Parameters affecting the maximum cell concentration of *Tetrahymena*

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Summary. In cultures with efficient aeration a maximum cell concentration (MCC) of 6×10^5 cells/ml (defined medium) and 5.5×10^6 cells/ml (broth) can be reached. By culturing within Millicells with excess supply of medium and efficient removal of waste products a physical limit for MCC of about 13×10^6 cells/ml is reached.
Key words. Tetrahymena; growth media; maximum cell concentration.

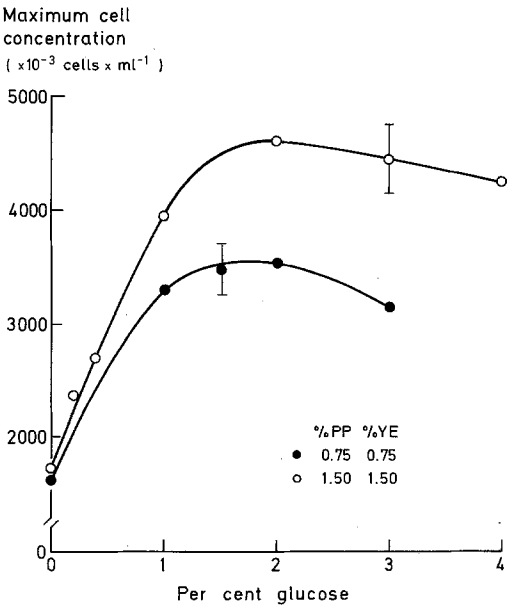
Tetrahymena has been a favorite species for many biochemical and cell physiological studies for a long time[1]. For 'imprinting' studies[2,3] and studies on chemotaxis[4,5] it is important to know in detail the interactions between the cells and the medium. Few reports have studied the parameters affecting the maximum cell concentration (MCC). An early study[6] established that the MCC of *Tetrahymena* is directly proportional to the concentration of nutrients. A value of 0.7×10^6 cells/ml was found. Kidder[7] found that *T. geleii = pyriformis* ferments glucose and maltose, but not sucrose or galactose. Browning[8] reported that the inhibitory effect of high concentrations of carbohydrate is prevented by addition of calcium ions (0.7 mM). A MCC of 608,000 cells/ml was obtained with 2.5% PP, 0.25% liver extract[1]. This value can be increased two-fold in a medium of 2% PP, 0.2% glucose, 0.1% YE, and 0.003% sequestrine with aeration. Recently, Saitoh and Asai[9] found that the MCC of a shaking culture of *T. pyriformis* is determined by the number of collisions and not due to production of waste materials or exhaustion of nutrients.

Materials and methods. T. hegewishi, strains KP 7 and WF 7, *T. setosa,* ATCC 30782 and *T. malaccensis,* strain MP 35 were obtained through the generosity of Dr E. Simon, University of Illinois. *T. pyriformis,* strain GL, was kindly supplied by Dr H. A. Andersen. All strains were kept at 28 °C in a medium (called PY) composed of 0.75% proteose peptone (PP) + 0.75% Bacto yeast extract (YE) + 1.5% glucose + 1 mM $MgSO_4$ + 50 µM $CaCl_2$ + 100 µM Ferric citrate. Defined medium (DM) was according to Holz[10] as modified by Rasmussen and Modeweg-Hansen[11]. It contains 0.25% glucose. Millicells-HA (0.45 µm culture plate inserts, 30 mm) were obtained from Millipore.

Cells were cultured in Erlenmeyer flasks with medium volumes of 10–100 ml or in scintillation glass vials. The inoculum was kept low ($< 10^4$ cells/ml). These cultures were left at 28 °C in humid atmosphere for several days with no change of medium. Cultures were shaken with an amplitude of 5 cm and a frequency of 1/s. In some experiments, cells were cultured in 'chambers', i.e. within Millicell filter discs. A cell suspension (10^4 cells/ml) was added and the Millicell was placed in a jar, giving a medium depth of 2 mm in the Millicell 'chamber'. A magnetic stirrer was present in the outer medium. Cells were counted in 0.1% NaN_3/0.9% NaCl with a Coulter counter.

Results. It takes several days at 28 °C for a *T. pyriformis* culture to reach a plateau with a defined MCC. The generation time in log phase is 2.7 h. The MCC was constant between 25 °C and 28 °C. The effect of the composition of the medium on the MCC was studied. Defined medium (DM) supports growth to 0.55 million cells/ml (table 1) and if present in the broth medium it adds this value to the MCC. The PY-medium supports 3.48 million cells/ml. There is nearly a direct proportionality between dilution of PY and decrease in MCC.

Cell growth in broth media containing PP + YE + further additions was analyzed with the purpose of determining the largest MCC. A concentration of 2% glucose gives the highest MCC and higher concentrations are non-optimal (fig.). Mannose and to some degree fructose can substitute



Effect of glucose on the maximum cell concentration obtained in two different media. *T. pyriformis* was grown in 0.75% PP + 0.75% YE + glucose (●—●) or in 1.5% PP + 1.5% YE + glucose (○—○). 800 µl cell suspensions (10^4 cells/ml) were cultured in glass scintillation vials (h = 2 mm) at 28 °C in a humid atmosphere. No shaking.

for glucose, whereas the non-fermentable carbohydrates (for *T. pyriformis*) galactose and sucrose cannot. Addition of 10 mM $CaCl_2$ increases the MCC by about 0.8 million cells/ml when the fermentable carbohydrates are present. In the absence of glucose a sigmoid curve arises when MCC is plotted against total concentration of PP and YE (equal concentrations). At MCC, growth is not limited because of a lack of ribosides, amino acids, vitamins or trace metals. Optimal values of MCC are independent of shaking if the depth of the medium is no greater than 2 mm.

PY-medium was used for the culture of different species of *Tetrahymena.* MCC and dry weight per cell at MCC were measured (table 1). Apparently all *Tetrahymena* stop dividing when the dry weight of cells/ml in the cultures is 10–12 mg/ml (table 1, line 4). If all of the constituents of the medium were used completely it would support 30 mg dry w/ml. During growth in log phase *Tetrahymena* adjusts its size and dry weight to the medium (table 1). How much of each component of the medium has been used up when untouched cultures reach MCC? Table 1 shows that glucose increases the efficiency and that a maximum efficiency of 41% is obtained with PY. What will happen if the cells are grown in PY, for example, with changes of the medium with regular intervals? Cells were harvested at a concentration of 2 million cells/ml, washed with fresh medium and the concentration was adjusted to 8 million cells/ml and left overnight. These cultures reached 11 million cells/ml, which may be the physical limit for growth.

Table 1. Efficiency of different media

Medium	Dry weight of medium (mg/ml) I	MCC ($\times 10^6$ cells/ml)	Dry weight of cells aat MCC (mg/10^6 cells)	Dry weight of cells at MCC (mg/ml) II	Efficiency (II/I · 100%)
2 × PY	60	3.85	4.0	15.4	26
	60	(5.5)[a]	4.0	22.0	37
PY	30	3.48	3.5	12.2	41
PY (5 different *Tetrahymena*)	30	2.1–4.6	2.6–4.7	9.6–12.2	32–41
2 × PY-glucose	30	1.27	3.1	3.9	13
PY-glucose	15	1.14	1.6	1.8	12
1/3 PY	10[b]	1.25	2.7	3.4	34
DM	8.6	0.55	2.5	1.4	16
1/10 PY	3	0.48	1.9	0.9	30

Dry weight of medium includes PP, YE and, if present, glucose. For DM it includes amino acids, ribosides, glucose, vitamins and salts. [a] By vigorous shaking. [b] Dilution in water.

Table 2. Maximum cell concentration with excess supply of medium

Medium	MCC ($\times 10^6$ cells/ml)	Vo (ml)	Change of medium	Usage of medium (%)	Growth period (days)	Growth temperature (°C)
PY without glucose	11.2	33	no	19	4	28
2/3 PY	9.5	2	once	–	5	22
1/3 PY	9.7	25	no	12	3	25
	12.9	60	no	5	3	25
1/10 PY	12.7	30	no	10	5	23
	9.1	32	no	22	9	25
	10.6	34	no	32	9	28
	9.1	35	no	9	9	28
	11.0	37	no	8	9	25
	10.3	38	twice	–	9	28
	10.0	84	no	3	5	23
DM	11.7	40	no	44	7	28
Average	10.7					

Tetrahymena pyriformis, GL cells (10^4 cells/ml) were added to Millicell 'chambers' placed in sterile jars with a medium reservoir of a certain size (Vo). Vo was adjusted to provide a depth of medium of 2 mm in the Millicell. The cultures were sealed and placed at 22–28 °C for 3–9 days. In some cases the medium was changed. 'Usage of medium' is calculated as follows: (example) 1 ml 1/10 PY medium supports at maximum $0.48 \cdot 10^6$ cells in batch cultures. If Vo = 35 ml, the medium can support maximally $35 \times 0.48 = 16.8$ million cells. If the Millicell at the end of the experiment had 10.5 million/ml in a volume of 800 µl it means a total of 8.4 million cells. The usage is thus $\frac{8.4}{16.8} \times 100 = 50\%$.

The same question is dealt with in table 2 where cells were cultured in Millicells. The final concentration of GL cells within the Millicell is on average 10.7 million cells/ml (range about 9–13) independent of the medium used, the size of the external volume of medium, change of the external medium, the temperature (within 22–28 °C) and the using-up of the medium as long as it is far from 100%. There is thus strong evidence for assuming that about 13 million cells/ml is the physical limit.

Discussion. The factors that determine the onset of the so-called stationary phase and define the MCC are not known. As stated by Elliott[4] the total number of cells present under different conditions varies strikingly. Dobra et al.[12] cultured three *Tetrahymena* species on solid agar and obtained by an optimal surface-to volume ratio a density of ciliates of 5.8×10^5 cells/cm^2, like a monolayer 'tissue'. By use of floating glass beads as dummy *Tetrahymena* cells, Saitoh and Asai[9] provided evidence for a collision frequency theory according to which growth inhibition is a result of cell-to-cell and cell-to-bead collision in shaken *T. pyriformis* cultures. The present study confirms the complexity of the interaction of different factors in the culture, and shows that an adequate supply of oxygen is of major importance. Counting of cells in test tube cultures or Millicell cultures with greater than optimal depth of medium support the theory that non-optimal aeration leads to reduced MCC. Our study shows that glucose (and other fermentable carbohydrates) 1) reduces the growth rate, 2) increases the MCC in media composed of PP and YE, especially when Ca^{++} is present, 3) increases the dry weight of log cells and 4) increases the efficiency of the media (table 1). In the absence of glucose or other carbohydrates the MCC is only 1.3 million cells/ml and the dry weight is much reduced (table 1). Elliott[4] found a maximum efficiency of 11% for a medium with 1% liver extract. We obtain figures like that in media without glucose. With an unlimited supply of medium, as is obtained using Millicells, any of the media used support growth to 10.7 million cells/ml (average). Including the cilia, the volume of the cell is probably 65,000 µm^3, which gives a theoretical MCC of 15 million cells/ml.

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Intrinsic forces alone are sufficient to cause closure of the neural tube in the chick [1]

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Summary. An isolated neural plate or a postnodal piece of early chick embryos, when cultured under appropriate experimental conditions, can undergo morphogenetic movements and form tubular structures closely resembling neural tubes of early chick embryos.
Key words. Chick embryo; neurulation; intrinsic forces.

Neurulation represents a situation whereby a flat neuroepithelial sheet (the neural plate) progressively changes its shape to become a hollow cylinder (the neural tube). This shape change is caused by forces originating from within neuroepithelial cells (intrinsic forces) and/or from outside the neuroepithelium (extrinsic factors)[2]. Waddington and Perry[3], working on the developing amphibian neuroepithelium, originally reported that an apicobasal alignment of microtubules coincide with a period of elongation of neuroepithelial cells. This and similar observations on other vertebrates such as birds and mammals[4, 5] have formed the basis for the hypothesis that microtubules are involved in the formation and maintenance of the elongated shape of neuroepithelial cells. Baker and Schroeder[6] proposed that immediately following the elongation of neuroepithelial cells, apical microfilaments constrict in a 'purse-string-like' fashion to generate a wedge- (or bottle-) shaped appearance. In support of this idea, numerous subsequent studies[4, 5, 7] have shown that chemical agents (e.g., cytochalasins, local anesthetics, calcium agonists and antagonists, and calmodulin inhibitors), which interfere with microfilament-dependent cellular processes in many other developing systems, inhibit apical constriction of neuroepithelial cells and cause a variety of neural tube closure defects in vertebrates. This finding strongly suggests that the major driving forces for closure of the neural tube originate from microfilament-mediated changes in the shape of neuroepithelial cells (especially apical ends). However, some investigators still believe that extrinsic forces (e.g., tension generated by pulling of the elongating notochord and mediad pushing forces exerted by the somites, perineural extracellular matrix and expanding surface ectoderm) play a key role in neural tube closure[5, 8]. To clarify this uncertainty, we present several lines of evidence to demonstrate that intrinsic forces (e.g., cytoskeleton-mediated cell shape changes, cell rearrangement and programmed cell division) alone are sufficient to bring about closure of the neural tube (especially the cephalic region) in the chick.

Materials and methods. Fertile White Leghorn eggs (Shamrock Poultry & Breeding Farm, North Brunswick, New Jersey) were incubated at 37.5 °C to obtain embryos at stages 4–6 of development[9]. Some embryos were explanted either dorsal side up or down using New's[10] technique and grown for about 15 h in nutrient medium (thin albumen). Others were removed from the vitelline membrane and used in the following two experimental series: In the first series, a piece of the neural plate ($0.5 \times 0.5\ mm^2$) was removed from the region just anterior to the primitive streak at each stage 5 or 6 embryo and grown in Medium 199 (Grand Island) at 37.5 °C in an atmosphere of 5% CO_2 in air. After 24–36 h of incubation, explants were fixed and processed for microscopy[11, 12]. In the second series, the area opaca was trimmed off and the remaining area pellucida was transected 0.6 mm posterior to Hensen's node as previously described[11]. The posterior portion, which is now referred to as the postnodal piece (PNP), was grown in nutrient medium (avian Ringer's solution-whole egg extract) with or without nerve growth factor (NGF)[12]. After 4 days of incubation, PNPs were fixed, serially sectioned at 4 µm, and examined microscopically for identifying recognizable (differentiated) structures.

Results and discussion. Nearly all (23 out of 24) chick embryos, which were explanted either dorsal side up or down using New's[10] technique, underwent neurulation indistinguishable from one another. This finding suggests that tension generated by the gravity and by the weight of the blastoderm has no effect on neural tube closure. Similarly, all (34) neural plate explants underwent morphogenesis to form tubular structures closely resembling the 'C'-shaped neuroepithelium or the closed neural tube of early chick embryos (fig. 1). This is probably the best evidence that the forces for neural tube closure originate from within individual neuroepithelial cells, because almost all of the outside influences, that could conceivably contribute to bending of the neuroepithelium, have been eliminated. As for PNPs, no axial structures were observed in any of the 18 untreated controls (fig. 2), a finding consistent with that of previous studies[11–13]. In contrast, all (36) of the PNPs grown in the presence of 10 ng/ml NGF were induced to undergo neuralization ranging from a local thickening of the epithelium (resembling a neural plate) to a completely closed neural tube (fig. 3). In addition, the induced neural tissue showed no preferential orientation and dimension, and in many instances, there were two or more regions with varying degrees of neuralization in a single PNP (fig. 3). We also have learned over the years that 1) the cephalic regions of early chick embryos are devoid of organized mesodermal structures (e.g., somites); they can nevertheless elevate their neural folds and 2) a section of the chick neural plate, when isolated, turned over and put back in place, proceeds to form a neural tube in an inverted orientation. These and other related findings strongly suggest that intrinsic forces alone are sufficient to bring about closure of the neural tube. There has been some speculation that mesodermal structures play a primary role in uplifting and apposition of neural folds[5]. Although