Roux's Arch Dev Biol (1992) 201:296–300

Roux's Archives of
Developmental
Biology

A quantitative method for separation of living *Hydra* cells

Martin J. Greber, Charles N. David, and Thomas W. Holstein

Zoologisches Institut der Universität München, Luisenstraße 14, W-8000 München 2, Federal Republic of Germany

Received March 17 / Accepted March 19, 1992

Summary. We describe a rapid method for the isolation of large numbers of living *Hydra* cells of defined cell type in an isotonic cell medium (Gierer et al. 1972). Intact animals are enzymatically dissociated into a single cell suspension and the various cell types separated in less than one hour by counterflow centrifugation elutriation. Cell loss is minimal. RNA isolated from various fractions can be probed with cell type specific cDNA-clones.

Key words: Cell separation – Counterflow centrifugation elutriation – Cell type specific gene expression – *Hydra*

Introduction

Hydra contains a variety of cell types (Bode et al. 1973; David 1973) which show position- and cell-type-specific patterns of gene activity (see David et al. 1987 and MacWilliams 1991 for review). In order to study the factors controlling this gene activity it is advantageous to have access to purified cell types. Cell types in *Hydra* are characterized by considerable size differences (David 1973). Counterflow centrifugation elutriation (Lindahl 1948) allows a very high resolution separation of living cells according to sizes (for review see Conkie 1986). Even small size differences during the cell cycle have been successfully used to enrich cells of different cell cycle phases and corresponding genes (Hayeles et al. 1986).

The present paper describes the elutriation method for *Hydra*, criteria for distinguishing cell types and evidence that the method quantitatively recovers cells from tissue. We also demonstrate the usefulness of the method by slot blot analysis with cell type specific cDNA clones. Application of the method to the study of cell type specific gene expression in *Hydra* will follow.

Offprint requests to: T.W. Holstein

Materials and methods

Animals and culture: *Hydra magnipapillata* (wildtype strain 105) was used in all experiments. Mass cultures were kept in M solution (Loomis and Lenhoff 1956) at $18 \pm 0.5°$ C and fed daily with *Artemia* brine shrimps. Experimental animals were starved for 24 h.

Dissociation of Hydra tissue: *Hydra* were enzymatically dissociated to single cells at 18° C by a 3 h treatment with Pronase E (from *Streptomyces griseus*; Serva, Heidelberg, FRG) at a concentration of 50 DMC-U ml^{-1} (Stei 1988; Schartl et al. 1989) in an isotonic cell culture medium (6 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4 \cdot 7H_2O$, 3.6 m*M* KCl, 12.5 m*M* N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), 6 m*M* sodium pyruvate, 6 m*M* sodium citrate, 6.0 m*M* glucose and 50 mg/ml rifampicin; p*H* 6.9 (Gierer et al. 1972). For 1000 polyps we used a total volume of 50 ml in 250 ml culture flasks (Greiner, Nürtingen, FRG).

Separation of Hydra cells. Pronase E dissociated *Hydra* cells (see above) were collected in a clinical centrifuge (4° C) at 100 g for 5 min; the supernatant was recentrifuged at 300 g (5 min), both pellets were resuspended and pooled in a total volume of 5 ml of isotonic cell culture medium. The cells were separated into size fractions using a Beckman J6 series centrifuge (18° C) and a new elutriator (JE 5.0) with a 5 ml chamber. Four 100 ml fractions were collected using rotor speeds of 4000, 2800, 1700 and 1100 rpm at 15 ml/min flow rate and two additional fractions using flow rates of 30 and 60 ml/min at 1100 rpm rotor speed. The cells of 1000 polyps (10^8 cells) were separated in one run. A Masterflex Digistaltic pump was used.

Microscopy. Separated *Hydra* cells were identified with a Leitz Dialux 20 microscope equipped with a Nomarski interference contrast (ICT 16/0.75 and ICT 40/0.75 lenses). Photography was performed with Kodak Ektachrome Tungsten 50 ASA film.

Isolation of RNA and dot blot analysis. Total RNA from various fractions of elutriated cells was isolated by the method of Chomczynski and Sacchi (1987) and used for dot blot analysis. Equal amounts (2 µg) of total RNA from each fraction were spotted on a nylon membrane (Biodyne B from Pall GmbH, Dreieich, FRG) using a Bio-dot apparatus (Bio-Rad Laboratories GmbH, Munich, FRG). Filters were hybridized with [^{32}P]dATP labeled (random primer labeling kit from BRL GmbH, Eggenstein, FRG) cell type specific cDNA clones. RNA was hybridized and washed at high stringency conditions according to Sambrook et al. (1989).

Results and discussion

Tissue dissociation and the identification of cell types

The classical approach for obtaining single cell suspensions from *Hydra* is the mechanical dissociation technique of Gierer et al. (1972). This method, however, cannot be used for obtaining single cells for elutriation because epithelial cells are very adhesive and rapidly form clumps in cell suspensions (Gierer et al. 1972; Technau and Holstein 1992).

To overcome this drawback we have used an enzymatic dissociation procedure that yields cells which have completely lost their adhesiveness and do not form clumps. Animals were incubated in a dissociation medium containing Pronase E (see Materials and methods). Under these conditions the pedal discs of the polyps rapidly lose their stickiness. After about one hour ectoderm pieces detach from the mesoglea and the animals begin to fall apart. Lysis of the mesoglea and detachment of the endodermal cells takes a further two hours.

A suspension of enzymatically dissociated cells is remarkably clean: There is no indication of cell debris and the cells appear healthy: endodermal cells (digestive and gland cells) have two intensively beating flagella; epithelial cells continuously form pseudopodia; nerve cells have strikingly long processes; sensory cells and nematocytes still have their delicate ciliary processes. A small number of commensal protozoans, e.g. *Naegleria gruberi*, can also be seen actively swimming in the cell suspensions.

During dissociation some cells round up and lose their characteristic morphology. Nevertheless all basic cell types can be identified (Fig. 1). *Endodermal epithelial (digestive) cells* are the largest cells (25–35 µm). They have highly refractile vacuoles of different size, which are conspicuously colored in phase and interference contrast optics. *Ectodermal epithelial cells* (20–30 µm) are also very large, but they have neither refractive nor coloured vacuoles, and one of the vacuoles may be very prominent (Figs. 1, 3). *Gland cells* are of medium size (15–25 µm) and have a large number of vacuoles of uniform diameter (Fig. 1). Using phase- or interference contrast optics, two gland cell types could be distinguished, one with very refractile and one with rather pale vacuoles, presumably representing the zymogen and mucous secretion types (Rose and Burnett 1968; David 1973).

Dissociated *interstitial cells* look similar to those in macerated cell preparations. They are relatively small (10–20 µm), have a large nucleus with a prominent nucleolus, and a uniform cytoplasm (Figs. 1, 3). *Differen-*

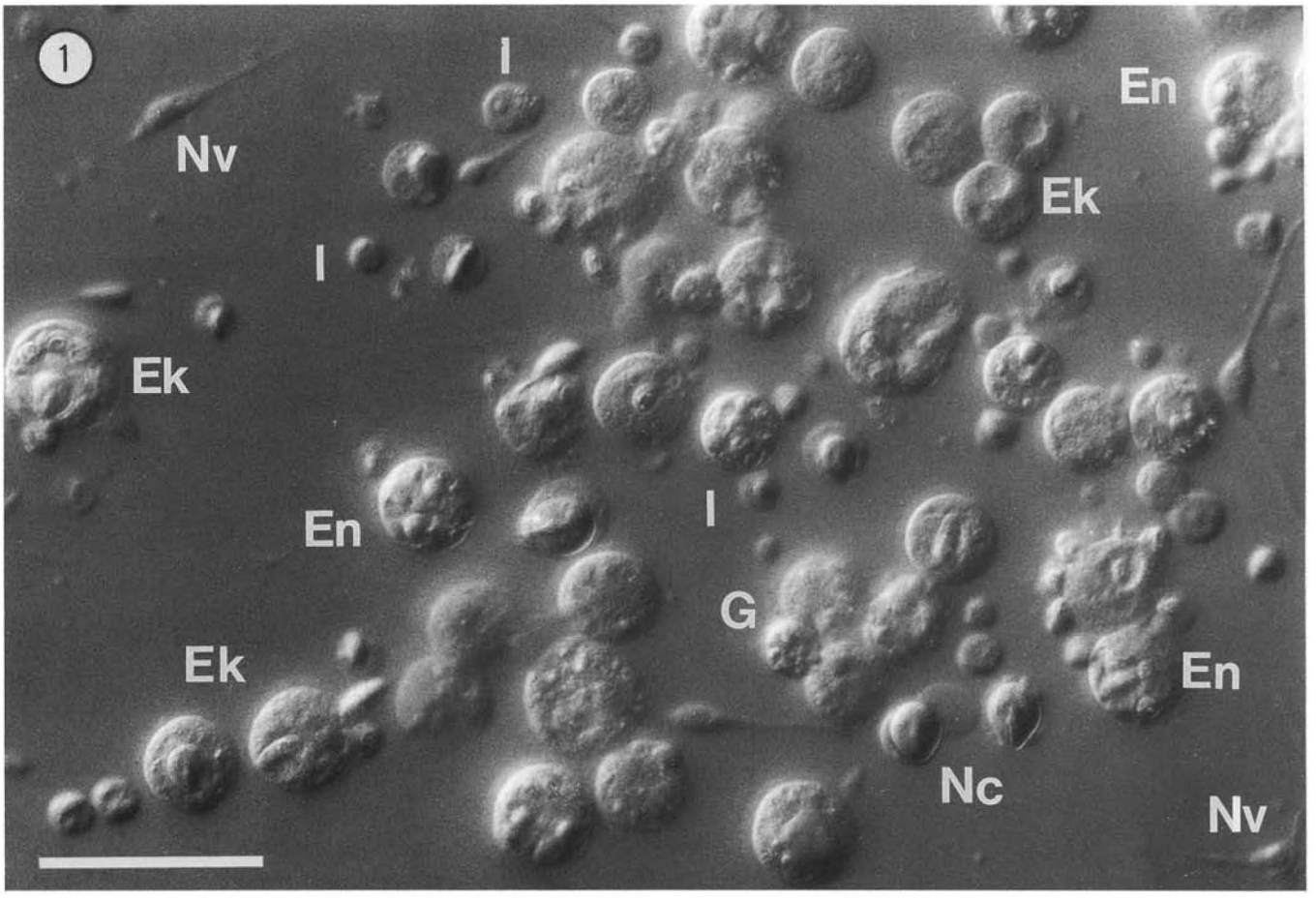


Fig. 1. Unfractioned cell suspension of enzymatically dissociated tissue (see Material and methods). *Ek* = ectodermal epithelial cells, *En* = endodermal epithelial cells, *G* = gland cell, *I* = interstitial (stem) cell, *Nc* = nematocyte, *Nv* = nerve cell; bar indicates 50 µm

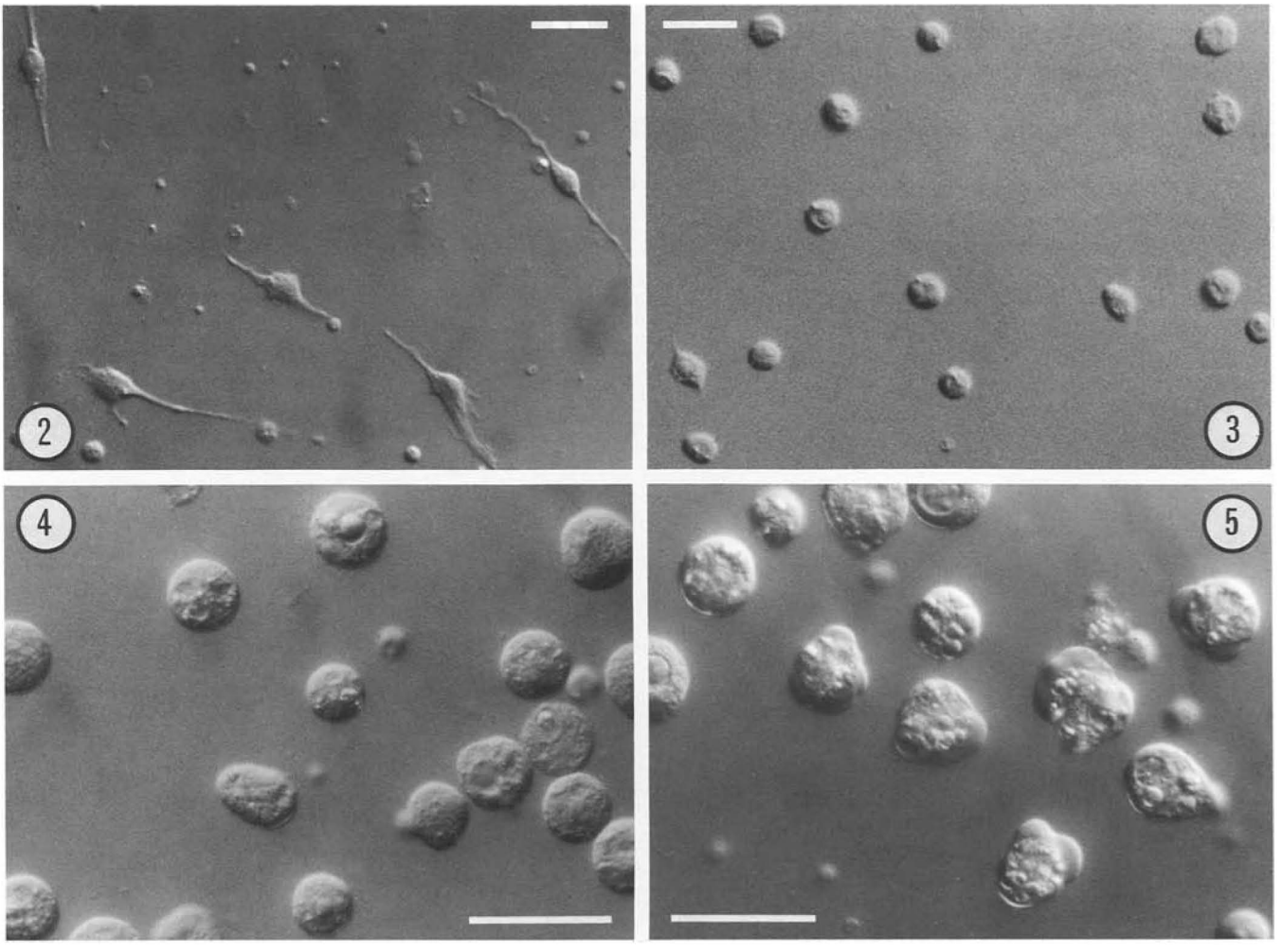
tiating nematocytes contain a prominent vacuole representing the anlage of the nematocyst capsule. In late differentiation stages is also possible to determine the nematocyst type (Holstein 1981). Nests of interstitial cells and differentiating nematocytes are dispersed into single cells as soon as suspensions are manipulated by pipeting or centrifugation. *Mature nematocytes* of the various types are easily distinguishable by their characteristic capsule morphology (Fig. 1). *Nerve cells* represent the smallest cell type in *Hydra* (>10 µm; Figs. 1, 2). They retain their fragile processes during all steps of manipulation.

Separation of Hydra cells by counterflow centrifugation elutriation

Dissociated *Hydra* cells (about 1.5×10^8 cells from 1000 animals) were separated by centrifugal elutriation with a 5 ml separation chamber of a Beckman JE-5.0 Rotor (see Materials and methods). During elutriation cells are subjected to two opposing forces within the separation chamber: the flow of the cell medium tending to carry them upward and the centrifugal force causing them to sink. Each cell comes to lie at a position where its sedimentation rate is balanced by the counterflow. By decreasing the rotor speed or increasing the flow rate cells can be washed successively from the chamber according to their size; the small first and the larger later.

Initial values for the separation parameters were estimated on the basis of previous size measurements of *Hydra* cell types (David 1973; Holstein and David 1990a), and by using a Beckmann rotor speed and flow rate nomogram. The parameters were then optimized empirically. We obtained a good separation for all cell types by collecting six size fractions in one run with rotor speeds ranging from 4000–1100 rpm and flow rates from 15–60 ml/min (see Materials and methods). Such a separation was completed in less than one hour.

We analyzed the quality of the elutriation by determing the distribution and quantity of cell types in the various size fractions using interference contrast microscopy. The size of the cells was rather uniform in each fraction, but it increased dramatically from fraction 1



Figs. 2–5. Elutriated fractions. Figure 2 Nerve cell fraction (no. 1), bar indicates 25 µm; Figure 3 interstitial cell fraction (no. 3), bar indicates 25 µm; Figure 4 ectodermal cell fraction (no. 5), bar indicates 50 µm; Figure 5 endodermal cell fraction (no. 6), bar indicates 50 µm

Table 1. Distribution of cell types in different elutriation fractions

Fraction	Total cell number (X 10^{-6})		Nerve	Inter-stitial	Differentiating Nematocyte	Mature Nematocyte	Gland	Ectodermal Epi	Endodermal Epi
I		%	86	37	3	2	–	–	–
	22.2	#	12.9	8.2	0.5	0.5	–	–	–
II		%	6	13	–	–	–	–	–
	3.8	#	0.9	2.8	–	–	–	–	–
III		%	8	43	73	25	–	–	–
	30.2	#	1.2	9.6	13.0	6.0	–	–	–
IV		%	–	6	16	45	57	31	–
	28.8	#	–	1.4	2.9	11.0	4.3	8.6	–
V		%	–	2	4	13	21	31	15
	17.5	#	–	0.4	0.7	3.2	1.6	8.4	3.2
VI		%	–	–	4	15	22	38	85
	34.5	#	–	–	0.7	3.5	1.7	10.5	18.1
Total	137	#	15.0	22.4	17.8	24.2	7.6	27.5	21.3
		%	11	16	13	18	6	20	16

Cells of 1000 *Hydra* were dissociated and separated by elutrial centrifugation into six size fractions (I–VI) (see Materials and methods). For each fraction 500–1000 living cells where counted and classified using Nomarksi interference contrast microscopy

to 6 (Figs. 2–5). This clearly demonstrates that size separation by centrifugal elutriation was efficient. Concentrated suspensions of the elutriated cells were also free of any cellular debris indicating that the cells are not damaged during the procedure.

Table 1 shows the quantitative distribution of cell types in elutriated fractions: Fraction 1 contains 86% of all nerve cells. There is also a significant number of small interstitial cells, which could be either nerve cell precursors or early S-phase interstitial cells (Holstein and David 1990b; Bode et al. 1990). Fraction 1 also contains a small number of differentiation and/or mature nematocytes (5%). In fraction 2 there are a few more interstitial cells, but the total number of cells is low. Fraction 3 contains 43% of the interstitial cells and 73% of the differentiating nematocytes. Gland cells are not detectable in the first three fractions, but 57% of them were found in fraction 4 and the remainder in fractions 5 and 6. Ectodermal epithelial cells are distributed in the three last fractions (about 30% in each); the majority of endodermal epithelial cells are in the last fraction (85%).

Table 1 shows that following elutriation of cells from 1000 dissociated *Hydra* 1.4×10^8 cells were recovered. This is in good agreement with the expected number of cells (1.6×10^8 cells, Sugiyama and Fujisawa 1977) and shows that the recovery of cells is nearly quantitative.

Figure 6 shows a comparison of our results using elutriation (Table 1) with previous maceration data on the frequency of cell types in *Hydra magnipapillata* strain 105 (Sugiyama and Fujisawa 1977). Both methods give similar results for most cell types: 17% ectodermal epithelial cells, 17–20% endodermal epithelial cells, 6% gland cells, and 30% nematocytes. However, we found more nerve cells (10%) and fewer interstitial cells (16%). This might suggest that our method preserves nerve cell morphology better, i.e. that in macerates some nerve cells are misidentified as small interstitial cells.

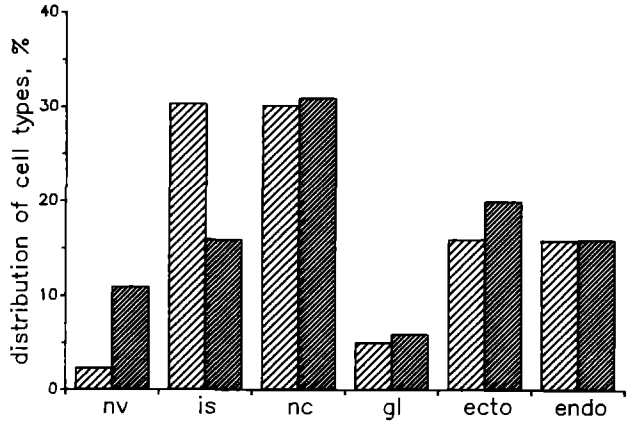


Fig. 6. Comparison of the distribution of cell types observed in this study (data from Table 1) (dark shaded) with previous data from *Hydra magnipapillata* (Sugiyama and Fujisawa 1977) (light shaded)

Applications of the centrifugation elutriation

Our method for the quantitative separation of *Hydra* cells may be useful: (1) for providing cell-type enriched fractions of living cells for biochemical determination of cellular components and for cell type specific DNA/RNA analysis, (2) for the isolation of cell-type specific morphogenetically active factors, (3) for the manipulation of specific cell populations for cell type specific gene expression and for cell lineage studies. The method can be combined with density centrifugation ("two dimensional centrifugation"), allowing a more refined characterisation of various cell types.

A disadvantage of the technique, which limits its application in reaggregation experiments, is the loss of adhesiveness of epithelial cells. Not all cell surface properties, however, are lost: several cell surface antigens can

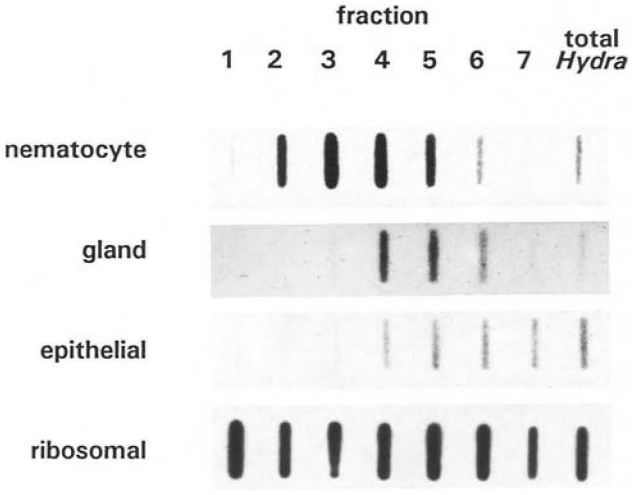


Fig. 7. Slot blot analysis of the elutriated fractions using specific cDNA clones (see text and Materials and methods): (*a*) (differentiating) nematocyte specific minicollagen N-Col 1, (*b*) gland cell specific cDNA, (*c*) regeneration specific (*epithelial*) cDNA, (*d*) ribosomal DNA

still be detected by monoclonal antibodies specific for epithelial cells (data not shown).

We tested the elutriation method for the analysis of cell type specific gene expression. Total RNA isolated from 7 size fractions and from whole *Hydra* was spotted on a nylon membrane and hybridized with cell type specific [^{32}P] labeled cDNA clones (see Materials and methods). We used a minicollagen cDNA specifically expressed in differentiating *Hydra* nematocytes (Kurz et al. 1991), a gland cell specific cDNA clone, whose expression pattern has been verified by in situ hybridization (Holstein and Kurz, unpublished work), and a cDNA clone specifically expressed in regenerating head tissue *Hydra* (Holstein, unpublished work) whose cell type specific expression, however, was unknown.

Figure 7 shows that the expression pattern for the nematocyte and gland specific clones correlates well with the known distribution of cell types in the various fractions (Table 1). Based on its expression pattern in Fig. 7 the regeneration specific cDNA clone is expressed in ectodermal and endodermal epithelial cells. This experiment demonstrates the usefulness of the method for the analysis of cell type specific patterns of gene expression.

Acknowledgements. We are indebted to T. Sugiyama (National Institute of Genetics, Mishima) for supplying *Hydra magnipapillata* strain 105 and to E. Fanning and H.-P. Nashener (both at the Department of Biochemistry, Munich, FRG), for access to their Beckmann J6B elutriator. We cordially thank H.K. MacWilliams for encouraging support and critically reading the manuscript.

References

Bode HR, Gee LW, Chow M (1990) Neuron differentiation in hydra involves dividing intermediates. Dev Biol 139:231–243

Bode H, Berking S, David CN, Gierer A, Schaller H, Trenkner E (1973) Quantitative analysis of cell types during growth and morphogenesis in *Hydra*. Roux' Arch Dev Biol 171:269–285

Chomczynski P, Sacchi N (1987) Single-Step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Conkie D (1986) Separation of viable cells by centrifugal elutriation. In: Freshney RI (ed) Animal cell culture. Oxford IRL Press, Oxford, pp 113–134

David CN (1973) A quantitative method for maceration of *Hydra* tissue. Roux' Arch Dev Biol 171:259–268

David CN, Bosch TCG, Hobmayer B, Holstein ThW, Schmidt T (1987) Interstitial stem cell in hydra. In: Loomis WF (ed) Genetic regulation of development. AR Liss, New York, pp 389–408

Gierer A, Berking S, Bode H, David CN, Flick K, Hansmann G, Schaller H, Trenkner E (1972) Regeneration of *Hydra* from reaggregated cells. Nature 239:98–105

Hayles J, Aves S, Nurse P (1986) *Suc1* is an essential gene involved in both the cell cycle and growth in fision yeast. EMBO J 5:3373–3379

Holstein ThW (1981) The morphogenesis of nematocytes in *Hydra* and Forskalia: an ultrastructural study. J Ultrastruct Res 75:276–290

Holstein ThW, David CN (1990a) Cell cycle length, cell size, and proliferation rate in hydra stem cells. Dev Biol 142:392–400

Holstein ThW, David CN (1990b) Putative intermediates in the nerve cell differentiation pathway in hydra have properties of multipotent stem cells. Dev Biol 142:401–405

Kurz E, Holstein ThW, Petri BM, Engel J, David CN (1991) Minicollagens in *Hydra* nematocytes. J Cell Biol 115:1159–1169

MacWilliams HK (1991) Models of pattern formation. *Hydra* and *Dictyostelium*. Semin Dev Biol 2:119–128

Lindahl PE (1948) Principle of a counter-streaming centrifuge for separation of particles of different sizes. Nature 161:648–649

Sambrook J, Fritsch EF, Maniatis Th (1989) Molecular cloning. A laboratory manual. (2nd edition) Cold Spring Harbor Laboratory Press, New York

Schaller H, Gierer A (1973) Distribution of the head-activating substance in hydra and its localisation in membranous particles in nerve cells. J Embryol Exp Morphol 29:39–52

Schartl M, Holstein ThW, Robertson SM, Barnekow A (1989) Preferential expression of a pp60$^{c\text{-}src}$ related tyrosine kinase activity in nerve cells of the early metazoan *Hydra* (Coelenterates). Oncogene 4:1185–1191

Stei A (1988) Entwicklung von Methoden zur Verwendung von Hydrazellen im Durchflußzytometer. Diploma-Thesis, Zoologisches Institut der Ludwig-Maximilians-Universität München: S 1–75

Sugiyama T, Fujisawa T (1977) Genetic analysis of developmental mechanisms in *Hydra*. I. Sexual reproduction of *Hydra magnipapillata* and isolation of mutants. Dev Growth Differ 19:187–200

Technau U, Holstein ThW (1992). Cell sorting during the regeneration of *Hydra* from reaggreated cells. Dev Biol 151 (in press).