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CELL CYCLE KINETICS AND DEVELOPMENT OF *HYDRA ATTENUATA*

III. NERVE AND NEMATOCYTE DIFFERENTIATION

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SUMMARY

The differentiation of nerve cells and nematocytes in *Hydra attenuata* has been investigated by labelling interstitial cell precursors with [^{3}H]thymidine and following by autoradiography the appearance of labelled, newly differentiated cells. Nematocyte differentiation occurs only in the gastric region where labelled nematoblasts appear 12 h and labelled nematocytes 72–96 h after addition of [^{3}H]thymidine. Labelled nerves appear in hypostome, gastric region, and basal disk about 18 h after addition of [^{3}H]thymidine. The lag in the appearance of labelled cells includes cell division of the precursor as well as differentiation since nerves and nematocytes have $2n$ postmitotic nuclear DNA content.

A cell flow model is proposed for interstitial cells and their differentiated products. Stem cells occur as single interstitial cells or in pairs. Per cell generation about 60% of the daughter cells of stem cell divisions remain stem cells and about 40% differentiate nerves and nematocytes. Nerves differentiate directly from stem cells in about 1 day. Nematocyte differentiation requires 5–7 days including proliferation of a cluster of 4, 8, 16 or 32 interstitial cells and differentiation of a nematocyst capsule in each cell. The numbers of interstitial cells and nematoblasts predicted by the cell flow model from the rates of nerve differentiation (900 nerves/day/hydra), nematocyte differentiation (1760 nematocyte nests/day/hydra) and stem cell proliferation (stem cell cycle = 24 h), agree with the numbers of these cells observed in hydra. The number of stem cells per hydra is 3000–6000 depending on assumptions about the time of determination. The ratio of nematocyte to nerve differentiation averaged over the whole hydra is 3:1. In the hypostome and basal disk interstitial cell differentiation occurs exclusively to nerve cells while in the gastric region the ratio of nematocyte to nerve differentiation is about 7:1.

INTRODUCTION

Because of its limited number of cell types and simple morphology, hydra is a useful system for investigating the control of cell proliferation and differentiation in a multicellular animal. The body column of a mature animal contains about 100000 cells representing five major types: epithelial, interstitial, gland/mucous, nerve and nematocyte. The tentacles contain an additional 29000 cells, of which 26000 are nematocytes (Bode *et al.* 1973). Epithelial, interstitial and gland/mucous cells are proliferating types while nerves and nematocytes arise by differentiation from interstitial cells (Lentz, 1966; Burnett, 1968). Although there are reports of other cell types differentiating from interstitial cells we have not found this to occur

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in our asexually budding laboratory culture (David & Campbell, 1972; C. N. David, unpublished observations). This paper is a quantitative study of the differentiation of nematocytes and nerves from interstitial cells. Our data, in conjunction with results on interstitial cell proliferation presented in a preceding paper (Campbell & David, 1974), make possible a quantitative description of the proliferation and differentiation of interstitial cells in hydra.

Hydra's body has the form of a cylinder made up of 2 epithelial cell layers – endoderm and ectoderm – surrounding a central gastric cavity. The tissue is specialized at one end to form a hypostome, surrounded by tentacles and, at the proximal end, to form a holdfast or basal disk. Embedded between epithelial cells in the ectoderm are large numbers of undifferentiated interstitial cells, differentiating nematoblasts, nematocytes and nerve cells. Growth, which occurs uniformly throughout the unspecialized body column, is balanced by cell loss through the export of tissue to developing buds, through the export of nematocytes in tentacles, and through cell death at the ends of tentacles and in the basal disk (Campbell, 1967*a*, *b*). Thus, in the body column, proliferation and differentiation occur at nearly constant rates in an unchanging tissue.

Interstitial cells in hydra occur singly or in clusters of 2, 4, 8 and 16 cells (David, 1973; David & Challoner, 1974). All classes of interstitial cells except 16's are proliferating actively (Campbell & David, 1974). Cells within a cluster divide in synchrony (Lehn, 1951; Campbell & David, 1974) and remain attached to one another following cytokinesis. Clusters of 2 thus divide to become clusters of 4, clusters of 4 divide to become clusters of 8, etc. (Slautterback & Fawcett, 1959). Clusters of 4, 8 and 16 interstitial cells differentiate nematocytes with all cells in a cluster synchronously forming the same type of nematocyst capsule (Lehn, 1951; Rich & Tardent, 1969; David & Challoner, 1974). Single interstitial cells or clusters of 2 form nerves (David & Challoner, 1974). 'Stem' interstitial cells, which do not differentiate but simply proliferate, must exist to provide for growth of the interstitial cell population and to provide a continuing supply of differentiating nematocytes and nerve cells. It is not known which nests contain stem cells since they are apparently not cytologically different from other interstitial cells. However, clusters of 2 are much more common than other nests and it has been suggested that these might contain most of the stem cells (David & Challoner, 1974).

Nematocyte and nerve cell differentiation have been assayed in the present experiments by following the appearance of newly differentiated labelled cells after labelling the proliferating interstitial cell precursors with [^{3}H]thymidine. These studies show that nerve cell differentiation occurs throughout hydra tissue from hypostome to basal disk. This is in contrast to nematocyte differentiation which occurs only in the gastric region (David & Challoner, 1974). Calculations of interstitial cell populations required to support the observed differentiation rates agree quantitatively with the populations observed in hydra tissue and, in particular, strongly support the suggestion that stem cells occur as single interstitial cells or clusters of 2.

MATERIALS AND METHODS

Hydra attenuata were used for all experiments. Hydra were cultured at 20 °C in tap water to which 10^{-3} M $CaCl_2$ and 10^{-5} M EDTA had been added. The animals were fed daily with *Artemia* nauplii and washed after 4 h. Under these conditions the cultures have a doubling time of 3·5 days (David & Campbell, 1972). Large hydra bearing several buds were selected for all experiments.

Classification of cells

Nerve cells, nematoblasts and nematocytes were identified and counted in maceration preparations under phase-contrast optics as previously described (David, 1973; Bode *et al.* 1973). Three categories of nematocyte were distinguished according to nematocyst capsule type: desmoneme, stenotele and isorhiza. Nematocytes mounted in the battery cells of the tentacles, those mounted in ectodermal epithelial cells of the body column and those in the final stage of differentiation in the body column were counted as mature nematocytes. These cells contain differentiated nematocyst capsules which are highly refractile and appear yellow under phase-contrast optics (fig. 4*g*, *h* in David, 1973). Earlier stages of nematocyte differentiation, in which the developing capsule appears only as a pearl-shaped vacuole in the cytoplasm and in which the capsule type is not yet recognizable, were classified as nematoblasts in the present experiments.

For each preparation 3–5 hydra were macerated together. Tentacles and developing buds were removed prior to maceration.

[^3H]thymidine labelling and autoradiography

Hydra were labelled with [*Me*-^{3}H]thymidine (20 Ci/mM) by injecting 0·1 μl containing 0·001 μCi of the isotope directly into the gastric cavity (David & Campbell, 1972). A single injection results in a pulse label of about 1 h duration. 'Continuous' labelling was achieved by repeating the [^{3}H]thymidine injection at intervals of about 8 h (the *S*-phase of interstitial cells is 11 ± 2 h). Labelled cells were identified in cell macerations by autoradiography using Kodak AR10 stripping film.

Nuclear DNA determinations

Measurements of nuclear DNA content in individual nerve cells and nematocytes were performed by quantitative microfluorimetry in maceration preparations stained with the fluorescent Feulgen reagent bisaminophenyl-oxdiazole (David & Campbell, 1972; Ruch, 1966).

RESULTS

Nematocyte differentiation

A group of hydra were continuously labelled with [^{3}H]thymidine. At each time indicated in Fig. 1 cell macerations were prepared and subjected to autoradiography. Neither nematoblasts nor nematocytes are labelled at the conclusion of the first pulse of isotope indicating that neither population contains proliferating cells. The interstitial cell populations, by contrast, begin to label immediately (Campbell & David, 1974). After 10–12 h some labelled interstitial cells begin to form vacuoles and become thereby the first labelled nematoblasts. By 50 h most of the nematoblast population is labelled and labelled desmoneme and isorhiza nematocytes begin to appear. By 72 h, when labelled stenoteles first occur, the nematoblast population is completely labelled.

Desmonemes differentiate in the body column and then migrate to the tentacles where they are utilized. Thus desmonemes found in the body column are a transient

population which is constantly being replaced by differentiation. If all new cells entering the differentiating pool after a certain time are labelled, as is the case in a continuous labelling experiment, the fraction of desmonemes labelled will increase linearly from 0 to 100 % in the time required to complete this stage of differentiation.

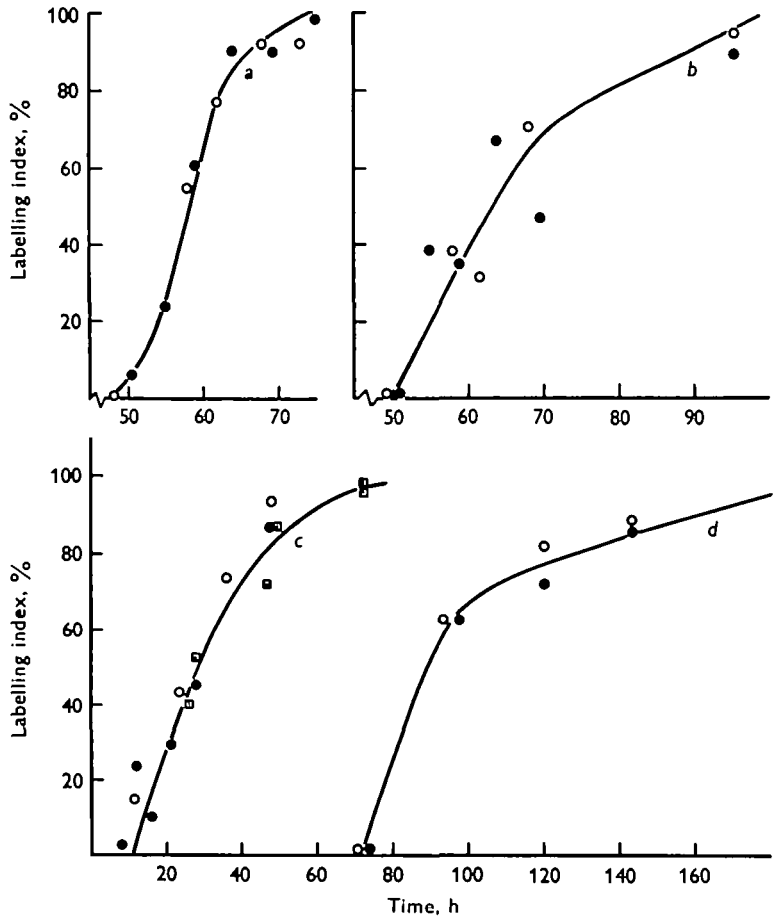

Fig. 1. Labelling kinetics of nematoblasts (*c*) and desmoneme (*a*), stenotele (*d*), and isorhiza (*b*) nematocytes in the body column of hydra continuously labelled with [^{3}H]thymidine. Hydra were labelled with 10 μCi/ml [^{3}H]thymidine (2 μCi/ml in one experiment) by repeated injections at intervals of 12 h starting at t = 0. At the times indicated the tentacles and developing buds were removed from 3–5 hydra and the body columns macerated. Following autoradiography of the maceration preparations, the labelling index was determined for nematoblasts, and desmoneme, stenotele, and isorhiza nematocytes. The different symbols represent results from independent experiments.

Fig. 1 shows that the actual desmoneme labelling curve is approximately linear and that desmonemes require about 12 h to complete the late stages of differentiation and migrate out of the body column into the tentacles. Small variations in the differentiation rate in individual animals could account for the slight deviations from linearity.

The isorhiza and stenotele labelling curves are, in contrast to the desmoneme curve,

distinctly biphasic. There is an initial period of rapid labelling followed by a longer period during which the labelling index slowly approaches 100 %. It is known that some isorhizas and stenoteles are mounted in the ectodermal epithelial cells of the body column upon completion of differentiation while others migrate to the tentacles like desmonemes. Thus for both isorhizas and stenoteles there are 2 populations of nematocytes in the body column – one differentiating and migrating to the tentacles and one consisting of nematocytes mounted in the body column. The initial rapidly labelling portion of the curves in Fig. 1 can be interpreted as the labelling of the differentiating and migrating populations while the slow approach to 100 % labelling reflects the slower replacement in the population of nematocytes mounted in epithelial cells of the body column. Thus, about 65 % of stenoteles and 70 % of the isorhizas in the body column are differentiating cells.

The data in Fig. 1 apply to well fed, rapidly growing hydra. In animals maintained on lower levels of feeding, all stages in the differentiation of nematocytes appear to be longer (unpublished results).

Nerve cell differentiation

The differentiation of nerve cells has been followed in hydra continuously labelled with [^{3}H]thymidine. The results are shown in Fig. 2. As in the case of nematoblasts and nematocytes, nerve cells are not labelled immediately following the initial injection of [^{3}H]thymidine, indicating that nerve cells are non-proliferating. After a lag of 18–20 h, the first labelled nerve cells appear. Newly differentiated nerve cells appear almost simultaneously in all parts of hydra from hypostome to basal disk, indicating that nerve cell differentiation is not limited to any particular region of the animal and that the differentiation time is about the same throughout. The rate of nerve cell labelling appears to be similar in hypostome and gastric tissue and only slightly less in the peduncle and basal disk.

Nerve cells are not known to migrate out of the body column after completion of differentiation as nematocytes do. Thus, if differentiated nerve cells live indefinitely, after one tissue doubling in the presence of [^{3}H]thymidine, 50 % of the nerve cells in the body column will be labelled. If old nerve cells die, some unlabelled cells will have disappeared in this time interval and a higher proportion of nerve cells will be labelled. The data in Fig. 2 indicate that, 3·5 days after labelled cells first appear in the differentiated nerve cell population, about 50 % of the nerve cells in the gastric region and hypostome are labelled. In both regions the epithelial cells have undergone one doubling in this time interval (David & Campbell, 1972). These observations indicate that differentiated nerve cells in these regions are long-lived. Nerve cells in the peduncle and basal disk are labelled at a slightly lower rate, in agreement with the observation that the epithelial cells proliferate slightly more slowly than in gastric and hypostomal tissue.

The rate of nerve cell labelling in the developing bud is much more rapid than in the body column (Fig. 3). Coincident with the outgrowth of the bud from the body column, there is a sharp increase in the number of labelled nerve cells compared to the parental tissue. This increase in labelling and the corresponding increase in

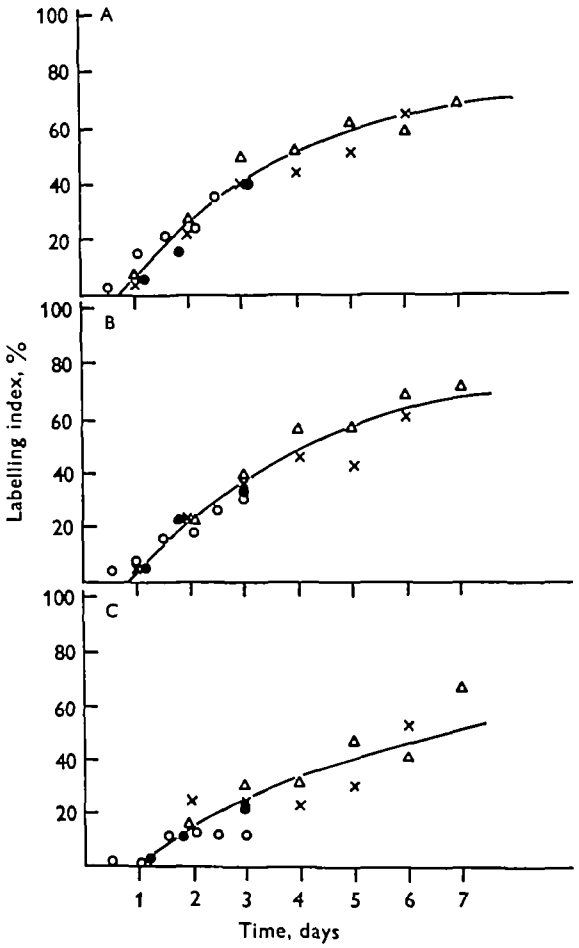

Fig. 2. Labelling kinetics of nerve cells in the hypostome, gastric region, and peduncle-basal disk (A, B, C, respectively) of hydra continuously labelled with [^{3}H]thymidine. Hydra were labelled with 10 μCi/ml [^{3}H]thymidine (2 μCi/ml in one experiment) by repeated injections at 12-h intervals starting at t = 0. At each time indicated 3–5 hydra were sampled. After removal of the tentacles and developing buds, the body column was dissected into hypostome, gastric region and peduncle-basal disk and the tissue pieces macerated. Following autoradiography, the labelling index of nerve cells was determined. The different symbols indicate results from independent experiments.

differentiation accounts for the increase in the numbers of nerve cells found in the developing bud by direct measurements (Bode *et al.* 1973).

Nuclear DNA content of nerve cells and nematocytes

The observed lag between injection of [^{3}H]thymidine and the appearance of labelled nerve cells and nematocytes is consistent with the idea that interstitial cells complete DNA synthesis and undergo mitosis before initiating differentiation. If this is so, nerve cells and nematocytes should have $2n$ postmitotic nuclear DNA content.

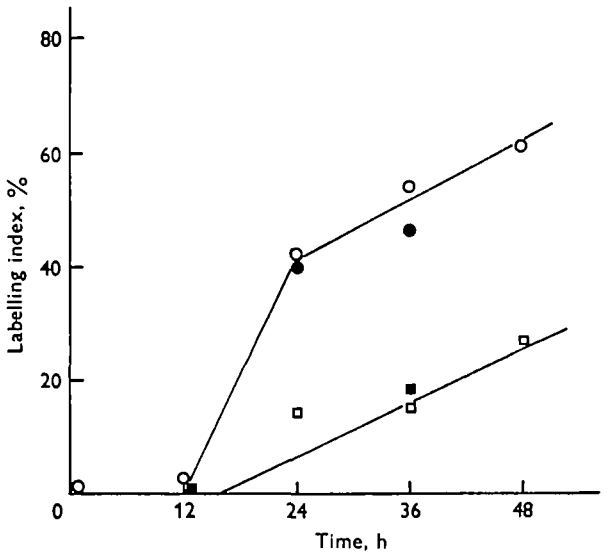

Fig. 3. Labelling kinetics of nerve cells in developing buds continuously labelled with [^{3}H]thymidine. Hydra were labelled with 10 μCi/ml [^{3}H]thymidine every 12 h starting at t = 0. Buds, whose evagination occurred between 12 and 24 h, were identified in individual hydra and used for samples at later time points. At 0 and 12 h, prior to the appearance of buds, the region from which the next buds would appear was sampled. For each sample 3–4 buds were excised from the body column and macerated; following autoradiography the labelling index of nerve cells was determined (○, ●). As a control the labelling index of nerve cells in the body column was also determined (□, ■). The open and filled symbols represent 2 independent experiments.

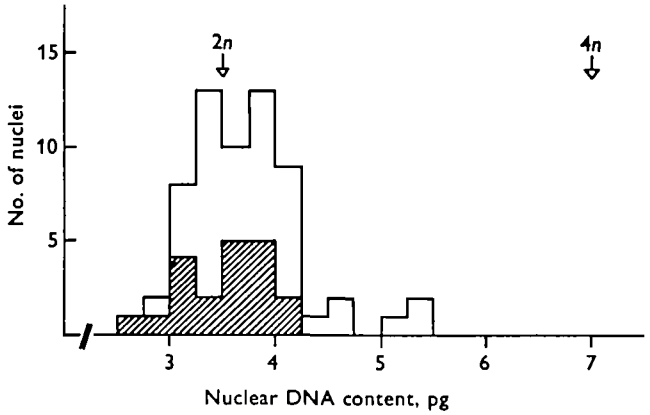

Fig. 4. Nuclear DNA contents of nematocytes and nerve cells. Hydra were dissociated to single cells by maceration and the resulting cell suspension dried on to microscope slides. The preparations were stained with the fluorescent Feulgen derivative bisaminophenyl-oxdiazole and the nuclear DNA content of individual nematocytes (clear bars) and nerve cells (shaded bars) determined by microfluorimetry; $2n$ and $4n$ DNA values were determined on mitotic interstitial cells.

Fig. 4 shows the distribution of nuclear DNA contents of nerve cells and nematocytes determined in maceration preparations stained with Feulgen reagent. More than 50 nerve and nematocyte nuclei were examined and all had $2n$ nuclear DNA contents.

A cell flow model for interstitial cells

Assignment of functions to various classes of interstitial cells. Nematocytes differentiate primarily in clusters of 4, 8, 16 and 32 cells. These clusters are derived from the corresponding clusters of interstitial cells. Following a final cell division there is a period of about 8 h during which the cells develop a vacuole and become classifiable as nematoblasts (Fig. 1). Precursors to nerve cells occur as single interstitial cells or nests of 2 since these are the only classes of interstitial cells found in the hypostome and basal disk where nerve cells differentiate (Fig. 2).

The above observations do not indicate which interstitial cells are the stem cells nor do they indicate whether we have identified all functions of interstitial cells in asexually budding hydra. A more quantitative analysis is, however, possible since we know the number of cells involved in various intermediate stages (David & Challoner, 1974) and their times of turnover (Campbell & David, 1974). For a given flow rate in the steady state, these data are inversely related to each other. Thus we may compare calculated with observed numbers of various cell types. In the following we present such a calculation. We calculate the total number of interstitial cell nests of each size class necessary for the observed rate of nematocyte production. Comparison of the result with the actual numbers of nests of each size class indicate that nests of 4, 8 and 16 interstitial cells are in fact necessary for nematocyte production. Single interstitial cells and nests of 2 cells are present in excess of those leading to nematocyte differentiation. These are quantitatively accounted for by the requirements for nerve cell production and the proliferating stem cell population.

The rate of nematocyte nest production. Table 1 gives the total number of desmoneme, isorhiza, and stenotele nematocytes in the body column of standard hydra. The rate of production of each type of nematocyte (cells/day) was estimated by dividing the number of cells in the differentiating population (see text above) by the time required to label this population (Fig. 1). These rates (cells/hydra/day) were then converted to nests/hydra/day for each nematocyte type using the observed occurrence of differentiating nematocytes in nests of different sizes.

To calculate the number of precursors necessary to maintain this flow rate the length of the differentiation pathway for nematocytes and the continuing growth of the body column must be considered. The production of a nematocyte nest requires 2–4 days of interstitial cell proliferation followed by 2·5–3·5 days of differentiation to transform the interstitial cell nest into mature nematocytes. During this 5–7 day period, hydra tissue grows substantially – our laboratory culture has a mass doubling time of 3·5 days – while maintaining a constant rate of nematocyte production. Thus hydra tissue must turnover, at any given time, more proliferating nests in early stages than nests in final stages of differentiation. Fig. 5 gives a graphic representation of this situation for nests of 16 desmonemes. The daily production rate per body column is 320 nests/day. By plotting time on the abscissa and the number of nests on the

Table 1. *Rates of nematocyte nest differentiation*

	Nematocyte/ epithelial cell*	Nematocytes/ hydra†	Turn-over time, h‡	Nemato-cytes/ hydra/ day	Nematocyte nests/ hydra/day§			
					4	8	16	32
Desmoneme	0·15 ± 0·02	3150 (100 %)	12	6300	—	20	320	32
Stenotele	0·14 ± 0·02	2950 (65 %)	24	1900	9	99	68	—
Isorhiza	0·03 ± 0·01	700 (70 %)	18	660	—	34	24	—

* Nematocytes in the body column (excluding tentacles and developing buds) counted in maceration preparations. Results are average of 8 independent determinations.

† Calculated for standard hydra having 21 000 epithelial cells in body column (excluding tentacles and developing buds). Percentage in parentheses indicates the fraction of each type in the differentiating and migrating population, determined as the rapidly turning over fraction in Fig. 1.

‡ Turnover time was determined as the time required to label completely the rapidly turning over fraction of nematocytes in the body column of hydra continuously labelled with [^{3}H]-thymidine (Fig. 1).

§ Calculated from the rate of nematocyte production and the measured distribution of differentiating nematocytes in nests (David & Challoner, 1974).

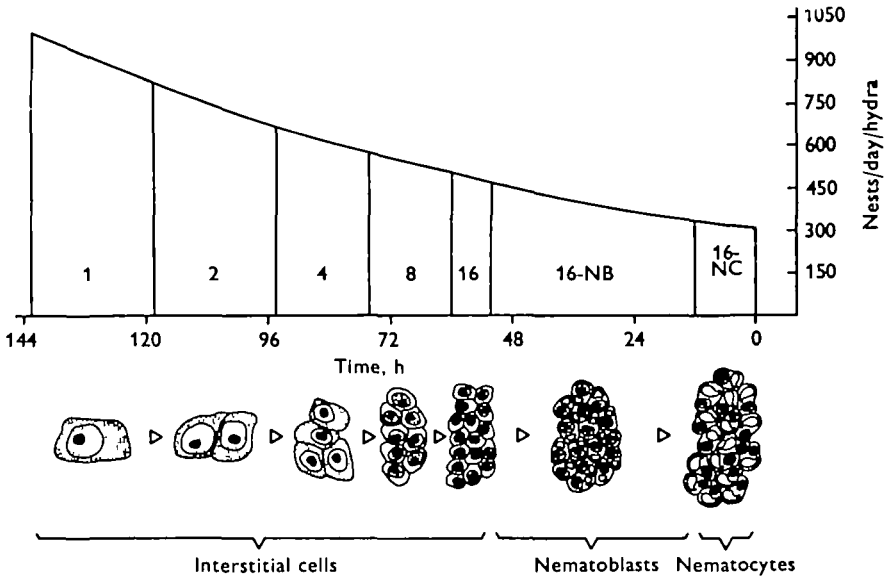

Fig. 5. Exponential projection of nests of nematocyte precursors in the body column of rapidly growing hydra. All stages from a single cell to a completed nest of 16 desmonemes are shown. The cell cycle time for each interstitial cell nest (Campbell & David, 1974) and the duration of the nematoblast and nematocyte stages of differentiation (Fig. 1) are plotted on the abscissa. The ordinate gives the number of nests/day/hydra. The curve shows the exponential growth of hydra tissue ($N/N_0 = e^{0.21t}$). The area under the curve between any 2 time points equals the number of nests at that stage of proliferation or differentiation. A summary of the number of nests at each stage for all nematocyte types and nest sizes is given in Table 2.

ordinate the area under the exponential growth function ($N/N_0 = e^{0.2t}$) gives the number of nests at each stage from 1 interstitial cell to 16 differentiated desmonemes. Table 2 summarizes the data from similar extrapolations for each type of nematocyte. The sum of interstitial cell nests involved in all types of nematocyte differentiation is given at the bottom of Table 2. For 4, 8, and 16 the sums are similar to the measured

Table 2. *Calculated numbers of nests of interstitial cells, nematoblasts and nematocytes in the nematocyte line*

The results are calculated, as shown in Fig. 5, from the rate of nematocyte nest differentiation (Table 1) and the turnover time of each intermediate. Only cells in the nematocyte line (Fig. 7) are included. The values are expressed per hydra. Numbers in parentheses indicate interstitial cells in 8-h pre-nematoblast stage (Fig. 5).

		Interstitial cell nests						Nemato-blast nests	Nemato-cyte nests
		1	2	4	8	16	32		
Desmonemes differentiating in nests of	8	49	40	26	(11)	—	—	41	10
	16	890	730	460	350	(170)	—	660	160
	32	100	84	54	43	35	(17)	66	16
Stenoteles differentiating in nests of	4	24	20	(6)	—	—	—	32	9
	8	300	250	160	(68)	—	—	360	99
	16	240	200	120	93	(47)	—	250	68
Isorhizas differentiating in nests of	8	88	72	45	(18)	—	—	74	26
	16	70	57	36	27	(12)	—	51	18
Total number of nests		**1760**	**1450**	**907**	**610**	**264**	**17**	—	—
Total number of cells		**1760**	**2900**	—	—	—	—	**21300**	**5600**

numbers of interstitial cell nests (Table 3*b*). This supports the exponential model and suggests that nests of 4, 8, and 16 interstitial cells are nematocyte precursors only. The significant excess of single interstitial cells and nests of 2 cells over the nematocyte line indicates that many of these cells are not nematocyte precursors, but stem cells and nerve cell precursors.

Rate of nerve cell production. A standard hydra contains 4900 nerve cells (Bode *et al.* 1973) excluding those in tentacles (where nerve cell differentiation does not occur). This population labels at an average rate of 18 % per day (Fig. 2) equivalent to the production of 900 nerve cells per day. To calculate the number of nerve cell precursors in hydra we must consider, as for nematocytes, the differentiation pathway and the continuous exponential growth of the body column. Nerve cells require about 18 h between the end of the last *S*-phase and the first appearance of nerve processes (Fig. 2). Since the G_2 phase of stem cells is about 12 h (Campbell & David, 1974) and the DNA content of nerve cells $2n$, one expects that determination is followed by one cell division and a 6-h period of differentiation to yield nerve cells. The production of 900 new nerve cells per day is thus expected to require the determination of 450 $\times f$ stem cells per day, where f accounts for the exponential growth of the tissue which occurs during the formation of nerve cells from stem cells. This correction factor is found, by calculations similar to those in the previous section on

nematocyte production, to be 1·23. Thus about 550 stem cells are determined per day per hydra to differentiate nerve cells. The number of differentiating cells in the 6-h period following the last mitosis is 230.

The above calculation includes exponential tissue growth, most of which goes into buds. Developing buds, however, have an increased rate of nerve cell differentiation (Fig. 3) above that due to exponential tissue growth. Because this appears to occur by local depletion of the stem cell pool in the bud hypostome (Bode *et al.* 1973) and thus, probably does not require additional determination and differentiation of stem cells in the body column, we have not included it in calculating the number of nerve cell precursors in the body column.

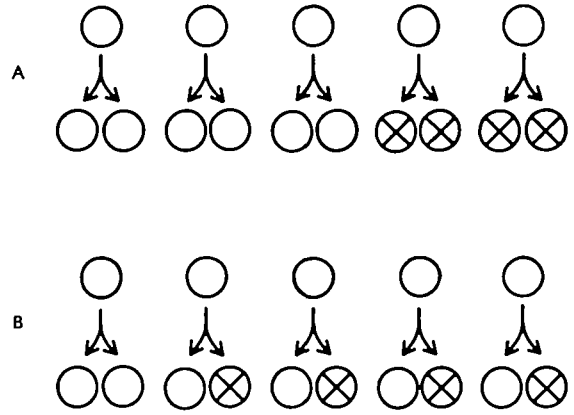

Fig. 6. Two models for stem cell proliferation and differentiation. In model A determination of a stem cell causes both daughter cells to differentiate (⊗). In model B one daughter cell differentiates (⊗) and the other remains a stem cell (○). Growth of the stem cell population occurs when a stem cell divides to yield 2 daughter cells which remain stem cells. If stem cells divide every 24 h, the stem cell population will double in 3·5 days in either model when, in each generation, 61 % of the daughter cells remain stem cells and 39 % differentiate.

Size of the stem cell pool. The continuous differentiation of nerve cells and nematocytes from interstitial cells implies that, among interstitial cells, there is a subpopulation of 'stem' cells which are capable of producing both new stem cells, as well as determined cells. This population of stem cells, while supplying precursors for nerve and nematocyte differentiation, must also double in size every 3·5 days to keep pace with the growth of hydra tissue. Two different models of stem cell proliferation and differentiation will be considered (Fig. 6). In model A determination of a stem cell is assumed to lead to 2 differentiated nerve cells or to a nest of 2 cells as the first intermediate in nematocyte differentiation. Model B assumes that a stem cell, if determined, divides to produce 1 stem cell and 1 determined cell, which may be either a nerve cell or serve as precursor to a nematocyte nest. In both models growth of the stem cell population occurs when stem cells divide to give 2 stem cells and no differentiated products.

The number of stem cells required to maintain the observed growth of the stem cell population and the rates of nerve and nematocyte differentiation can be calculated.

From the results above, stem cells occur as single interstitial cells or clusters of 2. Assuming an average cell cycle of 24 h (Campbell & David, 1974), this population doubles in 3·5 days if 61 % of the daughter cells of stem cell divisions remain stem cells. Thus, in any one stem cell generation, 39 % of the daughter cells can differentiate as nerve or nematocyte. In model A, 61 % of the stem cells in a given cell generation divide to produce 2 stem cells and 39% divide to give 2 differentiating products. In model B, 22 % of stem cells divide to give 2 stem cells while 78 % divide assymmetrically to give 1 stem cell and 1 differentiating product.

Table 3*a*. *Total calculated numbers of interstitial cells in nests of* 1 *and* 2

The results were calculated, on the basis of model A, from observed rates of nematocyte and nerve differentiation and requirements for growth of stem cell population.

Stem cells determined in present cell cycle to differentiate 2 nerves (1·23 × 900/2)	550
Stem cells determined in present cell cycle to proliferate a nest of nematocytes (Table 2)	1760
Stem cells required for maintenance and growth of the stem cell population 61/39 × (1760 + 550)	3600
Cells in nests of 2 determined to proliferate a nest of nematocytes (Table 2)	2900
Postmitotic cells differentiating nerves (0·25 × 900)	230
Total	**9040**

Table 3*b*. *Comparison of calculated and measured numbers of interstitial cells, nematoblasts and nematocytes in hydra*

	Interstitial cell nests			Cells in nests of 1 and 2	Nematoblasts and nematocytes
	16	8	4		
Calculated					
Tables 2 and 3*a*	281	610	907	9040	26900
Measured					
David & Challoner (1974) based on 21000 epithelial cells per body column	250 ± 170 (336)*	780 ± 270 (990)*	1240 ± 500 (1430)*	12800 ± 1300	22000 ± 4000†
Bode *et al.* (1973)	—	—	—	11200 ± 1700	29300 ± 4100

* Values in parentheses include intermediate nest sizes, i.e. 9–16, 5–8 and 3–4.
† Determined in same preparations used for counting interstitial cell nests.

Table 3*a* presents specific calculations, based on model A, of the numbers of stem cells and differentiating cells among single interstitial cells and nests of 2. Per day 1760 stem cells initiate nematocyte differentiation and 550 initiate nerve cell differentiation. Thus, in the steady state, 2310 stem cells are determined per day at some stage during their cell cycle to produce differentiated cells and a further 3600 (61/39 × 2310) stem cells are required per day to account for the maintenance and growth of the stem cell population. Since the cell cycle time of stem cells is about

24 h, a total of 5900 cells are required per hydra to reproduce stem cells and to produce differentiated cells. This number is invariant to assumptions about the stage of the cell cycle in which determination occurs. If it occurs in the middle of the cell cycle, about 4700 (3600 + 1760/2 + 550/2) cells would be undetermined stem cells and about 1100 (1760/2 + 550/2) would be determined cells. If determination occurs earlier, the proportion of determined cells is higher; if determination occurs late in G_2, it is lower.

Stem cells occur as single interstitial cells and in clusters of 2. However, to compare the calculated numbers of stem cells with the observed populations of interstitial cells, we must also include nests of 2 interstitial cells in the nematocyte line and postmitotic interstitial cells differentiating nerve cells. The sum of all cells calculated to occur as single interstitial cells and in nests of 2 is 9040 (Table 3*a*) which is slightly lower than the measured values of 11000–12500 cells (Table 3*b*). Model B (Fig. 6), which has rather different implications in terms of mechanism, leads to a similar number of total cells occurring as single interstitial cells and nests of 2. However, the number of undetermined stem cells is smaller because determination of nematocytes occurs one cell generation earlier than in model A. If determination occurs in the middle of the stem cell cycle, there are about 2400 undetermined stem cells and 6600 cells which are committed to nerve or nematocyte differentiation.

DISCUSSION

Differentiation of nerve cells

The differentiation of nerves from interstitial cells has been extensively described cytologically (Burnett & Diehl, 1964; Lentz, 1966; Davis, 1969, 1971). In the present experiments we have measured the rate of differentiation of [^{3}H]thymidine-labelled precursors to nerve cells. The initial lag in the appearance of labelled nerves (Fig. 2) and the observed 2*n* nuclear DNA content of differentiated nerve cells (Fig. 4) indicate that interstitial cell precursors undergo mitosis before differentiation into nerves. Nerve differentiation occurs throughout the body column from hypostome to basal disk (Fig. 2) at a rate sufficient to maintain the local proportion of nerve cells to epithelial cells despite continuous epithelial cell division. In the developing bud where the proportion of nerve cells to epithelial cells undergoes a marked increase (Bode *et al.* 1973), the rate of nerve cell differentiation is higher than in the parent body column (Fig. 3). [^{3}H]thymidine labelling of nerve cells has also shown that nerve cells survive for at least several times the mass doubling period of hydra tissue since, after 8 days of continuous labelling, 20 % of the nerve cells in the body column are still unlabelled (Fig. 2).

Differentiation of nematocytes

[^{3}H]thymidine labelling demonstrates a lag between the *S*-phase of interstitial cell precursors and the appearance of differentiating nematoblasts (Fig. 1). Since nematoblasts have 2*n* nuclear DNA content (Fig. 4), interstitial cell precursors must undergo a final mitosis prior to differentiating. The nematoblast stage, during which all 3 types

of differentiating nematoblasts appear almost identical, continues for 1·5–2·5 days (Fig. 1). It is followed by the nematocyte stage, lasting 12–24 h, during which the vacuole of the nematoblast differentiates into a mature nematocyst capsule and the

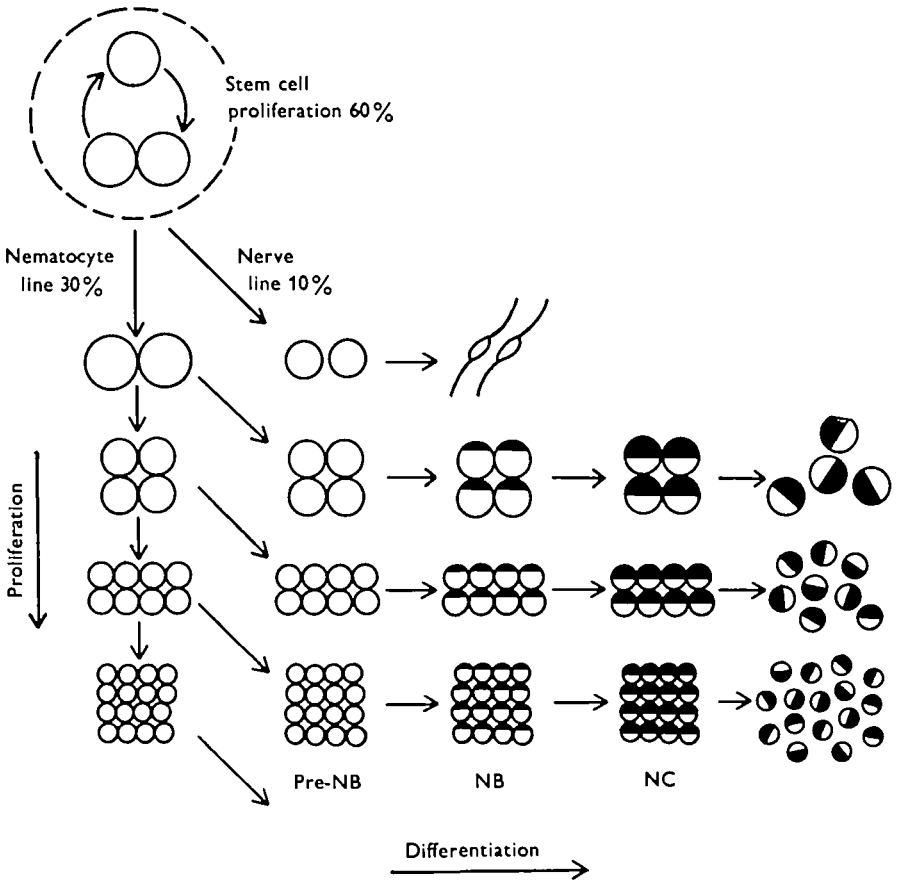

Fig. 7. Cell flow model of the proliferation and differentiation of interstitial cells in hydra. Stem cells, occurring as single interstitial cells or nests of 2, proliferate continously. In each cell generation about 60 % remain stem cells, about 30 % initiate proliferation of a nest of nematocyte precursors and about 10 % initiate nerve differentiation. Nerve differentiation occurs directly from the stem cell pool. In the nematocyte line, after one or more cell divisions, proliferation ceases and a nest commences differentiation of nematocyst capsules. During the Pre-NB stage, nests are morphologically indistinguishable from interstitial cells. Later a vacuole develops in the cytoplasm (NB stage) which differentiates into a mature capsule (NC stage) before the nests break up and single nematocytes migrate through the tissue into the tentacles. For clarity, nests of 32 differentiating nematocytes have been left off the diagram.

nematocyte migrates from the body column to the tentacles. This long postmitotic period of differentiation has been observed independently by Vögeli (1972). Using pulse-chase labelling with [^{3}H]thymidine he demonstrated a lag of 4–5 days between the administration of isotope and the occurrence of migrating nematocytes which were labelled.

We have measured the total rate of nematocyte production by the body column of a mature hydra to be about 8300 nematocytes per day (Table 1, corrected for exponential growth). This value is roughly similar to estimates of the nematocyte requirements of a mature hydra. H. Bode & R. D. Campbell (personal communication) estimate that continuing tentacle outgrowth and replacement of the nematocytes in tentacles of a mature hydra consume about 6000 nematocytes per day. A further 200–300 nematocytes mounted in the ectoderm of the body column are carried daily into the developing bud and must therefore be replaced. Finally some of the nematocytes produced in the body column may also migrate into the tentacles of the developing bud. The total requirements thus appear similar to the measured production rate.

Cell flow model for interstitial cells and their derivatives

In Fig. 7 we propose a model of the production of interstitial cells and their derivatives in hydra. Stem cells, which proliferate to maintain a continuous supply of precursors, occur as single interstitial cells or in nests of 2. Nerve differentiation occurs directly from the stem cell pool in about 1 day. Nematocyte differentiation requires 5–7 days which includes proliferation of a nest of 4, 8, 16 or 32 cells and capsule differentiation. Lehn (1951) and Rich & Tardent (1969) have also shown that nematocyte differentiation occurs in clusters of interstitial cells derived from single cells.

On the basis of the cell flow model, the measured rates of nematocyte and nerve differentiation and the turnover times of intermediate stages, it is possible to make predictions of the number of cells at each stage along each pathway. These predictions are similar to the actual frequencies of interstitial cells, nematoblasts, and nematocytes measured in hydra (Table 3*b*). The values for all classes of interstitial cells are about 20% lower than the directly measured values suggesting a systematic error. If the interstitial cell cycles were 2 h longer than estimated by Campbell & David (1974) – a difference well within the range of accuracy – predictions would match observations nearly perfectly. Although it is possible that some interstitial cells have functions other than those indicated in Fig. 7, our measurements suggest their number cannot be large.

Stem cell determination

Single interstitial cells and nests of 2 include stem cells and their immediate derivatives, committed to produce nematocytes and nerves. The total number of single cells and cells in nests of 2 required in the steady-state hydra does not depend significantly on assumptions about the time or scheme of stem cell commitment. For instance, the determination of a stem cell to form nerves might occur immediately prior to the differentiation itself; alternatively, stem cells might give rise to committed cells which form nerves only after a defined number of cell divisions. If the determination occurs earlier, fewer stem cells, but correspondingly more committed cells, will be present in the animal. In particular, the number of undetermined stem cells per animal would be higher in model A (about 5000) than in the 'disjunctive' model B (about 2500).

The ratio of nematocyte to nerve cell differentiation averaged over the whole body

column is about 3:1 model A and 2:1 model B. Taking into account the cell distribution in hydra (Bode *et al.* 1973), one can calculate that in the gastric region the ratio of nematocyte to nerve differentiation is about 7:1 whereas in the head and foot regions exclusively nerve cell differentiation occurs. This positional dependence of interstitial cell differentiation is one of the main features of the spatial organization of the animal. Models of pattern formation accounting for the continuous maintenance of a spatial pattern in the steady state by prepattern formation, differentiation and growth have been proposed elsewhere (Meinhardt & Gierer, 1974).

The results summarized in the cell flow model show that there are stem cells in hydra capable of producing both further stem cells and differentiated cells. However, it remains undecided whether individual stem cells can differentiate as nematocyte or nerve, depending on the signals they receive, or whether morphologically indistinguishable, determined nerve and nematocyte stem cells coexist in hydra. One might even imagine that desmonemes, stenoteles and isorhizas are each made by a separate stem cell line. If stem cells are multipotent, is the decision to produce, for example, desmonemes reached when a stem cell initiates proliferation of a nest or does determination for a particular nematocyte type involve subsequent events during nest proliferation (Fig. 7).

One approach to questions about the time and scheme of interstitial cell determination would be to clone individual cells and observe their differentiation capabilities. Another approach is to study non-steady-state animals. The pattern of interstitial cell differentiation changes, for instance, during the regeneration of a new hypostome at a cut surface in the gastric region. In particular, new nerve cells appear in large numbers about 24 h after hypostome excision (Bode *et al.* 1973) in tissue which previously produced mainly nematocytes. This suggests that nematocyte precursors may also be used to form nerves, and thus argues that stem cells in hydra are multipotent. This conclusion depends on the fact that nerve precursors do not migrate into the regenerating hypostome. Although Campbell (1967*b*) has shown that interstitial cells migrate very little in the normal gastric region, these findings do not necessarily apply in regenerating animals. In particular, it is known that interstitial cells can migrate rapidly under certain conditions, i.e. into regions in which the indigenous interstitial cell population has been destroyed by irradiation (Tardent & Morgenthaler, 1966).

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