Established Eurythermic Line of Fish Cells in vitro

Abstract. An established line of fish cells has been propagated in vitro for 21 months and 48 subcultivations. Important characteristics of the cells are described.

Established lines of animal cells have been employed for many purposes, notably in the study of viral diseases of man and other warm-blooded vertebrates. As far as we could determine, all cell lines thus far reported have been derived from tissues of warm-blooded vertebrates. Susceptible cell lines also have been needed in the study of viral diseases of fishes. The purpose of this report is to describe some characteristics of such a line.

Line RTG-2 originated in rainbow trout gonadal cells (*Salmo gairdneri*) obtained by primary cultivation of pooled normal gonads of fingerling yearling fish. Most of the gonads were macroscopically identifiable as developing ovaries, but because the fish were immature testicular tissue was presumed to have been present also. Tissues were dispersed with prolonged cold trypsinization. Original cultures were established in January 1960 in a previously described cord serum medium (*1*) at 19°C. Monolayers of mixed epithelial-like and fibroblast-like cells developed readily, and cells were first subcultured during the second and third weeks. Static cultivation has been used throughout.

The line has been subcultured 48 times over a period of 21 months. Transfers have been made at 1- to 2-week intervals depending upon such factors as initial cell density, temperature of incubation, and *p*H of medium. Media have not been renewed between transfers. Eagle's minimal essential medium (*2*) plus 10 to 15 percent fetal calf serum has supported excellent growth and for reasons of convenience and economy was judged to be the medium of choice. The line also has been routinely cultivated in the growth medium of Puck *et al.* (*3*), NCTC-109 medium of Evans *et al.* (*4*) with 10 percent cord serum, and other media originally designed for warm-blooded animal cells.

Subcultures grew best when initial *p*H was about 7.3. A nearly neutral medium was tolerated, and the *p*H of old cultures dropped to 6.8. A *p*H of 7.4 or higher was somewhat inhibitory.

Highest tested levels of antibiotics were 400 units each of penicillin and streptomycin and 50 units of nystatin per milliliter. Cultures have been grown in antibiotic-free media for 11 months.

High initial populations have not been necessary to maintain the cultures. Based upon cell counts, populations of 10,000 or fewer cells per milliliter have consistently been adequate; in fact, several hundred cells per milliliter are sufficient to start a subculture.

Sanford's method of estimating populations by enumeration of nuclei (*5*) could not be used because the reagents lysed RTG-2 cells and their nuclei, but samples of cell suspension could be counted accurately after heat fixation at 60°C for 30 minutes (*6*). Staining facilitated counting, and reproducible results could be obtained even after several days.

Cultures have been incubated at 4°C and at intermediate temperatures up to and including 26°C. The limit of heat tolerance was near 26°C, and cells contracted and ultimately died after 24 hours at 30°C. Glucose utilization and protein content at different temperatures was measured at 2-day intervals in triplicate cultures. Utilization of glucose from the medium was determined by the anthrone method of Trevelyan and Harrison (*7*), and protein content of cells from the same cultures was determined with the bromosulfalein method of Bonting and Jones (*8*). In comparison with data obtained at 24°C, cultures at 18°C required about 1.3 times longer to reach an equal level of protein. At 12.5°C the time increase factor was 2.2 and at 4°C it was 3.5 (Fig. 1). Similar values were found for utilization of glucose. Cultures at 18°C required 1.5 times longer and those at 12.5°C, 2.7 times longer to equal the utilization which occurred at 24°C. Cultures at 4°C had an appreciably slower rate and required 7.2 times longer.

The line tolerated long periods of incubation without feeding or attention. At 19°C cultures were viable and even showed mitoses after 5 months, but glucose had been completely utilized and the medium was slightly acid. At 4°C similar cultures show mitoses after 9 months and give every indication of being able to survive additional months of storage. Advantage of this attribute has been taken in handling stock cultures; they have been subcultured at 2-month intervals. Subcultures have been made by scraping, by a 10-minute cold digestion with 0.25 percent trypsin, but preferably by 10-minute cold dispersion with disodium versenate (20 mg/100 ml) followed by immediate "neutralization" with old medium.

The morphology of the RTG-2 cells has been influenced strongly by the medium, age of the culture, and by location within the cell sheet, but under most conditions the cells have been fibroblast-like. Based upon measurements of 200 versene-dispersed cells

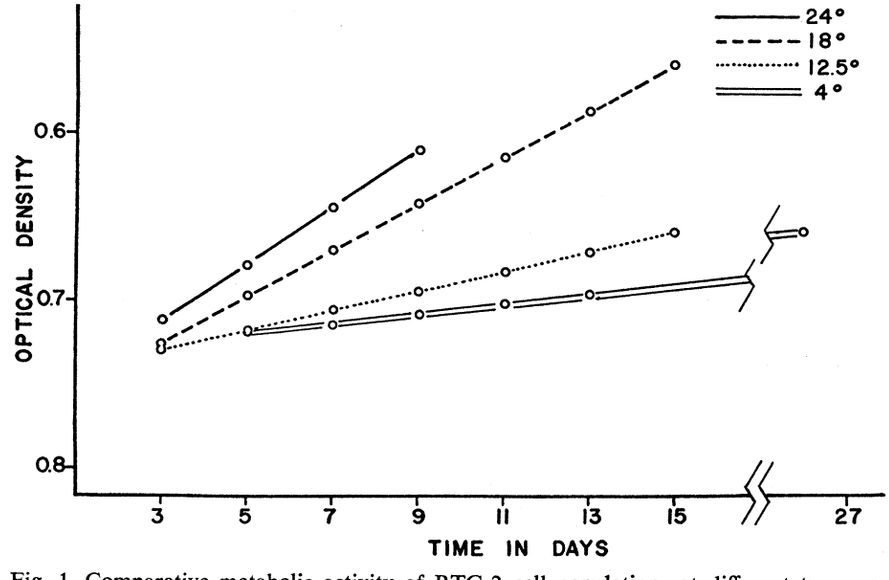

Fig. 1. Comparative metabolic activity of RTG-2 cell populations at different temperatures. Washed cells were used for determining comparative synthesis of protein. The bromosulfalein method of protein determination shows a decrease in optical density with increasing protein. Results were treated by least squares method to obtain the points shown. Medium from the same cultures was analyzed to determine the amount of glucose utilization (see text).

having a generally spherical form, median cell diameter was 15.6 μ, and the range was 5.2 to 23.4 μ.

Chromosomes were prepared for enumeration by procedures adopted from the methods of Tjio and Puck (*9*) and Hsu and Kellogg (*10*). Counts were made on 50 cells and the modal chromosome number was 59 with the range being 49 to 71 and 56 percent of the cells having 59 ± 2. The diploid number for the species is 60.

The line is highly susceptible to the virus of infectious pancreatic necrosis of trouts and supports a rise of virus titer of about $10^{8.5}$ per milliliter.

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References and Notes

1. K. Wolf *et al.*, *Science* **132**, 1890 (1960).
2. H. Eagle, *Science* **130**, 432 (1959).
3 T. T. Puck *et al.*, *J. Exptl. Med.* **108**, 945 (1958).
4. V. J. Evans *et al.*, *J. Natl. Cancer Inst.* **19**, 885 (1957).
5. K. K. Sanford, *Methods in Medical Research*, M. B. Visscher, Ed. (Year Book, Chicago, 1951).
6. T. L. Wellborn and K. Wolf, *Excerpta Med.* **Sect. I, 15**, 589 (1961).
7. W. E. Trevelyan and J. S. Harrison, *Biochem. J.* **50**, 298 (1952).
8. S. L. Bonting and M. Jones, *Arch. Biochem. Biophys.* **66**, 340 (1957).
9. J. H. Tjio and T. T. Puck, *J. Exptl. Med.* **108**, 259 (1958).
10. T. C. Hsu and D. S. Kellogg, Jr., *J. Natl. Cancer Inst.* **25**, 221, (1960).
11. Thomas L. Wellborn performed the biochemical determinations, and his contribution is gratefully acknowledged.

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Inhibitors of Deuterophoma tracheiphila in Citrus Varieties Resistant to "Mal Secco"

Abstract. Growth of colonies of the fungus *Deuterophoma tracheiphila*, the causal agent of the "mal secco" disease of lemons, is inhibited by two substances, C_1 and CS_2, which are found in resistant mandarin varieties but not in susceptible lemons and related species. Some properties of these substances are reported. CS_2 may be naringenin, but the stronger inhibitor, C_1, has not yet been identified.

The "mal secco" disease caused by *Deuterophoma tracheiphila* is the main limiting factor of lemon growing in many areas of the Mediterranean basin (*1*). The fungus may attack either through the roots or through the branches. Diseased trees die progressively by loss of leaves and subsequent drying of branches till their eventual death. Research for resistant rootstocks and lemon varieties is very active in countries where citrus trees are affected. Other citrus species such as mandarins, oranges, and grapefruit are generally not affected.

In the course of studies of physiological reactions of the lemon scion to fungal attack and on the reasons for the difference in susceptibility of different citrus species (*2*) natural inhibitors of fungal growth in resistant species have been detected. This is the first instance known to us of detection of such inhibitors of *D. tracheiphila*.

We first investigated the influence of water extracts of bark of different citrus species on the growth of fungus on carrot-agar dishes. The water extract was prepared by blending 50 g of bark, previously cut to small pieces, in 250 ml of distilled water. The extract was boiled for 30 min, brought to volume, and filtered through a Buchner funnel; the filtrate was further sterilized for 30 min at 15 lb pressure. The extract was mixed with agar; dishes were poured, inoculated, and incubated at 21°C for several days. Growth experiments are summarized in Table 1. Only measurements at 8 (or 9) and 12 days are reported.

Table 1 shows that there is a very clear inhibition of growth by Cleopatra extract, a lesser inhibition due to sour orange extract, and a slight initial promotion of growth by lemon extracts, especially by extract from the highly susceptible Eureka lemon. Cleopatra extract inhibited the normal spread of the colonies, causing an initial increase in height.

In order to separate and identify the factors responsible for growth inhibition, the water extracts (after a fivefold concentration) were chromatographed by descending procedure on Whatman paper No. 1 with butanol, acetic acid, and water (5 : 1 : 4) for 20 hours.

Table 1. Growth in diameter of fungal colonies of *Deuterophoma tracheiphila* as influenced by water extracts of bark. Growth is expressed in percentage of growth in water controls.

Fruit	Growth (%)					
	Expt. 1: 400 mg of bark extracted		Expt. 2: 1000 mg of bark extracted		Expt. 3: 1000 mg of bark extracted	
	9 days	12 days	8 days	12 days	8 days	12 days
Water control	100.0	100.0	100.0	100.0	100.0	100.0
Eureka lemon	107.6	108.2	112.0	100.0	101.4	90.7
Interdonato lemon					103.6	93.8
Monachello lemon					99.3	87.6
Sour orange			99.1	87.1	95.6	83.0
Cleopatra mandarin	58.5	64.1	61.2	54.1	54.3	46.1
Standard errors	0.5	4.5	1.9	1.5	1.7	1.1

Each spot consisted of the extract from 50 mg of bark.

The examination of chromatograms in ultraviolet light (320 mμ) showed a yellow fluorescent spot at R_F 0.87 in Cleopatra and sour orange extracts, and a yellow fluorescent spot at R_F 0.92 in Cleopatra extracts only. Both spots were absent from Eureka and other lemon extracts. Other differences between chromatograms were found to be irrelevant.

The chromatograms were marked under ultraviolet light so that the spots could be identified, and five main zones were eluted separately. Paper portions, corresponding to extract from 150 mg of bark spotted on the origin, were extracted with 5 ml of 80-percent ethyl alcohol. After 1 hour the alcohol was collected and the paper strips were washed with 2 ml of water. The combined elutions were evaporated on a water bath to a volume of 1 ml, added to carrot agar, and sterilized for 30 min. Four dishes were poured for each zone and extract, and inoculated with fungus. Only the elution from zone 1 (R_F 0.8 to 0.95) caused inhibition of growth. When measured 11 days after inoculation, growth over extract from Eureka lemon was 104 percent of control, but over extract from sour orange growth was only 87.5 percent of control, and over extract from Cleopatra mandarin growth was only 58.7 percent of control.

In another experiment, we tested the effect on growth of higher concentrations of the inhibitors from Cleopatra. Growth was increasingly inhibited at higher concentrations of the extract: it decreased from 82 percent of control with extract from 100 mg of bark to about 65 percent with extract from 500 mg and 59 percent with extract from 1000 mg. Colonies were measured 18 days after inoculation.

The area from R_F 0.80 to 0.95 was