planation for the disparity of the sex ratios must be sought.

Subsequent to the discovery of these virtually all-female species it was discovered that the phenomenon had been independently noted by others, namely Minton (*2*) and Tinkle (*3*), in the species *C. tessellatus*.

Parthenogenesis in vertebrates is extremely rare. The first report of the phenomenon occurring naturally in vertebrates was made by Hubbs and Hubbs (*4*). According to their account the poeciliid fish *Mollienisia formosa* occurs in the female condition only. But wherever it occurs it is sympatric with one or the other of two bisexual species of the same genus, *M. latipinna* or *M. sphenops*. They concluded that *M. formosa* was a natural self-perpetuating hybrid of these bisexual species, borrowing the males of these to initiate embryogenesis. They subsequently demonstrated experimentally (*5*) that the males of other species of *Mollienisia*, and less effectively other genera, *Gambusia, Allopoecilia, Limia,* and *Lebistes,* could also induce pregnancies. All such matings resulted in matriclinous young. They further suggested that the species probably is permanently diploid. Mayer (*6*) has confirmed their findings. Other investigators such as C. P. Haskins, E. F. Haskins, and Hewitt (*7*) have been working with this species also, and Miller and Schultz (*8*) are investigating two other all-female poeciliid fish.

One other example of natural parthenogenesis is known in vertebrates and is of particular interest in that it occurs in lizards. The existence of an all-female subspecies, *Lacerta saxicola armeniaca*, in Armenia was first pointed out by Lantz and Cyren (*9*). This phenomenon was corroborated by Darevskii (*10*) who at the same time recognized two additional all-female subspecies of the same species, and then in 1958 (*11*) proved conclusively that in all three races this is true parthenogenesis.

Mertens (*12*), in reviewing this work of Darevskii's, pointed out that peculiar sex ratios are known in some species of gekkos and suggested that possibly here too there may be parthenogenesis.

As can be seen from these accounts, natural parthenogenesis is so unusual in vertebrates that one should proceed with extreme caution before ascribing the lack of males in a species to this phenomenon. The presence of a male in *C. tessellatus* and the apparent presence of two males in *C. velox* and the apparent partition of *C. inornatus* into bisexual and unisexual moieties are conditions that mitigate against the immediate adoption of a parthenogenetic hypothesis. Ecological explanations should be carefully considered first.

During the latter part of the summer of 1959 periodic checks were made for hatchlings of *C. tessellatus* in the field. On 26 August, two juveniles and one adult were collected in Pueblo County, Colorado. Thereafter 60 additional hatchlings and 8 adults were captured. All of these specimens were females. It is possible that hatchling males might exhibit sexual disparity in their behavior and that they were already occupying separate or different habitats. Or they might have been active at different times of the day. These possibilities were kept in mind, and every effort was made to explore all of the obvious available habitats at all times of the day. This evidence, while not conclusive, strongly suggests that the skewed sex ratios in this and the other species mentioned have indeed a genetic explanation (*13*).

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References and Notes

1. W. E. Duellman, *Univ. Kansas Publs. Museum Nat. Hist.* **10**, 596.
2. S. A. Minton, Jr., *Southwestern Naturalist* **3**, 44 (1958).
3. D. W. Tinkle, *ibid.* **4**, 196 (1959).
4. C. L. Hubbs and L. C. Hubbs, *Science* **76**, 628 (1932).
5. ———, *Rec. Genet. Soc. Am.* **14**, 48 (1946).
6. H. Meyer, *J. Genet,* **36**, 329 (1938).
7. C. P. Haskins, E. F. Haskins, R. E. Hewitt, *Evolution* **14**, 473 (1960).
8. R. R. Miller and R. J. Schultz, *Science* **130**, 1656 (1959).
9. L. A. Lantz and O. Cyren, *Bull. soc. zool. France* **61**, 1959 (1936).
10. L. S. Darevskii, *Zool. sborn, AN Arm. SSR,* No. 10, 27 (1957).
11. ———, *Doklady Akad. Nauk S.S.S.R., Biol. Sci. Sect.* **122**, 877 (1958) (English translation).
12. R. Mertens, *The World of Amphibians and Reptiles,* H. W. Parker, trans. (McGraw-Hill, New York, 1960), p. 143.
13. I acknowledge the financial aid extended to me by the Council on Research and Creative Work of the University of Colorado, which made possible the preliminary investigations outlined in this paper. On the strength of the evidence uncovered by this work the National Science Foundation has contributed an additional grant (G 16244) to make possible a continuance of these studies for an additional year. I also wish to thank the following persons and the institutions they represent for allowing me to study specimens under their care. Charles M. Bogert and Richard G. Zweifel, American Museum of Natural History; Robert F. Inger, Chicago Natural History Museum; Hobart M. Smith, Museum of Natural History, University of Illinois; William E. Duellman, Museum of Natural History, University of Kansas; and Norman Hartweg and Charles F. Walker, Museum of Zoology, University of Michigan.

2 October 1961

Chronic Infusion of Tritiated Thymidine into Mice with Tumors

Abstract. A technique for the chronic infusion of mice is presented. Infusion of tritiated thymidine for 3 to 7 days labels most, but not all, of the cells of autochthonous breast tumors. Many of the unlabeled tumor cells are probably nonproliferating, but some may represent cells with exceptionally long intermitotic times.

The chronic infusion of tritiated thymidine into mice with autochthonous tumors indicates the relationship between cell division and tumor growth.

As shown in Fig. 1, a flexible 1-mm polyvinyl catheter is introduced through the skin of the back, carried subcutaneously to the midabdomen, inserted into the peritoneal cavity, and anchored by two sutures. Heavier, less-flexible plastic tubing is then threaded over the catheter and is anchored to the skin of the back with four sutures. The heavy tubing extends beyond the animal's reach and prevents access to the catheter. The catheter is connected to an infusion pump.

The pump supplied fluid at a rate of 0.06 ml/hr. At the concentration used, 50 μc of tritiated thymidine (Schwarz BioResearch 350 mc/mmole) was infused per 10-hour period. Mice have been infused continuously for 10 days without apparent ill effect, either from the tritiated thymidine or from the experimental manipulation.

As a preliminary study, C3H mice with 12 autochthonous breast tumors were infused for 3 to 7 days. Usually, the animals were allowed to survive 3 to 5 days after infusion was stopped. In one case, an animal with two tumors was killed immediately after a 4-day infusion. The tumors were removed and fixed in neutral formalin immediately after the mice were killed. Autoradiographs were made from 2-μ-thick sections by the dipping method (*1*).

These autoradiographs presented certain problems. For example, background was difficult to define in areas where most cells were heavily labeled. Exposures of up to 200 days—made to separate marginally labeled cells from background—obscured the cytological details of heavily labeled cells. More appropriate exposures for the majority of the cells resulted in some cells with a few grains over them, and there was no assurance that this was due to background, scatter from nearby cells, or marginal labeling. Another possible cause for apparently unlabeled cells

was absorption of the radiation by overlying tissue in excessively thick sections. By selecting appropriate exposures and avoiding thick sections, the large majority of nuclei were scored with confidence. Perhaps an uncertainty of plus or minus several percent in the data presented here is attributable to these technical limitations. In addition, the variable histology and growth patterns of these tumors required careful sampling techniques for counting and made the use of average values somewhat arbitrary.

With no obvious exceptions, the autoradiographs made after chronic infusion of tritiated thymidine were qualitatively similar to those produced by a single injection. There was no evidence of cytoplasmic labeling, and the incidence of labeled cells closely paralleled the mitotic activity in both normal and tumor tissues. The number of grains per labeled nucleus seen after infusion of 50 μc for 10 hours approximated the number of grains seen after a single injection of 50 μc.

In no case did infusions of 3 to 7 days label all the cells of a tumor, or even all the cells of an oil-immersion field within the tumor. In general, the longer the infusion ran, the higher was the incidence of labeling (see Fig. 2, top). Both areas of frequent labeling and areas of infrequent labeling were found adjacent to blood vessels. However, the regions with many labeled cells were likely to be the regions of high mitotic activity.

The two tumors fixed immediately after infusion showed 100-percent labeling of mitotic figures. This is not unexpected in view of the current concept that the synthesis of deoxyribonucleic acid (DNA) precedes mitosis by a matter of hours in the mitotic cycle (*1*). Nevertheless, it confirms the fact that tritiated thymidine is available to and utilized by all the premitotic cells of the tumor. It is also indication that the interval between the end of DNA synthesis and visible mitosis is rarely, if ever, 4 days long.

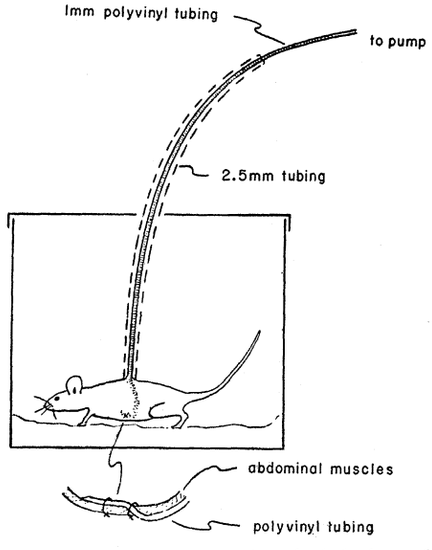

Fig. 1. A catheter for chronic infusion of mice.

The delay after infusion allowed the labeled cells to distribute randomly in the mitotic cycle. Although there was a risk that some cells would divide rapidly and would no longer be detectable, this delay and the measurement of labeled mitoses per mitoses permitted an estimate of the extent to which the proliferating tumor cells were labeled (*2*). A comparison of the two ratios—labeled mitoses per mitoses and labeled cells per cells—gives some indication that there are nonproliferating cells in the population (*3*). However, the calculation of growth fraction by a previously described formula (*3*) was not appropriate to these experiments because they extended over so long a time interval. As shown in Fig. 2, a small fraction of unlabeled mitotic figures was found in most of the tumors; this number was always less than the corresponding fraction of unlabeled cells. Apart from the outside possibility that these unlabeled mitotic figures represent technical defects in the method, they indicate the presence of small numbers of proliferating cells with intermitotic times at least as long as the period of infusion. The results indicate that a majority of the proliferating cells had intermitotic times of less than 3 to 7 days. Furthermore, the relationship between the values suggests that slightly longer infusion times would have labeled all the proliferating cells. If such is the case, then the disparity between labeled cells and labeled mitoses indicates the presence of a significant fraction of nonproliferating cells in the tumor.

Since the volumes of these mouse tumors double in about 1 week, it is most probable that slower-growing human neoplasms will yield smaller fractions of labeled cells after comparable periods of infusion. In connection with cancer chemotherapy and particularly the administration of halogenated pyrimidines, these results emphasize the difficulty of incorporating precursors into the DNA of all tumor cells. A tumor in which all cells are proliferating at a similar rate would presumably become totally labeled after an exposure approximating the mean intermitotic time. In terms of external growth, this would approximate the time necessary for the tumor to double in volume. The C3H tumors used in this study evidently do not fit such a simple model; possibly there is a wide variation in intermitotic time, but in addition there is a good chance that many of the tumor cells are not proliferating at all. In such tumors, the exposure times necessary to incorporate precursor into all cells become very much longer than

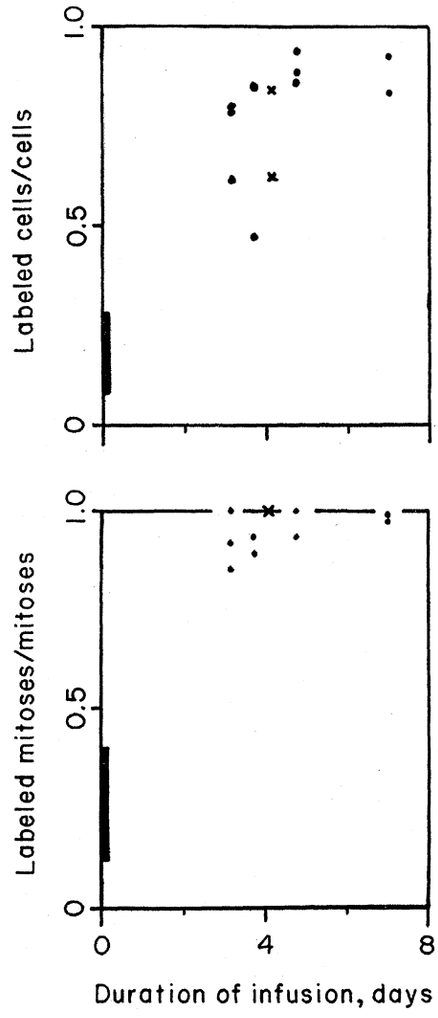

Fig. 2. Autoradiographic counts of C3H breast tumors labeled with tritiated thymidine. The bars on the *y*-axis represent the distribution of results after a single injection. Each entry within any one graph represents a single autochthonous tumor. The *x* refers to tumors fixed immediately after infusion.

either the mean intermitotic time or the doubling time of the tumor. Strictly speaking, it may not be possible to label every cell of a tumor regardless of how long the exposure is prolonged. The significance of nonproliferating tumor cells is an open question, depending on whether or not these cells still have the potential to proliferate and repopulate the tumor after therapy. In any case, the therapist is faced with the prospect of delaying the definitive step in tumor therapy while the tumor literally doubles before his eyes (*4*).

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References and Notes

1. M. L. Mendelsohn, F. Curtis Doban, Jr., Hugh A. Moore, *J. Natl. Cancer Inst.* **25**, 477 (1960).
2. M. L. Mendelsohn, *ibid.* **25**, 485 (1960).
3. ———, *Science* **132**, 1496 (1960), abstr.
4. This investigation was supported by grant No. C-3896 from the National Cancer Institute, U.S. Public Health Service. It was described in abstract in *Radiation Research* **14**, 485 (1961). The technical assistance of William Jenkins and Allan Reiskin is gratefully acknowledged.

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Food Production by Submerged Culture of Plant Tissue Cells

Abstract. In investigating the possibility of producing food by large-scale culture of plant tissue, several plant tissues were used. The yield for carrot tissue, which is especially capable of rapid growth, was an average of 4.6 grams per liter per day in 6 liters of medium. The results suggest that with improved techniques food could be produced by this method.

Within the past few years considerable progress has been made in the design of systems for accelerated food production. The goal of much of this work is to establish the requirements for a practical method of feeding men under unusual survival conditions or, ultimately, during prolonged space travel.

Our work (*1*) was undertaken as an exploratory study to determine the feasibility of producing food by tissue-culture techniques. That large amounts of plant material may be obtained by growing specific tissues of higher plants in liquid media has been established (*2*). The large-scale culture of plant tissue for food, however, has not been previously studied, which is rather surprising in view of the potentials of a successful system of this type.

Table 1. Yields of carrot tissue from submerged cultures in 6 liters of broth.

Culture No.	Days cultured (No.)	Age of inoculum (days)	Yield (wet weight)		
			Total (g)	g/lit.	g/lit. day
1	6	7	167.1	27.8	4.6
2	6	19	160.4	26.7	4.4
3	7	35	167.2	27.8	3.9
4	5	2	183.5	30.6	6.1
5*	5	2	122	20.3	4.0

* Malt extract used in place of coconut milk.

In seeking to discover the specific requirements for a large-scale food production system we used cultures of normal tissues of carrot and potato and of the stems of tomato, rose, grape, and tobacco (*3*). These tissues have been maintained on 0.6-percent agar "tobacco" medium at *p*H 5.7 to which coconut milk (15 percent by volume) and α-naphthaleneacetic acid (0.1 mg/lit.) were added. "Tobacco" medium without agar was used for the liquid cultures (*4*). All of the tissues grew well in submerged cultures, but, since the carrot tissue appeared to be especially capable of rapid growth, it was selected as the principal tissue for this study.

To scale-up liquid cultures to large volume we used Erlenmeyer flasks (300 ml), then Fernbach flasks (3 liters), and finally Florence flasks (12 liters). The inoculum for the largest flasks was prepared by transferring a piece of tissue from the agar to 100 ml of liquid medium in a 300-ml Erlenmeyer flask. This culture was agitated on a rotary shaking machine. When sufficient growth occurred (1 to 3 weeks), the contents were transferred to a Fernbach flask containing 1 liter of medium. This culture was also agitated on a shaker. After another period of growth, 200 to 300 ml of the culture were inoculated aseptically into 6 liters of medium in a 12-liter flask. The tip of a 100-ml volumetric pipette was cut off before sterilization to facilitate this transfer of tissue. To aerate and agitate the culture during growth, the flask was fitted with sterile tubing, by which compressed air, sterilized by passage through a 2-inch pipe packed with sterile cotton and two Seitz filter pads, could be injected into the flask. The pipe was connected to a compressed-air vent with a pressure regulator. Aseptic techniques were followed in all the procedures.

At first, cultures grown in the large flasks were frequently contaminated with mold and bacteria. To control this contamination without inhibiting the growth of tissue, antibacterial Tylosin (20 parts per million) (*5*) and antifungal Mycostatin (25 units) (*6*) were added to the liquid medium before inoculation. These two antibiotics—now used routinely in our tissue cultures of carrot, grape stem, and Queen Elizabeth rose stem—have produced no toxic effects.

The cultures on the shaking machine were incubated at 28°C while the culture in the 12-liter flask was of necessity subjected to the variations of room temperature. It is assumed that a constant optimum temperature would increase the growth rate and the yields of tissue.

The yields of tissue from five large-volume cultures are shown in Table 1. Our method of large-volume culture did not provide for removing any of the tissue or for supplementing the medium. Consequently, the tissues were harvested after 5 to 7 days in culture because heavy growth almost stopped agitation by the aeration system.

Inocula from a young, actively growing culture contributed to an improved yield and decreased the lag phase of growth in the large flask (culture No. 4). Culture No. 5, with malt extract in place of coconut milk, was inoculated at the same time as No. 4 with material from the same Fernbach flask. By the use of a Y-tube the two 12-liter flasks were aerated simultaneously.

Our yields from 6 liters of medium averaged 4.6 g/lit. per day. They are slightly higher than the yields obtained by Tulecke and Nickell from cultures of *Gingko*, Holly, and *Lolium* tissue grown in 10 liters of medium in 20-liter carboys (3.1 g/lit. per day). Their pilot-plant tank cultures of rose stem gave a higher yield (9.7 g/lit. per day). However, they say this figure is subject to interpretation, since the culture period was only 2 days and a heavy inoculum was used in 134 liters of medium.

We feel that these yields are not indicative of the best results that could be obtained under better conditions of