phosphate. Thus myokinase activity remained slight under these conditions with this muscle (*8*). Although the individual changes are small and the variance between the left and right muscles of each pair is relatively high, it is clear from taking together all the various conditions used (Table 1) that stretching an activated muscle caused a reduction in the breakdown of ATP ($P < .001$), even though the tension developed was about 70 percent greater than the isometric tension at rest length. Thus, the changes in series B and D were less than in A and C, respectively, and in series E and G the reduced breakdown of ATP left more ATP and less inorganic phosphate in the stretched muscles than in their pairs which had been stimulated isometrically for the same length of time. The value of P can be calculated both from the distribution of the signs of the observed changes or by combining the probabilities of the individual results by the method of Fisher (*14*), taking account of the signs of the differences.

The reduction in ATP breakdown was about half, and thus net resynthesis certainly did not occur; otherwise, on the basis of series A, C, and H, the changes in ATP and Pi contents, respectively, would have been +0.3 and −0.3 μmole/g in series F, zero in series B and D, and +0.66 and −0.66 μmole/g in series E and G.

This conclusion that the breakdown of ATP is reduced by stretching an activated muscle is in apparent contradiction to the recent results of Aubert and Maréchal (*15*) who found an increased breakdown at 0°C of phosphorylcreatine in iodoacetate-treated sartorius muscles of the frog which were stretched several times when activated. However they had used rapid stretches and obtained results quite similar to ours with slower rates of stretch comparable to that used by us (*16*).

Our results indicate that the net ATP (phosphorylcreatine) breakdown found previously (*8, 12, 17*) really is associated with work and not with a function of developed tension × time. The stretched muscles in Table 1 absorbed an average of 400 g-cm of work per gram. A comparison of the ATP requirements for positive work (*9*) and for this negative work shows that under these conditions the ATP usage during negative work was only about 1/13th that required during positive work. This is in good agreement with the finding that the extra oxygen needed in man for negative work can be less than 1/10th that needed for positive work (*18*).

These results are relevant to the conclusion of Hill and his colleagues concerning the reversal of the chemical processes of muscular activity during negative work (*1–4*). If their conclusion is correct and applicable to sartorius muscles previously treated with FDNB, and if ATP were the direct final energy source for muscle contraction, then it should have been resynthesized. This did not occur; so it seems that ATP is, after all, not the direct final energy source for muscular contraction. The finding of a reduction of ATP breakdown, but not a resynthesis in these experiments was predicted by a recent molecular theory of muscle contraction (*19*) which is based on the view that ATP is used to extend potential linkages between myosin and actin, which in the presence of calcium, form actual linkages, contract, and do work by the energy of hydrogen and hydrophobic bond formation to make α-helices, this process being repeated cyclically during muscular contraction. Stretching an activated muscle should thus break hydrogen bonds and reduce the breakdown of ATP which has now been observed.

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Amphibian Cell Culture: Permanent Cell Line from the Bullfrog (Rana catesbeiana)

Abstract. *A line of fibroblast cells has been established from tongue tissue of the bullfrog* (Rana catesbeiana). *The cells are near-triploid and were subcultured 57 times during the 2⅔ years of their existence. Some of their characteristics are described.*

Among vertebrate classes permanent cell lines have been established from mammals, birds, and teleosts. The sole report concerning repeatedly subcultured amphibian cells is that of Freed (*1*), who cultured frog cells through at least ten passages. Our report describes a permanant frog cell line which is over 2⅔ years old and is now in its 57th passage.

Line FT was initiated in July 1961 from bullfrog (*Rana catesbeiana*) cells obtained by a primary cultivation of a macroscopically normal-appearing tongue from an adult female. Tissue was dispersed by treatment with trypsin in the cold for 16 hours, and original cultures were established at 20°C in medium having a freezing point of −0.57 to −0.61°C. Methods and media were those (*2*) used for cold-blooded vertebrates. While these procedures are entirely appropriate for fresh-water teleosts, we subsequently found that our results with amphibian cells were better when solutions and media measured

had a freezing point between –0.45 to –0.5°C.

Cells which attached and proliferated were dominantly fibroblast-like, but ciliated cells were also present and active throughout the first week in culture. During the 3rd and 4th weeks, subcultivations were made, but the number and quality of cells declined, and it was feared that the culture would be lost. Reduction of osmotic pressure was associated with renewed vigor. Auclair (*3*) had prepared original cultivations of frog renal cells in mammalian-type medium diluted with water. We used NaCl-free Earle's balanced salt solution (BSS) to dilute media 15, 25, and 35 percent. Tongue cells responded favorably to all dilutions, but the better results were obtained at the two greater dilutions and after the *p*H was adjusted to between 7.6 and 7.8.

Cells grew in a variety of commercial media intended for mammalian cells, but the osmotic pressure of such media was reduced by dilution. The medium of choice had a freezing point of –0.5°C and consisted of 25 percent NaCl-free Earle's BBS, 10 percent whole egg (chicken) ultrafiltrate, 10 percent fetal bovine serum, and 55 percent Eagle's minimal essential medium (*4*).

Up to 1000 units each of penicillin and streptomycin and 50 units of nystatin per milliliter could be used, but for routine purposes media contained 100 or 200 units each of penicillin and streptomycin per milliliter. Stock cultures have been propagated on antibiotic-free media since the 14th subcultivation—or for the last 22 months.

Subcultivations have been made by scraping, or preferably by treatment for 5 to 7 minutes with a cold solution of the disodium salt of EDTA (20 mg/100 ml), and then followed by immediate neutralization of the EDTA with growth medium.

The FT cells have been incubated at 4°C, and at intermediate temperatures up to and including 37°C, but they did not survive direct transfer from 25°C to 37°C, and 25°C is considered nearly optimum. This temperature is slightly higher than that used by Shah (*5*), but it is the same as that used by Auclair (*3*) and Freed (*1*). Using the acid dichromate method of Bailey and Meymandi-Nejad (*6*) to measure oxidizable carbon, we determined the growth of FT cells at different temperatures. Seed stock for this work was grown at 25°C. Replicate tubes were seeded with approximately 14,000 cells per milliliter and incubated overnight at 25°C before being transferred to incubators at the various temperatures.

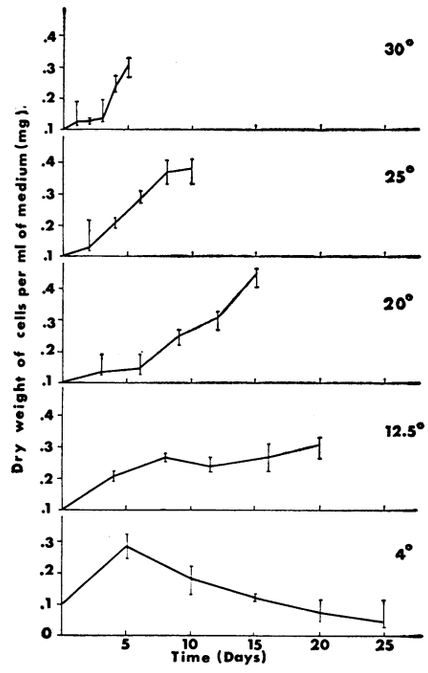

Fig. 1. Growth at different temperatures of FT cells from the tongue of a bullfrog (*Rana catesbeiana*). Curves represent mean oxidizable carbon as determined with an acid dichromate reagent. The range of three determinations for each point is shown by the vertical lines.

Following a schedule based upon preliminary results, triplicate tubes of cultured cells were taken from each incubator at regular intervals, and biochemical determinations were made. As shown in Fig. 1, a gradation in metabolic activity occurred. The data obtained from cultures at 4°C were seemingly aberrant, but daily observation showed progressive loss of cells from the glass thus explaining the "loss" of oxidizable carbon. The curve at 4°C proved reproducible, and when declining cultures were returned to incubation at 25°C the cells returned to normal activity and repopulated the available surfaces. A similar recovery was noted in cultures returned to 25°C after being kept at 12.5°C for 8 months without handling. Survival over longer periods was not tested.

At 25°C newly seeded cells attach to the glass, resume mitosis, and form a monolayer. Unless dense cultures are divided, cell sheets soon show contraction, cells slough, and varying degrees of necrosis ensue. The usual reduction in *p*H aggravates the situation, but in spite of such destructive changes, cells in mitosis can usually be found in lightly populated areas. Viable cells usually survive several months under such adverse conditions of progressively lower *p*H and crowding, and they can be recovered by dispersing in fresh medium. The degenerative changes can be avoided by dividing confluent cultures before contraction and sloughing occur.

The FT cells are readily cultivated in hard glass tubes or bottles and in soft glass prescription bottles. Unless they are suitably vented, the *p*H of cultures in Leighton tubes (16 by 85 mm) decreases causing the cells to contract and slough.

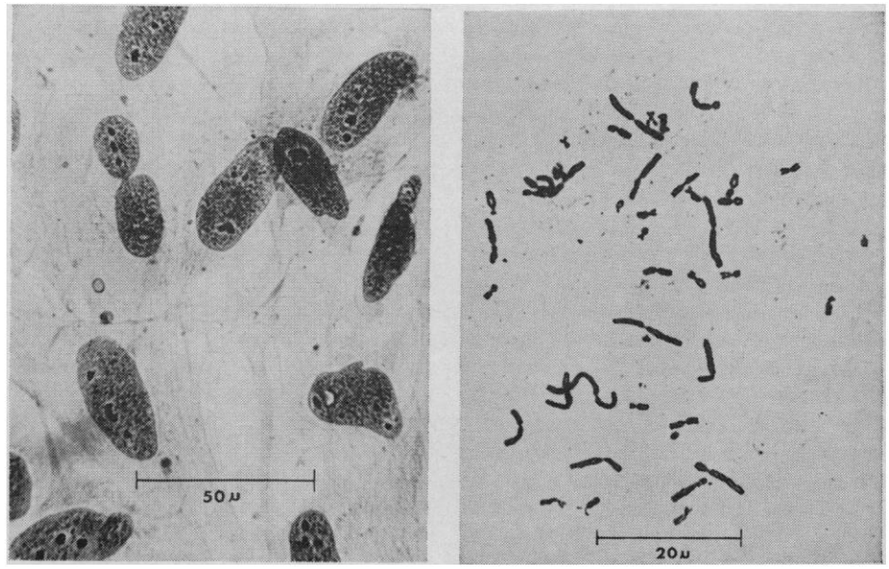

Fig. 2 (left). Cells of permanent line of bullfrog (*Rana catesbeiana*) tongue fibroblasts in 51st subcultivation. (Right). Chromosome spread from a cell in comparable passage shows 42 chromosomes, the modal number. The 2N number for this species is 26.

Attachment of cells in mitosis has been particularly tenuous. Colchicine solutions (0.4 μg/ml) appeared to intensify dislodging and this necessitated modified methods of chromosome counting to prevent loss of mitotic cells. Colchicine was added to media in tubes showing active mitosis; they were incubated for 2.5 hours, then each received an equal volume of NaCl-free Earle's BSS.

After 15 minutes at 25°C, the cells were scraped free and concentrated by centrifugation at 200*g* at 4°C for 10 minutes. The medium was smoothly decanted, two drops of aceto-orcein were added with mixing, and squash preparations were made immediately. This procedure prevented loss of dislodged mitotic cells.

With colchicine, a modal number of 42 chromosomes was found among 65 cells in 46th to 51st passage. The range of chromosome numbers was 24 to 79, the mean being 44, and 70 percent of the counts being in the group 44 ± 3. Since the diploid number for this species is 26, the cells are near-triploid (Fig. 2). The mean for 15 counts made without colchicine was 42, and this was considered ample evidence for intrinsic heterploidy, the usual condition found in established cell lines.

The FT cells are large, having a mean diameter of 25 microns for spherical cells, with the range being 10 to 42 microns. They do not support growth of IPN virus, the agent of infectious pancreatic necrosis of trouts (*7*), and this feature has been used as part of a method for presumptive identification of this virus.

The FT cells are readily preserved by freezing at −80°C, but they have not been cloned.

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Plasmodium berghei: Cyclical Transmissions by Experimentally Infected Anopheles quadrimaculatus

Abstract. *A number of strains of* Plasmodium berghei *were isolated from sporozoites of* Anopheles dureni. *Laboratory-bred* Anopheles quadrimaculatus *fed on carriers of the newly isolated strains showed overwhelming midgut infections and moderate or mild salivary gland infections. Successive cyclic transmissions by the bite of experimentally infected* A. quadrimaculatus *in laboratory-bred tree rats* (Thamnomys surdaster) *were carried out.*

Within the biocene of certain forest galleries of Upper Katanga in the Congo, a sylvatic mosquito, *Anopheles dureni* Edwards, transmits *Plasmodium berghei* (*1*) among tree rats (*Thamnomys surdaster*).

Sporozoites in the salivary glands of wild-caught *A. dureni* were recorded by Vincke and Lips (*1*), and Vincke (*2*) in 1946. These sporozoites obtained in mosquito dissections during the rainy season (December to March) served for the isolation of a number of strains of *P. berghei* in the last 15 years. However, all these plasmodial strains were induced by syringe inoculation of harvested sporozoites. No cyclical transmission by the direct bite of a naturally or experimentally infected *A. dureni* has ever been recorded. The few successful transmissions of *P. berghei* with other unrelated anopheline species originated from sporozoites of crushed mature oocysts inoculated into experimental rodent hosts (*3*).

We now report successive cylical transmission of *P. berghei* in the laboratory by the bite of experimentally infected *Anopheles quadrimaculatus.*

During the period from the 20 December 1963 to 3 January 1964, 2300 engorged *A. dureni* were caught in the forest gallery of Kisanga in the vicinity of Elisabethville. The mosquitoes were kept at 22°C in small cages within cooling containers with the relative humidity at 90 percent. These climatic conditions resemble those of the natural environment at the height of the transmission season and of mosquito infections in nature. The *A. dureni* were brought by plane to our laboratories in New York and kept under similar conditions in an insectary (*4*). Dissection of the weak and dying mosquitoes revealed a high incidence of infection, there being 24 percent oocysts in the midguts (81 positive of 334 dissected) and 10.5 percent sporozoites in the salivary gland (35 positive of 334 dissected).

Twelve new strains of *P. berghei* were isolated from these sporozoites by intraperitoneal inoculations into laboratory-bred *Thamnomys* (*5*), Syrian golden hamsters, young albino rats, and white mice. The plasmodial strains, each derived from different naturally infected *A. dureni,* were designated as N.K. (New York-Kisanga) strains, maintained by blood transfer and preserved at low temperatures (*6*). Incubation periods, from sporozoite inoculation to the appearance of parasites in the blood, varied from 72 hours to 8 days in the infected animals.

Batches of laboratory-bred *A. quadrimaculatus* were allowed to feed on mouse number M2293 on the third day of its patent blood infection after inoculation of the sporozoites from *A. dureni* (N.K.$_1$). The engorged mosquitoes were kept at 22°C. Mature oocysts developed within 8 days after the infective blood meal, but sporozoites in the salivary glands were encountered only from the 13th day onward. Of 36 dissected *A. quadrimaculatus,* 28 (77 percent) showed very heavy (200 to 400) oocyst midgut infections, 12 (33 percent) showed light or medium salivary gland infections.

The remaining 21 mosquitoes were divided into two groups; they were allowed to engorge on two laboratory-bred *Thamnomys,* BTR-65 and BTR-67. Parasitemia ensued in both these animals, 4 and 7 days respectively, after the bite. Three further successive cyclical transmissions by the bite of infected *A. quadrimaculatus* in laboratory-bred tree rats have thus far been carried out. These cyclical passage strains were designated as N.K.$_4$BI, N.K.$_4$BII, N.K.$_4$BIII, and N.K.$_4$BIV.

Another sporozoite isolated strain (N.K.$_1$) has been successfully transmitted through three cyclic generations