoplasm usually did not

TABLE 5-I

PROPORTION OF CELLS SHOWING LABEL AFTER HYBRIDIZATION IN SITU

Treatment	Chub Cells Number of Grains per Cell: Rodlet Cells (N = 100) 0	1-4	>4	Other Cells (N = 100) 0	1-4	>4
Chub Genomic DNA (23°C) Expt. 11*	13	21	66	8	21	71
Expt. 6b★	7.4	19.2	73.6	0.3	0	99.7
Expt. 6a♦	11.7	32	56.3	1.5	12.2	86.5
45°C	3	12	85	4	16	80
50°C	16	34	50	8	25	67
55°C	36	42	22	32	32	36
Rainbow Trout DNA† (23°C)	42	46	13	36	55	9

*Preparation with patchy distribution of label and few rodlet cells; unlabeled cells in both rodlet cell and "other cell" categories probably an overestimate.

★Autoradiograph for 13 days.

♦Autoradiograph for 6 days.

†No more than 7 grains seen on any rodlet cell nucleus; no more than 5 grains seen on any other cell nuclei.

TABLE 5-II

NUMBER OF GRAINS PER CELL AFTER POST-HYBRIDIZATION WASHING OF PREPARATIONS AT VARIOUS TEMPERATURES

Chub cells hybridized with chub genomic DNA (^{125}I-dCTP)
(N = 20)

Treatment	Number of Grains over Nucleus: Rodlet Cells (Range)	Somatic Cells (Range)	Significance Rodlet Cells vs. Somatic Cells
Control (23°C)	28.8 (7-50)	39.1 (12-75)	n.s. (t = ±1.23)
Washing Experiment			
45°C	14.2 (4-46)	15.2 (8-40)	n.s. (t = ±1.16)
50°C	14.3 (1-50)	14.1 (5-28)	n.s. (t = ±0.12)
55°C	3.4 (0-15)	3.7 (0-18)	n.s. (t = ±0.13)
Rainbow Trout DNA X Chub Cells (23°)	1.1 (0-5)	2.1 (0-6)	

RESIDUAL LABEL AFTER WASHING EXPERIMENT

Area: 0.004 mm^2 (4 X 10^3 µm^2)

Treatment	Average Grain Counts: Over Background (Range)	Over Imprints (Range)
Control (23°C)	8.4 (6-16)	50.4 (20-153)
45°C	9.4 (5-16)	54.3 (8-108)
50°C	9.4 (2-23)	45.4 (30-107)
55°C	1.6 (0-6)	17.3 (5-35)
Rainbow Trout DNA X Chub Cells	4.3 (1-8)	5.3 (1-12)

stain. In other somatic cells, the nuclei and sometimes the cytoplasm remained intact.

Comparison of counts of numbers of grains over nuclei washed at different temperatures after hybridization are given in Table 5-II. In the controls, rodlet cells showed a mean of 28.8 grains per nucleus, while somatic cells had 39.1 grains per nucleus. Under conditions of washing at 45° and 50°C, the mean number of silver grains over rodlet cells declined to 14.2 and 14.3, respectively, and the number over somatic cells to 15.2 and 14.1 grains, respectively. When the washing temperature was raised to 55°C, a mean of 3.4 and 3.7 grains were seen over rodlet cells and somatic cells, respectively. None of the differences between the numbers of grains over rodlet cell nuclei vs. somatic cell nuclei in the same preparation was statistically significant.

When comparisons are made between the relative number of grains over background and imprint areas (10 areas, each ~400 μm^2), values for the "empty space" in control, 45° and 50° preparations are virtually constant at 8.4-9.4 grains/400 μm^2. In addition, the grain counts over the imprints (both inside and outside the individual cells) were comparable at 45.4-54.3 for the same three conditions. In the 55°C washing experiment, the background declined to 1.6 grains per area, and the label over the imprints declined to 17.3 grains per area. When the results of hybridizing rainbow trout DNA to chub cells are examined, the label of both background and imprint areas are essentially the same, at 4.3 and 5.3 grains per area, respectively.

Chub cells hybridized only slightly to rainbow trout genomic DNA, with an average of 1.1 and 2.1 silver grains per nucleus for rodlet cells

and somatic cells, respectively (Table 5-I). When background values for this experiment were examined, the imprints showed a mean of 5.3 silver grains per field, while the empty background was 4.3 grains per field. Using the "4-grain" criterion stated above for the identification of label, only 13 individual rodlet cells and 9 somatic cells could be classed as labeled, and the group as a whole was "unlabeled". However, 59.0% and 64.0% of rodlet cells and somatic cells, respectively, showed one or more silver grains over the nucleus.

The label over all nuclei was discrete, with some label slightly outside the bounds of the nuclear envelope. All areas of slides were not equally labeled, but where the label was consistent, rodlet cells were labeled as heavily as other cells known to belong to the fish (Fig. 5-2).

When chub DNA was applied to human, frog and ciliate cells, there was virtually no label seen. The human leukocytes (Fig. 5-3) generally showed one grain over the nucleus. The background in these preparations was very low. By the terms of the estimation of the label for this experiment, such a nucleus is "unlabeled", but the consistency of the label may represent a small degree of hybridization. A similar pattern was seen in the frog. In the few ciliates which were encountered in preparations, a maximum of four grains was seen over the micronucleus (Fig. 5-4); by the criteria defined for this experiment, the ciliates were unlabeled.

The oncogene, myc 1-81, applied to chub preparations, showed very few grains over any nuclei. Some grains were seen on the cytoplasm of rodlet cells; in a few cases the label appeared to be aligned with the rodlets, but too few cells were labeled sufficiently to describe a consistent

pattern.

DISCUSSION

If a rodlet cell is labeled with as many grains over the nucleus as a cell known to contain the genome of the fish, it must have, qualitatively, the same genome. This is true especially in view of the lack of label seen when chub genomic DNA is applied to other teleost cells, or to more genetically distant organisms. Although 74.0% of rodlet cells were labeled with more than 4 silver grains over the nucleus, versus 99.7% for somatic cells, this lighter label might be attributed to the thickness of the "capsule" surrounding the cell. A piece of DNA may be impeded by the capsule, since it is a rather large molecule. Supporting this suggestion is the observation that some rodlet cells where the rodlets had been discharged, and where the "capsule" is fully contracted, therefore thickest, showed the lightest label.

Hybridization *in situ* is probably the least precise method commonly used for estimating the degree of affinity between two types of DNA. It is frequently used in localizing unique sequences to particular chromosomes (*e.g.*, Harper and Saunders, 1981) and in these preparations the total number of grains at particular loci is pooled for a large number of cells to estimate the real label. In this study, the whole genome was used, with appropriate controls, to estimate the relative affinity of label on rodlet cells and normal fish cells. If it had been possible to isolate rodlet cells in relatively pure form, precise measurements of the degree of affinity might have been made using the Southern blot procedures (Southern, 1975), but to date such purification

has not been possible.

Hybridization *in situ* is not especially sensitive when low degrees of affinity are present. Brahic and Haase (1978) estimated that a maximum of 10% of the potential label is visualized in radioautography of hybridized DNA. In their experiments, they estimated that enhancement with ammonium acetate in the washes and emulsion resulted, for their experiments, in the visualization of the entire possible label; *i.e.*, the entire 10% of the actual label present. Other procedures, without enhancement, reduced the label to about 10% of the enhanced value. For the chub rodlet cell experiment, therefore, considering the difficulty of visualizing the actual label achieved, it might be estimated that false negative results would be more likely than false positive results.

With the washing experiment, the importance of potential leaching of DNA from nuclei becomes evident: Table 5-II shows that the number of grains/nucleus declines by more than 50% in both somatic cells and rodlet cells after 45° and 50°C washes. However, for estimations of total label *vs.* background, Table 5-II indicates that control, 45° and 50° experiments show the same number of grains over the imprints. Since the grains are not counted over the nucleus, they must be present over the cytoplasm and between cells. Whether the leaching represents excess nick-translated genomic DNA or includes complexes leached from cell nuclei by the washing procedure is unknown.

A further contradiction appears in the 45° experiment when compared to the control condition. Table 5-I indicates that, even though the number of grains/nucleus declines with the washing experiment, the percentage of "labeled" (more than 4 grains per nucleus) rodlet cells actually is greater (85% vs. 66%). Certainly some of the variation is

due to the success of hybridization for individual slides. It may also reflect the fact that cells in the control preparations were often so heavily labeled that the cytoplasm was effectively obscured, making unequivocal identification of labeled rodlet cells impossible. The 45° wash may have removed enough excess label that such cells could have been more easily classified.

Another factor which may affect the visualization of nuclear label is the length of time the autoradiograph is exposed before development. For example, in Experiments 6a and 11 (Table 5-II) preparations were exposed for 13-14 days, and yet there was considerable disparity of label, especially for the somatic cells.

The presence of label over the cytoplasm of rodlet cells after myc 1-81 may represent either leaching of DNA from the nucleus, entrapment of labeled nucleic acid on the surface of the cell, or a real hybridization event. The genomic DNA of the chub never labeled the cytoplasm if the nucleus was not also heavily labeled. Inevitably, some of the grains in the emulsion were seen over the cytoplasm, but the maximum energy of ^{125}I is 0.021 Mev (Quimby et al., 1970), so scattered label is not unexpected. However, with the complete lack of label over the nucleus, the label over the rodlets becomes intriguing. Oncogenes are sequences which are part of the normal genome of vertebrates which are thought to be evolutionarily conserved (Shilo and Weinberg, 1981). There is no reason to expect that the DNA of rodlets would be more likely to share sequences with oncogenes than with the genome of the fish.

THE ANSWER

Hybridization in situ shows that the nuclei of rodlet cells label to the same degree as nuclei of known somatic cells, when both are treated with autologous genomic DNA, and that this label is much heavier than is seen on any cells of other teleost species, protozoa, or human leukocytes. Such evidence leads to rejection of the hypothesis that the rodlet cell has a genome qualitatively different from that of the cells of the fish.

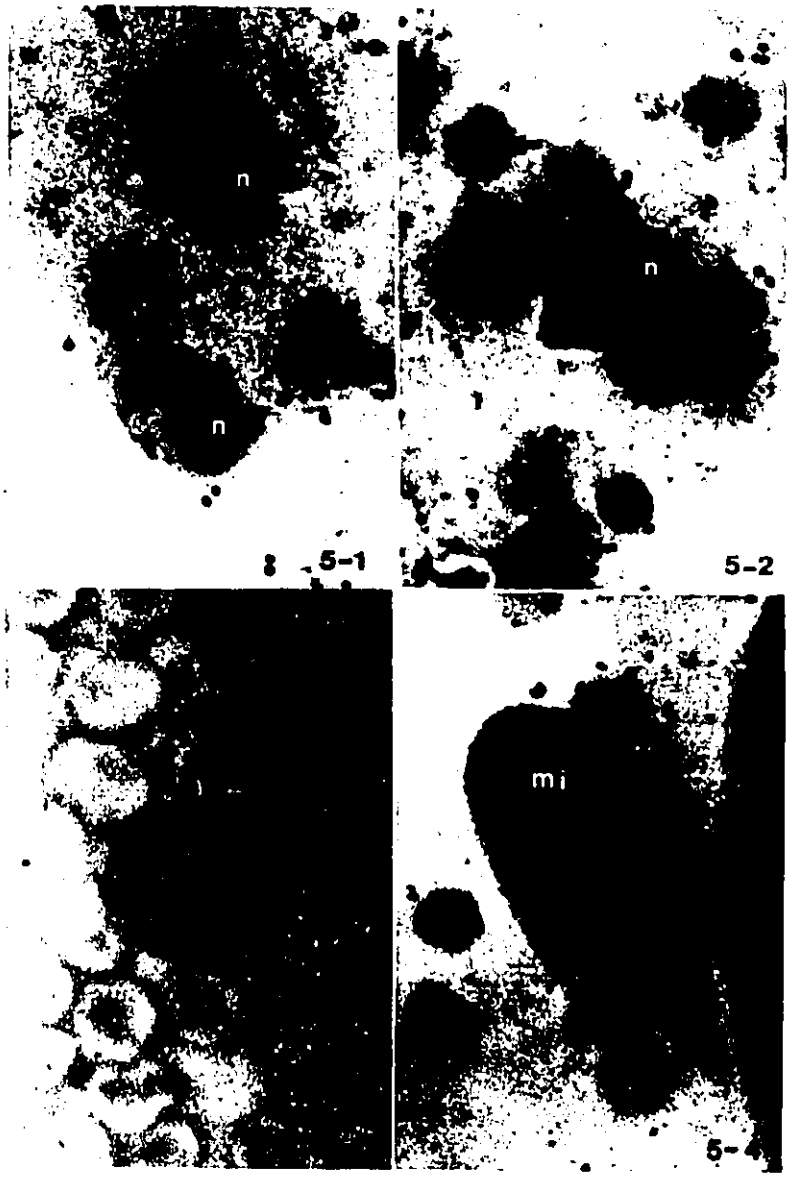
PLATE VI

Fig. 5-1: Rodlet cells (rq) from gill imprint preparation of Semotilus atromaculatus, showing nuclei (n) with label of silver grains after hybridization in situ. A labeled erythrocyte (arrow) lies adjacent to the rodlet cells and also shows label. Hematoxylin and eosin. X 1000

Fig. 5-2: Rodlet cell (rc) from imprint preparation of Semotilus atromaculatus gill after hybridization in situ showing heavily labeled nucleus (n), and cytoplasm free from label. Labeled nuclei of gill epithelial cells or other cells larger than erythrocytes (arrows) are also present. Hematoxylin and eosin. X 1000

Fig. 5-3: Human neutrophil from blood smear showing one silver grain (arrow) at the edge of the polymorphic nucleus (n) after hybridization in situ. Surrounding erythrocytes (rbc) are largely unlabeled. Hematoxylin and eosin. X 1000

Fig. 5-4: Ciliate in gill imprint of Semotilus atromaculatus showing macro- and micronuclei (ma, mi) with scattered label after hybridization in situ. Some adjacent teleost cell nuclei are lightly labeled (arrows). Hematoxylin and eosin. X 1250



5-1 5-2

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CHAPTER VI

NUCLEASE EXPERIMENTS

CHAPTER VI

NUCLEASE EXPERIMENTS

INTRODUCTION

Histochemical studies (e.g., Barber and Westermann, 1975; Leino, 1982) of rodlet cells have established that the elongate cytoplasmic rodlets show variable but generally positive reactions to protein and polysaccharide stains, but not to Feulgen procedures for DNA. However, Barber et al. (1979) reported that rodlet cores gave red fluorescence after acridine orange, and stained pink with methyl green/pyronin, suggesting the presence of single-stranded nucleic acids, either RNA or single-stranded or short polymers of DNA (Pearse, 1983). Incubation of Spurr's-embedded ultrathin sections in RNAse A solutions caused the rodlet cores to become less electron-dense, suggesting, on the basis of this plus the light-microscopic histochemical reactions, that RNA was a component of the rodlet core (Barber et al., 1979).

Bernhard (1969) reported that EDTA applied to uranyl acetate-stained thin sections selectively removed the stain from electron-dense, DNA-containing structures such as heterochromatin, but left RNA-containing structures untouched. Viehberger and Bielek (1982), applying this technique to rodlet cells in Cyprinus carpio, reported that the heterochromatin of the rodlet cell nuclei faded, and so did the cores of the rodlets. Using Epon-embedded material, they were unable to duplicate the experiments of Barber et al. (1979). Since the rodlet cores were

Feulgen-negative as well, these authors concluded that "at least the [DNA of the] core of the rodlets is constituted of condensed chromatin with extraordinary configurations".

Bendayan (1981) established that nucleic acids could be precisely localized and identified in thin sections of Epon-embedded tissue, by DNAse I or RNAse A complexed to colloidal gold particles. When DNAse I was applied to thin sections of rat pancreatic acinar cells, the gold particles were seen over euchromatin, the nucleolus and mitochondria in the cell. RNAse A-gold labeled heterochromatin, the nucleolus and the ribosomes of the cell. The RNAse A-gold showed virtually no label on mitochondria or euchromatin, and neither nuclease-gold preparation labeled the protein-containing zymogen granules.

THE QUESTION

In view of the discrepancies among the findings of Barber et al. (1979), Viehberger and Bielek (1982), and the histochemical experiments of Leino (1982), do rodlets, or a portion of them, contain nucleic acids? If so, which acids are present, and where are they located?

In order to resolve the reported discrepancies, and to determine precisely the types and distribution of nucleic acids in rodlet cells, the following experiments were undertaken:

1) Re-examination of the light microscopic techniques of Barber et al. (1979), using acridine orange and methyl green-pyronin, with and without nuclease pre-treatment.

2) Applications of both RNAse A and DNAse I to Spurr's and Epon resin-embedded gill tissues, in an attempt to duplicate the experiments

of Barber et al. (1979) and Viehberger and Bielek (1982); also the application of EDTA solutions to uranyl acetate-stained thin sections to confirm the experiments of Viehberger and Bielek (1982) and Bernhard (1969).

3) Use of the nuclease-gold technique of Bendayan (1981) to establish the nature and distribution of nucleic acids in the rodlet cell.

MATERIALS AND METHODS

Animals

Northern creek chub (Semotilus atromaculatus) and white sucker (Catostomus commersoni) were obtained from a commercial bait supplier (B. Conroy, Toronto, Canada), and were acclimated for at least one week in aquaria fed by running, dechlorinated tap water. The fish were maintained on a commercial fish pellet.

LIGHT MICROSCOPY

Histochemical Methods

Touch preparations of gill tissues were fixed in 99.9 mol% pure methanol. In order to demonstrate the presence, in rodlet cells on the slide, of DNA, RNA, single-stranded DNA, or protein, respectively, the fixed preparations were treated in the following ways: 1) Preparations were digested at 37°C for 2 hours with one of: a) 1 mg/ml DNAse I in phosphate buffer pH 6.0, b) RNAse A in phosphate buffer pH 7.5, c) S1 nuclease in acetate buffer at pH 5.0, d) 0.1 mg/ml Proteinase K

at 37°C for 1 hour, e) untreated, as controls. They were then stained with acridine orange at pH 6.0 and examined with a Leitz epifluorescence microscope. 2) Preparations treated as above were stained with methyl green and pyronin (Humason, 1972), and examined in ordinary light microscopy. Cells of interest were photographed using Fujichrome HR400 high resolution colour film.

ELECTRON MICROSCOPY

Preparation of Tissues

Details of tissue preparation are found in Appendix IV.

Enzyme-Gold Experiments

Fresh solutions of gold and enzymes were prepared for each experiment; detailed methods for the preparation of DNAse I-gold and RNAse A-gold (Bendayan, 1981) are given in Appendix IV.

All preparations were carried out in glassware cleaned with a heavy-duty detergent (Extran, BDH Chemicals), rinsed in hot tap water and distilled water, baked at 85°C for at least 24 hours to destroy exogenous DNAse, and then siliconized (Sigmacote, Sigma Chemical Co.) and allowed to dry at 85°C.

The following nuclease-gold preparations were used in the experiments:

1) DNAse I-gold was used in phosphate-buffered saline (PBS) at pH 6.0.

2) RNAse A-gold was used in PBS at pH 7.5.

3) In addition to the methods of Bendayan (1981), S1 nuclease-gold

was prepared according to methods outlined below and used in acetate-buffered saline (ABS) at pH 5.7.

Methods for S1 Nuclease

Colloidal gold was prepared by adding 5 ml of 1% w/v sodium citrate to 100 ml of boiling 0.01% gold chloride (tetrachloroauric acid), and allowed to boil gently for 5 minutes. The solution was allowed to cool, and 10 ml of the cooled colloidal gold was added to 0.2 mg commercial SI nuclease (Boehringer Mannheim) in 0.1 ml double distilled water, and stirred gently on a magnetic stirrer for 5 minutes. The solution was checked for stabilization using 10% NaCl (Bendayan, 1981); the S1 nuclease-gold was never completely stable, so approximately 2 mg/ml polyethylene glycol (MW 20,000) was added before the solution was centrifuged at 18700 X g at 2°C in a Beckman J-20 rotor for 30 minutes. The dark red sediment was resuspended in 3 ml ABS plus polyethylene glycol (0.2 mg/ml), pH 6.0. Thin sections of Epon-embedded gill as prepared for other nuclease-gold experiments were placed on formvar-coated nickel grids, hydrated on a drop of ABS + PEG at pH 6.0, and then treated with the S1 nuclease-gold at room temperature for 30-45 minutes. After incubation, the sections were washed carefully in three changes of ABS and three changes of distilled water over a period of 5 minutes before being examined, unstained, in the electron microscope.

Controls for Enzyme-Gold Experiments

1) In order to ascertain the specificity of the enzyme-gold label for the appropriate nucleic acids, for each of the above, some sections were pretreated with nuclease for 30 minutes at room temperature before being treated with nuclease-gold for 30-45 minutes at room temperature.

Grids were carefully rinsed in PBS and double distilled water after treatment, and were examined either unstained or stained with aqueous uranyl acetate and lead citrate.

2) In order to establish whether stabilized gold without complexed nucleases will bind to tissues previously treated with nucleases alone, and hence to confirm the specificity of the nuclease-gold label for the preparations, stabilized gold solutions were prepared from 10 ml aliquots of colloidal gold by the addition of 50 mg polyethylene glycol (MW 20,000) to 10 ml of the appropriate pH-adjusted solutions before centrifugation, and resuspending the sediment in 3 ml of PBS + 0.2 mg polyethylene glycol per ml. Sections were preincubated in a drop of nuclease in PBS of the appropriate pH (pH 6.0 for DNAse I and S1 nuclease and pH 7.5 for RNAse A) for 30 minutes at room temperature before being treated with the stabilized gold for 45 minutes at room temperature. RNAse A-treated sections were exposed to stabilized gold at pH 8.0, and DNAse I and S1 nuclease-treated sections were exposed to stabilized gold at pH 6.0. The sections were rinsed in distilled water and examined unstained.

Nuclease Digestion Experiments

1) Thin sections of Epon-embedded tissues were routinely used for enzyme-gold experiments, and in experiments duplicating the work of Viehberger and Bielek (1982) and Bernhard (1969) (q.v.).

2) The above Epon-embedded tissues were also used for enzyme digestion experiments in parallel with Spurr's-embedded unosmicated tissues to duplicate the experiments of Barber et al. (1979). The following experiments were performed:

i). The thin sections were placed on a drop of 0.01% (10 mg/ml) RNAse A in Tris-HCℓ buffer, pH 7.5, and were incubated in a moist chamber at 37°C for 30 min-3 hr. The grids were then rinsed in distilled water, stained with uranyl acetate and lead citrate and examined in the electron microscope.

ii) Thin sections were also incubated in DNAse I in phosphate-buffered saline (PBS) (0.9% NaCℓ in M/10 Na_2HPO_4 + NaH_2PO_4) pH 6.0 at 37°C for 30 min to 5 hr, rinsed in distilled water and stained with uranyl acetate and lead citrate.

iii) Some preparations were incubated on distilled water for 3 hours at 37°C and then stained with uranyl acetate and lead citrate.

3) Based upon the methods of Bernhard (1969), EDTA treatment of DNA-containing structures was carried out as described by Viehberger and Bielek (1982):

i) Epon sections were stained for 10 minutes with uranyl acetate, then exposed to 0.2 M aqueous EDTA for 30 minutes, rinsed in distilled water and stained for 1 minute in lead citrate.

ii) Some preparations were treated first with EDTA and then stained with uranyl acetate and lead citrate.

iii) Some preparations were treated first with EDTA and then subjected to DNAse I-gold treatment.

Observation of sections was made on a Philips EM-300 electron microscope.

RESULTS

LIGHT MICROSCOPY:

Acridine Orange Experiments

Acridine orange at pH 6.0 combined with fluorescence microscopy causes double-stranded nucleic acids (usually DNA) to fluoresce yellow-green, and single-stranded nucleic acids (usually RNA) to fluoresce red. The latter is a metachromatic reaction, brought about by the steric interaction of dye molecules on the basis of the single strand.

Reactions of rodlet cells treated with acridine orange pH 6.0 and examined with epifluorescence microscopy are shown in Table 6-I. The rodlet cells (Fig. 6-1) had bright yellow-green nuclei; rodlet cell cytoplasm showed rodlets in the cytoplasm were outlined in orange-red. In the sucker rodlet cores were consistently faintly red. In the chub the rodlet cores were discernible, but difficult to photograph, and appeared to be faintly orange, but the colour was not sufficiently intense to make an unequivocal decision. Certainly the rodlet cores were not yellow-green. Other cells in the preparation showed yellow-green nuclei, and varying degrees of reddish colouration in the cytoplasm. Where rodlets had been expelled from the cell prior to fixation and staining, the peripheral substance was unstained, distinguishable from the slightly reddish background as an almost black structure with a slender strand of pale-staining material along its length.

After treatment with RNAse A (Fig. 6-2), the preparation showed complete loss of the red-staining moiety of the cytoplasm in all cells. Rodlet cells were distinguishable with difficulty; the nucleus of the cell

TABLE 6-I

REACTIONS TO ACRIDINE ORANGE (AO) WITH AND WITHOUT ENZYME DIGESTION

TREATMENT	MOST CELLS IN PREPARATION			RODLET CELLS			
	Nucleus	Cytoplasm	Capsule	Nucleus	Cytoplasm	Rodlet Core	Rodlet Sheath
No Digestion/AO	yellow-green	various intensities of red; RBCs unstained	blue-green	yellow-green	red	faintly red	unstained
RNAse A/AO	yellow-green, reticulate	unstained	blue-green	yellow-green	blue-green	blue-green	unstained
DNAse I/AO	unstained	*brownish, or unstained	blue-green	unstained	blue-green	blue-green	unstained
S1 Nuclease/AO	reticulate, some RBC nuclei unstained	brownish, or unstained	blue-green	gen. unstained	blue-green	blue-green, or indeterminate	unstained
Proteinase K/AO	unstained; only outline remains	unstained; amorphous	blue-green	unstained only outline remains	blue-green	unstained	unstained

*Commercial DNAse preparations usually contain small amounts of RNAse.

was yellow-green, as were other nuclei in the preparation, and the "capsule", cytoplasmic areas which had stained red without RNAse A digestion, and rodlet cores were a faint blue-green. The rodlet cores showed the same faint colouration when viewed in isolation.

After DNAse I treatment (Fig. 6-3), all the nuclei in the monolayered areas of the preparation were virtually unstained, although occasional flecks of yellow-green material remained in areas that would usually be considered to contain heterochromatin. The reddish colouration of the cytoplasm of various cell types, including rodlet cells, was reduced, but the rodlet cells showed a blue-green colouration in the capsule, RNA-containing cytoplasm and the rodlet cores, especially when the cells were seen in close proximity to other cells in the preparation. Isolated rodlets had unstaining peripheral substance and faintly blue-green cores.

The appearance of preparations treated with S1 nuclease (Fig. 6-4) was substantially the same as those treated with DNAse I, except that nuclei seemed reticulated, presumably where the heterochromatin was intact. Rodlet cells showed loss of red colouration in the cytoplasm, and the capsule and cytoplasm outside the rodlets were pale blue-green. The rodlet cores still inside the cell varied in their colours, ranging from clearly blue-green to faintly orange. Isolated rodlets showed cores with the same variability in colouration; generally, the cores were blue-green, however. One rodlet cell which was partially collapsed still contained one rodlet which appeared pale blue-green in its entirety, peripheral substance and core alike.

Preparations treated with protease (Fig. 6-5) showed that neither nuclei nor cytoplasm of any cell type stained well with acridine orange.

Some erythrocyte nuclei appeared unaffected by the digestion, but most were unstaining shells with a few flecks of yellow-green material. The cytoplasm of most cells consisted of faintly brown, amorphous material. Rodlet cells were recognizable by their general morphology: a pale blue-green border, spherical basal unstaining area where the nucleus had been, and faintly blue-green cytoplasm between the rodlets. There was no evidence of rodlet cores in the preparations.

Methyl Green-Pyronin Experiments

Rodlet cells stained with methyl green/pyronin (Table 6-II) showed a green nucleus, and largely unstained cytoplasm. Areas stained faintly pink could be discerned adjacent to the nucleus and in long strands between the nucleus and the cell apex. It was not clear, in the intact cell, whether the strands represented rodlet cores, inter-rodlet cytoplasm, or both. Rodlets liberated from the rodlet cell showed an unstaining peripheral substance and pink rodlet cores (Fig. 6-6).

Preparations treated with RNAse A (Fig. 6-7) appeared to be very similar to the undigested preparations. The nucleus of the rodlet cell stained green with methyl green, and the cytoplasm showed faintly pink strands which were difficult to localize to the rodlet cores. Unfortunately, it was not possible to locate a rodlet cell in the preparation in which the rodlets were lying free of the cell.

DNAse I-treated preparations (Fig. 6-8) showed fading of the cell nuclei; many cells contained either unstaining nuclei or nuclei in which only the periphery of the nucleus showed green after methyl green. The cytoplasm of rodlet cells was largely unstaining, and rodlet cores were discernible only because they were slightly refractile. In rodlets

TABLE 6-II

REACTIONS TO METHYL GREEN-PYRONIN (MG-P) WITH AND WITHOUT ENZYME DIGESTION

TREATMENT	HOST CELLS IN PREPARATION			RODLET CELLS			
	Nucleus	Cytoplasm	Capsule	Nucleus	Cytoplasm	Rodlet Core	Rodlet Sheath
No Digestion/MG-P	green	pink; varying intensities or unstained (RBCs)	unstained	green	faintly pink	faintly pink	unstained
RNAse A/MG-P	green	unstained, or faintly pink	unstained	green	v. faint pink	?faintly pink (no free rodlets seen)	unstained
DNAse I/MG-P	unstained, faintly pink, or with green rim	varying intensities of pink	unstained	unstained to sl. green	v. faint pink	unstained; free rodlets recognizable by shape only	unstained
S1 Nuclease/MG-P	green	v. faint pink	unstained	green	unstained, greyish	Whole cell: cores unstained. Free rodlets: faint, indeterminate colour	unstained
Proteinase K/MG-P	unstained; only outline remains, exc. some RBCs	unstained to opaque pink	unstained	almost unstained to faintly green	unstained	Whole Cell:unstained cores; free rodlets not seen	unstained

lying free of the rodlet cell, neither peripheral substance nor rodlet core was stained; the rodlets were identifiable by shape alone.

When preparations were treated with S1 nuclease (Fig. 6-9), the appearance of the cell was very similar to the DNAse-treated material, except that the cell nuclei were stained green, without the apparent "leaching" of material from the centre of the nucleus. Rodlet cell cytoplasm was largely unstained, and cores were seen only as refractile structures. Isolated rodlets showed faint cores of an indeterminate colour.

ELECTRON MICROSCOPY:

For these experiments, "specific" label is defined as gold grains found over areas identified by Bendayan (1981) as containing the appropriate nucleic acid, and with very little "background" label in the form of scattered or clumped gold grains in other cellular or matrix areas. For DNAse I-gold, the "specific" label in Bendayan's experiments was largely confined to the euchromatin and nucleolus of the nucleus and to mitochondrial cristae. For RNAse A, label was found on the heterochromatin and nucleolus of the nucleus, and over ribosome-containing areas such as rough endoplasmic reticulum of the cytoplasm. Where these areas were labeled in the sections under study, other consistently-appearing label in the specimen was also classed as "specific" label. Reactions to enzyme-gold treatments are seen in

Table 6-III.

The reactions of the rodlet cores to DNAse I-gold are shown in Figs. 6-10 to 6-12. The undigested DNAse I-gold sections showed a heavy label which appeared to be precisely located at the periphery of the rodlet core (Fig. 6-10), sometimes showing evidence of a helical arrangement (Fig. 6-11). In addition, the euchromatin of the nuclei in the section was also labeled, and a small amount of label was seen in the nucleolus, and occasional grains were seen over the mitochondria. When the label in some experiments was very light, often only the cores of rodlets were labeled. Pretreating sections with 0.01% DNAse I before applying the DNAse I-gold solution resulted in virtual abolition of the rodlet core label, and reduced the label on the euchromatin (Fig. 6-12).

The results of pretreating sections with nucleases before applying the enzyme-gold procedure are given in Table 6-III.

Sections treated with RNAse A-gold showed moderate to heavy reaction along cytoplasmic strands containing rough endoplasmic reticulum between the large, electron-lucent cisternae or vacuoles (Fig. 6-13), and at the cell periphery where ribosomes were also visible. In addition to cytoplasmic label, nuclear heterochromatin and the nucleolus also showed gold grains. Predigestion of sections with RNAse A for 30 minutes resulted in a vast increase in nonspecific label; however, the rodlets were never heavily covered with grains, and there was no specific label of rodlet cores (Fig. 6-14).

Treatment of RNAse A-predigested sections with DNAse-gold resulted in a very heavy label of structures ordinarily specifically

TABLE 6-III

REACTIONS TO ENZYME-GOLD TREATMENTS

TREATMENT	REACTIONS Euchromatin	Heterochromatin	Nucleolus	Ribosomes	Rodlet Cores	Rodlet Sheath	Comment
DNAse I-Gold	scattered label	no label	light label	no label	label at periphery of rodlet core	no label	consistent; cores label when other elements do not
RNAse A/DNAse-Gold	labelled	labelled	labelled	labelled	labelled	no label	usually very heavy label
DNAse/DNAse-Gold	lightly labelled	no label	virtually no label	no label	few grains at core periphery	no label	label largely absent from DNAse-Gold areas
S1/DNAse-Gold	heavy label	light label	light label	no label	heavy label	no label	
RNAse A-Gold	no label	heavy label	mod. label	heavy label	no label	no label	consistent; rodlets never labelled.
RNAse/RNAse-Gold	labelled	labelled	labelled	labelled	non-specific label	non-specific label	loss of specific label, > non-specific
DNAse/RNAse-Gold	light label	light label	light label	light label	no label	light label	non-specific
S1/RNAse-Gold	light label	light label	?light label	light label	light label	no label	some non-specific label

Table 6-III cont'd

S1 Nuclease-Gold	labels junction of euchromatin and heterochromatin		lightlabel	light label	labels periphery of core	no label	core label specific but more scattered than DNAseI-gold
RNAse/S1-Gold	light label on both euchromatin and heterochromatin		no label	no label	light label	no label	
DNAse/S1-Gold	as above		scattered	no label	heavy label at core periphery	no label	
S1/S1-Gold	no label	few grains	no label	no label	few grains	no label	

identified by both enzymes: rodlet cores, euchromatin, heterochromatin, ribosomes, the nucleolus. Other cytoplasmic structures, such as the rodlet sheath, and contractile "capsule", and mucous granules were free of label (Fig. 6-15), even though the cumulative effect of the treatment appeared to be an increase in nonspecific label (Fig. 6-16). Predigestion of sections with DNAse I followed by RNAse A-gold (Fig. 6-17) resulted in nonspecific label which appeared evenly scattered over all portions of the section, including mucous granules, which were not labeled with RNAse-predigested DNAse I-gold preparations.

When S1 nuclease-gold was applied, the label was less intense but quite specific, and found at the periphery of the rodlet core (Fig. 6-18). Oblique sections of the rodlet gave some indication of a spiral distribution of the label (Fig. 6-19), and cores sectioned near the apical region of the core were more heavily labeled than areas associated with the thickened peripheral substance. In addition, S1-gold label was found on nuclear heterochromatin, often just at the junction point between it and the euchromatin, and occasionally in the cytoplasm over ribosomes (Figs. 6-18, 6-19).

Treatment of S1 nuclease-predigested sections with DNAse-gold resulted in heavy labeling of the periphery of the rodlet core, and of euchromatin and heterochromatin (Fig. 6-20); occasional grains appeared over ribosome-containing areas.

Predigestion with any of the enzymes, followed by treatment with unconjugated, stabilized colloidal gold at pH values ordinarily used in enzyme-gold procedures, resulted in no specific label for any specimen. A few scattered grains of gold were seen on the sections.

Repetition of RNAse A digestion experiments with Spurr's and Epon-embedded materials showed that rodlet cores in the Spurr's resin faded markedly (Fig. 6-21) after enzyme treatment, while Epon-embedded cores and peripheral substance were very electron-dense (Figs. 2-3, 6-22). DNAse I treatment of Epon-embedded sections for up to 5 hours followed by normal uranyl acetate and lead citrate staining showed slightly increased electron density of the entire rodlet, but no loss in density of the core itself (Fig. 6-12).

EDTA experiments duplicated the results of Bernhard (1969) and Viehberger and Bielek (1982), in that uranyl acetate-stained preparations subsequently exposed to EDTA and then stained in lead citrate showed loss of electron density in the heterochromatin of the nucleus and in the cores of the rodlets (Fig. 6-23). When EDTA treatment preceded all staining, no change could be observed in the electron density of the preparations, and EDTA-treated rodlet cells subsequently exposed to DNAse I-gold showed strong labeling of the periphery of the rodlet cores. In general, the core region was almost never disrupted after EDTA treatment. A central fibrillar region was seen in EDTA-treated rodlet cores stained with lead citrate, although the periphery of the core was electron-lucent; unstained, unosmicated rodlet cores were generally entirely electron-lucent. Treatment of EDTA-exposed rodlet cells with DNAse I-gold resulted in a large increase in nonspecific label, but the periphery of the rodlet core was also strongly labeled.

DISCUSSION

The results of the acridine orange experiments appear somewhat

ambiguous at first glance. All nuclease digestion experiments resulted in a more discernible rodlet core, while the undigested, stained chub preparation showed very faintly staining cores in the epifluorescence microscope, although the larger rodlets of sucker preparations were more easily defined. In most cases, the rodlet cores seen in the digested preparations showed the same blue-green colour as other cytoplasmic elements of the rodlet cell; this could be autofluorescence or perhaps a nonspecific fluorescence induced by the enzyme treatment. Certainly the rodlet cores in the undigested chub preparations were not blue-green; that they were difficult to define as red in colour may be attributed to two conditions: with the predominantly red coloration of the ribosome-containing strands of cytoplasm surrounding the rodlets, fine cores of a more faint red would be difficult to differentiate visually. Also, for the microscope on which these observations were made, the clearest objective was a 50X water-immersion lens. Attempts to use higher-powered objectives were frustrated by problems with resolution, probably due to glare from the intense illuminating beam. As well, the high-intensity beam through the oil immersion objective caused the preparation in the field to fade rapidly. The red fluorescence seemed to be most sensitive, and it was difficult to focus the specimen and to take the photograph before the colours had faded. With the digested specimens, the removal of the red fluorescence of the cytoplasm might have allowed the rodlet cores to be more easily discerned, in addition to the possibility that digestion increased the blue-green fluorescence of proteinaceous structures such as the capsule, the remaining cytoplasm and the rodlet cores.

The presence of protein in the rodlet core has been established

histochemically (Leino, 1982) and here by the fact that the protease digestion of the acridine orange-stained preparation eliminated the blue-green fluorescence of the rodlet cores. Certainly the blue-green colour of the rodlet core after digestion by the nucleases is not an indication of the presence of DNA; almost always the rodlet core was the same colour as the capsule and the peri-rodlet cytoplasm. The apparent blue-green of the RNAse A-digested cores would suggest that the cores were RNAse-sensitive. All nucleases were applied for two hours in a moist chamber at 37°C; for the RNAse, it might have been a long enough period to allow any small amounts of DNAse present in the stock enzyme to digest material as well. The apparent reddish colour of some rodlet cores after S1 nuclease would suggest that the nuclease had not digested the cores. The methyl green-pyronin procedure showed unequivocally that the undigested cores of isolated rodlets were pink, indicating either RNA or DNA that is either single-stranded, depolymerized or in very short polymers (Wilner, 1965; Pearse, 1983). Since the pink colour is removed with DNAse I and also S1 digestions but not with RNAse A digestion, the suggestion that rodlet cores contain RNA, originally proposed by Barber *et al.* (1979), is not supported by the methyl green-pyronin experiments. The present observations, combined with the results of the acridine orange experiments, indicate that the cores consist of DNA and protein. The fact that S1 nuclease digestion in both techniques appeared to reduce but not obliterate the specific staining of the rodlet core suggests either that the rodlet core DNA is not entirely single-stranded or that S1 nuclease preparations digest single-stranded DNA *in situ* more slowly than *in vitro*, and more slowly than DNAse I or RNAse A. Bendayan

(1981) did not use S1 nuclease-gold in his determinations; these experiments represent the first reports of another nuclease complexed to colloidal gold. The S1 nuclease-gold label varies from DNAse-gold in that it labels heterochromatin of rodlet cell and other nuclei. Close examination (Fig. 6-18) suggests that the actual position of the label may be at the junction of euchromatin and heterochromatin. S1 essentially gives a "hybrid" reaction between DNAse-gold and RNAse-gold: it labels areas consistently labeled by an enzyme known to attack single-stranded nucleic acid (RNA), and it fails to label regions attacked by enzymes for double-stranded nucleic acid (in this case, DNA). However, the periphery of the rodlet core, now known to contain only DNA, is also labeled by S1-gold. This suggests that the DNA of the rodlet core is not in the same molecular configuration as, say, the euchromatin of the nucleus.

To declare that the DNA of the rodlet cores is either entirely or partially single-stranded would be premature. Although the label obtained with S1 nuclease-gold certainly supports such a contention, positive control experiments have not yet been done, and while S1 nuclease, used *in vitro*, preferentially digests only single-stranded DNA. (Wiegand *et al.*, 1975), forming acid-soluble oligonucleotides, in "high concentrations" double-stranded DNA may also be attacked. The results of the present experiments argue against the S1 nuclease's being present in "high" concentrations, since it fails to label the euchromatin of the nucleus, but the possibility cannot be dismissed without further study. As well, throughout the experiments with DNAse I-gold, rodlet cores have always shown label, even when the enzyme-gold preparations were insufficiently concentrated to label nuclear euchromatin. Bendayan (1983, personal communication) indicated that, in his hands, single-stranded DNA

viruses always labeled very strongly and preferentially with DNAse I-gold. The pattern of the S1-gold label, somewhat like RNAse-gold as well, since it labels heterochromatin and occasionally rough endoplasmic reticulum, also suggests a single-stranded nucleic acid may be involved.

S1 nuclease-gold yields a light label in these experiments perhaps because the best quality of commercial S1 enzyme is still bound to protein, and sold as "units per mg". S1 nuclease pretreatment followed by DNAse I-gold showed an increased label of rodlet cores, nuclear heterochromatin and rough endoplasmic reticulum, the latter two structures not to the same degree as was found with RNAse+DNAse-gold. While DNAse I-gold binds to the periphery of rodlet cores in its own right, the apparent enhancement of the label may mean that it is possible to demonstrate other enzyme-substrate reactions for which purified enzymes are not yet available.

Viehberger and Bielek (1982) reported rodlet cores faded after 6 hours' DNAse I digestion (at pH 7.5) followed by uranyl acetate and lead citrate staining; Monneron and Bernhard (1966) could find no consistent reaction of DNAse on glutaraldehyde-fixed nuclear chromatin even when sections were incubated for 24 hours at 38°C. In the present studies, no fading of stained rodlet cores was seen with DNAse I incubation up to 5 hours at pH 6.0, a level much closer to its actual isoelectric point (pI), and further, unosmicated, undigested and unstained preparations of rodlet cells of S. atromaculatus often showed electron-lucent rodlet cores similar to those reported by Viehberger and Bielek (1982) after DNAse I digestion. In view of the apparent peripheral distribution of DNA in rodlet cores found with the enzyme-gold procedures, it is difficult to explain why the entire rodlet core should

be sensitive to DNAse action. Indeed, Viehberger and Bielek reported the presence of a "scaffolding" of protein in the centre of the rodlet core after EDTA treatment to demonstrate DNA; the present studies concur with this even though they do not agree with the wholesale loss of electron density of the rodlet core and heterochromatin after DNAse.

The use of EDTA in these experiments to demonstrate DNA-containing structures in EM (Fig. 6-23) agrees with results reported by Viehberger and Bielek (1982). They reported that only a light shadow, "possibly a proteinaceous scaffold" remained in the centre of the rodlet. They may be correct; labeling of EDTA-treated rodlet cores with DNAse I-gold showed the enzyme-gold complex still at the periphery of the core and protein has been shown (Barber et al., 1979; Leino, 1982) to be present in the cores of rodlets. However, Viehberger and Bielek (1982) appear not to have realized the peculiar distribution of DNA in the rodlet, and did not attempt to explain what they meant by the "extra-ordinary configuration" they claimed the rodlet core DNA must possess. Fading of the rodlet cores and nuclear heterochromatin seen after EDTA treatment is based upon the chelation of uranium atoms in the previously applied uranyl acetate (Bernhard, 1969); it apparently does not affect the actual nucleic acid, and certainly does not change its accessibility for subsequent procedures.

The apparent loss of specificity of label with RNAse predigestion may represent the complexing of RNAse and DNAse-gold so that both RNA and DNA-containing structures are revealed. Prior application of the enzyme to the section may allow RNAse to bind to RNA molecules at the surface of the section, and the DNAse subsequently binds to the RNAse as

well as to its usual sites in the section. Figure 6-15 indicates quite clearly that the embedding matrix of the section is free of label, as are the protein-containing regions such as the rodlet peripheral substance and some mucous granules. That this phenomenon represents an enzyme-enzyme complex and not merely the in situ binding of enzyme to unconjugated colloidal gold particles is indicated by the failure of merely stabilized colloidal gold to bind to enzyme-treated sections. However, the precise nature of the complex is not known, although it must be relatively strong to resist post-labeling processing.

THE ANSWER

The nuclease-gold experiments show the presence and distribution of DNA at the periphery of the cores of the rodlets, and in the euchromatin of rodlet cell and other nuclei in the section. The presence of DNAse I-gold label in the euchromatin of the nucleus corresponds to the labeling pattern reported by Bendayan (1981) for pancreatic acinar cells. Both DNAse I- and S1 nuclease-gold confirm the presence of DNA, and both show the same distribution of the nucleic acid in the rodlet core. The loss of nuclease-gold label by predigestion with the same nuclease (Table 6-IV) also confirms the specificity of the procedure.

Cross-sections of rodlet cells treated with DNAse I-gold show unequivocally that the label is confined to the periphery of the rodlet; where it has been possible to cut the rodlet longitudinally, some evidence of a helical arrangement of the DNA is demonstrated (Fig. 6-11). This is not conclusive, since the label is lost when the DNA passes out of the plane of the section surface, as the nuclease-gold procedure

is not a penetrating technique (Bendayan, 1981).

There is no evidence of any nucleic acid in the outer peripheral substance of the rodlet; this area was never labeled in nuclease-gold experiments.

Using RNAse A-gold, there was no evidence of label in the cores of rodlets. The label was present over the heterochromatin and nucleolus in the cell nucleus, and over cytoplasmic ribosomes, corresponding to the pattern observed by Bendayan (1981) for pancreatic acinar cells. The failure of the RNAse-gold to label the rodlet cores also contradicts the findings of Barber et al. (1979), who suggested, on the basis of other histochemical procedures, that RNA was present in the cores of rodlets, although they also indicated that short DNA polymers or depolymerized DNA could give the same reaction. However, repetition of the RNAse treatment of Spurr's-embedded material confirms that, with this resin, the rodlet cores do fade; as well, it is not clear with Epon-embedded RNAse A-treated material, whether the rodlet core fades or whether the whole rodlet simply acquires a much greater affinity for uranyl acetate and lead citrate. In Spurr's-embedded material, the reaction was clearly perceived as a less dense core in a rodlet with peripheral substance of only slightly increased electron density. Monneron and Bernhard (1966) reported that ribonuclease applied to glutaraldehyde-fixed, unosmicated Epon-embedded sections had an inconsistent effect. With some preparations, it acted to increase the contrast between nuclear "chromatin" (? = heterochromatin) and nucleolar and ribosomal RNA. The RNA did not disappear from sections even if the RNAse was associated with protease. Certainly both the experiments of

Barber et al. (1979) and the present studies show a marked reaction of rodlets to RNAse digestion, whatever the embedding medium, and possibly dependent upon the medium. Why the reactions occur as they do remains unresolved.

PLATE VII

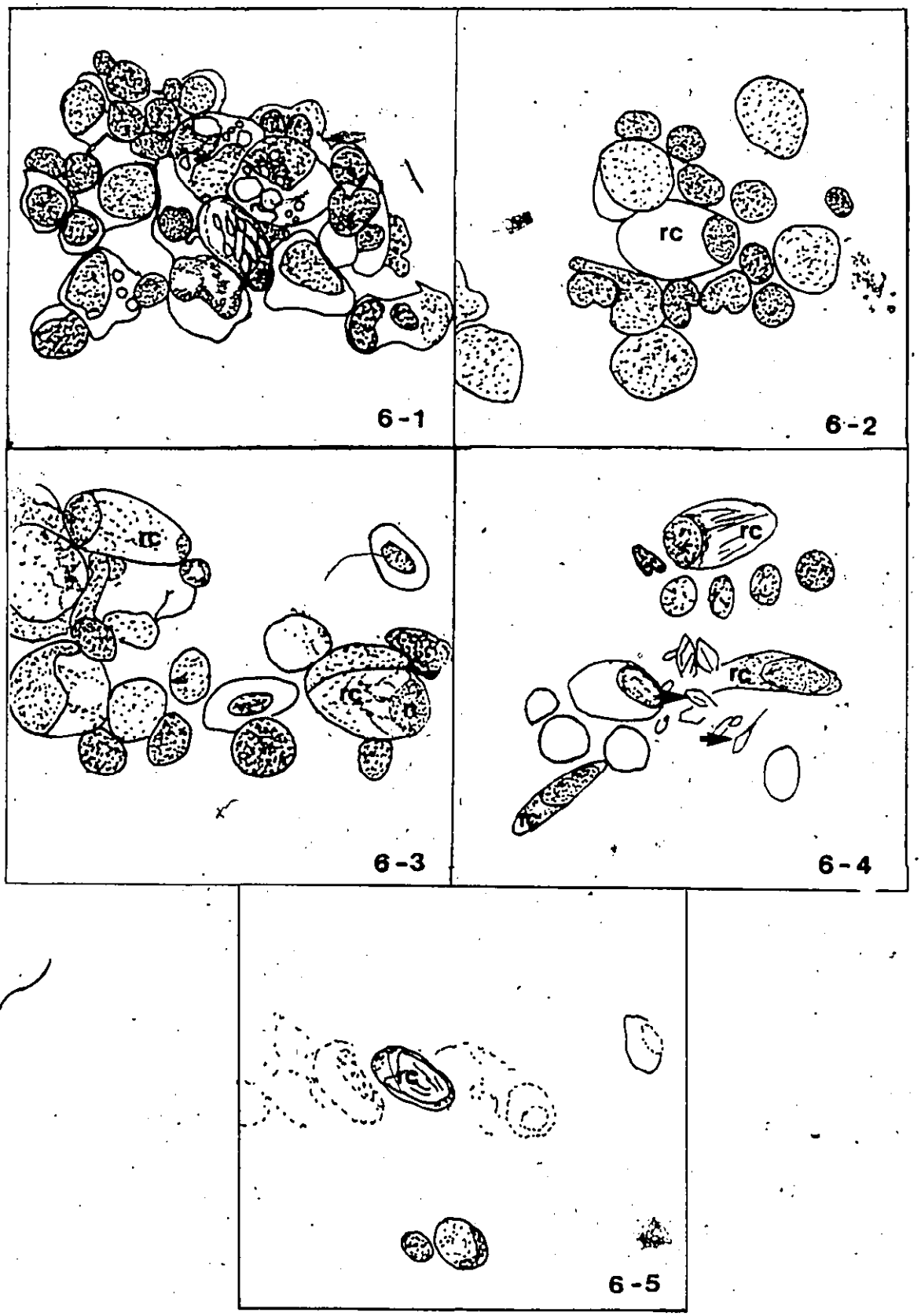
Fig. 6-1: Imprint of gill tissue of Semotilus atromaculatus showing rodlet cell with yellow nucleus (n) and reticulate cytoplasm having a faintly red fluorescence. Acridine orange, fluorescent light. X 750

Fig. 6-2: Gill imprint of S. atromaculatus showing rodlet cell (rc) after digestion by RNAse A. The nucleus (n) is yellow; structures that would fluoresce red are either unstaining or coloured faintly blue-green. Acridine orange. X 750

Fig. 6-3: Rodlet cells (rc) in gill imprint of S. atromaculatus digested with DNAse I. The nuclei of all cells have lost yellow fluorescence and appear pale orange; one rodlet cell nucleus (n) is virtually unstained. The rodlet cell cytoplasm shows that cores are still discernible, but of an indeterminate colour. Acridine orange. X 750

Fig. 6-4: Imprint of gill tissue from S. atromaculatus showing three rodlet cells (rc), two of which are collapsed, showing discharged rodlets (arrows). The preparation has been treated with S1 nuclease and while the entire preparation has lost considerable fluorescence, one rodlet cell shows a bright yellow nucleus. The cytoplasm of the undischarged rodlet cell shows an orange, reticular fluorescence, but the rodlet cores are either of an indeterminate colour, or faintly orange. Acridine orange. X 750

Fig. 6-5: Imprint of gill tissue of S. atromaculatus after digestion with Proteinase K. The integrity of most cells has been destroyed, and most specific fluorescence has been lost. A rodlet cell (rc) shows a generally blue-green fluorescence. Acridine orange. X 750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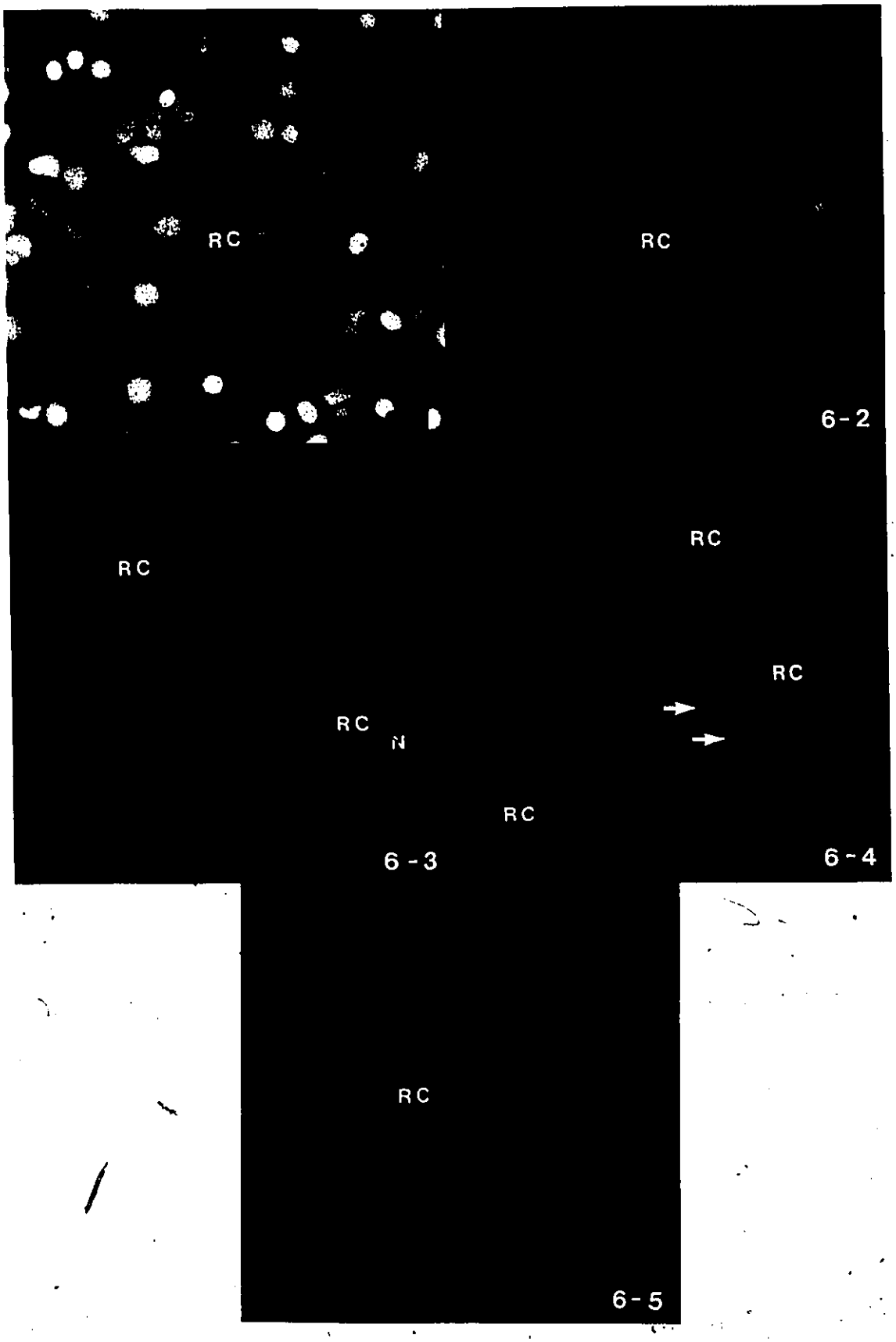


COLOURED PICTURES
Images en couleur

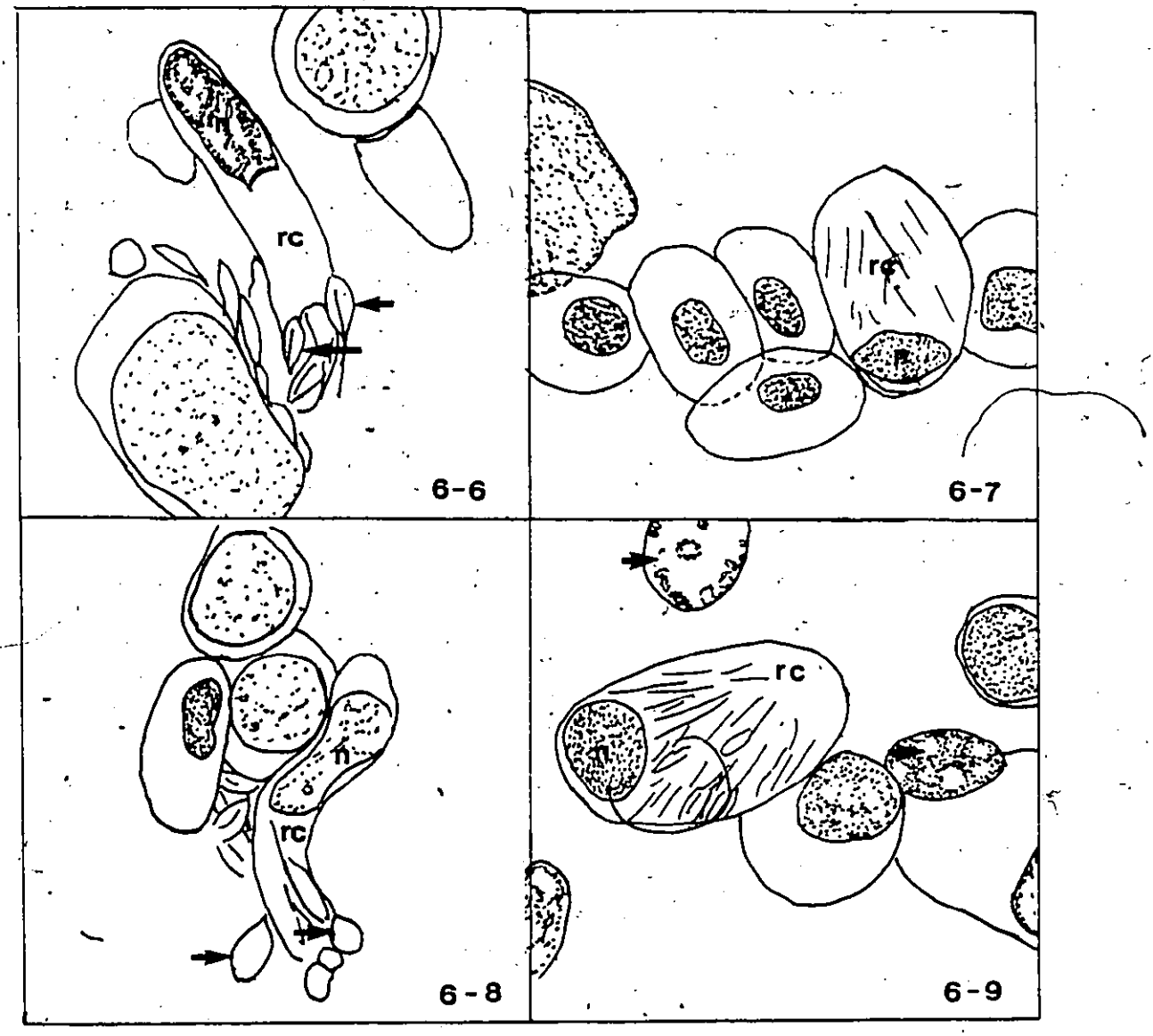
PLATE VIII

Fig. 6-6: Imprint of gill tissue of S. atromaculatus, showing collapsed rodlet cell (rc) with discharged rodlets (arrows). The nucleus (n) of the rodlet cell stains blue-green; the rodlet cores are faintly pink. Methyl green and pyronin. X 1500

Fig. 6-7: Rodlet cell (rc) from gill of S. atromaculatus after RNAse digestion, showing blue-green nucleus (n) and pink striae in cytoplasm; it is not clear whether the striae are rodlet cores or the inter-rodlet cytoplasm. Adjacent erythrocytes show blue-green nuclei and pink-staining cytoplasm. Methyl green and pyronin. X 1500

Fig. 6-8: Collapsed rodlet cell (rc) with discharged rodlets (arrows) from gill tissue of S. atromaculatus. The preparation has been digested with DNAse I; the nucleus of the rodlet cell (n) is virtually unstained, and the rodlet cores have disappeared. The cytoplasm of the rodlet cell and that of an adjacent erythrocyte are faintly pink. Methyl green and pyronin. X 1500

Fig. 6-9: Rodlet cell from gill imprint of S. atromaculatus after digestion by S1 nuclease. The nucleus of the rodlet cell stains blue-green (n), the nuclei of adjacent cells are reticulate (arrows), and the cytoplasm of the rodlet cell shows unstained, slightly refractile striae, although the cytoplasm of some adjacent cells is pink. Where individual rodlets are discernible in the rodlet cell cytoplasm, no core is visible. Methyl green and pyronin. X 1500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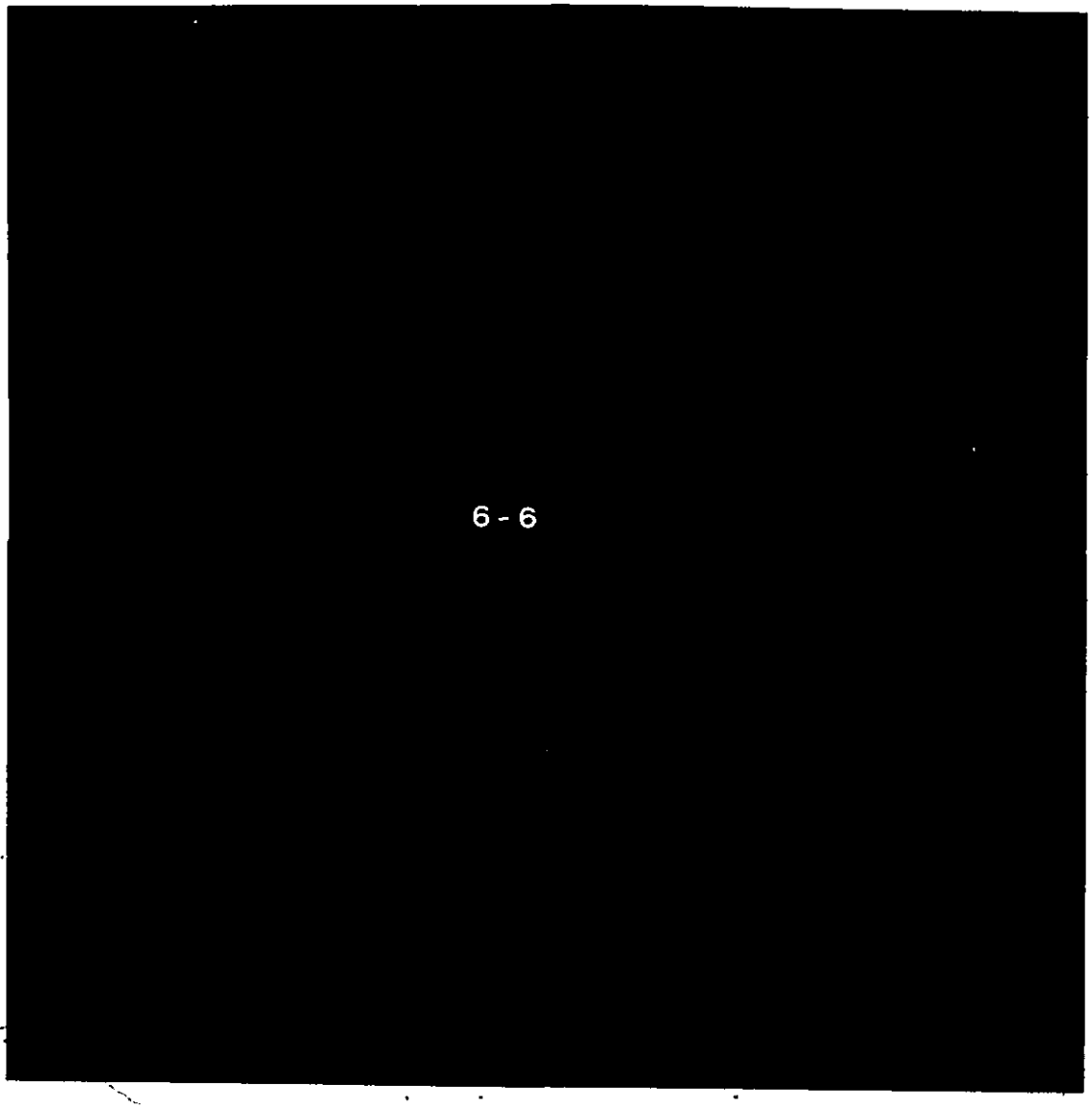


COLOURED PICTURES
Images en couleur

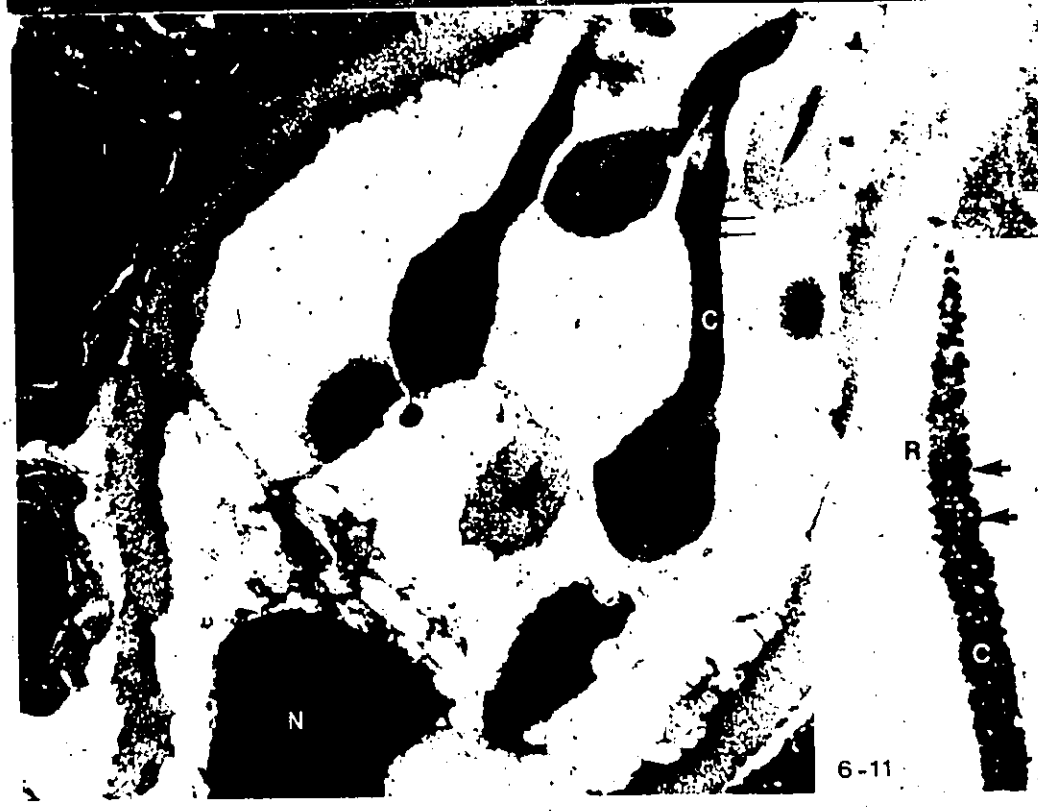
PLATE IX

Fig. 6-10: Electron photomicrograph of cross-section of rodlet cell showing thickened cell border (B) and rodlets (R) with cores (arrows) labeled by DNAse I-gold. Note that the label is confined to the periphery of the rodlet core, and that adjacent peripheral substance and other cytoplasmic structures are unlabeled. An adjacent cell nucleus (N) shows label on the euchromatin, and there is a small amount of label scattered over the cytoplasm of the adjacent cell. Epon section; unosmicated, unstained preparation. X 17,800

Fig. 6-11: Electron photomicrograph of oblique section of rodlet cell showing the cell nucleus (N), rodlets (R) and the electron-dense core (C) with a heavy label of DNAse I-gold. The label on the cores is seen to have a spiral distribution (arrows), and to extend the length of the rodlet. Epon section; unosmicated preparation; uranyl acetate, lead citrate stain. . X 12,500

Inset: Enlargement of a portion of a rodlet (R) showing the spiral distribution of the DNAse I-gold label (arrows) around the rodlet core (C). X 29,000

6-10



6-11

PLATE X

Fig. 6-12: Electron photomicrograph of oblique section of rodlet cell digested with DNAse I and subsequently treated with DNAse I-gold. The rodlets (R) show cores with very light label (arrows). Note also that label is absent from other areas of the cytoplasm of the cell. Epon section; unosmicated preparation; uranyl acetate, lead citrate stains. X 25,000

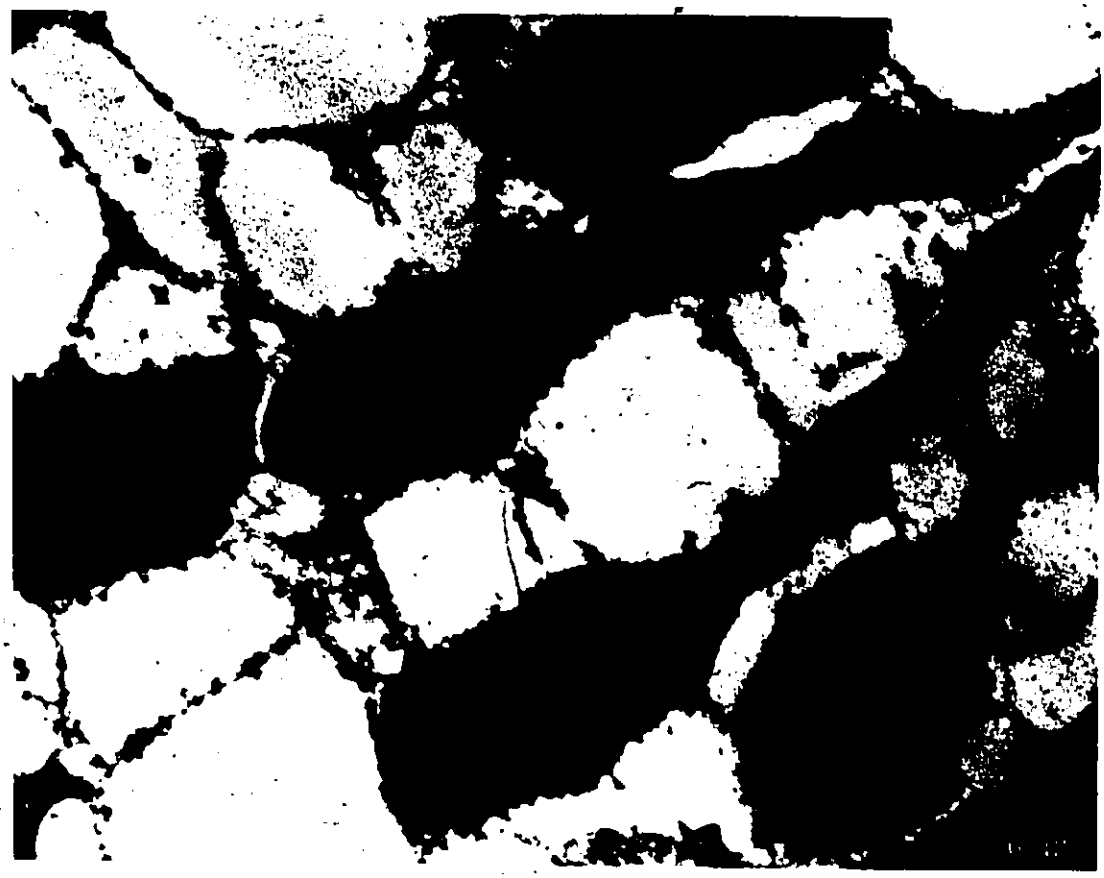


PLATE XI

Fig. 6-13: Electron photomicrograph of cross-sections of two rodlet cells treated with RNAse A-gold. The rodlets (R) are entirely unlabeled, but the cytoplasmic strands which contain ribosomes are heavily labeled (arrows), as is the inner surface of the rodlet cell border, which also contains ribosomes. The nucleus of the rodlet cell (N) is lightly labeled, as are the nuclei of adjacent cells; the label is frequently associated with the heterochromatin of the nucleus. Epon section; unosmicated preparation; uranyl acetate and lead citrate stains. X 8900

Fig. 6-14: Electron photomicrograph of oblique section of rodlet cell treated first with RNAse A, and subsequently with RNAse A-gold. The label is specific in that it appears over areas which are electron dense, such as the rodlet (R) and the nucleus (N) of the rodlet cell. The cores (C) of the rodlet are unlabeled, and appear electron lucent. Epon section; unosmicated, unstained preparation. X 12,500

PLATE XII

Fig. 6-15: Electron photomicrograph of oblique section of rodlet cell digested with RNAse A before being treated with DNAse I-gold. The gold grains are generally seen over electron-dense areas with the nucleus (n) being especially heavily labeled. A discrete label is also evident at the periphery of the rodlet core, and what appears to be nonspecific label is scattered over the cell border and the peripheral substance of the rodlet (R). Note the thinning of the cell border at the base of the cell (arrow). Epon section; unosmicated preparation; uranyl acetate and lead citrate stains. X 12,200

Fig. 6-16: Electron photomicrograph of mucous cell and rodlet cell in the gill of S. atromaculatus. The preparation has been digested with RNAse A and then treated with DNAse I. The nuclei of cells in the micrograph are heavily labeled (N), and gold grains are seen over the rodlet cell border (B) and on the rodlet cores (arrows). However, the mucous granules (M) are devoid of label as, generally, is the space surrounding the gill (L). Epon section; unosmicated preparation; uranyl acetate and lead citrate stains. X 11,400

PLATE XIII

Fig. 6-17: Obliquely-sectioned rodlet cell treated first with DNAse I and then with RNAse A-gold, showing nonspecific labeling. The electron lucent rodlet cores (c) contain a slightly more dense central area (arrow), and are surrounded by peripheral substance (ps). The border of the rodlet cell (b) is adjacent to a mucous cell containing electron lucent granules (m) and a portion of nucleus (n). Epon section; unstained. X 15,600

Fig. 6-18: Cross-section of rodlet cell treated with S1 nuclease-gold. The rodlets show S1-gold label at the periphery of the rodlet core (arrow). The label is lighter on cores where the peripheral substance is thick (ps). A small portion of the rodlet cell nucleus (n) shows label on the heterochromatin. The fibrillar border (b) of the rodlet cell has a few grains of label on the inner surface. Epon section; unstained. X 15,600

PLATE XIV

Fig. 6-19: Electron photomicrograph of a portion of an obliquely-cut rodlet cell treated with S1 nuclease-gold. The cell border (b) is largely unlabeled, although a few grains may be seen along the inner surface of the border. The label is seen at the periphery of the rodlet cores (arrows), and shows evidence of a helical pattern on one core. In the nucleus (n), the S1-gold label is found either on the heterochromatin or at the junction of the euchromatin and heterochromatin. Epon section; unosmicated, unstained section. X 15,600

Fig. 6-20: Electron photomicrograph of two rodlet cells (rc) cut in cross-section. The preparation was digested with S1 nuclease and then treated with DNAse I-gold. The label on the nuclei (N) is concentrated in the euchromatin and at the edge of the heterochromatin, and the label on the rodlets is confined to the periphery of the cores (arrows). The remainder of the cell is essentially unlabeled. Epon section; unosmicated, unstained preparation. X 11,400

PLATE XV

Fig. 6-21: Electron photomicrograph of a portion of a rodlet cell from Catostomus commersoni treated with RNAse A. Note the rodlet core (C) has become less distinct with respect to the rodlet peripheral substance (R), although the cell border (B) retains its normal density. Spurr's-embedded section; unosmicated preparation; uranyl acetate and lead citrate stains. X 31,600

Fig. 6-22: Electron photomicrograph of a rodlet cell from the gill of S. atromaculatus in which the preparation was digested with RNAse A before staining. The rodlets (R) have become electron-dense, and the core is not distinguishable from the peripheral substance. The remainder of the preparation shows normal contrast. The fibrillar border is clearly evident (arrow). Epon section; unosmicated preparation. Uranyl acetate and lead citrate stains. X 15,300

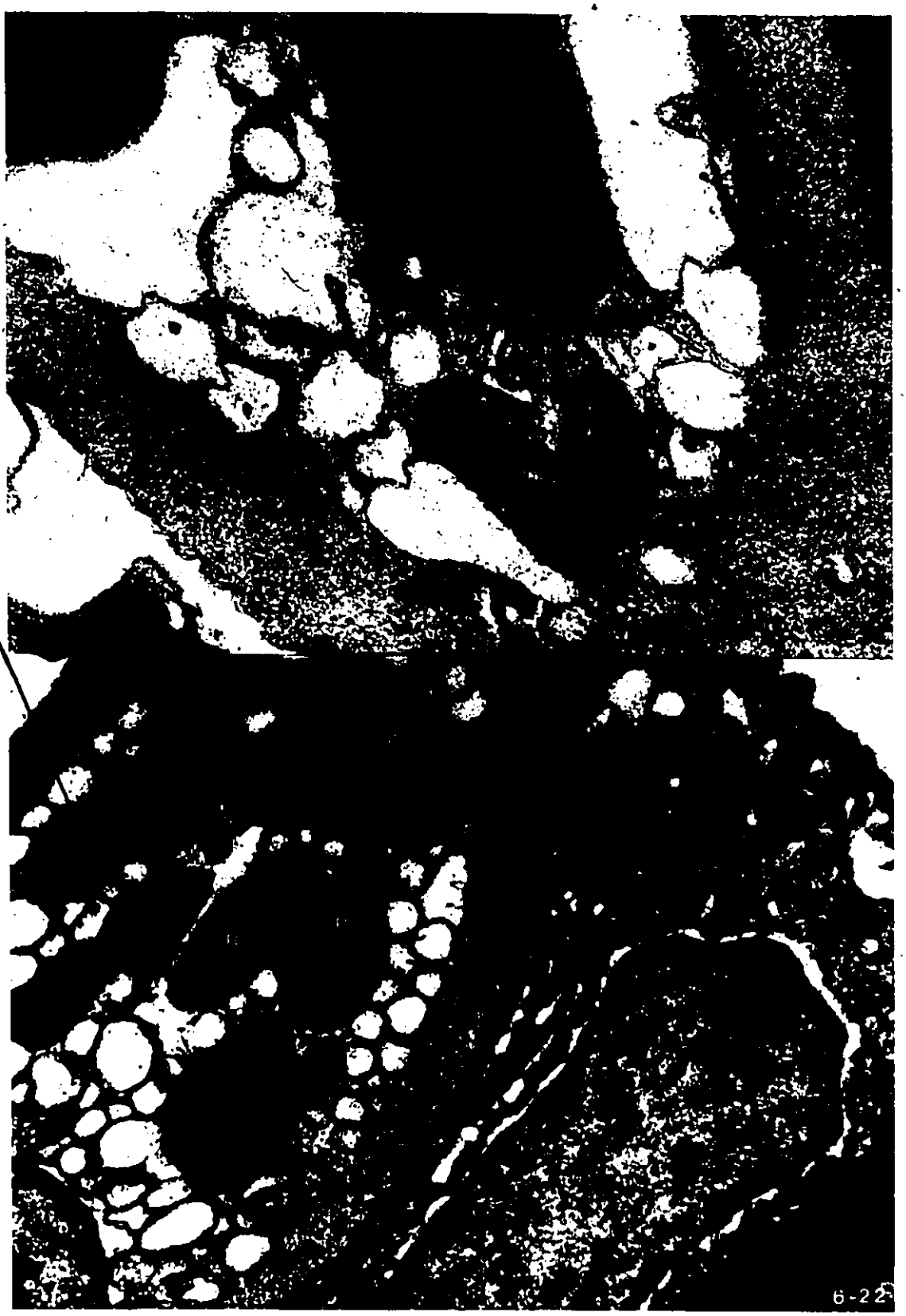
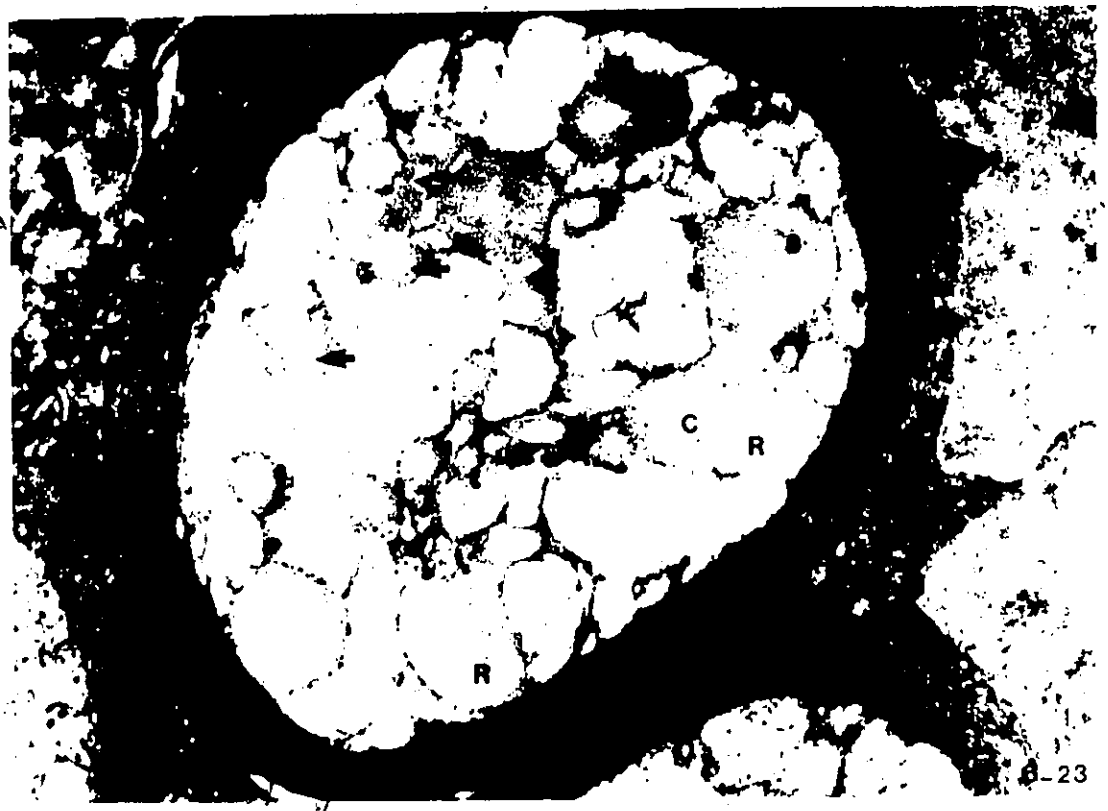


PLATE XVI

Fig. 6-23: Electron photomicrograph of a cross-section of a rodlet cell stained in uranyl acetate, then treated with EDTA after the method of Bernhard (1969). The rodlet peripheral substance (R) appears to be of normal electron density, but the rodlet cores (C) have remained electron lucent except for a faintly distinguishable central region (arrows). Epon section; unosmicated preparation; uranyl acetate, EDTA, lead citrate stains. X 20,400



B-23

CHAPTER VII

GENERAL DISCUSSION

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GENERAL DISCUSSION

Clearly the rodlet cell is a more complex entity than has hitherto been suspected. In examining the evidence on the identity of the rodlet cell, the following areas will be considered:

1) the evidence which supports the hypothesis that the rodlet cell is a normal component of teleost epithelia;

2) the evidence supporting the hypothesis that the rodlet cell is other than a normal teleost cell;

3) the nature of the rodlet;

4) unanswered questions and prospects for future research.

THE CELL:

1) Evidence for the Rodlet Cell as a Normal Component of Teleost Epithelia

a) From the literature, it is clear that most investigators have regarded the rodlet cell as a normal cell of unknown function (e.g., Leino, 1974; Desser and Lester, 1975; Morrison and Odense, 1978); its intercellular existence, association with adjacent cells by means of desmosomes or tight junctions (Leino, 1974; Desser and Lester, 1975), and apparent failure to cause an inflammatory or other reaction characteristic of pathogens or parasites (see, for example, Modin, 1981) are not individually conclusive proof of "normalcy", but taken in combination,

these morphological factors provide a strong argument.

b) Another argument for rodlet cells as "normal" teleost components is the common morphology with some variations in size that rodlet cells show among species. All rodlet cells show the refractile cell border, which is contractile. They all have a basal nucleus, and are characteristically found in epithelia (Leino, 1974; Morrison and Odense, 1978). While the numbers of rodlets within any rodlet cell seem to be variable, all rodlets have approximately the same club or arrow shape, with the slender region pointing toward the cell apex. Developmental stages of rodlet cells are recognizable only after the fibrillar cell border is discernible, even though thin, and only after one or two characteristic rodlets are present in the cytoplasm; some authors (Mattey et al., 1979; Flood et al., 1975) claim to recognize rodlet cells without the rodlets, but proof is lacking. Insofar as developmental stages for rodlet cells have been established, all investigators agree that in the early stages, the rodlet cell contains large amounts of RER surrounding dilated cisternae, a Golgi complex, and scattered mitochondria. All rodlet cells from a species are approximately the same size, but the size may vary from one teleost species to another (Table 2-I). For example, those of Catostomus commersoni have been independently reported to measure up to 25.4 µm X 8-9 µm (Dawe et al., 1964; Chaicharn and Bullock, 1967; Barber and Westermann, 1975; Desser and Lester, 1975), while those of another cyprinid, Carassius auratus, were measured at 6.0 µm X 3.3 µm (Flood et al., 1975). This type of observation can be made of other normal cells in a species; e.g., teleost erythrocytes frequently show specific variations in size and shape of both nucleus and overall

dimensions, but all members of any species have a common morphology of blood cell.

c) Another common factor in the argument of the rodlet cell's being a normal component revolves around the cell nucleus: rodlet cells have never reliably been seen in mitosis (the claims of Al-Hussaini, 1964, have not been substantiated by any other author). If the rodlet cell is a normal cell, it is an end-stage in development. If the rodlet cell were a "parasite", one might expect some evidence of multiplication. Indeed, Vickers (1962) reported that the numbers of rodlet cells increased in goldfish intestine after treatment with cobalt, but he gave no quantitative data, and mitotic figures were not mentioned.

d) Evidence here from microdensitometry experiments and hybridization in situ supports the hypothesis that the rodlet cell arises in the fish, in that the DNA content of rodlet cells is not significantly quantitatively different from known somatic cells of the fish. This is true for three species with different somatic 2C DNA contents, and for two different methods of measuring the amount of stain complexed by the DNA.

e) Qualitatively, from the present experiments with hybridization in situ the nucleus of the rodlet cell also shows compelling evidence that it shares the same genome with cells known to be fish cells. The rodlet cells show a pattern of label not significantly different from the other cells, and the label is washed out of the rodlet cell at approximately the same rate as from the somatic cells. Chub genomic DNA hybridized to cells of other teleost species, protozoa or human cells were not labeled significantly above background. Therefore, the nuclei

of rodlet cells of chub have a genome more similar to chub cells than to protozoa, human or other teleost cells (Figs. 5-2, 5-3, 5-4).

These microdensitometry and hybridization experiments represent the first experimental attempts to acquire factual rather than speculative information on the nature of the DNA of the rodlet cell nucleus, and they support the hypothesis that the rodlet cell has a normal teleost origin.

2) Evidence for the Rodlet Cell's Being Other than a Normal Teleost Cell

Although the above evidence in favour of the rodlet cell's being a normal teleost component is logical, there are other reasonable arguments involving morphology, function, distribution and ecology which may be invoked against that view.

a) The mature rodlet cell does not resemble, overall, any known type of somatic cell in spite of its apparently normal nucleus, conventional Golgi complex and ribosomes. The uniqueness of rodlet cells lies in the morphology of the rodlets, the shape and function of the contractile cell border, and the distribution of mitochondria in the mature cell which is not reminiscent of any group of identified cells.

The contractile border appears to function largely to expel the rodlets onto the free surface of the epithelium; the thinner basal region with its reported dense-cored vesicles, local expansions and vacuoles has been proposed as possibly a nutrient-absorptive structure, although nothing has been established (Kimura, 1973; Barber et al., 1979). Leino's (1974) argument that because rodlet cells form junctional complexes with adjacent epithelial cells, they must be normal teleost

cells, cannot be justified. Michalke and Lowenstein (1971) reported the presence of junctional complexes between cells of different origins. Certainly rodlet cells form desmosomal junctions in gill epithelia; Desser and Lester (1975) reported the presence of tight junctions between rodlet cells and adjacent gill raker and opercular epithelial cells.

The fate of rodlet cell mitochondria is puzzling. When the apices of rodlet cells treated with DNAse-gold are examined, none of the whorled structures is labeled (Fig. 6-11), indicating the probable absence of DNA, but Paterson and Desser (1981) reported the presence of recognizable mitochondria in this region of the rodlet cell in Notropis cornutus, and other authors (Desser and Lester, 1975; Flood et al., 1975) have noted changes in the apical mitochondria with maturity of the cell, and their replacement or degeneration to whorled, membranous structures. These do not appear to be fixation artefacts, since they are common to rodlet cells of several species described by different authors. Why the involution apparently occurs is unknown. Figures 2-3, 2-5 show tubular structures associated with the cell apex in S. atromaculatus; these have never been previously described, and their importance and function are also unknown.

The rodlet cells of salmonids show a peculiar horseshoe-shaped nuclear morphology (Plehn, 1906; Bullock, 1963; Kimura, 1973; Modin, 1981); salmonid epithelial cells in, for example, the intestine have oval to spherical nuclei. The salmonid rodlet cell nucleus appears to be a morphological departure from normal cells. It could be argued that the common rodlet cell nuclear morphology seen in this family may be characteristic of a salmonid-specific parasite. The possibility of a variety of species-specific rodlet cell parasites was also raised by

Ciullo (1975).

b) The somatic distribution of rodlet cells raises many doubts about their status as "normal" components in fish. The common denominators seem to be that rodlet cells are reliably associated with epithelia, that they are situated in the epithelium with the apex of the cell at the free surface, and that rodlets are discharged onto the free surface with the contraction of the cell border. Beyond that, rodlet cells have been reported from most internal and external epithelia, mesothelia, and vascular endothelium. Epithelial surfaces serve vastly different purposes: some are columnar and absorptive, such as the intestine and archinephric duct; some are squamous and/or protective, such as vascular endothelium, mesothelium and fin epithelium.

It has been argued (Desser and Lester, 1975) that the somatic distribution of rodlet cells is too broad for them to be parasites, since parasites are usually species-specific and/or site-specific. However, as broadly distributed as they are, rodlet cells are not always found in all locations in an individual fish, they seem to show some seasonality in occurrence (Leino, 1974; Ladd, unpublished), and there is evidence that they are found in particular somatic sites which vary with the teleost species. No common function for rodlet cells has been established, however, since proposals that the rodlets may be either lubricant, sensory or osmoregulatory in nature are defeated by their broad distribution.

c) The ecological studies show a global distribution of rodlet cells in a wide variety of teleost families, so geography is not a limiting factor. However, environment and feeding patterns may play a

role in the prevalence of rodlet cells. Their presence seems not to depend upon whether the species is solitary or schooling, marine or freshwater, polar or tropical, but rather whether it is demersal and omnivorous, detritus-feeding or herbivorous, when compared to a representative, mixed population of marine and freshwater fish (Table 3-III). It is unlikely that a normal teleost cell of such broad somatic and geographical distribution would show incidence related to behaviour and food choices. On the other hand, these factors may be very important in the transmission of parasitic diseases.

d) Another characteristic of the distribution of rodlet cells is their tendency to be found in sites where the osmolarity of the liquids flowing above is quite constant. Mattey et al. (1979) and Fearnhead and Fabian (1971) independently proposed an osmoregulatory function for rodlet cells on the basis of an apparent loss of rodlet cells from fish that had been transferred from seawater to brackish water. Rodlet cells are not often found in gills of marine fish (Morrison and Odense, 1978; Westermann et al., 1984); they are more likely to be seen internally in the kidney, gut and blood vessels. This may mean that they are sensitive to osmotic changes, not that they somehow control the fish's response to osmotic changes. Such behaviour supports the "parasite" hypothesis in that chemosensory mechanisms play a large role in host-parasite relationships, e.g. in schistosomiasis, and a change in osmolarity might signal the onset of favourable conditions for transmission of infective stages.

Clearly there are cogent arguments for rodlet cells being either normal cell or parasite. Where are the discrepancies? If the rodlet cell

is a normal cell, why does it appear so sporadically? There is no known epithelial cell type having a normal function which appears sporadically, sometimes in large numbers, and sometimes not at all. Certainly normal cells show variations in morphology related to functional activity, e.g. chloride cells of the gill, but such cells are always present in some form. It could be argued, of course, that rodlets might also be present in some cells at all times, but with a morphology not recognized; rodlet cells are not reliably recognized without rodlets and/or the thickened cell border.

If the rodlet cell is a normal cell, what is its function? Cells found in digestive tract, gill and cutaneous epithelia might conceivably be thought to have mucous cell properties, considering their location at the free surface. However, the "secretory" products of mucous cells and rodlet cells are quite different: the mucus droplets diffuse smoothly onto the surface but rodlets do not dissolve, and may retain their morphological integrity for several days after discharge from the rodlet cell (Barber, unpublished observations). Rodlet cells are also found in vascular endothelium, and bile, pancreatic and archinephric duct epithelia; certainly in the vascular endothelium, mucous cells have never been considered a component part. To call the rodlet cell an osmoregulatory cell is negated by its variable incidence and its location in epithelia associated with fluids which undergo little osmotic change, such as blood.

If the rodlet cell is a normal cell, why does its incidence parallel certain habits of the species, such as activity and food choices?

If the rodlet cell is a normal cell, why does the rodlet core contain DNA? All of the previously proposed normal functions ignore the

presence of the rodlet core, and all must be rejected because of the rodlet core and the fact that it contains DNA.

On the other hand, if the rodlet cell is a "parasite", why does its nucleus share the genome of the fish both qualitatively and quantitatively, and why is the rodlet DNA apparently not related to the DNA of the rodlet cell nucleus, and hence, to the fish?

If the rodlet cell is a "parasite", what is its life cycle, and how does it undertake its development in the fish?

If the rodlet cell is a "parasite", why is it so broadly distributed both somatically and phylogenetically?

If the rodlet cell is a protozoan parasite, why does the rodlet core show reactions which are not consistent with the presence of double-stranded DNA?

None of these discrepancies is reconciled unless the rodlet cell and its rodlets are regarded separately. Once the rodlets and the cell itself are examined individually all controversies may be resolved.

THE RODLET:

3) The Nature of the Rodlet

Morphologically, the rodlets do not resemble any known vertebrate cytoplasmic structure. The early authors (e.g., Thélohan, 1892b; Laguesse, 1895; Labbé, 1896) regarded rodlets as coccidian sporozoites. With the advent of electron microscopy (Mourier, 1970; Leino, 1974), the coccidian hypothesis was rejected. More recently, rodlets were termed "rhoptries", subcellular structures found in apicomplexan Sporozoan parasites (Mayberry et al., 1979), but this identification has also been effectively refuted (Paterson and Desser, 1981).

Rodlets have been regarded as secretory products of both exocrine and endocrine gland cells; many authors have considered the rodlet as a type of mucous secretion, especially when identified in the digestive system. Inevitably, this sort of reasoning fails to note that rodlets on the free surface to not dissolve or diffuse, and it ignores the existence of the rodlet core.

To account for the nature of the rodlet core is crucial in any argument regarding the status of the rodlet cell.

In evaluating the present experiments, a new perspective on the rodlet becomes evident: the rodlet is not the biphasic structure that has been described for years, but is at least a triphasic structure. The EDTA experiments show the core is divisible into a central region and an electron-lucent periphery (Fig. 6-23), and the DNAse-gold confirms that the nucleic acid is restricted to the narrow rim between the central region and the peripheral substance (Fig. 6-10). Viehberger and Bielek (1982) called the central region a "proteinaceous scaffolding", although they thought the DNA occupied the entire rodlet core. Support for their description comes from Leino (1982, Fig. 18), in which trypsin-digested rodlet cores show an electron-lucent central region with an electron-dense periphery, confirming a proteinaceous structure in the centre of the rodlet core. Leino also comments that the cores' histochemical reactions to coupled tetrazonium procedures and their strong acidophilia indicates that, unlike the peripheral substance, they contain concentrated protein with substantial amounts of tyrosine, tryptophan and histidine, perhaps also some basic amino acids, and little polysaccharide.

The distribution pattern of the DNAse-gold shows not only the position of the nucleic acid at the periphery of the rodlet core, but also indicates the relative amounts of DNA at points along the rodlet core. For instance, the DNAse-gold label is seen throughout the entire length of the core, but is consistently heavier at the apical end, where the core is thicker. The abluminal end of the rodlet, where the peripheral substance is most abundant, is labeled with DNAse-gold, but the label is sparse (Fig. 6-10). The significance of this distribution pattern is unknown, but it is consistent.

The regionalization of the core suggests that the structure is organized, and that the DNA is on the periphery of a stiff support structure, protected from the environment for most of its extent by a very thin layer of glycoprotein. Where the glycoprotein layer is thickest, the DNA seems to be sparse (Figs. 6-10, 6-18, 6-19). It also argues strongly against the rodlets being randomly packaged cellular debris. When cellular debris is identified, such as the secondary lysosomes in macrophages, the bodies seen are highly variable in size, density and distribution. Such a pattern is not seen in the rodlet cell. The rodlet cell is clearly a structure designed to deliver packaged DNA from one cell into the environment with considerable energy. Such a consistent, complex structure is unlikely to be merely being thrown away. The end result is that a DNA-containing structure is free in the environment; stages in parasite development show very similar patterns.

The third portion of the rodlet is the peripheral substance. What is known about it? Histochemically, the material is a neutral to slightly acidic glycoprotein containing 1,2-glycol groups, primarily

neutral sugars and perhaps traces of sialic acid (Leino, 1982). Leino also argues that, on the basis of its reaction to acid dyes and to protein stains such as coupled tetrazonium, the protein moieties of the peripheral substance are not completely masked by the prosthetic groups as they appear to be in most mucous cells. The proteinaceous component of the peripheral substance contains few basic amino acids; therefore, it is probably not a histone. The peripheral substance is not amenable to pepsin and trypsin digestion, unlike the rodlet core. Leino (1982) argues this is because the carbohydrate prosthetic groups protect the protein from the enzyme action. There appear to be no lipids in rodlets; reactions for phospholipids are variable (Morrison and Odense, 1978; Leino, 1982). Little is known of the physical properties of the peripheral substance. Light microscopic observations on rodlets freshly discharged from living rodlet cells indicate the peripheral substance may be sticky, since rodlets tend to remain in the microscope field as surrounding materials drift through.

The central issue is the origin of the DNA in the rodlet core. Only two possibilities exist: either it is endogenous, or it is exogenous.

If the rodlet is an endogenous structure, the stimulus for its formation is unknown. Where does the DNA found in the rodlet core arise? Is the DNA mitochondrial in origin? During rodlet cell development, when only a few recognizable rodlets are present, mitochondria are present throughout the cytoplasm. In the mature rodlet cell, the mitochondria, or their membranous remnants, seem to be confined to the apex of the cell. While it might be postulated that mitochondrial DNA becomes translocated into a sheathed, elongate structure, there is no morphological

or molecular biological evidence that mitochondria transform into rodlets or that their DNA is somehow reoriented into these extrusible structures. If the rodlet is an endogenous structure, does the nucleus, for some reason, rid itself of "excess" DNA by means of the rodlet? If this were true, one might expect to see reduced quantities of DNA in rodlet cells when compared to a standard 2C genome such as that of the erythrocyte. In fact, while one experiment showed that in two teleost species, the amount of DNA in the rodlet cell nucleus was indeed less than in the erythrocytes of the species, the third species measured showed more DNA in the rodlet cell than in the erythrocyte. Subsequent experiments also indicated that rodlet cells had significantly more DNA than corresponding erythrocytes, but that the DNA content was not significantly different from known somatic cells of the fish whose nuclei were more nearly the same size as the rodlet cell's. In addition, no mechanism has ever been proposed for such a translocation of nuclear DNA. If such an event occurs, why is the "package" so elaborate, with its glycoprotein coat, stiff proteinaceous core scaffolding and helically disposed nucleic acid? Why, also, would the cell's morphology appear to be directed entirely toward the expulsion of these elaborate structures from the cell? Invertebrates, such as nematodes, which lose nuclear DNA in a piecemeal fashion (Goldstein, 1977) do not have rodlets or anything resembling them. Waste cell products are generally digested by lysosomal action, and the metabolites either recycled, stored in the cell, or removed by exocytosis. Lysosomal enzymes have never been identified histochemically in the cytoplasm of rodlet cells, although Kimura (1973) reported the presence of densely-staining bodies in the basal vacuolated region of the cell border in rodlet cells of

Salmo gairdneri; he considered these might be lysosomes involved with nutrient acquisition. Certainly the rodlets themselves contain no lysosomal enzymes (Morrison and Odense, 1978; Leino, 1982), and they are expelled in toto by contraction of the cell border.

To postulate the endogenous origin of the rodlet, hence, to consider the rodlets and rodlet cells as normal teleost components, requires too many unlikely mechanisms to be tenable. One is obliged to accept the idea of a cell with no known function, occurring apparently randomly and variably in several types of epithelia, but only in epithelia. Then, that the cells, which are also an end-stage in development, contain nuclei producing DNA in excess, and finally, that the excess DNA is then elaborately packaged and extruded by means of cell modifications which have arisen simply to accomplish this task. (Such a postulate fails to obey Occam's Razor, the economy of natural processes, and scientific logic.)

The alternative is an exogenous origin for the rodlet core. A corollary of this hypothesis is that the rodlet, or at least its core, invades an epithelial, or other, cell and somehow converts the synthetic apparatus of the host cell's cytoplasm to making the rodlets, the contractile border and the rodlet peripheral substance. Evidence in favour of this argument lies in the DNA present in the rodlet, and the fact that it bears no apparent relationship to the DNA of the cell nucleus. The mechanism for this is entirely unknown. There is no detectable evidence the rodlet invades the nucleus; in fact, the microdensitometry experiments argue against it. Presumably the rodlet DNA would function in the cytoplasm.

Rodlet cells are not recognized as such until there is at least

one ensheathed rodlet present, and no rodlet cell has ever reliably been identified without the beginnings of the contractile cell border. Figure 2-4 clearly shows evidence that the cell's Golgi complex gives rise to the rodlet peripheral substance. The origin of the rodlet core is unknown; it is unlikely that the core with its complex structure simply condenses out of sheath material with the passage of time. Some of the evidence against a "condensation" hypothesis is histochemical, and the fact that the rodlet core gives reactions not found in the peripheral substance, notably for single-stranded nucleic acids (Barber et al., 1979), and fails to react to some stains of the peripheral substance (Leino, 1982).

Observations of red fluorescence in rodlet cores after acridine orange, and pink staining with methyl green-pyronin are histochemical evidence for single-stranded nucleic acids, and usually are taken to indicate RNA (Pearse, 1983; Humason, 1972). However, no RNA is found in rodlet cores with RNAse A-gold, although ribosomes and the rodlet cell nucleolus are labeled. In addition, the rodlet core does label with S1 nuclease-gold, an enzyme specific for single-stranded DNA (Wiegand et al., 1975). Certainly only one nucleic acid has been found in the rodlet core, and that is DNA of some sort. Since the DNA is Feulgen negative, fails to hybridize to genomic DNA sequences found in the fish, gives an orange reaction to acridine orange and a pink reaction with methyl green-pyronin, and appears to react to S1 nuclease, it probably has a conformation and nucleotide sequences unlike that of somatic DNA. Viehberger and Bielek (1982) considered that the DNA of the rodlets could be assumed to be in a "totally different configuration" than the chromatin of the

nucleus of the rodlet cell, on the basis of its negative Feulgen reaction, both in light and electron microscopy. Somatic cell DNA consists largely of B-DNA which is a right-handed helix with major and minor grooves in the molecule, as a consequence of the arrangement of bases. Is it possible that rodlet DNA with its "totally different configuration" is an example of something like left-handed DNA?

In 1979, Wang et al. described the physical properties of left-handed DNA (Z-DNA), which consists of purine-pyrimidine repeating pairs of nucleotides, especially poly (CpGp) on one side of the helix. The steric interaction of the C-G molecules results in a hexameric, left-handed spiral with 2 base pairs in every 60° of rotation of the helix. Z-DNA shows variations from B-DNA in that the helix is super-coiled, with 12 base-pairs per turn rather than 13, with the ribose of the cytosine nucleotide on the outside of the molecule rather than on the inner portion of the helix, with a zigzag pattern to the backbone and a major groove that "really does not exist as a groove. . . but has been transformed into a convex surface on the upper part of the molecule". B-DNA has consistent major and minor grooves. The guanine residues of Z-DNA are stacked upon the O1' oxygen atoms of the preceding deoxyribose residues, since the cytosine-deoxyribose components of the helix are twisted. Z-DNA has been reported to be potentially present in sequences from humans (Hamada and Kakunaga, 1982), in which a repeating sequence of poly (dT-dG)·poly (dC-dA) has been found as part of a human cardiac muscle actin gene. As well, Z-DNA has been reported as very short polymers in SV-40, a double-stranded DNA virus (Nordheim and Rich, 1983).

While it was first described as a laboratory-grown curiosity,

the appearance of Z-DNA in living organisms suggests more variability in DNA molecules than had hitherto been suspected (Wang et al., 1979).

In view of the physical structure of Z-DNA described by Wang et al. (1979), some predictions might be made as to its affinities in some histochemical procedures: the nucleotides in Z-DNA are in pairs, with part of the cytosine nucleotide exposed at the surface of the helix; acid hydrolysis first breaks the sugar linkages of polymeric bonding. It also ruptures the glycoside linkages between the sugars and the purine bases (Pearse, 1983). Hydrolysis of Z-DNA with 1N HCl might destroy the polymer removing the exposed, twisted deoxyribose sugars. Either destruction might occur, or the cytosine-guanine complex could be so tightly bound together that the entire molecule resists hydrolysis. In either case, the net result would be a negative Feulgen reaction.

The yellow-green fluorescence of Z-DNA-containing compounds has been established as resulting from the orderly arrangement of acridine orange dye molecules in a ratio of one dye molecule for every three base pairs. Their dimethylamino groups are associated with the negatively charged PO_4 groups, and the acridine ring is in contact with the purine and pyrimidine rings of the upper and lower bases of a pair, thereby establishing a stable complex of nucleic acid and dye in which the dye molecules are too far apart to interact. The result is that the fluorescence characteristics are those of the acridine orange monomer: an orthochromatic yellow-green. For random-coil, single-stranded nucleic

acids, almost every phosphate molecule has an attached dye molecule, and adjacent molecules interact, forming polymers on the phosphate chain, and resulting in red metachromasia (Pearse, 1983). Z-DNA has a repeating, tightly coiled sequence in which part of one of the nucleotides is exposed to form a convex surface instead of a major groove. The nucleoside residues are also rotated 180°, so they are essentially "upside down". It may be that acridine orange could bind to every second molecule in the Z-DNA coil; this might be close enough for interaction of dye molecules to occur, resulting in polymer formation and metachromasia. If this could occur, it would mean that a double-stranded DNA molecule could, in effect, yield a "single-stranded" reaction. Or, since Z-DNA has been recorded only as short sequences in normal B-DNA molecules, and appears to tend to unwind adjacent right-handed sequences, it potentially provides short regions in which one polymer is separated from the other. This might result in enough "single-stranded" nucleic acid for both acridine orange and methyl green to give "RNA" reactions.

If rodlet core DNA contains some sort of odd configuration such as Z-DNA, the light microscopic histochemical reactions observed for the rodlet cores might be explained in this fashion. In addition, the nuclease digestion experiments of Viehberger and Bielek (1982) showed that DNAse I, which acts on double-stranded DNA, was the only nuclease that completely destroyed the rodlet cores; normal sequences adjacent to Z-DNA in SV-40 virus are reportedly very sensitive to DNAse I

(Nordheim and Rich, 1983). Other evidence supporting such a speculation is lacking, although future research might be directed toward elucidating the structure of rodlet DNA with Z-DNA in mind. The remaining possibilities for the DNA in the rodlet core are that it is single-stranded, or that the B-DNA is in very short, right-handed polymers. The only known single-stranded DNA genomes are very small viruses, e.g. Phage 174 (Watson, 1976), which code for only a few repeated proteins. Unless the rodlet core represents an unprecedented aggregation of single-stranded virions, helically arranged and without capsids, the rodlet cannot be a single-stranded virus. Therefore, it is unlikely that if the rodlet does indeed contain single-stranded DNA, it is a virus. It would have to be an entirely new concept in living organisms. On the positive side of the viral question, both viruses and the rodlet appear to contain a single type of nucleic acid. If the rodlet DNA is very short, separate polymers of double-stranded B-DNA, it is difficult to speculate upon why the core is Feulgen negative, since other procedures indicate the DNA is retained in position on the core during processing.

The somatic and individual variation of rodlet cell incidence supports an exogenous origin for rodlet cores. If the rodlet core is an infectious agent, the extruded rodlets may be able to drift in the fluid above the epithelial free surface until they encounter a suitable epithelial or subepithelial cell (a vascular pericyte, for instance) which the stiff, sharp rodlet core can penetrate. If the fluid above

the free surface is the water of the external environment or the contents of the intestine, the rodlets may be transported away from the fish and drift toward the bottom. The free rodlet may adhere to the substratum, or to plants, bottom invertebrates or detritus which become food for some of the demersal fish. That this mechanism of transmission is possible, is borne out indirectly through the evidence of Table 3-III.

The fate of the peripheral substance is unknown. It may simply be left on the cell surface as the rodlet core penetrates the cytoplasm; alternatively, the peripheral substance may be digested if the rodlets are ingested or released into the digestive tract, and the naked core may invade the epithelium. Such a phenomenon could account for the groups of rodlet cells reported in some sites by some authors, e.g. Plehn (1906); Smallwood and Smallwood (1931); if released rodlets penetrated adjacent epithelial cells, one would soon see a "colony" of rodlet cells (Fig. 2-7) (see Fig. 3 of Barber and Westermann, 1975).

If the rodlet loses its peripheral substance in the process of infecting a cell, there is no morphological evidence to prove it. The rodlet core might assume a crystalloid appearance or disintegrate rapidly, leaving little evidence for the electron microscopist. As indirect support for this mechanism, it is possible to say that mature rodlets, complete with peripheral substance, have never been seen in cells which lack the contractile "capsule"; it is unlikely that the rodlet invades as a complete structure.

To compare the rodlet with other infectious organisms is difficult because it shares characteristics with very few. The rodlet is not a bacterium; there is no cell wall, and no evidence of mitosis in rodlets.

viruses, unless they are very small viroids, are invariably surrounded by a structurally regular protein coat, and the nucleic acid of the virus is intimately bound to the coat molecules. Rodlets seem to have an amorphous, variably thick peripheral substance, and the DNA appears to be more associated with the protein scaffolding internal to it than to the peripheral substance surrounding it (Figs. 6-10, 6-11). There has been no evidence from the enzyme-gold experiments that rodlets contain RNA as well as DNA.

SUMMARY

In summary, these studies have established the following facts about the rodlet cell of teleost fish:

1) Rodlet cells have been reliably reported only from teleosts, and the Siluriformes may not have them. While there are size ranges which appear to be related to the species of fish, rodlet cells show many common elements of morphology in the teleosts from which they have been reported. In the case of the Salmonidae alone, the rodlet cell nucleus appears to have a unique morphology. This study represents the first time an attempt has been made to examine all the available literature on the topic of rodlet cells.

2) In examining some ecological parameters of teleosts containing rodlet cells, it has been found that they are distributed worldwide, in fresh and saltwater environments, of a variety of behavioural habits, and with a variety of dietary proclivities. A positive association between reports of rodlet cells and diet in a species exists for fish which feed on plants or bottom debris, and especially for those which

are classed as omnivorous. Solitary, pelagic plankton feeders such as postlarval nototheniids and myctophids may not have rodlet cells. This is the first time teleost ecological parameters have been examined in conjunction with rodlet cell incidence.

3) The basal nuclei of rodlet cells have been shown, both qualitatively and quantitatively, to contain amounts of DNA which are not significantly different from the DNA of cells known to belong to the fish. This is the first time factual, rather than speculative, evidence has been acquired on the nature of the DNA of the rodlet cell nucleus, and it is the first time modern molecular biological techniques have been applied to the solution of some of the basic questions that have been asked of rodlet cells since their first description in 1892.

4) The rodlet core contains DNA. This has been firmly established, and while the precise conformation of the DNA on the rodlet core has not been determined, it is clear that rodlets contain only one nucleic acid, and that it is DNA.

5) With the localization of the DNA to the periphery of the rodlet core, using nuclease-gold techniques, and after reapplying established chelation techniques, the rodlet is now identified as a triphasic structure, rather than a biphasic one. This is the first time the rodlet has been recognized as such. It is the first time that the distribution of the DNA has been sh[illegible], and that a helical pattern along the core has been postulated. It is the first time that possible variations in abundance of DNA along the rodlet core has been suggested, and it is the first time the entire rodlet core has not been implicated as the site of nucleic acid localization.

6) As a consequence of the above findings, it is now clear that the rodlet cell must be regarded as both normal cell and "parasite", in other words, as a structure with two separate origins. The experimental observations support the hypothesis that the rodlet itself is a structure whose DNA is exogenous; since it is found inside a teleost cell, it must be an invasive organism. There is no information yet on the mechanism of invasion, duplication of rodlet cores, or life history of the rodlet, and the nature of the teleost cells invaded is not known. The time course of these events is also unknown.

7) Appendix I represents the first attempts to concentrate isolated rodlet cells from tissues.

4) Unanswered Questions and Future Studies

The fundamental question remains: what is the rodlet? Since it is neither bacterium, eukaryote, nor obvious virus, does the possibility exist that the rodlet represents a whole new type of organism? Is the rodlet some developmental stage in the life cycle of a protozoan parasite recognized in another morphology in a different animal? Wolf and Mackiw (1984) have demonstrated that a myxozoan parasite of rainbow trout and an actinosporean of tubificid worms, formerly in two separate taxonomic classes, are in fact life stages of the same organism. At the moment there is no evidence to suggest the rodlet is indeed a life history stage of some already-described organism, but the possibility cannot be dismissed. Why are rodlet cells apparently restricted to teleost fish, and do other classes of vertebrates act as hosts for related infectious agents? Is it possible that rodlets represent a longstanding association between the teleost cell and a formerly

free-living body which has now assumed quasi-organelle status? Rodlet-infected cells appear to cause little or no reaction in adjacent cells, and no obvious pathological manifestations in the host fish. Until the border contracts, the cell itself appears to be an integral part of the epithelium. No investigations have ever been conducted on possible prokaryotes which might be ancestral to the rodlet.

Rodlets, for instance, cannot be classed as an X-cell type of parasite (Dawe, 1982). The X-cell is a component of pseudobranch lesions generally in Pleuronectiform fish from the North Atlantic and North Pacific Oceans. Microscopic examination shows that it is a tiny eukaryotic cell surrounded by hypertrophied cells, and these cell types comprise the lesion. Dawe (1982) has recorded mitotic divisions in X-cells, and has proposed that they induce the lesion formation. Neither rodlets nor the rodlet cell itself have been seen in division, they do not induce a pathological host reaction, and the rodlets are not eukaryotic organisms. The mechanism of production of the rodlet core is still a complete mystery. Also, past and present studies have given little indication of the rate at which rodlet cells or rodlets are produced, and nucleotide uptake studies might be a profitable avenue of approach.

Future studies on rodlet cells ought to be prefaced by the development of a reliable method for isolating and concentrating rodlets and/or rodlet cells from a population of freed cells, such as are found in a macerated gill. Once the rodlets are available in reasonably pure form, molecular biological experiments would be conducted to determine the DNA structure, sequences and orientation. In addition, it would be

possible to address the metabolic and infection questions using tissue culture methods combined with isolated rodlets.

1) The most obvious question to be directed toward rodlet identification relates to the actual nature of the DNA in the rodlet: what is its conformation: is it single-stranded, double-stranded, left-handed, right-handed, a combination, or short polymers of DNA? From that information, it may be possible to deduce the rodlet's affinities. Electrophoretic methods exist for determining the length of DNA strands; centrifugal methods exist for estimating molecular weight; biophysical methods such as X-ray diffraction analysis exist for determining molecular orientation. Single- or double-strandedness can be determined in vitro by means of reactivity to S1 nuclease.

2) There are several functional questions which could be examined: how does the rodlet invade the cell, and what type of teleost cell is invaded; how does the rodlet DNA direct the synthesis of other rodlets in the cell; how does the rodlet obtain the nucleotides to build rodlet DNA? In what order do synthetic events occur? These questions are more difficult to address than the molecular questions. They might be tackled by means of tissue culture, in that isolated rodlets could be added to teleost cells in culture, and various time-course studies done. Such a system could be treated with radioactive nucleotide triphosphates to determine de novo synthesis of rodlet DNA.

4) There are some experiments that have been begun, either as part of this study or by others. The basic information on how long it takes a rodlet cell to form is lacking. Preliminary evidence using a 3-day pulse labeling of Catostomus commersoni with ^{3}H-thymidine indicates that

no identifiable rodlet cells in the gill were labeled in any location, whether basal nucleus or cytoplasmic rodlet, although adjacent cells showed silver grains in the autoradiograph. Fearnhead and Fabian (1961) and Mattey et al. (1979) both performed experiments in which they moved euryhaline fish from marine to brackish water conditions, noting that the rodlet cells which had been abundant in individuals in the marine environment declined almost to zero in the brackish water situation. Both research teams apparently stopped experimentation at that point. As part of a time-course experiment, it might be useful to continue the experiment in two phases: to leave the fish in brackish water and, using ^{3}H-thymidine as a marker, attempt to determine the time span of reinfection of the fish by rodlets. As well, it would be useful to return the fish to sea water, and follow the same reinfection process with ^{3}H-thymidine.

3) Are rodlets species-specific, or are the rodlet cells' appearance and variable dimensions the result of an influence of host DNA on rodlet production? Until the mechanism of formation of rodlets is known, this question can only be a subject of speculation.

Finally, although the question of the affinities of the rodlet may remain speculative, the proposed DNA studies suggest how rodlets might be classified phylogenetically. Certainly with its unusual nucleic acid conformation, an understanding of the rodlet should contribute to knowledge of the variability of DNA in living organisms.

APPENDICES

APPENDIX I

Numerous attempts were made to isolate rodlet cells from gill tissues in order to obtain an enriched preparation for use in *in vitro* experiments.

REMOVAL OF CELLS FROM GILL TISSUE

1) Northern creek chub, *Semotilus atromaculatus*, weighing approximately 60 g, were injected with 100 IU heparin (Hepalean 10,000 IU/ml) approximately 20 minutes before killing the fish. It was found that red blood cells were a major component of gill maceration preparations if this step was not performed.

2) The fish was killed with a pair of large scissors, severing the spinal cord and the dorsal aorta just behind the head. The heart and gill region were then perfused with 10 ml Ca^{++}, Mg^{++}-free saline containing 10 IU heparin/ml to wash out as many erythrocytes as possible from the gills.

3) The gills were excised as separate arches, rinsed in heparinized saline, and placed in 10 ml heparinized ice-cold saline in a siliconized 50 ml beaker on a magnetic stirrer. Using a 2 cm Teflon-coated stirring magnet, and with the stirrer set at fairly low speed, the gill was macerated for 7-10 minutes to dislodge the rodlet cells.

4) Using a bright-line haemocytometer, the preparation was checked for numbers of rodlet cells. Values ranged up to 3×10^5 ml^{-1}. As a

rough estimate, one drop of the macerate could be examined at 250X on a microscope slide under a 30 mm coverslip; four or five rodlet cells in such a preparation were considered indicative of numbers adequate to proceed with the isolation experiments. Experiments with trypan blue indicated that, providing the pH of the saline was above pH 7.3, providing the fish was freshly killed and providing the saline used was aerated and on ice, rodlet cells excluded trypan blue, i.e. they were alive.

5) The macerate was filtered through 2 layers of a 250 µm nylon mesh to remove clumps of cells.

The following methods were used to attempt to separate rodlet cells from other cell types in the macerate:

1) density gradient centrifugation using sucrose or Percoll (Pharmacia);

2) low concentrations of the detergent Triton-X in an attempt to lyse the cells in the macerate without destroying rodlet cells;

3) elutriation experiments using a Beckman JE-6B Elutriator. The experiments were performed singly and in combination with each other and with and without trypsin/pronase digestions.

DENSITY GRADIENT CENTRIFUGATION

Prior to these experiments, one of the undergraduate thesis projects (Ladd, unpublished) had been directed toward separation of rodlet cells using the compound Percoll (Pharmacia, Stockholm), an osmotically neutral colloid. Using Percoll gradients in the ultracentrifuge at 20,000 X g in nitrocellulose

tubes for extended periods had resulted in the creation of several bands of material. Rodlet cells were found in all the bands, along with other cells and clumps of cells. A major problem with this method had been the glutinous nature of the bands. In the early experiments, the cells had been removed from the gill using a trypsinized saline solution. With the above methods, none of the experiments had resulted in the concentration of rodlet cells in any particular fraction. Experience suggests that his problems were due to several factors: too many cells in the preparation, high speed centrifugation, no heparin, and extended time periods in the gradient.

With Percoll in saline, the same sticky bands of cells occurred. Resuspending the material and layering it over a four-layer gradient (15%, 20%, 25%, 30% Percoll) in 15 ml glass centrifuge tubes spun at approximately 1000 g, yielded rodlet cells and other cell types at all levels, whether or not heparin was used in the saline. The particular batch of Percoll used may have been defective because of its opacity when diluted with saline. It was impossible to discern any bands visually in the tube; it would appear that there was something wrong with the Percoll, since others have apparently not had the same difficulty.

Using sucrose, discontinuous gradients ranging from 50% to 20% were prepared in Ca^{++}, Mg^{++}-free saline and placed in siliconized glass centrifuge tubes. The macerate was layered either on top of the tube or at the bottom of the tube and the tube was centrifuged at approximately 1000 X g in a clinical centrifuge for 30 minutes. During this period, the friction of the centrifuge bucket passing through the air resulted in heating of the preparation, and for some experiments, the centrifuge

was moved to a cold chamber at 4°C. No obvious differences were noted between experiments conducted at room temperature and those at 4°C. Most cells stratified at 35-45% sucrose; the one real advantage of the sucrose as a gradient material was that all the erythrocytes were removed. Unfortunately, the cells that were left behind were an indiscriminate mix of rodlet cells and other cells; the rodlet cells were found in all layers, including the supernatant. The high osmolarity of the sucrose/saline mixture also resulted in the deformation of most cell types. As well, the cell bands were inevitably glutinous, and even agitation of the bands in heparinized saline failed to dislodge more than a few rodlet cells and other cell types from the glue. When the saline was centrifuged very gently, a few rodlet cells remained in the supernatant along with some of the other cells, and the rest were at the bottom of the tube along with the glue. The cells remaining in the supernatant were not sufficient in number to warrant further processing.

In some experiments, 10% fetal calf serum was added to the saline in an attempt to cause the non-rodlet cells to clump and hence to make them easier to remove from solution. There was no noticeable difference in the behaviour of cells in the preparations with or without fetal calf serum.

The use of enzymes such as trypsin and pronase in tissue culture and density gradient centrifugation is well-known (e.g., Cornell-Bell and Munro, 1982). Trypsin was prepared initially from dry powder as a 1:250 solution in saline pH 7.4 but later as a 1:10 dilution from a commercial liquid preparation which was frozen at -20°C between experiments. It was felt that the trypsin might make non-rodlet cells more sensitive

to osmotic shock, and since the rodlet cell has a thickened cell border or capsule, it might not be as sensitive to osmotic changes. Treating the macerate with trypsin followed by exposure of the solution to a hypotonic saline destroyed the rodlet cells as well as other cells. Using trypsin 1:250 (GIBCO) in a 1:10 dilution during maceration does not selectively destroy other cells, leaving rodlet cells. It might help to prevent the formation of the "glue" that frustrated all the density gradient experiments, although the early experiments in which cells were removed from the gill tissues by trypsin would tend to negate such an idea.

In addition to centrifugation, the density gradient experiments were also tried at 1 g, although the time course of the experiment was probably too long (72 hours).

When the chub rodlet cells proved refractory, the experiments were repeated using preparations from Catostomus commersoni, the white sucker, because rodlet cells in this species are larger. With a sucrose gradient, the upper layer of cells contained more rodlet cells than the lower layers, but the rodlet cells even in the upper layer represented <10% of the total number of cells.

DETERGENT EXPERIMENTS

1) Triton X-100 was prepared in dilutions from 1:1000 to 1:100,000 and applied to macerated gill tissue. In the microscope, rodlet cells could be seen to be morphologically intact after 30 minutes when the detergent was used at 1:1000, and rodlet cells and most other cell types

were intact when lower concentrations were used. Unfortunately, the rodlet cells were shown to pick up trypan blue after the detergent treatment.

2) When macerates were treated with detergent diluted to 1:1000, the centrifuged preparation became a glutinous mass in which rodlet cells were the only recognizable entities, but they were inextricable, and also picked up trypan blue. Heparin added to the saline before detergent treatment reduced the amount of agglutination, but did not prevent the formation of the glutinous material.

3) Enzyme treatment combined with detergent experiments: with Triton -X alone, the macerate became a glutinous mass studded with rodlet cells. When pronase was added to the macerate without detergent, many cells, including rodlet cells, survived. When Triton X (1:1000 dilution) was added along with pronase, the glutinous mass was much reduced, but all cells were dead, although rodlet cells were still the most notable component in the preparation. If the detergent was added after pronase digestion of the rough macerate, all the cells were destroyed. The same observations were made with trypsinized preparations. Collagenase, an enzyme used in some laboratories on campus to separate liver cells from one another (G. Sweeney, personal communication), was not tried.

Presumably the detergent breaks down the lipids in the cell membrane, and it is only the thick cell border that permits the rodlet cell to retain its shape over the experimental period. To test this hypothesis, pellets of detergent-treated and untreated cells were fixed in glutaraldehyde, dehydrated, and embedded in Spurr's resin. Semi-thin sections of the pellet showed that the detergent-treated material had

widely-spaced rodlets and pale-staining nuclei. Considerable cellular debris was present in both preparations, and in the detergent-treated material, rodlet cells were the only recognizable cell type. In the untreated macerate, the rodlet cells had dark-staining nuclei and pale rodlets lying close together inside the capsule.

ELUTRIATOR EXPERIMENTS

The elutriator is a refrigerated centrifuge which functions on the principles of countercurrent pressures. The cells to be separated are placed in a chamber which rotates at precisely determinable speeds. Then fluid is added through the base of the chamber at controlled rates. The pressure of the centrifugal force exerted by the rotating centrifuge may be balanced by the fluid pressure of the eluting fluid, and each is variable. Consequently, by varying the speed of the centrifuge and the rate of fluid flow within known limits, it is possible to select the sizes of live cells that are to be removed from the preparation. These float away with the eluting fluid, and are collected in numbered test tubes. The material in the test tubes is centrifuged to precipitate the cells, and the supernatant decanted.

Several types of experiment were performed with gill macerates:

1) The rough macerate in unheparinized saline was processed through the elutriator. This was unsuccessful because as soon as the cells came into close proximity to one another, they agglutinated, and the solid mass of material retained all the cells.

2) With heparin, the cells could be loaded and elutriated, but the majority of them came off in the first fraction (small or dead cells).

Rodlet cells could be reliably obtained in certain fractions, but these fractions also contained a variety of cell types of the same apparent diameter.

3) Cells from Experiment 2 were elutriated at high flow rates and high centrifuge speed with a view to shearing cells without a heavy cell border. This was rather unsuccessful; almost all the cells remained intact, the rodlet cells were no more isolated than before, and the fluid volume was vastly increased.

4) Experiments using the detergent Triton X-100 were performed. The detergent was applied at 1:1000 dilution in an attempt to lyse non-rodlet cells. In one experiment, where the detergent was applied just before the cells were loaded into the elutriator, a very few, uncontaminated rodlet cells were isolated in two fractions. Unfortunately, there were very few of them, and attempts to duplicate the results were unsuccessful.

5) Experiments were performed using the eluted material from two fish in quick succession, pooling the collected eluates from each experiment, adding dilute Triton X-100 just before reloading the cells, and re-elutriating the preparation. Most of the rodlet cells in the second elutriation were killed and therefore came off in the first fraction; the remainder were present in the usual fractions, but were not clean.

The main problem with isolating rodlet cells in any kind of centrifugal field is the shape of the rodlet cell. Because of its thick cell border, the rodlet cell fails to "round up" when it is isolated from its neighbours. Unlike epithelial or blood cells, except for

erythrocytes, which essentially become spheres, the rodlet cell maintains its elongate oval shape. Consequently, rodlet cells present an infinity of apparent diameters to the centrifugal field, and migrate in the field according to whatever their apparent diameter is, unlike the spherical cells which tend to obey Stokes' Law ($v = (2r^2g/9n)(p-p_o)$. It was found in the elutriator that rodlet cells usually behaved as cells with diameter of approximately 8 μm, probably because the stream of saline passing among them forced them to present the narrow profile to the centrifugal force.

If there were some controllable way of killing all the other cells and keeping the rodlet cells alive, the problem would be fairly easy to solve, since all dead cells are removed from the elutriator in the first fraction, no matter what their size. One would simply have to kill the cells, load them into the machine and collect one fraction at fairly low speed. The fraction then remaining in the chamber would be largely rodlet cells. These could then be put onto a Ficoll gradient and purified. However, the problem has been to find some way of concentrating rodlet cells into a fraction.

All experiments that showed the least indication of progress were repeated. Unfortunately, the most promising ones could not be duplicated. Considerable time was spent in these unsuccessful attempts to isolate rodlet cells with a view to applying some *in vitro* methods for identifying the DNA in the rodlet cell nucleus, and tissue culture experiments, etc. It became obvious that for completion of the study in a reasonable amount of time, some other avenues, notably hybridization *in situ* and the enzyme-gold procedures which did not require the isolation of relatively

pure preparations of rodlet cells, ought to be explored. The isolation experiments were therefore abandoned.

GENERAL COMMENTS

Perceived problems: too many cells in each gradient; not enough gradient material in each tube; failure to use trypsin or pronase to keep the leaking proteins chewed up; failure to experiment with several different kinds of osmotically neutral colloids; e.g., Ficoll; generally too-high centrifugation rates; too few rodlet cells to begin with to compensate for high losses during processing.

Isolation of the rodlet cells or of the rodlets themselves is necessary if detailed study of the rodlet DNA is to be undertaken. It may be possible to do this if some combination of the above procedures is used; possibly a very low proportion of trypsin (GIBCO) used in the macerating fluid, and continued right through the experiment, followed by elutriation of the macerate with Ca^{++}, Mg^{++}-free heparinized saline, and then density gradient centrifugation of the rodlet cell-containing fractions using an osmotically neutral colloid, either fresh Percoll or freshly-prepared Ficoll, in siliconized glass tubes of at least 50 ml capacity. The rodlet cell-enriched fraction(s) could then be centrifuged, and the rodlets released in a hypotonic solution. They might then be concentrated using the methods of Bloebel and Potter (1966) (John Bergeron, personal communication) and then used for various experiments. To perform experiments such as $CsCl_3$ centrifugation, microgram quantities of DNA are required; the same is true for nick-translation procedures and

spectrophotometric measurements. However, if the rodlets could be consistently isolated, it would not be difficult to carry out some definitive studies.

APPENDIX II

SAMPLE STATISTICAL CALCULATION FOR
MICRODENSITOMETRY EXPERIMENTS

Calculations to Determine DNA Content of Cells

Estimating the amount of DNA using the Leitz Microspectrophotometer requires the use of two monochromatic wavelengths, calculations based upon Beer's Law and insertion of correction factors such as those in Patau (1952) into the formula. Patau (1952) estimates absorbance as $A = 1 - T$, where T is the fraction of light transmitted through the specimen. The two absorbances thus estimated are designated L_1 and L_2, for the shorter and longer wavelengths, respectively. The ratio $L_2:L_1$ is designated as "Q", and is used as a basis for the correction factor "C" published as a table (Patau, 1952). The DNA estimation is the product of the area of the aperture ("B"), the correction factor ("C") and L_1. The result is expressed as "units".

In the estimations expressed here, comparative calculations of measurements of a population of cells showed that using Beer's Law ($\log_{10}(I_i/I_o)$) gave marginally smaller variations in the estimates of DNA than did Patau's 1 - T. With present-day calculators, the mechanical operations involved with using Beer's Law are more feasible than they were in 1952.

Estimating DNA values from the Vickers Scanning Microdensitometer is simply a matter of subtracting the measurement of the background absorbance from the absorbance of the specimen.

Calculations for both microdensitometers were carried out using the "units" obtained from the machine; only as the final step were the "units" calculated as pg DNA/2C nucleus.

SAMPLE CALCULATION

Leitz Microspectrophotometer

DNA CONTENT OF SUCKER ERYTHROCYTES

#	Area	$\frac{\lambda_2}{I_2}$	I_0	$\frac{\lambda_1}{I_1}$	I_0	L_2	L_1	Q	C	DNA
1	150	31.2	50.1	34.1	50.0	0.206	0.166	1.237	1.878	46.82
2	130	28.3	50.1	31.7	50.0	0.248	0.198	1.253	1.848	47.55
3	130	27.3	50.0	30.3	49.9	0.263	0.217	1.213	1.975	55.63
4	130	30.3	49.9	33.1	50.0	0.217	0.179	1.209	1.975	46.96
5	126	27.6	49.7	30.8	50.1	0.255	0.211	1.209	1.975	52.58
6	126	28.4	47.9	32.3	50.0	0.227	0.190	1.196	2.012	48.11
7	130	29.0	50.1	31.5	50.0	0.237	0.201	1.183	2.091	54.55
8	130	28.8	50.0	32.0	50.0	0.240	0.194	1.236	1.878	47.32
9	154	32.2	50.0	34.6	50.0	0.191	0.160	1.195	2.012	49.54
10	165	33.3	50.1	35.6	49.9	0.177	0.147	1.210	1.975	47.79

MEAN DNA CONTENT: $\bar{X}$ = 49.68 units

CALCULATION FOR STANDARD ERROR OF THE MEAN

#	DNA (X)	$(X - \bar{X})$	$(X - \bar{X})^2$
1	46.82	-2.861	8.186
2	47.55	-2.138	4.571
3	55.63	5.943	35.317
4	46.96	-2.723	7.414
5	52.58	2.895	8.379
6	48.11	-1.576	2.483
7	54.55	4.861	23.630
8	47.32	-2.365	5.593
9	49.54	-0.141	0.020
10	47.79	-1.894	3.589

$\text{SUM } (X - \bar{X})^2 = 99.182$

$S^2 = \text{SUM } (X - \bar{X})^2/n - 1$

$= 99.182/9 = 11.020$

$\text{S.E.M.} = S/\sqrt{n} = \pm 1.050$

The DNA content of erythrocytes of Catostomus commersoni measured with the Leitz Microdensitometer is 49.68 ±1.050, or ±2.11%.

Similar calculations were carried out for rodlet cells and teleost cells larger than erythrocytes (see definition) for all species examined.

For rodlet cells in the same experiment,

$n = 10$, $X = 54.25$, $S^2 = 68.344$, $\text{S.E.M.} = \pm 2.614$

t Test

$S_p^2 = S_1^2(n_1) + S_2^2(n_2)/n_1 + n_2$

For sucker erythrocytes compared to sucker rodlet cells,

$S_p^2 = 11.020(10) + 68.344(10)/20 = 39.6682$

$S_p = \pm 6.299$

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

$= 54.25 - 49.68/6.299\sqrt{0.2}$

$= \pm 1.619$

The difference is not significant at 18 degrees of freedom $((n_1 - 1) + (n_2 - 1))$.

Vickers Scanning Microdensitometer

DETERMINATION OF DNA CONTENT FOR SUCKER ERYTHROCYTES*

#	Sum of 5 Measurements	Total Background	DNA "Units"
1	33662	954	32708
2	34736	5462	28274
3	33970	5254	28716
4	34532	4456	30076
5	36949	5715	31234
6	37650	4763	32887
7	38379	6424	31955
8	37611	6860	30751
9	40345	9534	30811
10	39974	6822	33192

MEAN DNA CONTENT ($\bar{X}$) = 31060.4; n = 10

S.E.M. = $\pm$535.24 or $\pm$1.72%

*All statistical manipulations are the same as for the Leitz Microspectrophotometer.

APPENDIX III

HYBRIDIZATION IN SITU

The method follows largely that of Brahic and Haase (1981). DNA extraction procedures are those of T. T. Chen (personal communication).

Slide Preparation

1) Wash pre-cleaned microscope slides in 95% ethanol and wipe dry with lint-free cheesecloth.

2) To reduce the amount of background staining, the cleaned slides may be immersed for 3 hours at 65°C in 450 mM NaCl/45 mM sodium citrate, pH 7.0 + 0.2% each of Ficoll, polyvinylpyrrolidone and BSA.

3) Treated slides were rinsed for a few seconds in double distilled water and allowed to dry.

4) 22 mm X 50 mm coverslips were dipped in silicon glass coating compound (Sigmacote, Sigma), and wrapped in aluminum foil, baked at 150°C for 2 hours to kill nucleases. The coverslips were kept wrapped in aluminum foil until required.

5) All glassware used in these experiments was cleaned in a heavy-duty detergent (Extran, BDH Chemicals), rinsed in hot tap water, then in distilled water (several changes) and dried overnight at 80°C or for at least 2 hours at 150°C. All glassware was siliconized before baking.

2) The aqueous phase from the centrifuged preparation was removed to a clean glass centrifuge tube, and the chloroform/phenol extraction procedure was repeated at least twice, or until the recovered aqueous phases, which contained the DNA, were clear and colourless.

3) The DNA and RNA in the final extract were precipitated in 2 volumes of absolute ethanol at -20°C overnight. The next day, the preparation was spun in a clinical centrifuge for 20 minutes, the ethanol decanted and the precipitate dried using a gentle stream of compressed air.

4) The nucleic acids were dissolved in DNA buffer (TNE: Tris-HCℓ pH 8.0 50 mM/EDTA 10 mM/NaCℓ 10 mM), and the RNA was removed either by adding 100 µg RNAse A/ml, and allowing the mixture to digest for 2 hours at 37°C, or by making the mixture 100 mM in NaOH and digesting it at 65°C for 15 minutes. The reaction was stopped, in the second case, by adding HCℓ to 100 mM. Whichever method was used, the DNA was extracted with phenol:chloroform and precipitated in 2 volumes of absolute ethanol at -20°C overnight, and the precipitate recovered by centrifugation and drying.

5) The purified DNA was dissolved in Nick-Translation (NT) buffer, an aliquot taken and the amount of DNA estimated in a Beckman UV Spectrophotometer set at 260 nm. Approximately 1 µg of DNA was used in each nick-translation procedure. Double-strength NT buffer (2X NT): Tris-HCℓ, pH 7.9 100 mM/$MgC\ell_2$ 10 mM/b-mercaptoethanol 20 mM/ BSA 200 $\mu g \cdot ml^{-1}$. Heat to 65°C for 20 minutes before use.

Tissue Preparation

1) Gills were excised from freshly-killed fish (either Semotilus atromaculatus or Catostomus commersoni) and touched lightly to cleaned and treated slides. The preparations were air-dried, then fixed in Carnoy's fluid (absolute ethanol: glacial acetic acid, 3:1) for 20 minutes, and allowed to dry.

2) Preparations were heated for 30 minutes at 70°C in 300 mM NaCℓ/30 mM sodium citrate pH 7.4, then rinsed in distilled water for 1 minute and treated with 1 µg/ml Proteinase K in 20 mM Tris-HCℓ pH 7.5/ 2 mM $CaCℓ_2$ for 15 min at 37°C. The slides were rinsed in distilled water and then dehydrated to 95% ethanol and allowed to dry.

3) Nuclear DNA was denatured by treating the preparations in 0.07 N NaOH in 300 mM NaCℓ for 3 minutes at room temperature, rinsing in distilled water and dehydrating through 95% ethanol. The slides were then ready for hybridization. These preparative steps were carried out immediately before hybridization was to be performed.

Nick-Translation Procedure

Extraction of DNA:

Genomic DNA was obtained from the blood of S. atromaculatus, C. commersoni and Salmo gairdneri.

1) Approximately 1 ml of blood was treated with 2 ml 100 mM EDTA in 0.5% SDS (Sodium dodecyl sulphate) pH 8.0 for 20 minutes at room temperature. Equal volumes of chloroform and distilled, buffer-saturated phenol were added to the mixture, shaken gently for 10 minutes and then centrifuged for 20 minutes in a clinical centrifuge at top speed.

Nick Translation:

^{125}Iododeoxycytidine tri-phosphate (^{125}I-dCTP) was obtained from New England Nuclear (Montreal, Canada); cold nucleotide triphosphates (dATP, dGTP, dTTP) (Boehringer Mannheim) were prepared as 330 µM aqueous solutions. DNAse I (Sigma) was prepared as a 1 mg/ml solution in 0.01 N HCℓ, divided into 50 µl aliquots, and frozen until required. An aliquot was diluted to 500 µl with DNAse buffer (10 mM Tris-HCℓ, pH 7.5/5 mM $MgC\ell_2$/1 mg·ml^{-1} BSA), and kept at 0°C for 2 hours to activate the enzyme. From this stock, 2 µl was diluted with DNAse buffer to 200 µl. 1-2 ng activated DNAse I was used for each nick-translation procedure.

DNA polymerase (Kornberg polymerase) was obtained commercially (Boehringer Mannheim), as were calf thymus DNA (Sigma), and transfer RNA (tRNA) (Sigma).

Nick translations were carried out in double strength Nick-Translation Buffer (2X NT).

1) 1-2 µCi (2-3 µl) ^{125}I-dCTP was placed in an 0.5 ml Eppendorf tube and the ethanol vehicle removed either under vacuum desiccation or freeze-drying; the procedure took about 30 minutes.

2) To the ^{125}I-dCTP, 50 µl 2X NT buffer, 8 µl cold nucleotide mixture, 1 µg genomic DNA and 1-2 ng DNAse I solution were added, and the mixture brought to a total volume of 100 µl with distilled water. The mixtures in the Eppendorf tubes were mixed gently, and incubated 13 minutes at 37°C.

3) 2 µl DNA polymerase were added, mixed gently, and the preparations were incubated at 11-13°C for 90 minutes. The polymerization reaction

was stopped with 30 µl 100 mM EDTA.

4) 100 µg tRNA (10 µl of 10 mg tRNA/ml water) were added, and the preparation was made 300 mM in Na acetate before 2 volumes of absolute ethanol were added. The ethanol mixture was allowed to precipitate at -20°C overnight, and then the precipitated nucleic acids were pelletized by centrifuging the Eppendorf tubes at 11,500 X g in an Eppendorf centrifuge. The supernatant was removed and pooled, and the precipitate gently dried with a stream of compressed air.

5) The nick-translated DNA was counted in a Hewlett-Packard Scintillation Counter with 3" KI crystal. A known volume of ^{125}I-dCTP was used as a standard, and the efficiency of the machine calculated. From this the number of dpm could be estimated. The nick-translated DNA was dissolved in sufficient hybridization buffer (50% formamide v/v; 2X SCP (2X SSC + 0.04 M NaH_2PO_4, pH 6.0); 10% dextran sulphate; 100 X excess, sonicated calf thymus DNA as a carrier ($\sim$µl mg·ml^{-1})) to give 5 X 10^5 dpm in 30 µl.

6) Nick-translated DNA in hybridization buffer was denatured by heating at 100°C for 5-7 minutes, and then cooled quickly on ice before being applied to the slides.

7) 5 X 10^5 dmp nick-translated DNA was applied to prepared slides. The slides were covered with the siliconized coverslips, thus spreading the DNA preparation over the surface of the slide. Finished preparations were placed on damp paper towel in an enamel tray, and when all preparations had been completed, the try was covered tightly with plastic food wrap (Saran Wrap, Dow Chemical) and incubated at 26-30°C for 60-65 hours.

8) After hybridization, the coverslips were removed and discarded, and the preparations washed in 3 changes of 50% formamide/300 mM NaCl and 30 mM Na citrate over 24 hours, then rinsed in 2X SSC, and dehydrated through 70% ethanol in 300 mM NH_4 acetate, and two changes of 95% ethanol.

Radioautography

Hybridized slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with 600 mM NH_4 acetate, allowed to dry for 2 hours at 28°C, and then bleached for 4 hours in 3% H_2O_2 vapour at room temperature. The bleached slides were placed in light-proof boxes with a packet of Drierite, and stored at 4°C for 10 days to 3 weeks. Completed radioautographs were developed in Kodak D-19 developer (10 g/250 ml water) for 2 minutes, and fixed for 10 minutes in Kodak acid hard fixer. The developed preparations were washed gently in running tap water for 10 minutes, rinsed in distilled water and allowed to dry.

Preparations were stained either in Harris's haematoxylin and aqueous eosin, or Scarba's red and fast green FCF (Humason, 1972).

APPENDIX IV

METHODS FOR ENZYME-GOLD PROCEDURES

The enzyme-gold techniques are based upon the work of Bendayan (1981) and Paiement and Bendayan (1983).

Tissue Preparation

Gill of Semotilus atromaculatus was excised from freshly-killed fish, and the filaments minced in a drop of fixative on a clean glass slide. The tissue was then fixed in 1% glutaraldehyde in 10 mM phosphate buffer at 4°C overnight, rinsed in cold buffer for 15 minutes, and dehydrated continuously through 100% ethanol in the cold. In continuous dehydration, the fixed tissue was placed in a 1.5 ml Eppendorf tube with a hole in the bottom made by a clean dissecting needle. The hole was small enough to allow slow leakage of the dehydrating solutions (∿1 drop every 3 seconds). At least 4 volumes of 70%, 95% and absolute ethanol were allowed to pass through the specimens, and the entire procedure was carried out on ice. Following dehydration, the specimens were transferred to a glass vial, and treated for 15 minutes in propylene oxide, then infiltrated with and embedded in Epon resin. Tissues were not post-fixed in osmium.

Pale gold to gold sections were cut on a Sorvall MT-1 ultramicrotome and mounted on Formvar-dipped nickel grids. It was not found to be necessary to coat grids with formvar and carbon, as reported by Bendayan (1981).

Preparation of Colloidal Gold

Glassware: All glassware was cleaned in heavy-duty liquid detergent (Extran, BDH Chemicals), rinsed in hot water and distilled water and dried at 80°C overnight, siliconized (Sigmacote, Sigma) and dried further at 80°C.

Chemicals: DNAse I (Type DN.EP, Sigma, or highest purity enzyme from Boehringer Mannheim), RNAse A (Type XII-A Sigma) and S1 Nuclease (Boehringer Mannheim, 5 X 10^4 units/mg protein) were used. One percent sodium citrate was prepared in quantity and divided into 5 ml aliquots which were stored at -20°C until used. Gold chloride was liquified by placing it in an oven at 37°C for a few minutes, and 8.5 µl of the melted chemical was added to 100 ml double distilled water to make a solution of 0.01%.

1) 0.01% aqueous gold chloride (10 mg tetrachloroauric acid in 100 ml double distilled water) was brought to boiling and 5 ml 1% sodium citrate added with rapid stirring. The mixture was boiled gently for 5 minutes to allow all the gold chloride to be converted to colloidal gold.

2) The solution was cooled to room temperature, and 10 ml aliquots taken for enzyme coupling.

3) DNAse-Gold: 0.2 mg DNAse I was dissolved in 0.1 ml double distilled water and placed in a siliconized 25 ml Erlenmeyer flask with a small magnetic stirring rod. An amount of 10 ml cooled colloidal gold was added (pH 6.0) and the mixture gently stirred for about 5 minutes. To check the stability of the enzyme-gold preparation, 0.5 ml of the suspension was combined with a few drops of 10% NaCl.

Preparations in which the colloidal gold was bound to the enzyme retained the pink-red colour of the gold; unstable suspensions, in which the enzyme had not bound to the gold, flocculated and became dark blue.

4) The stabilized suspensions were centrifuged in polycarbonate centrifuge tubes at 15,000 X g for 30 minutes in a Beckman centrifuge with a J-20 rotor. The supernatant was carefully drawn off, and the dark red precipitate was suspended in 3 ml phosphate-buffered saline pH 6.0, to which 0.2 mg/ml polyethylene glycol (MW 20,000) had been added.

5) RNAse-Gold: 0.5 mg RNAse A was dissolved in 0.1 ml double distilled water, and placed in the bottom of a siliconized Erlenmeyer flask with a small magnetic stirring bar. The colloidal gold suspension from the original preparation was adjusted to pH 9.2 with 0.2 M K_2CO_3 before being added to the enzyme. The mixture was stirred gently for 5 minutes, checked for stability with 10% NaCl solution, and the remainder centrifuged as described above. The dark red precipitate was suspended in 3 ml phosphate-buffered saline at pH 7.5.

6) S1 Nuclease-Gold: S1 nuclease is reported to act best at pH 4.5 (technical information sheet, Boehringer Mannheim, supplied with the preparation); this is too low for the colloidal gold, which tends to flocculate spontaneously below pH 5.0. In these experiments, 0.2 mg enzyme in 0.1 ml double distilled water was bound to colloidal gold at pH 6.0, as for DNAse I; the suspension was never completely stable with 10% NaCl, and to assist stability, 2 mg/ml polyethylene glycol was added to the suspension before it was centrifuged. The dark red sediment was suspended in 3 ml acetate-buffered saline

pH 6.0 and used immediately.

Labeling of Nucleic Acids

1) The sections were hydrated by placing them on a drop of PBS or ABS (pH 6.0 for DNAse I and S1 nuclease; pH 7.5 for RNAse A) for 5 minutes.

2) Sections were incubated 30-45 minutes at room temperature (23°C) on the enzyme-gold preparation and then rinsed in 3 changes of PBS or ABS, and 3 changes of double distilled water over a period of 10 minutes. The critical manipulation in this procedure was the method of removing excess fluid from the grid between solutions. Any agitation of the grid in the washing solution removed the label; consequently, the specimen was lifted from the drop of solution with forceps, and held tightly as the hand was bumped lightly against the work surface to flick the excess liquid from the grid.

3) Bendayan (1981) routinely stained grids with uranyl acetate and lead citrate before examining them in the electron microscope; we found that better contrast between the label and the specimen was obtained if the specimens were unstained, and it was possible to distinguish cytoplasmic components in rodlet cells even without staining. Examining unstained preparations also reduced the likelihood that a light label would be lost through overmanipulation of the specimens.

4) When specimens were stained, they were dried for 5 minutes between the last post-incubation rinse and the beginning of the uranyl acetate procedure, and were rehydrated for 3 minutes before being stained with uranyl acetate for 10 minutes and lead citrate (1 minute). In

the staining procedure, care was taken to handle the specimens gently during washing; and the same single "flick" of excess liquid from the grid was used.

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APPENDIX V

Introduction

After the final draft of this thesis had been submitted, criticisms of the statistical methods employed in Chapter 4 arrived from Dr. Peter Hodson, one of the members of my supervisory committee. Dr. Hodson suggested that a more satisfactory statistical test for the microdensitometry data would be a two-way analysis of variance with multiple comparisons. Appendix V records the results of these statistics. I am much indebted to Dr. Peter D. M. Macdonald of the Department of Mathematical Sciences, McMaster University, for his assistance in the analyses.

Program and Computing Package

The two-way analysis of variance was carried out using the central computing facility of McMaster University and the 'GLIM' (Royal Statistical Society, London) software program. The procedures for multiple comparisons within an unbalanced design were modified from Zar (1984), as described in Table II. Data used in the two-way analysis of variance were the same as reported in Chapter 4 of the thesis for Experiments 2 and 3, carried out on the Leitz Microdensitometer and the Vickers M85 Scanning Microdensitometer, respectively.

The Analyses

1) Data were compared for cell type and species in the following way: the statistical model was fitted for cell type with and without allowances

for species differences and for species with and without allowance for differences in cell type. As well, a fit was estimated for the interaction of cell type and species having allowed for both categories of variations individually. The 'noise' in the observations (the remainder of the unfitted data) was recorded as 'residual' deviance; it represents the reliability of the technique employed, and a small residual mean square indicates a reliable technique for the observations documented.

2) The data were manipulated in two ways: as 'pg DNA/nucleus', the values were inserted into the computer and the statistical manipulations performed directly. As 'ln pgDNA/nucleus', a data transformation procedure intended to ensure more constant error variance throughout the experiment (Snedecor, 1961), the same data were also manipulated in the analysis of variance.

3) Multiple comparisons were made among all pairs of cell-type categories adjusted for species differences, using data treated as described above.

Results and Discussion

The results of the two-way analysis of variance and multiple comparison tests for untreated data (pg DNA/nucleus) and data expressed as ln (pgDNA/nucleus) are found in Tables I and II, respectively.

The results show highly significant differences for 'cell type' with and without accounting for species, and for 'species' with and without accounting for cell type, in three of the four determinations in Table I. Only in the Leitz experiment using the natural logs of the data, did the 'cell type' category show $f = 0.01 < P < 0.05$. In all experiments the mean squares of the 'species' variant were much higher than the values found for

the 'cell type' variation. In all experiments, the interaction term shows a maximum probability of $0.01 < P < 0.05$; in half the calculations, the term is not significant. The residual mean square is very low in all experiments; in other words, the measuring techniques used were very reliable. Since the expression 'residual value/residual DF' is used as the denominator of the expression for computing 'f', the larger the residual DF, the larger 'f' will be, and the more likely statistical significance will be found in a comparison. It should be noted that the mean squares of the interactions for the treatment are very low; this suggests that the 'cell type' and 'species' categories are essentially independent variables. Confirmation of this is also seen as the mean square values for 'cell type corrected for species' and 'cell type uncorrected for species' are very similar, and the same is seen for the species categories.

Even though the 'cell type' category is found to show significant differences for one cell type from another, several points must be made:

1) This information does not contradict the findings for the Student 't' test on the same data. Table 4-IV & V shows that when erythrocytes, rodlet cells and larger cells are examined for a species using the 't' test within the same set of data used for the present calculations, there are significant cell type differences seen within all species groups, usually between the rodlet cell/larger cell group and the erythrocytes. In fact, the only insignificant comparison for all species is for the larger cells vs. the rodlet cells. Therefore, to find significant differences in cell type within a species group in the analysis of variance procedure is to be expected. The analysis of variance alone does not tell the observer precisely where the differences lie, only that they exist. Furthermore,

TABLE _

TWO-WAY ANALYSIS OF VARIANCE

	DEVIANCE	DF	MEAN SQUARES	F VALUES	LEVEL OF SIGNIFICANCE
Leitz Expt. Data as pg DNA/nucleus					
Fitting Cell Type before Species					
CELL TYPE	10.68	2	5.34	6.868	0.001
SPECIES	29.27	2	14.635	18.825	0.001
INTERACTION	8.485	4	2.1213	2.728	0.01 P 0.05
RESIDUAL	100.3	129	0.7775		
TOTAL DEVIANCE	148.735	137			
Fitting Species before Cell Type					
SPECIES	29.48	2	14.74	18.96	0.001
CELL TYPE	10.47	2	5.235	6.733	0.001
INTERACTION	8.485	4	2.1213	2.728	0.01 P 0.05
RESIDUAL	100.3	129	0.7775		
TOTAL DEVIANCE	148.735	137			
GRAND MEAN 3.586 +/- 0.0887 pg DNA/nucleus					
Leitz Expt. Data as ln(pg DNA/nucleus)					
Fitting Cell Type before Species					
CELL TYPE	0.6341	2	0.317	4.666	0.01 P 0.05
SPECIES	3.355	2	1.6775	24.68	0.001
INTERACTION	0.5915	4	0.1479	2.178	N.S.
RESIDUAL	8.766	129	0.06795		
TOTAL DEVIANCE	13.3466	137			
Fitting Species before Cell Type					
SPECIES	3.341	2	1.6705	24.583	0.001
CELL TYPE	0.6473	2	0.3237	4.763	0.01
INTERACTION	0.5915	4	0.1479	2.178	N.S.
RESIDUAL	8.766	129	0.06795		
TOTAL DEVIANCE	13.3458	137			
GRAND MEAN 1.232 +/-0.0266 ln(pg DNA/nucleus)					

Table I cont..

	DEVIANCE	DF	MEAN SQUARES	F VALUES	LEVEL OF SIGNIFICANCE
Vickers M85 Expt. Data as (pg DNA/nucleus)					
Fitting Cell Type before Species					
CELL TYPE	42.31	2	21.305	25.a2+	<0.001
SPECIES	189.9	2	94.95	115.31	<0.001
INTERACTION	[illegible].058	+	2.2645	2.75	0.01 P<0.05
RESIDUAL	127.9	15e	0.820		
TOTAL DEVIANCE	3ee.eee	164			
Fitting Species before Cell Type					
SPECIES	187.1	2	93.55	+14.103	0.001
CELL TYPE	44.78	2	22.39	27.309	0.001
INTERACTION	9.058	4	2.2e45	2.7e1	0.01 P<0.05
RESIDUAL	127.9	156	0.819e		
TOTAL DEVIANCE	3ee.838	164			
GRAND MEAN +.097 +/-0.11e6					
Vickers M85 Expt. Data as (ln(pg DNA/nucleus))					
Fitting Cell Type before Species					
CELL TYPE	2.137	2	1.159	29.20e	0.001
SPECIES	10.72	2	5.36.	135.12e	0.001
INTERACTION	0.24e3	4	0.0e1e	1.552	N.S.
RESIDUAL	e.138	15e	0.0397		
TOTAL DEVIANCE	19.4713	164			
Fitting Species before Cell Type					
SPECIES	10.51	2	5.255	132.479	0.001
CELL TYPE	2.527	2	1.264	31.853	0.001
INTERACTION	0.24e3	4	0.0e1e	1.552	N.S.
RESIDUAL	e.166	15e	0.0397		
TOTAL DEVIANCE	19.4713	164			
GRAND MEAN 1.349 +/-0.02e6 ln (pg DNA/nucleus)					

TABLE 11

MULTIPLE COMPARISONS WITH AN UNBALANCED DESIGN

Group 1 = erythrocytes
Group 2 = nuclei cells
Group 3 = larger cells

COMPARISON	DF	ESTIMATED DIFFERENCE IN EFFECT[1]	S.E.[1]	T RATIO	SIGNIFICANCE[2]
Leitz Experiment - data as pg DNA/nucleus					
GROUPS 1 VS 2	160	1.000	0.1630	6.14	P < 0.05
GROUPS 1 VS 3	160	1.136	0.1687	6.73	P < 0.05
GROUPS 2 VS 3	160	0.1357	0.1630	0.743	N.S.
Leitz Experiment - data as ln(pg DNA/nucleus)					
GROUPS 1 VS 2	160	0.1507	0.0397	3.821	P < 0.05
GROUPS 1 VS 3	160	0.2622	0.0366	7.162	P < 0.05
GROUPS 2 VS 3	160	0.0115	0.0396	0.289	N.S.
Vickers M85 Experiment - data as pg DNA/nucleus					
GROUPS 1 VS 2	133	0.6574	0.1782	3.222	P < 0.05
GROUPS 1 VS 3	133	1.167	0.1981	5.891	P < 0.05
GROUPS 2 VS 3	133	0.0106	0.1933	0.096	N.S.
Vickers M85 Experiment - data as ln(pg DNA/nucleus)					
GROUPS 1 VS 2	133	0.1256	0.0556	2.258	N.S.
GROUPS 1 VS 3	133	0.1556	0.0546	2.850	P < 0.05
GROUPS 2 VS 3	133	0.0300	0.0567	0.529	N.S.

1 Computed by GLIM

2 Critical Value = $q_{0.05,\ DF,\ 3}/\sqrt{2}$ = 2.- for DF= 133 or DF= 160

in the data from Experiment 2 (Leitz), where the lowest significant mean squares are seen in analysis of variance, the 't' test information shows no significant differences among all cell types in one of the three species.

2) The values seen for the 'f' term reflect the large number of measurements in the total experiment. Fewer measurements would have resulted in a lower number of DF, and therefore a lower 'f' value. At some point, the statistical result may reflect differences in the compared values that really do not exist in the population, i.e. the stray 5 cells out of 100 that through measurement error or mitotic condition show a significant difference in DNA content from the overall population, although they really are part of it. In support of this argument, it should be noted that within all the data measurements, mature erythrocytes showed the smallest S.E.M. Their nuclei are approximately all the same size, and they are all post-mitotic cells. The 'larger cell' category on the other hand consists of cells with a variety of sizes of nuclei which could be in S without being so recognized; cells in anaphase or metaphase were not measured for the experiment.

3) Multiple comparisons within an unbalanced design (Table II), using the data from the same experiments, show convincingly that for all species there is no significant difference between cell types 2 and 3, i.e. between rodlet cells and 'larger cells', although there may be significant differences between each of these and cell type 1, the erythrocytes. The multiple comparisons show that for the Vickers M85 experiment, when logarithmic values are used, there is no significant difference between rodlet cells and erythrocytes, although a difference exists for 'larger cells' and erythrocytes. Furthermore, the |T ratio| shows consistently a smaller value for Groups 1 vs.

2 than for Groups 1 vs. 3, even though both Groups 1 and 3 are known to be teleost cells. These comparisons are made with 160 and 133 degrees of freedom, respectively; hence they may be considered quite reliable.

The two-way analysis of variance does give a more reliable estimate of the overall significances, or lack of same, in the data from the Leitz and Vickers experiments than does the Student 't' test because it considers all the readings together, rather than subdividing them into a multiplicity of categories. However, the analysis of variance, combined with the multiple comparison test, agrees precisely with the Student 't' test results of Chapter 4, in that rodlet cells and larger cells show no statistically significant differences for any of the species studied.

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