We isolated DAGE and TG from the liver by thin-layer chromatography (*3*) and established the purity of each fraction (*3, 4*). The following specific gravities (water at 25°C) were obtained: DAGE, 0.908 at 25°C and TG, 0.922 at 25°C. Though these differences are not great, 1 g of DAGE gives 14 percent more lift in seawater (specific gravity 1.026) than 1 g of TG.

The difference in the specific gravities of the two classes of lipids encouraged us to study the changes in the ratios of DAGE to TG that would result from an artificial increase in the body weight of experimental animals. Thirteen male dogfish *Squalus acanthias*, caught in Puget Sound in July, were maintained for 2 days in circular tanks 2 m in diameter and 1.3 m deep. The water in the tanks was exchanged and aerated continuously. The fish were 80 to 90 cm long and weighed 2.7 to 3.4 kg. Lead weights (114 g) were suspended between the pectoral fins of seven fish selected at random. They were then returned to the tank with the unweighted fish. After 50 hours, all the fish were killed, and the livers were excised. Each liver was homogenized in a blender for 10 minutes. The resulting emulsion was centrifuged at 8°C for 30 minutes at 8000 rev/min. The DAGE were separated from TG by thin-layer chromatography. The layers were then charred with chromic-sulfuric acid, and the spots representing each glycerolipid were analyzed by photodensitometry (*5*). Peak areas were measured to establish the ratios of DAGE to TG (Table 1).

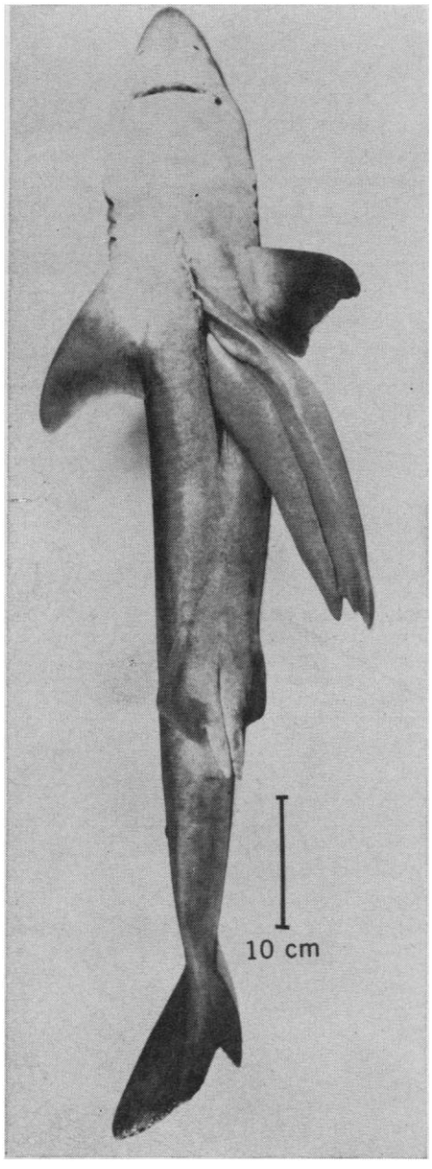

Fig. 1. Dissected dogfish *Squalus acanthias* illustrating the large liver in comparison to the body size.

Triglycerides predominate in the livers of each unweighted fish, whereas the proportion of DAGE is significantly higher in each weighted fish. We postulate, therefore, that the ratios of DAGE to TG in the weighted group are increased to offset the increase in body weight. No correlation exists between the ratios of DAGE to TG and the weights of the fish, the liver weights, and the percentage of oil. Individual anatomical features might contribute substantially to the variations observed (*6*).

We postulate that a regulatory mechanism, involving the selective metabolism of DAGE and TG, is used by *Squalus acanthias* in the maintenance of neutral buoyancy during vertical migrations. This regulatory mechanism may serve as a substitute for the commonly found gas-filled swim bladder.

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References and Notes

1. F. Snyder, in *Progress in the Chemistry of Fats and Other Lipids* (Pergamon Press, Oxford, 1969), vol. 10, part 3, p. 287.
2. M. Tsujimoto and Y. Toyama, *Chem. Umsch.* **29**, 27 (1922).
3. D. C. Malins, J. C. Wekell, C. R. Houle, *J. Lipid Res.* **6**, 100 (1965).
4. D. C. Malins, *ibid.* **9**, 687 (1968).
5. ———, in *Progress in the Chemistry of Fats and Other Lipids* (Pergamon Press, Oxford, 1966), vol. 8, part 3, p. 343.
6. E. D. S. Corner, E. J. Denton, G. R. Forster, *Proc. Roy. Soc. Ser. B Biol. Sci.* **171**, 415 (1969).
7. G. W. Snedecor, *Statistical Methods* (Iowa State College Press, Ames, 1956), p. 90.

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Animal Cells: Noncorrelation of Length of G_1 Phase with Size after Mitosis

Abstract. *The interval between mitosis and initiation of DNA synthesis (G_1) varied over a fourfold range for Chinese hamster ovary cells, an established line. This was not because of size differences. Synchronous cells of different sizes began DNA synthesis at similar times after mitosis. A novel technique of centrifugation for separating cells according to size is described.*

Individual cells of synchronous mitotic mammalian cultures proceed through the mitotic cycle with different rates (*1*). Cells obtained by shaking monolayers growing in glass bottles (*2*) are initially more than 90 percent in metaphase, and yet they enter the DNA synthesis phase of the cycle (S) between 3 and 12 hours after division (Fig. 1). Thus, highly synchronous mitotic cells exhibit G_1 phases which vary over more than half of the cell cycle. The remainder of the mitotic cycle appears to vary less, the time of DNA synthesis through mitosis ($S + G_2 + M$) being relatively constant for many mammalian cell lines (*1, 3*). Thus the variation in G_1 may account for much of the variation in intermitotic times.

The duration of G_1 might vary due to genetic, nutritional, or cell volume fluctuations within the population. We have examined the possibility (*4, 5*) that individual cells have G_1 durations dependent on initial cell mass. Velocity sedimentation of synchronous cells in early G_1 through Ficoll density gradients yields fractions of cells which differ in volume. These fractions are grown on glass cover slips and are exposed briefly to tritiated thymidine early and late during the period of DNA synthesis. Autoradiography of these cells indicates that there is no close relation

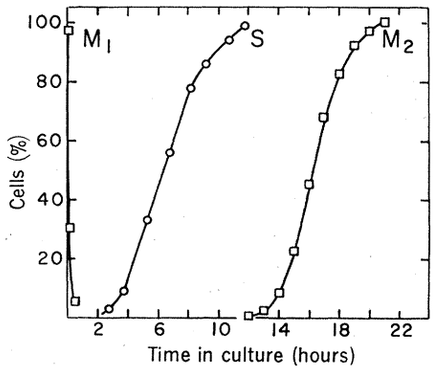

Fig. 1. Cells in M_1, S, and M_2 phases. Cells in first and second mitoses were determined by counting mitotic figures of chromosomes. Cells that have entered S were assayed by counting cells with nuclear grains in autoradiography after continuous incubation with tritiated thymidine.

between initial cell volume and length of G_1.

If a cap (Fig. 2) with a small hole through the center of its radial axis is inserted into a tube, then a cell sample can be layered within a smaller area than the entire width of the tube. Cells radiating from this hole will not have paths intersecting with the walls, and thus the cells will not stick to the walls (*6*). Small numbers of mammalian cells can be centrifuged without significant loss and with less damage to the cells.

Chinese hamster ovary (CHO) cells (*7*), obtained from D. F. Petersen and R. A. Tobey (Los Alamos Scientific Laboratory), were grown in Ham's F-10 medium (*8*) supplemented with penicillin and streptomycin (Grand Island Biological Co.) at concentrations of 100 units and 100 μg per milliliter, respectively, and with fetal calf serum (5 percent) and calf serum (10 percent) (Baltimore Biological Laboratory). Cells were grown with $2 \times 10^{-2} M$ Hepes buffer (Calbiochem) without a special CO_2 atmosphere. No contamination with pleuropneumonia-like organisms was detected by tests in two independent laboratories nor in our own routine tests.

The method of harvesting synchronous mitotic cells by shaking is basically that of Tobey *et al.* (*2*). The medium added for shaking cells and for subsequent incubations was a 1 : 1 mixture of fresh medium with conditioned medium that had been over the growing cells to be shaken for 24 hours. Conditioned medium was used to minimize possible synchronizing effects of fresh medium (*9*). Synchronous mitotic cells were incubated at 37°C for 30 minutes to allow cells to complete mitosis. Disruption by pipetting was necessary to separate daughter cells. These cells were centrifuged 5 minutes at 800 rev/min, resuspended, and layered on gradients in 0.2 to 0.5 ml of Ham's F-10 medium.

Density gradients were prepared with Ficoll (Pharmacia, molecular weight about 400,000) in F-10 medium that contained serum. Cells centrifuged through Ficoll in F-10 without serum did not attach to cover slips nor did they grow. Usually about 2×10^5 cells were layered in the adapter cap of the Ficoll gradient (Fig. 2) and were centrifuged 16 minutes at 900 rev/min (International size 2). High concentrations of cells could not be sedimented without considerable clumping.

Cell samples were obtained from layers of the gradient by siphoning with a capillary inserted through the cap to a depth of about 2 cm in the tube. Samples of 0.5 to 1 ml were usually diluted 1:1 with F-10 medium and placed in 5-ml beakers with glass cover slips at the bottom. The beakers were placed in small plastic cups mounted at the top of a rubber adapter in a swinging bucket and were centrifuged 10 minutes at 1000 rev/min (Sorvall GLC-1). The solution was then removed by pipette, and medium was added for incubation. The same procedure was used to prepare cover-slip cultures of the dilute solutions of synchronous cells obtained by shaking, so that it was not necessary to sediment the cells to concentrate and replate them. The cells on cover slips were cultured and later removed and prepared for autoradiography.

The early dose of ^{3}H-thymidine was from 4 to 6 hours in two experiments and from 3 to 5 hours in the third experiment. Labeling was nearly the same for both small and large cells. A

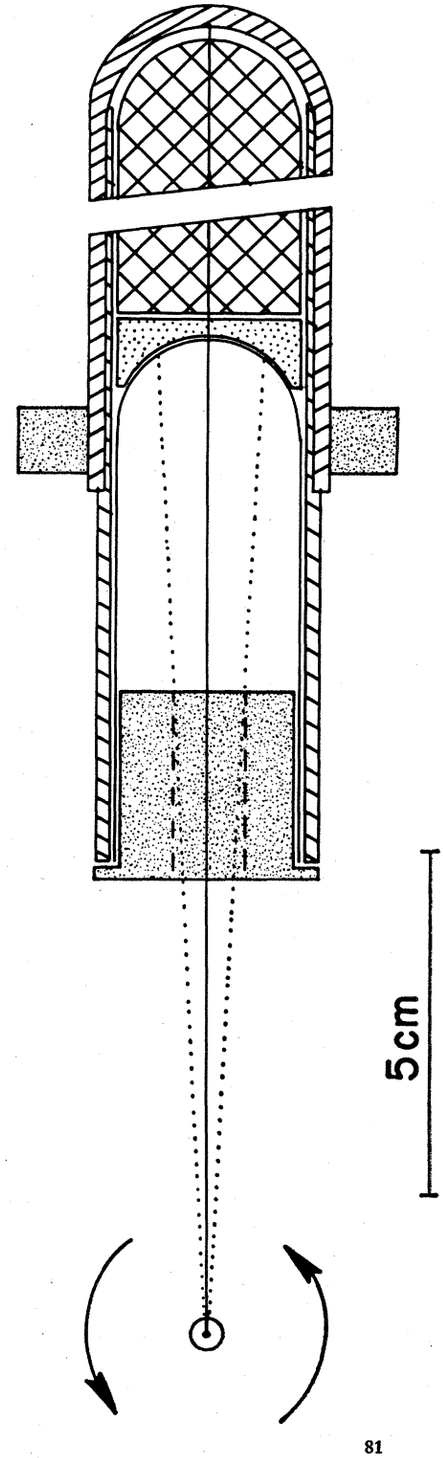

Fig. 2. Aluminum adapter cap and extension sleeve for density gradient centrifugation with swinging buckets. To obtain maximum cell separation by decreasing the initial distance of the cells from the center of rotation of the centrifuge (*14*), tubes were placed in aluminum holders. The holders were also built to support the adapter cap inserted in the tube. A Lucite plug and rubber base support the bottom of the cellulose nitrate tube. A 24-ml linear Ficoll gradient (10 to 20 percent) was prepared in a cellulose nitrate tube (2.5 by 7.5 cm, Beckman). Just above this volume was placed the aluminum cap with a hole bored through its center. A 5 to 10 percent gradient was then formed to fill the hole to within about 1 cm of the top of the cap. Cells were layered in the hole of the cap. The arrangement gave complete recovery of sedimented cells whereas most cells stuck to the tube walls due to collision (*6*) when the cap was not used. Dotted lines indicate the limiting radial paths of cells during sedimentation.

small decrease in labeling was noted for the smallest cells; in a fraction representing less than 10 percent of the total cells a decrease of two-thirds was observed. Therefore there was a small dependence on size, but no large correlation between cell size and length of G_1 (Fig. 3).

The late dose of ^{3}H-thymidine was from 8 to 10 hours in two experiments and from 9 to 11 hours in the third experiment. No significant differences in labeling were detected for the cells of different sizes (Fig. 3).

The early dose was timed so that only large cells would have become labeled if a correlation between G_1 and size existed. Likewise, a decrease in labeling of large cells might have been seen in the late dose. The absence of large differences in labeling patterns between large and small G_1 cells suggests that, for these cells, only a small correlation may exist between cell volume and length of G_1. The large heterogeneity in length of G_1 (Fig. 1) therefore is not primarily due to variations in cell size.

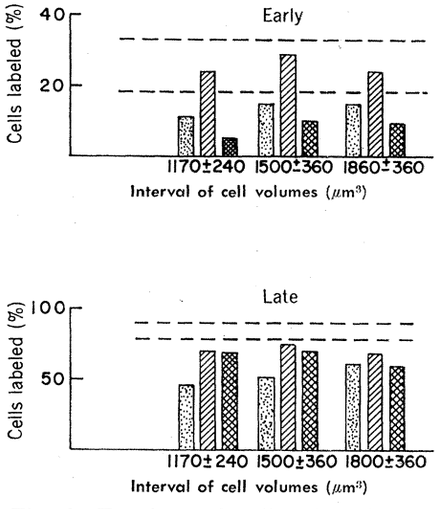

Fig. 3. Fractions of cells of each size which are in S. Shadings indicate individual experiments. Each bar represents blind counts of at least 400 cells. Dotted lines indicate the labeling of unfractionated cells for the experiments with highest and lowest labeling. Sizes of rounded cells prior to attachment to glass and flattening out were estimated by photographing cells and weighing paper cutouts of their profiles. These areas were converted to absolute volumes based upon an approximate measure of the cell diameters. Volume intervals are expressed as the average, plus or minus the standard deviation. Sized early G_1 cells were exposed for 2 hours during early and late S with 2 to 3 μc of thymidine-[methyl-^{3}H] per milliliter (New England Nuclear, final specific activity, 0.64 to 0.96 μc/nmole). After each dose of labeled thymidine, the cells were washed with cold calcium- and magnesium-free phosphate-buffered saline containing 1 mmole of unlabeled thymidine and fixed with an acetic acid, ethanol mixture (1:3). Cover slips were dried, mounted face up on slides with Permount (Fisher), and dipped in Kodak NTB-2 emulsion. These were developed after 2 to 7 days of exposure and were stained with Giemsa.

Killander and Zettterberg (*4*, *5*) have suggested that a correlation does exist between the mass of a cell after mitosis and duration of its G_1 phase. They observed a smaller variation in mass of mouse L cells at early S phase than immediately after division. Furthermore, they found a large variation in age of the early S-phase cells (*4*). This suggested a critical mass for S and the possibility that G_1 of small cells would last longer than G_1 of large cells in order to reach this mass. In another investigation (*5*), different populations were derived from the same cell line. Populations with smaller average masses after mitosis had longer average G_1 periods. These two experiments only indirectly suggest a correlation between duration of G_1 and cell size. The first results are statistical, and the second results are for cells of different populations with presumably different genetic compositions (*5*). Our experiments with CHO cells do not support this hypothesis.

Petersen and Anderson have studied synchrony in wild-type and clonal CHO cells (*10*). Because they found no large differences, they concluded that genetic dispersion in the population could not account for the observed dispersion of growth rates. They and their colleagues (*11*) also concluded from mathematical considerations that cell volumes alone could not account for experimental growth patterns. Thus, both cell size and genetic contents do not account for the observed heterogeneity in cell growth. Nutritional limitations may account for the dispersion, as in *Tetrahymena* (*12*), but this factor is difficult to test with mammalian cells.

Two further hypotheses might account for variable duration of G_1. Cells might normally enter a G_0 state for variable periods between mitosis and G_1 (*13*). The G_0 state is proposed for nondivision but potential proliferation. The capacity to enter G_0 might be lost in more malignant cells, as suggested by greater desynchrony of CHO than of HeLa cells (*10*). The second hypothesis accounts for heterogeneity among daughter cells by asymmetric distribution of scarce cellular components which are unequally segregated at random, and not in parallel with total cell mass. Components associated with nuclei might be in this class, and their quantity within cells might determine growth rates.

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References and Notes

1. P. González and R. M. Nardone, *Exp. Cell Res.* **50**, 599 (196→ M. D. Enger, R. A. Tobey, A. G. Saponara, *J. Cell Biol.* **36**, 583 (1968).
2. R. A. Tobey, E. C. Anderson, D. F. Petersen, *J. Cell Physiol.* **70**, 63 (1967).
3. J. E. Sisken, in *Cinemicrography in Cell Biology*, G. G. Rose, Ed. (Academic Press, New York, 1963), p. 143.
4. D. Killander and A. Zetterberg, *Exp. Cell Res* **38**, 272 (1965).
5. ———, *ibid.* **40**, 12 (1965).
→ C. W. Boone, G. S. Harell, H. E. Bond, *J. Cell Biol.* **36**, 369 (1968).
7. J. H. Tjio and T. T. Puck, *J. Exp. Med.* **108**, 259 (1958).
8. R. G. Ham, *Exp. Cell Res.* **29**, 515 (1963).
9. G. Todaro, Y. Matsuya, S. Bloom, A. Robbins, H. Green, in *Growth Regulating Substances for Animal Cells in Culture*, V. Defendi and M. Stoker, Eds. (Wistar Inst. Press, Philadelphia, 1967), p. 87.
10. D. F. Petersen and E. C. Anderson, *Nature* **203**, 642 (1964).
11. E. C. Anderson, G. I. Bell, D. F. Petersen, R. A. Tobey, *Biophys. J.* **9**, 246 (1969).
12. D. M. Prescott, *Exp. Cell Res.* **16**, 279 (1959).
13. O. I. Epifanova and V. V. Terskikh, *Cell Tissue Kinet.* **2**, 75 (1969).
→ T. G. Pretlow, II, and C. W. Boone, *Science* **161**, 911 (1968).
15. We thank J. R. Mycock and R. J. Pariser for technical contributions and D. D. Cunningham and E. Robinson for assistance. Supported by USPHS grant AI-04409. T.O.F. is an NSF predoctoral fellow.

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Sex Control by Bees: A Voluntary Act of Egg Fertilization during Oviposition

Abstract. *The alfalfa leaf-cutter bee,* Megachile rotundata, *stops abdominal contractions briefly during oviposition of female eggs but not during oviposition of male eggs. Sperm stored in the spermatheca probably is pumped onto the micropyle of the egg during this pause. The stimulus inducing fertilization seems to be associated with the depth of the nesting tunnel.*

Bees and other Hymenoptera control the sex of their offspring through arrhenotokous parthenogenesis. With some Hymenoptera, egg fertilization apparently is a random process, but in others, fertilization appears to be controlled by the female. In the honey bee, unfertilized eggs resulting in males are