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FISH CELL GROWTH RATES

Quantitative Comparison of RTG-2 Cell Growth at 5–25°C

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SUMMARY

Growth rates of RTG-2 cells were determined on cultures seeded at 12,500, 25,000, 50,000, 100,000, and 200,000 cells per ml and incubated at temperatures 5°C through 25°C. The optimum growth rate was found to be about 20°C at all seeding levels. Population doubling times were determined at each temperature—the shortest being 2 days at 20°C.

The RTG-2 line, an 11-year-old fibroblast-like cell culture derived from gonads of normal rainbow trout, *Salmo gairdneri* (1), has been used in research and in diagnostic procedures with fish viruses (2–9); in addition, it supports limited replication of an amphibian virus (10) and of two arboviruses from homeotherms (11). RTG-2 cells are in the repository of the American Type Culture Collection as Certified Cell Line 55.

The original description of RTG-2 cells included data on rates of protein synthesis and glucose utilization at 4–24°C—the approximate limits of the cells' growth range. Thus far, there has been no information on rates at which cell numbers increase. Poikilothermic cell cultures grow at rates which are dependent upon temperature. If growth rates are known, the information can be used to plan work with the cultures. To the best of our knowledge, enumeration data on animal tissue cell growth rates through a wide temperature range have not been previously reported.

The purpose of this study was to measure population growth rates of RTG-2 cells at five different seeding densities through the range of 5–30°C.

MATERIALS AND METHODS

RTG-2 cells in passages 130 to 188 were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and containing 100 I.U. of penicillin, 100 μg of streptomycin, and 25 I.U. of nystatin per ml.

Growth rates were determined on static cultures in 16 × 125 mm tubes seeded with 1 ml of cell suspension. Cells were dispersed with a solution of 0.1% pancreatin in Versene (ethylenediaminetetraacetate, 1:5000) in physiological saline prepared according to Merchant, Kahn, and Murphy (12). Cells were kept in suspension in the above medium at 0°C throughout counting, diluting, and standardizing prior to planting. The initial cell suspension was standardized at 200,000 ± 5% cells per ml. Dilutions were made in volumetric glassware, counted, and adjusted if necessary to 100,000, 50,000, 25,000, and 12,500 cells per ml, and seeded with calibrated pipettors.

As determined by exclusion of 0.05% trypan blue dye (12), viability of seeding suspensions of cells was 95% or better.

Although we carefully standardized seeding populations, the base line for all growth curves was the number of cells which settled out of suspension and attached during a preparatory initial 8-hr incubation at 20°C. Replicate samples were randomly selected from each seeding density, and then counted prior to incubation at test temperatures of 5°C, 10°C, 20°C, 25°C, and 30°C.

We wanted to learn the time that cultures required to become confluent at the various temperatures. For that purpose "confluency" was found to be equivalent to 275,000 cells in 1 ml of culture medium.

Preliminary work established the approximate

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length of time that the five seeding densities required to reach 275,000 cells at each temperature; then the elapsed times were divided into six equal parts for scheduling counts.

Among the lines which we have handled, the RTG-2 cells are among the most cohesive, especially as cultures mature. Mature RTG-2 cultures could not be harvested and adequately dispersed for accurate counting in a Coulter counter. Therefore, we made multiple hemacytometer counts on a minimum of five replicate cultures for the six scheduled times during each growth period.

For counting, medium was aspirated from the cultures, and 0.9 ml of the Versene-pancreatin mixture was added to the cell sheet. After 10 min, cells were washed off and dispersed with gentle pipetting, 0.1 ml of fetal bovine serum was added to neutralize enzyme action, and tubes were held at 0°C until counted. Such suspensions showed no significant loss in numbers during 2 hr.

For greater sample size, the usual hemacytometer cell-counting procedure was considerably amplified. Four complete hemacytometer fields, each having 0.9 mm^3 of cell suspension, were counted for each replicate tube. The mean (20 counts) was calculated and plotted on semilogarithmic paper for every point. Growth curves were fitted by eye.

Data for the basic growth curves were collected from three main trials and from several supplemental trials; the latter provided specific intermediate points and confirmed others.

Plots of growth curves were used to estimate variation in time required to reach confluency and to estimate population doubling times during exponential growth.

Results

RTG-2 cells grew well at 5°C through 25°C, but did not survive 30°C for long. Growth rates increased in regular fashion from 5–20°C. At 25°C, growth was slower than at 20°C, but faster than at 15°C (Fig. 1).

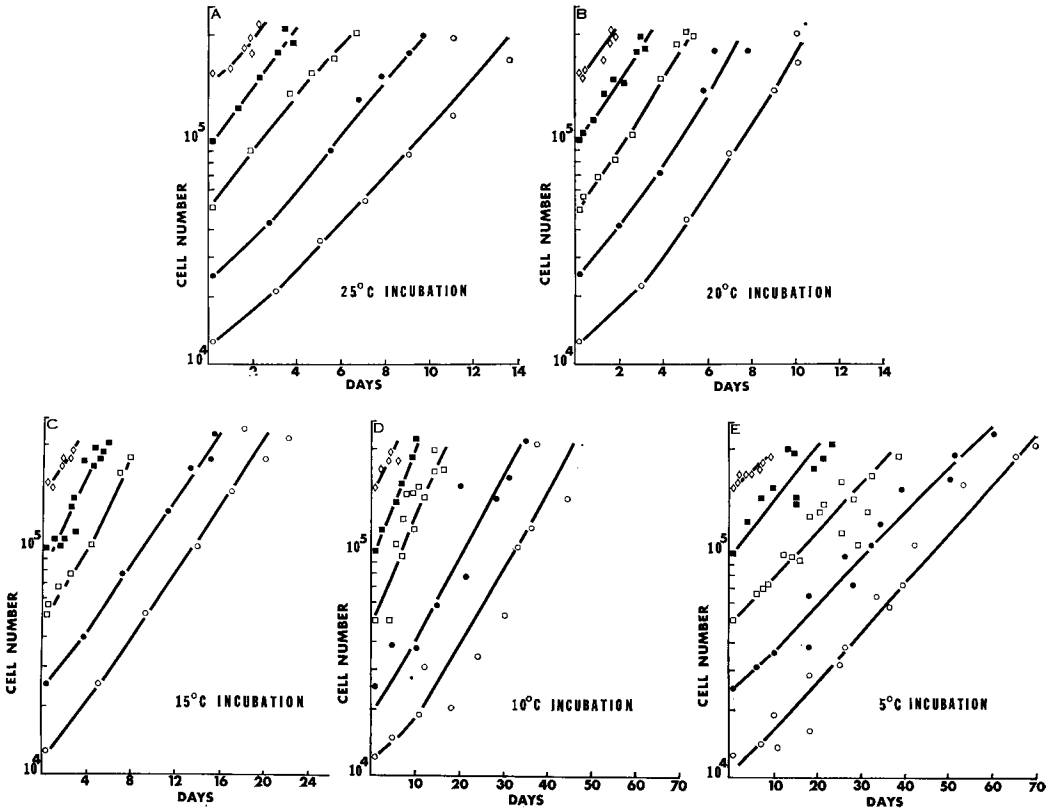

FIG. 1. Comparative growth rates of RTG-2 cells in 16 × 125 mm culture tubes seeded at 200,000, 100,000, 50,000, 25,000, and 12,500 cells per ml and incubated at 25°C (*A*), 20°C (*B*), 15°C (*C*), 10°C (*D*), and 5°C (*E*).

TABLE 1

A SUMMARY OF TIMES REQUIRED FOR FIVE DIFFERENT SEEDING DENSITIES OF RTG-2 CELLS TO ATTAIN 275,000 CELLS PER ML AT FIVE DIFFERENT TEMPERATURES OF INCUBATION

Initial Seeding Density	Mean Time at Temperature of Incubation of				
	25°C	20°C	15°C	10°C	5°C
cells/ml	*days*				
200,000	1.8 ± 0.2	1.5 ± 0.2	2.4 ± 0.5	5.0 ± 1.5	8.0 ± 1.0
100,000	3.3 ± 0.3	3.1 ± 0.3	4.8 ± 1.0	9.3 ± 1.0	18.0 ± 3.5
50,000	6.2 ± 0.5	4.8 ± 0.2	7.3 ± 1.0	14.0 ± 2.0	37.0 ± 7.0
25,000	9.3 ± 2.0	7.5 ± 0.8	14.0 ± 1.0	34.0 ± 3.0	53.0 ± 5.0
12,500	13.0 ± 2.0	10.5 ± 0.6	19.8 ± 0.6	45.0 ± 4.0	67.0 ± 5.0

Plots of growth at higher temperatures were quite regular for all seeding densities. Variability in the data from 15°C to 5°C increased with lowering of temperature. Nevertheless, the plots of growth at each temperature tended to be parallel for all seeding densities.

The shortest time required to reach 275,000 cells was 1.5 ± 0.2 days, and that occurred at 20°C in cultures seeded with 200,000 cells. Cultures seeded with only 12,500 cells and incubated at 5°C required the longest time, 67 ± 5 days, to attain 275,000 cells (Table 1).

Approximate population doubling times were derived from exponential portions of the growth curves, and at the two highest temperatures showed less than 10% variation for all seeding densities (Table 2). The shortest doubling time occurred at 20°C—a uniform 2 days at all seeding densities. At lower temperatures the mean doubling time increased inversely with temperature. Increase in mean doubling time was approximately 2-fold for each 5°C drop in temperature, and at 5°C the mean value was about 14 days. Again, the variation was greater among data taken at lower temperatures.

TABLE 2

APPROXIMATE POPULATION DOUBLING TIMES OF RTG-2 CELLS AT SEVERAL SEEDING DENSITIES AND FIVE DIFFERENT TEMPERATURES

Seeding Level	Doubling time at Temperature of Incubation of				
	25°C	20°C	15°C	10°C	5°C
cells/ml	*days*				
100,000	2.4	2.0	3.8	7.0	10.0
50,000	2.5	2.0	3.5	5.5	14.5
25,000	2.5	2.0	4.0	8.0	16.0
12,500	2.7	2.0	4.1	8.8	13.5
Mean	2.6	2.0	3.8	7.6	13.8

A total of 135 sets of five-tube replicate cultures was counted, and the mean deviation was ±10.8%, a value very close to the generally accepted 10% accuracy for hemacytometer counts of animal cell populations. The maximum variation was 44%, and the minimum was 1.9%.

For reasons which we cannot explain, cells incubated at 10°C did not extend processes normally and were noticeably spindly; nevertheless, mitoses were common. This peculiar morphological response was not observed at other temperatures.

DISCUSSION

Optimum temperature for RTG-2 cells was found to be about 20°C. Consistent with what had been reported earlier and consistent with the biology of the rainbow trout itself, continued exposure to 30°C was lethal.

Other cell cultures from *S. gairdneri* have given temperature response data which are in agreement with ours. Fryer, Yusha, and Pilcher (13) used second passage embryonic cells and found 35°C to be lethal; their cells were incubated at temperatures down to 4°C, but growth occurred only at 25°, 23°, and 18°C, and the latter was the most favorable. Li and Jordan (14) used ovarian cells and found that 28°C was not tolerated but that there was good growth at 18°C and 23°C. The lowest temperature tested was 8°C, at which there was "a decline in proliferation." Both groups of investigators used media which were different from ours, and made periodic changes, a practice which we find unnecessary and even undesirable for fish cell lines.

While it may appear unusual, the requirement of 67 days for lightly seeded cultures to reach confluency is wholly consistent with growth and survival of similar cultures at 4°C for 2 years with neither attention nor changes of medium (15).

Several factors which influenced culture growth

became evident during this study; they highlight the fact that reproducibility of the data depends on duplicating the culture conditions. The composition of the medium is obviously important. At one time, we decided that the rather extensive manipulations incurred a higher than normal risk of contamination. We supplemented a batch of medium with 100 μg of kanamycin per ml and found that it halved the growth rate. The work had to be discarded. Other media, sera, and percentages of serum would undoubtedly also have significant effects.

At the time that we used them, bottle cultures of cells were generally confluent and mature. Seeding suspensions prepared from bottle cultures in exponential growth phase responded somewhat more rapidly than mature cultures, and this factor must be considered in planning work with our data.

Although they differed by a factor of 16, the lightest and heaviest seeding densities responded essentially the same at each temperature. Accordingly, it is likely that our data can be used for work with larger cultures.

There is no need to abide rigidly by the tabular data. If a need arises, cells which have been at one temperature may be moved to any other in the growth range. In this way, a large stock of lightly seeded cultures may be kept at 5°C. As need for them arises, quantities of them can be moved to 20°C to accelerate their development to the desired level of confluency.

The data obtained provide a methodical approach to flexibility in poikilotherm cell culture.

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