interband readings were above 30 percent. As with the filaments, the direction of minimum intensity is parallel to the chromosome axis, within ± 5 degrees. Considering the size of the effect and the direction, the results are consistent with the interband regions of polytene chromosome, being a parallel array of uncoiled DNA. Presumably, in the band and puff regions, the DNA filaments are so tangled and looped as to preclude observation of any dominant orientation (*6*).

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6. The trypsin-treated chromosomal filament bundle stains "brighter" than the intact chromosome (Figs. 1 and 2). This may be due to the masking of DNA binding sites by nuclear protein, a phenomenon which may in itself prove of interest in studying the association of DNA and protein. However, both absorption and emission by acridine orange occur at wavelengths at which the major cellular constituents are quite transparent. Therefore the optical properties of nuclear protein should not affect the determination of DNA configurations, a problem that arises in techniques involving birefringence and ultraviolet dichroism. For a review of earlier studies of chromosome structure by these latter techniques, see M. Alfert, *Int. Rev. Cytol.* **3**, 142 (1954).
7. We thank Drs. H. Swift and R. Zirkle for discussions, and J. Costa and J. Hanacek for skilled machine work. Supported by PHS grant CA 02739. J.W.M. is a predoctoral trainee in biophysics, PHS grant 5 T1 GM 780; and R.B.U. is a recipient of a PHS Research Career Development Award.

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Synchronization of Mammalian Cells with Tritiated Thymidine

Abstract. *Short exposures of mammalian cells to tritiated thymidine of high specific activity destroys the proliferative capacity of mammalian cells. Since the killing is limited to cells that have synthesized DNA in the presence of the labeled compound, an exposure duration of less than one generation can yield a synchronized population.*

The potential usefulness of synchronized or partially synchronized mammalian cells for the investigation of various cellular properties has long been recognized, and several different techniques have been used to produce such populations (*1–7*). In several of these the cell was subjected to nonphysiological conditions for a certain period, and this may very well have resulted in alteration of the processes being studied in the synchronized population. For example, synchronization achieved by fluorodeoxyuridine (FUdR) treatment and subsequent reversal with thymidine (TdR) leads to conditions of unbalanced growth (*2, 3*). The method originated by Terasima and Tolmach (*4*) and later modified by Sinclair and Morton (*5*) and Robbins and Marcus (*6*) does not appear to subject the cells to nonphysiological conditions, but the yield of cells which can be easily obtained is limited. In this report we outline an alternative method of producing large populations of partially synchronized populations of L-cells based on the use of tritiated thymidine (H^3TdR).

The incorporation of H^3TdR into the DNA of mammalian cells can destroy their capacity for continuous proliferation (*8*). Therefore it seemed possible to produce a synchronized population of mammalian cells through the use of H^3TdR of high specific activity. The basis of the method can be understood with reference to Fig. 1. Immediately upon addition of the label to an L-cell population in which the durations of G_1, the phase prior to DNA synthesis was 5 hours; of S, the DNA synthetic phase, 8 hours; of G_2, the phase following DNA synthesis, 4 hours; and the mitotic phase M, 1 hour, all cells which were actively synthesizing DNA (that is, S-phase cells) should immediately take up the label. If the label were left in the culture for a period of 3 hours, then cells would flow from G_1 into S and become labeled, and labeled cells should flow from S into G_2. If the exposure to the H^3TdR were extended to 6 hours, then all of S, G_2, M, and part of G_1 would be filled with labeled cells, and the only unlabeled cells would be those few in a narrow phase interval at the end of G_1. If the concentration of H^3TdR and the specific activity were sufficient, then all of the labeled cells should have lost proliferative capacity, and the only viable cells in the population would be those few cells at the end of G_1. If at this time a large quantity of unlabeled TdR were added, then the cells at the ends G_1 would not take up label, and they would remain viable, constituting a partially synchronized population.

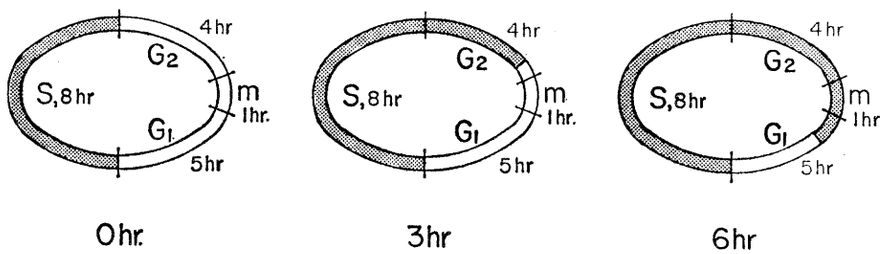

Fig. 1. Scheme of the cycle of L60T cells, showing the fraction of the cycle occupied by labeled cells after a short exposure, a 3-hour exposure, and a 6-hour exposure to H^3TdR.

For the method to be successful it must be possible to incorporate sufficient radioactivity into the cell in a short time to bring about the loss of proliferative capacity, and it must be possible to terminate immediately the uptake of labeled thymidine by the addition of unlabeled thymidine. Also the amounts of H^3TdR or of TdR must not affect cells in the population which have not incorporated the labeled material. Our experiments indicate that for mammalian cells in vitro all of these conditions can be satisfied.

We used L60T cells, a subline of L cells adapted to growth in a medium containing no exogenous thymidine, but capable of using an exogenous source when supplied (*3*). They were grown in suspension culture in spinner flasks at 37°C in growth medium 1066T—, consisting of 1066 (Connaught Medical Research Laboratories)

(*9*), with the thymidine and coenzyme concentrate omitted, but containing 10 percent horse serum. Under these conditions the cells have a generation time of between 15 and 18 hours. Colony counts were made in plastic petri dishes (*10*) in medium 1066 supplemented with 10 percent horse serum and 10 percent fetal calf serum. Cell counts were obtained with a Coulter counter (*11*). Tritiated thymidine (6.7 c/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). The techniques used for autoradiography have been described (*12*). For the determination of H^3Tdr incorporation, cells were filtered with suction on a 0.45-μ Millipore filter. The filter was washed twice with 5 ml of 1066, twice with 5 ml of cold trichloroacetic acid, twice with 5 ml of cold alcohol, and then thoroughly dried and placed, cell side up, on the bottom of a counting vial containing 15 ml of liquid scintillation fluid (0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene; 6 g of 2,5-phenyloxazole; 1 liter of toluene).

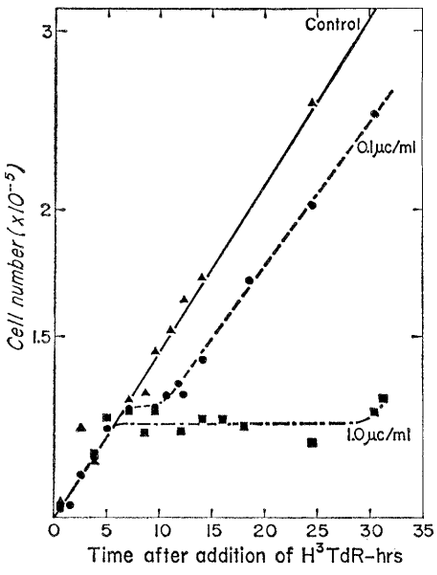

Fig. 2. The growth curves of L60T populations in 1066T— medium, and when exponential growth had been established (10^5 cells/ml), supplemented with H^3TdR (0.1 and 1.0 μc/ml).

To assess the amounts of H^3TdR required to inhibit proliferation of L-cells, growth was measured in different concentrations of H^3TdR (Fig. 2). Cell counts were made on these cultures at intervals. In the presence of 0.1 μc of H^3TdR per milliliter, growth was approximately the same as in the control culture for a period of approximately 7 hours; then there was a short lag followed by a period of logarithmic growth, the number of cells being doubled in approximately 21.5 hours. In the presence of 1.0 μc/ml, growth was similar to that of the control for 5 to 6 hours and then ceased.

The experiment described in Fig. 2 indicated that the addition of 1.0 μc of H^3TdR per milliliter prevented the continuous proliferation of L cells. It would be expected that the extent of this inhibition would be increased as the period of contact between the H^3TdR and the cells increased. This was examined by measuring the decrease in number of cells capable of forming colonies after exposure to H^3TdR for varying intervals. For this purpose, H^3TdR (1.0 μc/ml) was added to a culture of cells at time zero, and at intervals thereafter portions of cells were removed, diluted at least 100-fold, and plated in 1066 plus serum. One, four, and eight hours after the addition of H^3TdR large portions of cells were removed from the population, and 100 μg of TdR per milliliter was added. This concentration of TdR effectively prevented the uptake of H^3TdR but had no detectable effect on the subsequent growth of the cells, an observation in contrast to those of Puck (*13*) and Bootsma *et al.* (*14*) who found that

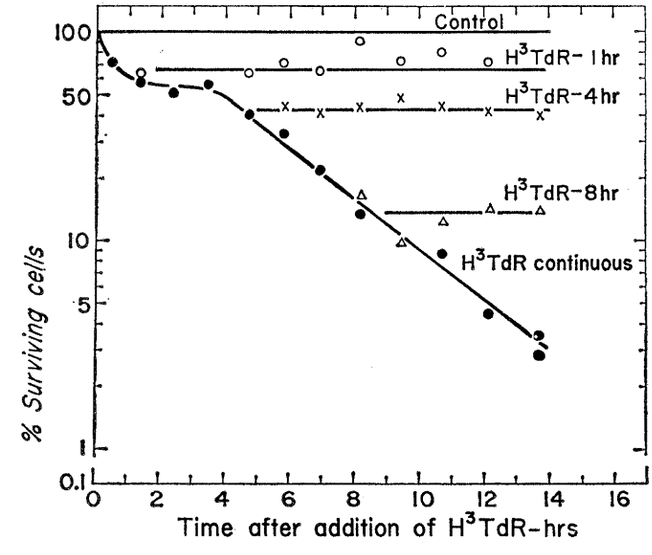

Fig. 3 (left). The percentage of surviving L60T cells as a function of time in a culture exposed to 1.0 μc of H^3TdR per milliliter. The curves designated H^3TdR -1 hr, -4 hr, and -8 hr had TdR added after 1, 4, and 8 hours of exposure to H^3TdR. The curve designated H^3TdR continuous was exposed to H^3TdR over the duration of the experiment.

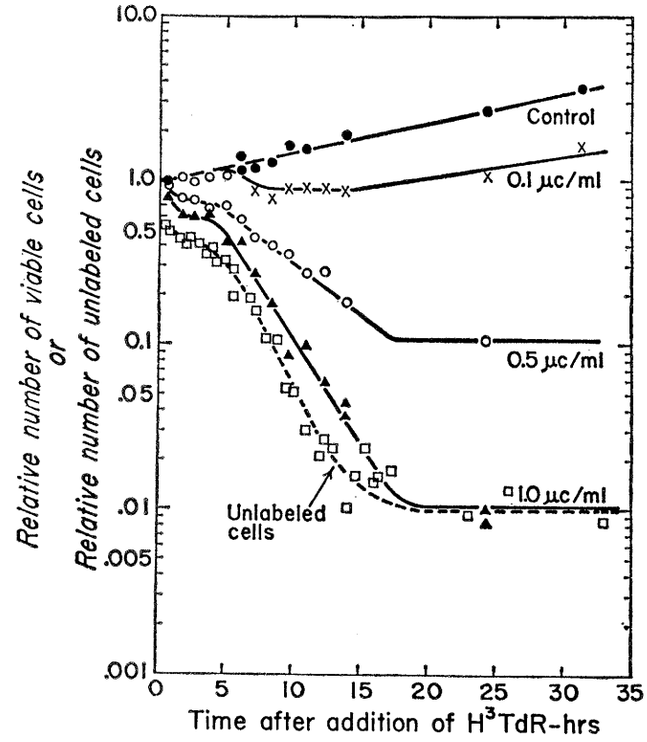

Fig. 4 (right). The solid curves show the relative number of colony-forming cells as a function of time in cultures exposed to 0, 0.1, 0.5, and 1.0 μc of H^3TdR per milliliter. The dashed curve shows the relative number of unlabeled cells in these same populations. The data used to construct the dashed curve was obtained from all three labeled cultures.

high concentration inhibited cell growth. At intervals after the addition of TdR, samples were removed from these portions and plated (Fig. 3). Within 1.5 hours after addition of the label the fraction of surviving cells fell from 100 percent (actual plating efficiency, 62 percent) to 55 percent. The survival remained constant at this value for about 2 hours and then began to drop exponentially, reaching a value of 3 percent 14 hours after the addition of H^3TdR. In the samples to which the TdR was added 1, 4, and 8 hours after the H^3TdR it is apparent that the number of cells killed decreased as soon as the unlabeled precursor was added.

The shape of the survival curves (Fig. 3) can be explained as follows. In the first minutes after the addition of the label all of the cells in the S phase would be rapidly killed, and this should result in a precipitous drop in plating efficiency. However, during the next 4 hours, the duration of the G_2 phase, the killing of cells as they moved from G_1 into S would be partially balanced by the division of cells as they moved from G_2 into mitosis. Finally G_2 would be depleted of cells capable of division, and then the number of viable cells in the population should decrease rapidly.

If the killing of cells is due only to incorporated H^3TdR then there should be a close correspondence between the number of cells which incorporate label and the number which lose proliferative capacity. To examine this question we have attempted to compare the number of cells in the population which have lost proliferative capacity with the number of labeled cells. For this, separate subcultures, derived from the same parent culture immediately before the beginning of the experiment, were exposed to H^3TdR at concentrations of 0, 0.1, 0.5, and 1.0 μc/ml. At intervals thereafter portions were removed from each culture, and the number of viable cells was determined by assaying for colony formation, and the number of labeled cells was determined autoradiographically (Fig. 4).

Since there was no detectable difference between the values obtained for the three concentrations, no attempt was made to separate the data. Immediately upon addition of the label approximately 50 percent of the cells were unlabeled and by 15 hours less than 1 percent of the cells were unlabeled. It can also be seen that the curve of unlabeled cells appears to lead the curve of surviving cells in the population given 1.0 μc/ml by about 30 to 60 minutes. This suggests that while a concentration of 1.0 μc/ml is sufficient to kill every cell in the population, if exposed for a sufficient length of time, a cell may have to incorporate H^3TdR for about 30 to 60 minutes in order to accumulate a lethal burden of H^3TdR. It is likely that if the concentration in the culture had been raised above 1.0 μc/ml, then the lag time between the curve of unlabeled cells and the curve of surviving cells could have been reduced.

Having determined that large amounts of H^3TdR with high specific activity would result in almost immediate loss of proliferative capacity of cells it was now possible to test whether synchrony could be achieved by this method. For this purpose H^3TdR (1.0 μc/ml) was added to two cultures. Three hours after this addition, TdR (100 μg/ml) was added to one culture, and 3 hours later it was added in the same amount to the other culture. At intervals after the addition, portions of cells were removed from each population, and the number of surviving cells was determined by colony assay. The data indicate that in both populations division began about 12 hours after the addition (Fig. 5). The fact that this 12 hours is equal to the sum of G_2 and S also indicates that the progression of the unlabeled cells around the cycle was not affected by either the H^3TdR or TdR concentrations used. In both populations the duration of the wave of division was somewhat longer than would have been predicted from a knowledge of the duration of the cell cycle. This was probably due to a combination of two factors, (i) a generation time slightly longer than the predicted 18 hours, and (ii) the fact that the cell phases are not the same length for every cell in the population so that there is a progressive loss of synchronization with time. However, the curves in Fig. 5 do indicate that it is possible to obtain a useful degree of synchronization. After a 6-hour exposure to H^3TdR approximately 30 percent of the cells in the population are viable, and almost all of these will divide during the first wave of division.

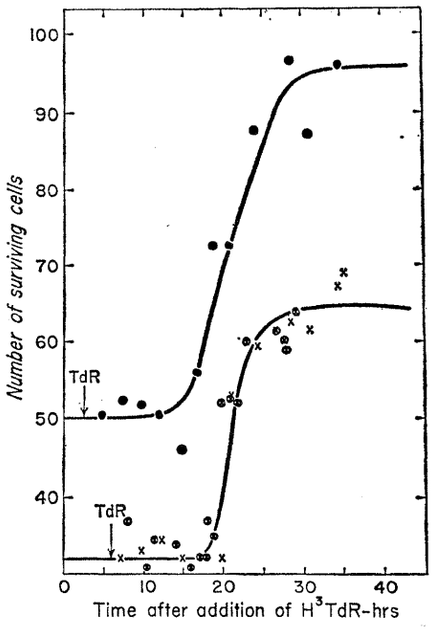

Fig. 5. The relative number of colony-forming cells as a function of time in cultures exposed to H^3TdR (1.0 μc/ml) with TdR (100 μg/ml) added after 3 or 6 hours.

Our data suggest that a method of cell killing due to the incorporation of large amounts of H^3TdR can be made to yield a population of mammalian cells having a useful degree of synchronization of cell division. Furthermore it would appear that the amount of H^3TdR required is such that only those cells which have incorporated the drug will be damaged and that there is little, if any, damage produced by emitted radiation in cells which have not incorporated the label. Also it appears that the amounts of TdR required have little or no effect on the population. The principal advantages of the technique are that it is technically easy and that it can produce large synchronous populations without subjecting the synchronized fraction of the population to conditions which are likely to bring about unbalanced growth or to conditions which are likely to damage the test population in any other way.

The principal disadvantage of this method of synchronization is that the synchronized population is always associated with a large population of cells, which, while they are incapable of indefinite proliferation, may still be capable of carrying on other functions. This means that it is not possible to use the technique for most biochemical studies.

Up to now the method has been

used to investigate the response to ionizing radiation (*7*) and ultraviolet light (*15*) of cells irradiated in various parts of the cell cycle and to determine the capacity of cells to repair sublethal radiation damage as a function of their age in the cell cycle (*7*).

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Thermoregulation in a Brooding Female Indian Python, Python molurus bivittatus

Abstract. *At varying environmental temperatures, measurements of body temperatures and gas exchange of a female Indian python* (Python molurus bivittatus) *show that during the brooding period this animal can regulate its body temperature by physiological means analogous to those in endotherms. Ambient temperatures below 33°C result in spasmodic contractions of the body musculature with a consequent increase in metabolism and body temperature.*

Internal heat production and temperature regulation in the Indian python during incubation have been suspected since 1832 when Lamarre-Picquot read a communication before the French Academy in which he stated that the python, after laying eggs, coils about them and produces sensible heat as an aid to incubation (*1*). A committee of the French Academy rejected Lamarre-Picquot's statements as being "hazardous and questionable." Later observations in zoological gardens appeared to support the original observations (*2, 3*), but the lack of adequate thermometers and the failure to consider all external sources of heat cast doubt on these earlier studies. Temperature measurements by one of us (H.G.D.) on brooding pythons in the New York Zoological Park in 1960 and 1961 showed conclusively that these snakes were able to maintain, for extended periods, body temperatures up to 7.3°C higher than the substrate or ambient air temperatures and that the maintenance of this temperature differential was correlated with the rate of spasmodic contractions of the body musculature (*4*).

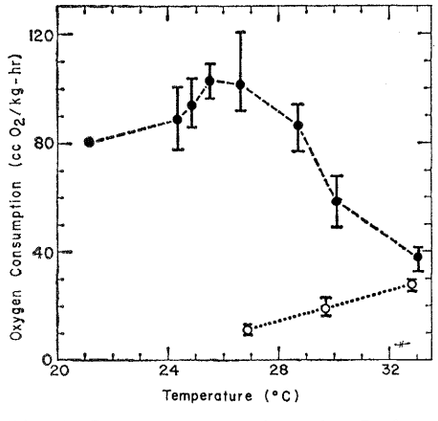

Fig. 1. Oxygen consumption of a *Python molurus* at different ambient temperatures. Upper curve: animal during brooding. Lower curve: the same animal during nonbrooding periods. Vertical lines, range; circles, means.

About 15 February 1965 a 14.25-kg, 2.7-m Indian python (NYZP specimen No. 630514) laid 23 eggs, all of which later proved to be infertile. The eggs with the snake coiled about them were transferred to a respiration chamber located in a temperature-controlled (± 1°C) room on 18 February. Oxygen consumption and carbon dioxide production were recorded constantly in an open circuit system by a Beckman Model F3A3 Paramagnetic Oxygen Analyzer and a Model 15A Infrared Carbon Dioxide Analyzer (*5*). Temperatures were recorded from copper-constantan thermocouples taped to the skin at several points so that some of the thermocouples would lie between tightly appressed coils of the snake. Spasmodic contractions of the body musculature were counted visually. The animal was allowed to acclimate (as evidenced by a new steady rate of gas exchange and new level of body temperature) for at least 48 hours after each change in the temperature of the room before data were taken.

The female remained coiled around the eggs for a period of approximately 30 days. We placed the same individual in the apparatus 40 days after the completion of the brooding period; data taken during this period were used for "nonbrooding" values. After oviposition and the incubation period the animal weighed 10.34 kg, a decrease in weight of 27.4 percent. Calculations of oxygen consumption were based on the weight of the snake at 14.25 kg during brooding; during nonbrooding, 12.37 kg.

The oxygen consumption of the nonbrooding python was characteristic of an ectothermic animal, decreasing with decreasing temperature (Fig. 1, lower curve); but the oxygen consumption of

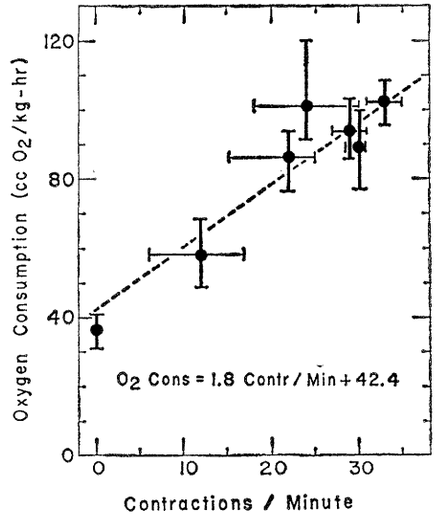

Fig. 2. Correlation of the rate of spasmodic body contractions with the rate of oxygen consumption in a brooding Indian python. Dashed line and regression equation calculated by method of least squares. Vertical lines, range of oxygen consumption; horizontal lines, range of contraction rate; circles, means.