A KINETIC ANALYSIS OF MYOGENESIS IN VITRO

MICHAEL C. O'NEILL and FRANK E. STOCKDALE

From the Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

Conditions which yielded reproducible growth kinetics with extensive, relatively synchronous differentiation are described for chick muscle cultures. The effects of cell density and medium changes on the timing of cell fusion were examined. Low-density cultures which received a change of medium at 24 hr after plating show the highest rate of cell fusion, increasing from 15 to 80% fused cells in a 10 hr period. These optimal culture conditions were employed to reexamine two questions from the earlier literature on muscle culture: (a) can cells which normally would fuse at the end of one cell cycle be forced to go through another cell cycle before fusion; and (b) how soon after its final S period can a cell complete fusion? In answer to the first question, it was found that if the medium is changed, many cells which would otherwise fuse can be made to undergo another cell cycle before fusion. In the second case, radioautographs were made from cultures incubated with tritiated thymidine for various times at the beginning of the fusion period. These show labeled nuclei in myotubes as early as 3 hr after the beginning of the incubation period. This indicates that cells can fuse as early as the beginning of the G_1 period, and suggests that there is not an obligatory exit from the cell cycle or a prolonged G_1 period before cell fusion and differentiation during myogenesis.

INTRODUCTION

The embryonic skeletal muscle culture system has long been recognized as having potential as a model system in development (Holtzer, 1961; Konigsberg, 1963). Muscle cells differentiate in culture and can be maintained in the differentiated state. Terminal differentiation is marked by cell fusion and specific protein synthesis and, as is the case in vivo, DNA synthesis ceases with the end of the S period preceding fusion (Lash et al., 1957; Firket, 1958; Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Herrmann et al., 1957).

Thus, in a period spanning only a few hours the cell makes the transition from a relatively undifferentiated, albeit determined, dividing myoblast into a part of the nondividing, very highly specialized syncytial myotube. Despite the sharpness of this transition, delineated by substantial morphological and biochemical changes, culture conditions have not always permitted the true potential of the system to be realized.

The primary reason for this lies in the "mixed" nature of these cultures. This problem has often been described in terms of the inevitable presence of a large percentage of nonmyogenic "fibroblasts" in the muscle cultures. This problem can be minimized to the point that the fibroblast level remains below 10% of the initial population. Nonetheless, the cultures remain "mixed" in another sense. The asynchrony of fusion in mass culture results in a mixture of cells in two states—those cells which have undergone terminal differentiation through fusion into myotubes, and those which have not and remain as dividing myoblasts. Terms such as

"rapid fusion" and "sudden onset of fusion" notwithstanding, the common experience under normal conditions has been cultures in which the cells fuse over a period of several days (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; and Yaffee, 1971; however, see Coleman and Coleman, 1968). Fused cells (myotubes) in a culture, therefore, differ widely with respect to the time at which they form. In kinetic studies, this asynchrony either obscures or altogether precludes the measurement of the events preparatory to or resultant upon fusion.

In this paper, we describe conditions which yield highly reproducible growth and fusion kinetics. As much as 70–80% of the cells fuse within a single generation time. The effects of cell density and feeding on the timing, rate, and extent of fusion were examined. The controlled kinetics reported here have enabled us to reexamine critically the evidence for certain conclusions about differentiating muscle cells. We find that the kinetics of cell fusion suggest that there is not a final cell cycle before fusion with a prolonged G_1 period distinguishable from other G_1 periods, and that cells which would otherwise fuse after a particular cell cycle may undergo another cell cycle.

MATERIALS AND METHODS

Cultures

Fertilized eggs of White Leghorn stock chickens were obtained from Kimber Farms (Niles, Calif.) or from Donsing Hutchery. Cultures were prepared according to the following procedure, with the use of cells from 11 day chick embryo thigh or 12 day breast muscle. (No differences could be detected between cells from the two sources.) The dissection was performed at room temperature in sterile Hanks' solution. The tissue was cleaned of connective tissue and minced to a size of 1–2 mm³. The Hanks' solution was then removed, and 4 ml of 0.1% trypsin (Difco Laboratories, Detroit, Mich., 1:250) in Saline G was added. The tissue was dispersed in the trypsin and then placed into a 37°C incubator for 30 min. At the end of this period, 5 ml of complete medium (described below) was added. The tissue was pipetted repeatedly with a Pasteur pipette, with the tip kept below the liquid level and the rate of expulsion kept low enough to avoid bubbles and frothing. Then the cells were pelleted by centrifugation in an International Clinical Centrifuge (International Equipment Company, Needham Heights, Mass.) for 5 min at 500 g. The cells were resuspended in complete medium with repeated pipetting, were repelleted, and resuspended again. The cell suspension was filtered through a double nylon cloth filter (Nitex, Tobler, Ernst, and Traber, Inc., New York, 10 μ pore size). The cells were "preplated," as described below, after the filtration step. They were then counted in a hemocytometer and diluted to the final nominal concentration for plating. The cells were plated on 60-mm Falcon Plastics tissue culture plates (Div. B-D Laboratories, Inc., Los Angeles, Calif.) which had been treated with collagen. Collagen treatment consisted of washing the plates with a collagen solution and allowing them to dry in air. The collagen solution is made by autoclaving 0.5 mg of collagen (Worthington Biochemical Corp., Freehold, N.J.) per ml of distilled water followed by centrifugation for removing the residue. The medium for these experiments was MEM (Grand Island Biological Co., Grand Island, N.Y.), horse serum (Grand Island Biological Co.), and embryo extract in the ratio 88:10:2. Embryo extract was prepared sterile by passing decapitated 11-day embryos through a 20 ml syringe and diluting 1:1 with MEM or Hank's solution. This mixture was stored frozen until use. Before use, the extract was centrifuged at 760 g for 10 min. The supernatant constitutes embryo extract.

In our experience, collagen is essential for good differentiation in culture (Hauschka and Konigsberg, 1966). Collagen from several different sources and several different methods of preparation were all found to be effective (Sigma Chemical Co. collagen was an exception). Failure to obtain a high level of differentiation is almost always due to the horse serum, as pointed out by others (Kaighn et al., 1966). Changing serum lots often changes cell fusion kinetics. Several serum lots must be screened in order to find one that is satisfactory.

The process of "preplating" the cells is a standard routine in our laboratory. This is a modification of the method suggested by Kaighn et al. (1966) and Yaffee (1968). After filtration, the cells are plated at concentrations up to 8 $\times$ 10⁶/ml in a volume of 8 ml of complete medium on a collagen-coated 100 mm dish and incubated at 37°C for 10 min. After this incubation, the cells remaining in the supernatant are counted and plated. As noted by the authors cited above, the cells identified morphologically as fibroblasts settle and attach more rapidly than myoblasts. Under our conditions, three-fourths of the 10–20% of the cells remaining unfused after the peak fusion period appear to be fibroblasts. This selective procedure reduces the proportion of these cells markedly, allowing maximum fusion levels of 85% without substantially lowering the yield. The densities reported in the figure legends are densities computed from actual cell counts on the dish at

the times indicated. These densities are reported in preference to nominal plating densities because of possible variations in plating efficiency as a function of density and other variables. The actual density at 15 hr after plating (very little cell division occurs before this time) was generally found to be 40–50% of the nominal plating density.

Thymidine Labeling

Where tritiated TdR (thymidine-methyl-^{3}H, 20 Ci/mmole) or 2-thymidine-^{14}C (Schwarz Bio Research Inc., Orangeburg, N.Y.; 48 mCi/mmole) was used for pulse labeling for 1- or 2-hr periods, it was added to rinsed dishes in 1 ml of MEM at a concentration of 0.5, 1, 2, or 5 μCi/ml as noted in the particular legend. For longer labeling periods, the label was added in 3 ml of complete medium, either 1 or 5 μCi/ml.

If the cultures were to be used for radioautographs, they were rinsed several times with Hanks' solution at the end of the incubation period and then fixed with Bouin's solution. Otherwise, the cells were harvested with 0.1% trypsin and precipitated with cold 0.5 N perchloric acid (PCA). The precipitate was successively pelleted in a table top centrifuge and washed three times with 0.5 N PCA. A nucleic acid hydrolysate was made by heating the precipitate in 0.6 ml of 0.5 N PCA for 15 min at 100°C. Of this, 0.05 ml was counted in 15 ml of Bray's scintillation fluid (Bray, 1960) in a Nuclear-Chicago Mark 1 counter (Nuclear-Chicago, Des Plaines, Ill.). An aliquot of the remaining extract was used for the DNA measurement where such measurement was made.

DNA Measurement

The DNA measurements were made on hot PCA extracts of two or more cultures, depending on the density. The Keck modification of the Cerriotti reaction was used for all DNA measurements; however, the amyl acetate extraction step in this procedure was omitted (Keck, 1956). The total assay volume was 0.8 ml. All samples were read at 460 mμ and 530 mμ in addition to the 486 mμ maximum so as to check for nonspecific absorption. A Gilford Model 240 spectrophotometer (Gilford Instrument Company, Oberlin, Ohio) was used.

Cell Counts

Unless otherwise specified, cell counts were made on cultures fixed with Bouin's solution and stained 3 min with Delafield's hematoxylin solution (Matheson, Coleman, and Bell, Cincinnati, Ohio). For each point, at least four fields containing a minimum of 400 cells or myotube nuclei were counted. The average count was about 1000 cells and/or nuclei/point. Random fields were counted. Overstaining was the only ground accepted for the rejection of a field for scoring. Counts were made at a magnification of 625 with a Zeiss microscope. The area of a 60 mm plate (51 mm I.D.) is 2020 mm^2; approximate conversion of cells per plate to cells per square millimeter is, therefore, obtained by dividing the former figure by 2000.

Radioautography

After fixation with Bouin's, the cultures were rinsed several times with water. Then, the bottoms of the dishes were punched out, halved, and attached to slides with Band-Aid "sheer strip" tape. After dipping in Kodak NTB II emulsion, which was preheated to 38°C, the slides were stored in a slide box with Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) at 4°C until development. The slides were developed for 10 min in ice-cold Kodak D-19 developer, washed with water, and fixed 10 min with Kodak fixer, washed, and stained for 3 min with Delafield's hematoxylin.

RESULTS

Kinetics of Cell Fusion

Fig. 1 shows the fusion kinetics of cultures at four different cell concentrations plated at different times over a 2 month period. It can be seen that the time of initiation, the rate, and the extent of fusion are highly reproducible. The conditions used in these cultures (low density with a change of medium at 24 hr after plating) produce the most rapid rate of fusion we have been able to obtain (Figs. 2–4). A plot of the rate of fusion often shows a biphasic pattern (inserts, Fig. 1).

Fig. 1 shows that the onset of rapid fusion is abrupt, and we wished to determine if the process terminated with equal sharpness. Tritiated thymidine was added to cultures which had already reached maximum fusion (48 hr) at 53, 64, 77, and 89 hr, and the cultures were all fixed at 113 hr. Radioautographs were made, and the percentage of myotube nuclei which were labeled was scored for each interval. The result is shown in Table I. Approximately 25% of the nuclei in myotubes at 113 hr after plating fuse subsequent to the main fusion period. A composite curve (Fig. 5) of the rate of accumulation of nuclei in myotubes shows that most of the fusion which is to occur takes place in a single burst spanning but a single generation time.
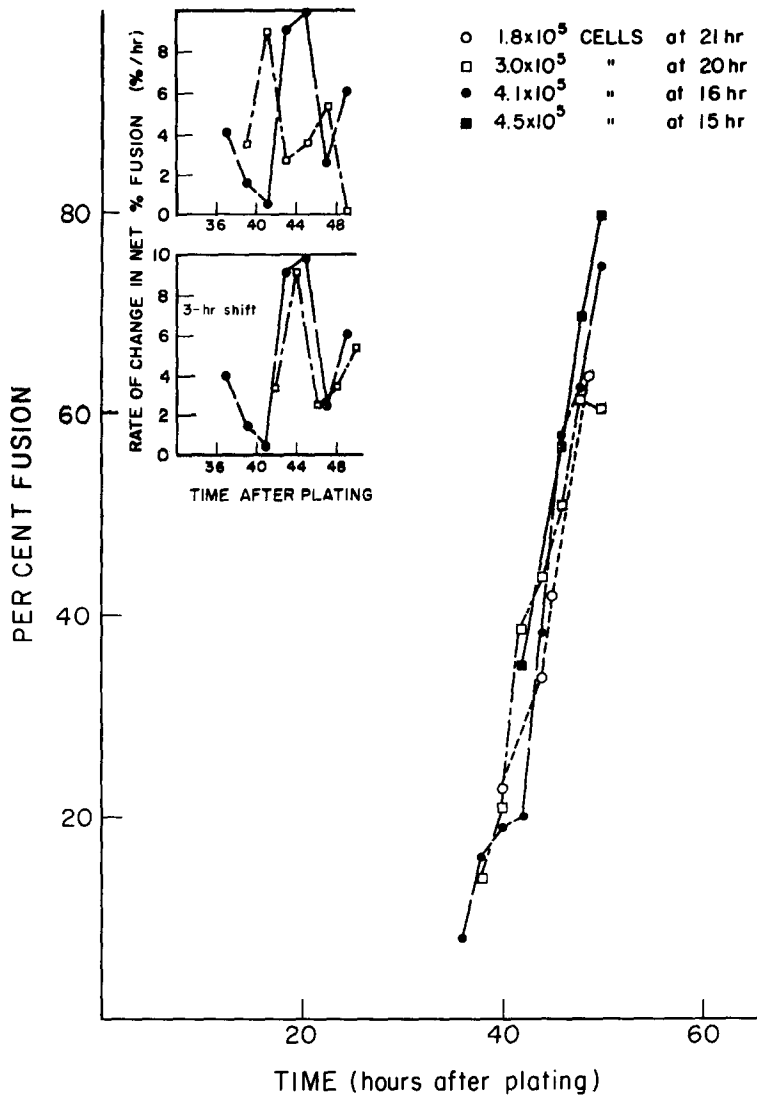
FIGURE 1 Fusion kinetics of several different series of low-density cultures with a change of medium at 24 hr. The cultures were grown as described in Methods. The curves represent four different platings at intervals totaling 2 months. The cell densities stated in the figure were computed from cell counts of the living cultures made at the times indicated; a Wild M40 inverted microscope with phase optics, × 300, was used. The medium was replaced with fresh medium 24 hr after plating. The inserts show the derivative rate curves for the cultures represented by the solid circles and the open squares. In the lower figure of the inserts, an arbitrary time shift of 3 hr has been executed on one of the curves (squares) to show that, once phased, the patterns are very similar.

Effects of Medium Change

Fig. 6 shows how fusion is affected by changing cell density and feeding schedules. These curves represent a set of cultures made from the same cells plated over a density range of 0.35–5.2 ×

10^6 cells. The medium on these cultures was not changed. It can be seen, by comparing the curves of low-density cultures of this experiment with those of Fig. 1, that not changing the medium 24 hr after plating affects both the time of initiation and the rate of fusion. The cells in cultures without
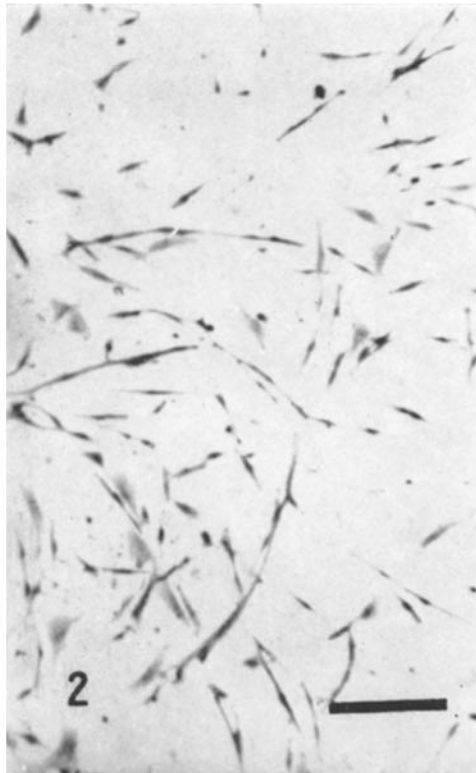
FIGURE 2 This culture was fixed at 36 hr after plating. The density at 15 hr was 6×10^5 cells per 60 mm dish. The medium was changed at 24 hr. Scale marker is 150 μ. $\times$ 105.

FIGURE 3 A higher magnification of the same culture shown in Fig. 2. Very little fusion has occurred. Scale marker is 30 μ. $\times$ 285.

FIGURE 4 A sister culture from the same plating as that in Figs. 2 and 3. This culture was fixed 12 hr later, at 48 hr. Extensive fusion has occurred during the intervening period of rapid fusion. Scale marker is 75 μ. $\times$ 185.
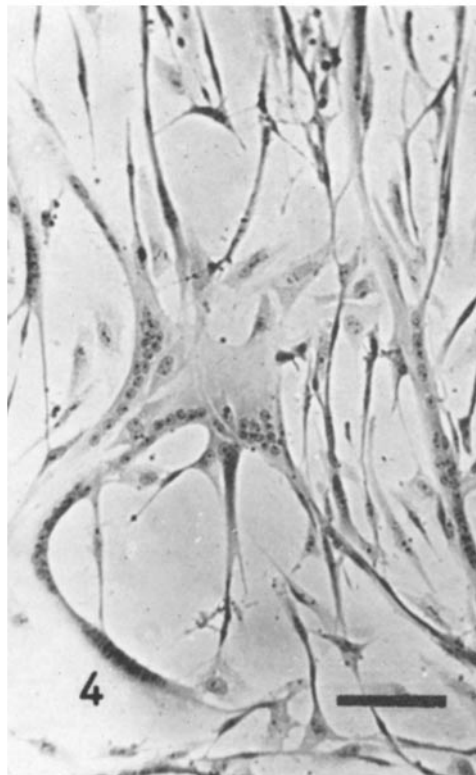
a medium change begin to fuse 8–12 hr earlier and at a slower rate than those which received a change of medium. The maximum extent of fusion, however, is not related to whether or not the medium is changed. The lower rate of fusion in the cultures without a medium change is compensated for by a longer period of fusion.

As might be expected, the effect of medium changes is not independent of cell density. When the density is raised to a level twice the highest density used in Fig. 1, the delay in the initiation of

TABLE I

Kinetics of Fusion Subsequent to the Primary Fusion Period

Time and length of labeling period	Total No. of myotube nuclei labeled	Calculated net increase in fusion
	%	%
53 to 113	25.5	
64 to 113	14.3	
77 to 113	2.83	
89 to 113	0.42	
53 to 64		10.9
64 to 77		11.5
77 to 89		2.4

Cultures were labeled with thymidine-^{3}H at 53, 64, 77, and 89 hr and then incubated until all were fixed at 113 hr. Radioautographs were prepared and scored for the per cent of myotube nuclei which were labeled corresponding to each interval. Differential values were calculated.

fusion due to a medium change is substantially reduced or eliminated (Fig. 7). Here again, however, the fusion rate is biphasic for the fed cultures but not for the unfed cultures.

Effects of Cell Density

Fig. 8 A shows that cells plated at different densities begin to behave differently during their first generation in culture. In this experiment, radioautographs were prepared from cultures pulsed for 1 hr with tritiated thymidine at later and later times of incubation. The radioautographs were scored for the per cent of the total number of nuclei which became labeled during each pulse. Increasing percentages of cells at all densities begin to incorporate tritiated thymidine at 12–15 hr after plating. But at high densities, a smaller percentage of cells is labeled at a given time. For cells with a generation time of 12 hr, a G_1 period of 4 hr, and an S period of 6 hr (Bischoff and Holtzer, 1969; Marchok and Herrmann, 1967; O'Neill and Strohman, 1969), it can be calculated that 47% of the cells in a logarithmically growing culture should be in the S period at any given time (see Cleaver, 1967). Only the lowest density cultures approach this value, and at the highest density a maximum of 26% of the cells are in the S period. These data suggest that cell density alters the length of the S period or the average generation time.

We observed, as have others (see Bischoff and Holtzer, 1969), that cells in cultures plated at confluent density do not fuse well. The cultures
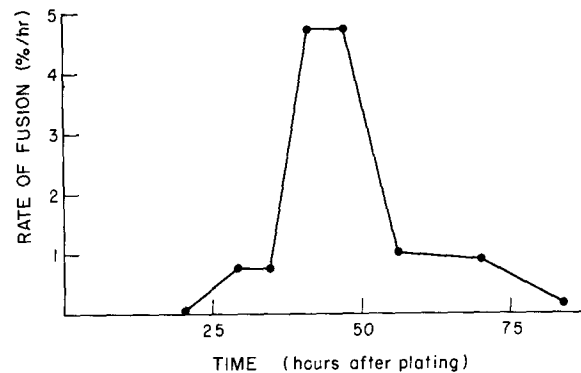

FIGURE 5 Composite fusion rate curve for low-density cultures which received a change of medium at 24 hr. The curve shown is a composite derived from the data of Fig. 1 and Table I. Points are plotted in the center of the interval bracketed by the relevant data points. Percentages are in terms of the cell number (approximated for Fig. 1 data) at 113 hr.
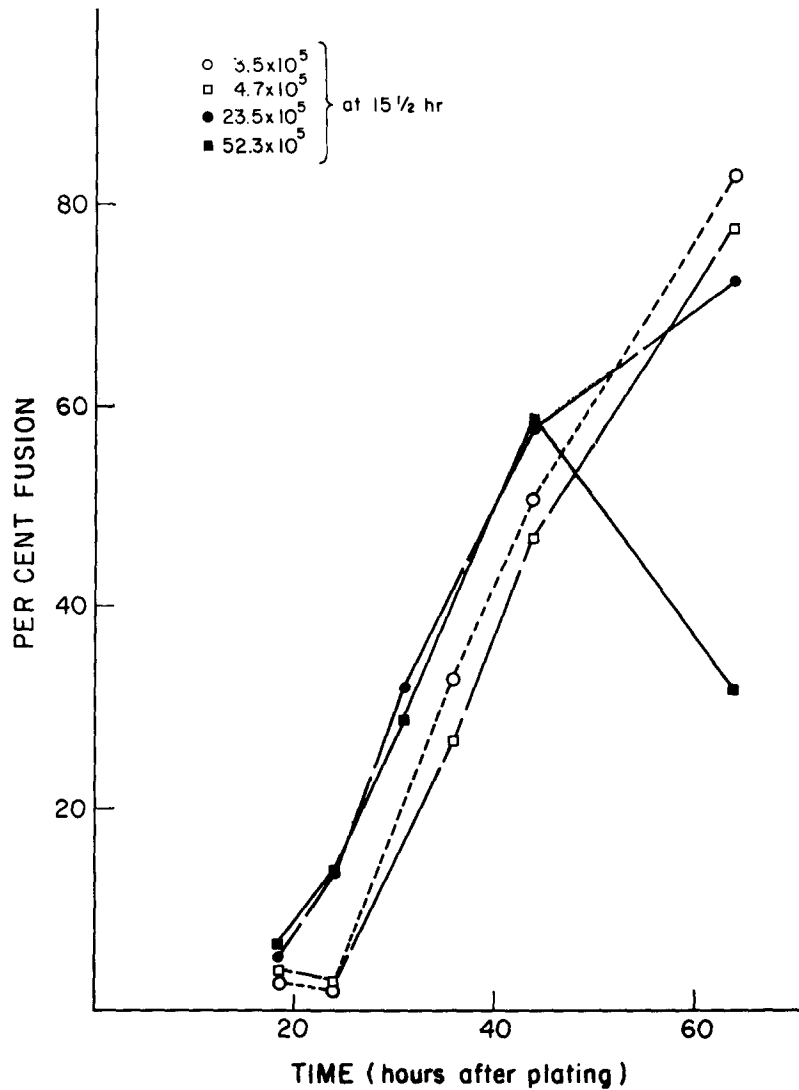
FIGURE 6   Fusion kinetics of both low- and high-density cultures in the absence of a change of medium. All the cultures used in this experiment were plated at one time from the same preparation of cells. The medium was not changed in the course of the experiment. Otherwise, the conditions were as described for Fig. 1.

with 5.2 × 10⁶ cells in Fig. 6 degenerated soon after fusion. At still higher densities, fusion is very much reduced. This may result from the simple inhibition of cell movement and/or from the failure of cells to cycle, or from depletion of the medium. Good differentiation in very high-density cultures may require semiperfusion conditions with specialized media (Kruse and Miedema, 1965; Todaro et al., 1965).

Kinetics of DNA Synthesis and Thymidine Incorporation

Fig. 8 B shows that in muscle cultures there is a lag period in DNA synthesis and then a logarithmic synthesis of DNA beginning 12–15 hr after plating. The curve for total DNA accumulation in low-density cultures indicates that the cells complete their first doubling in about 24 hr. As one would
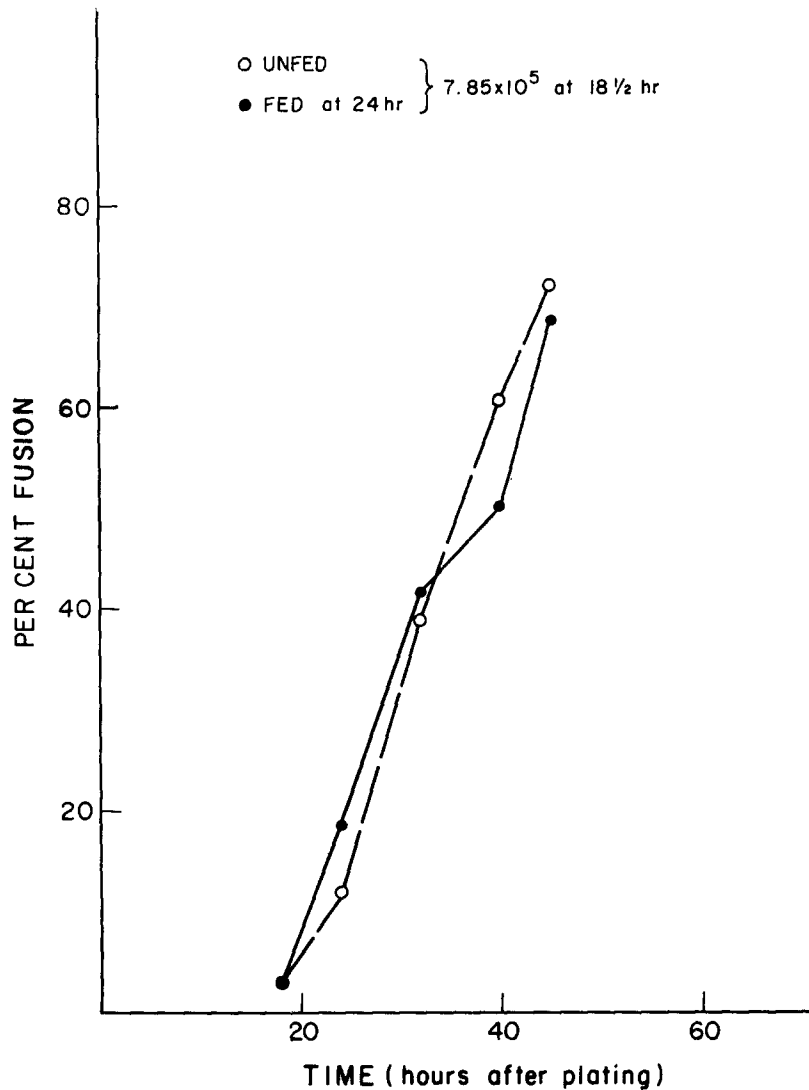
FIGURE 7 Effect of fresh medium on high-density cultures. Cultures represented by solid circles had their medium changed 24 hr after plating, and cultures represented by open circles did not.

conclude from the labeling indices (Fig. 8 A) and the rate of DNA accumulation (Fig. 8 B), the cells in higher density cultures are somewhat slower to double. Even so, radioautographs show that 70% of the cells in cultures with 2×10^6 cells at 24 hr have synthesized DNA by the time fusion begins (24 hr), versus 80% for cultures with 1×10^6 cells. Therefore, the large majority of cells divide at least once before fusion, even under our conditions of early fusion; and, if low-density cultures receive a change of medium at 24 hr, the cells will start a

second round of replication before fusing at a later period (Figs. 8, 9, 11).

The over-all rate of DNA synthesis is drastically reduced during the fusion period (Fig. 9). The increase in total DNA during the fusion period is usually less than 30%, and no increase was detected in this particular experiment. The incorporation of tritiated thymidine into DNA can provide an accurate indicator of DNA synthesis which is both convenient and sensitive. This incorporation can, however, be misleading (Smets,
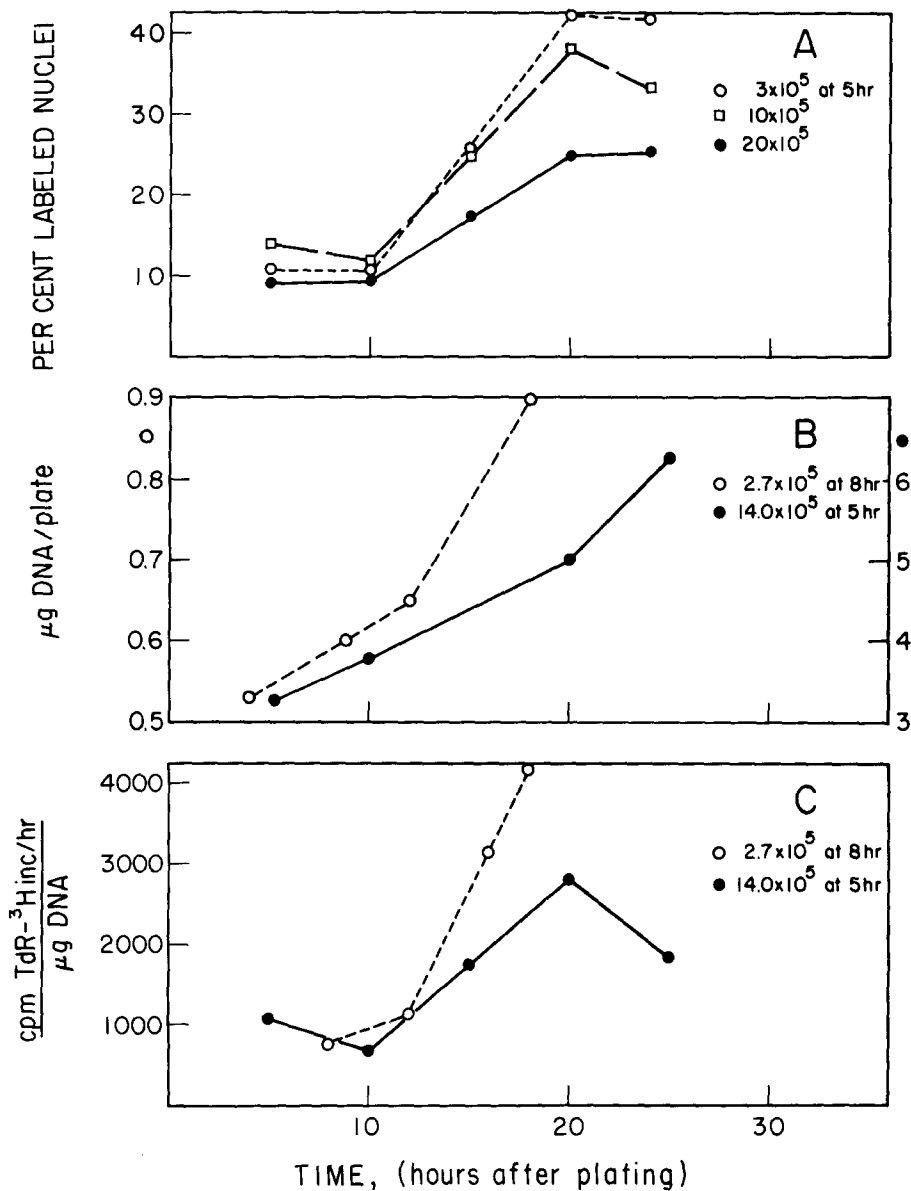
FIGURE 8 A Effects of cell density on early growth kinetics. At each point, the medium was removed from cultures of each density and was replaced with 1 ml of MEM containing 5 μCi of thymidine-^{3}H. After a 1 hr incubation at 37°C, the cultures were fixed. Radioautographs were made as described in Methods. The percentage of the total number of scored nuclei which were labeled is shown for each time point.

FIGURE 8 B Two separate determinations of the pattern of early DNA synthesis in the cultures are shown. DNA was determined as described in Methods. For the lower density, determinations of three separate samples of four plates each were averaged for each point. For the higher density, a single sample of four plates per point was assayed.

FIGURE 8 C Early kinetics of tritiated thymidine incorporation. Thymidine incorporation rate was measured in the same cultures represented in the two curves of Fig. 8 B. For the lower density cultures, the incubation was with 2 μCi/ml thymidine-^{3}H for 1 hr. For the higher density cultures, the incubation was with 5 μCi/ml thymidine-^{3}H for 1 hr. Thymidine incorporation per hour was normalized to the DNA values shown in Fig. 8 B.
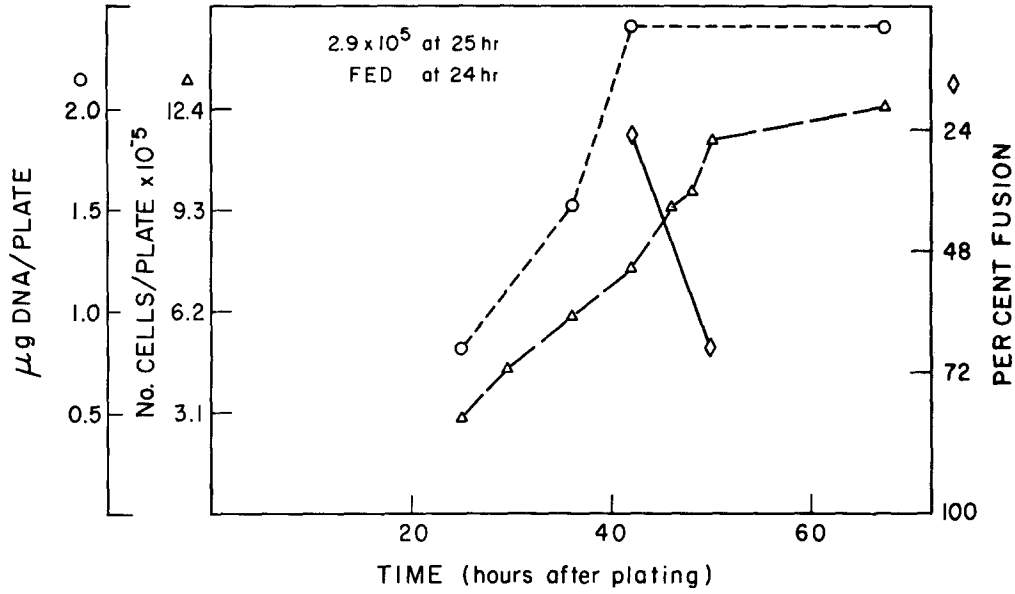
60

FIGURE 9   Growth curve before and during fusion. Scoring of cell number and per cent fusion in this curve was done on living cultures as described in the legend to Fig. 1. Cultures were scored at the times indicated, and then were harvested immediately for DNA determinations. The medium was changed on all cultures at 24 hr after plating.
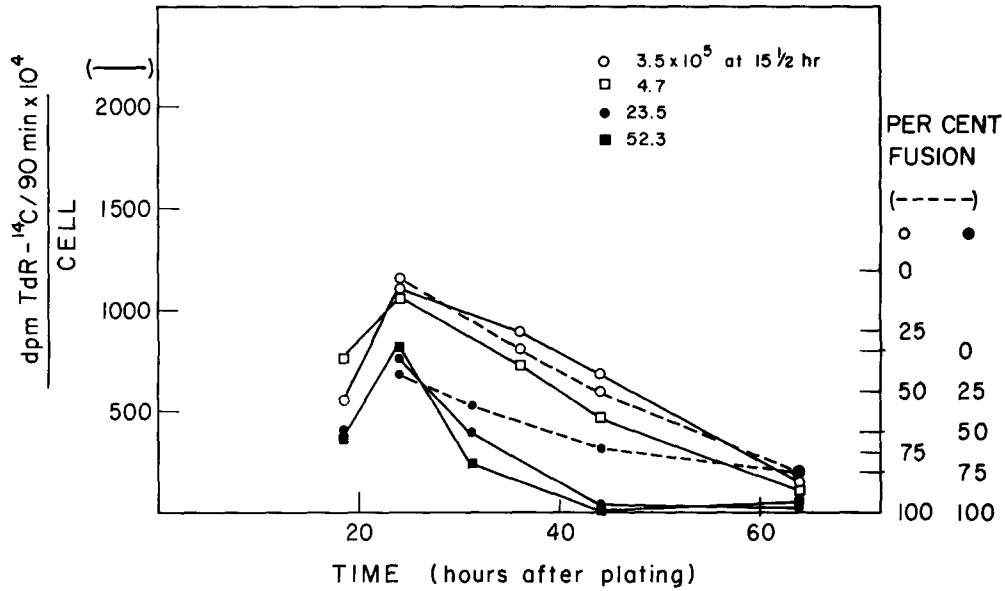

FIGURE 10   The decline in thymidine incorporation relative to fusion. Cultures from the same series used in Fig. 6 were incubated for 2 hr at each time point in MEM containing 0.5 μCi/ml thymidine-^{14}C and then harvested immediately and extracted as described in Methods. Fusion data from Fig. 6 are replotted here (----) for the 3.5 × 10^5 and 23.5 × 10^5 densities for comparison with the thymidine data (———).
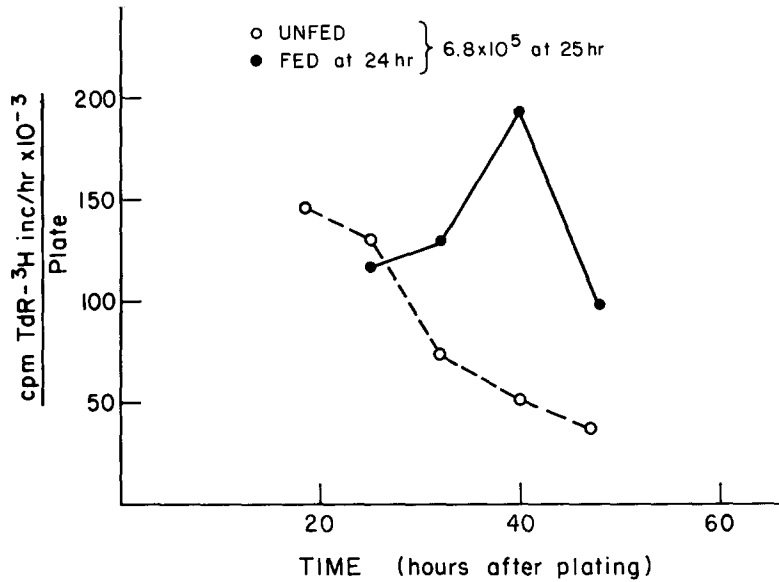
FIGURE 11 Effects of medium change on thymidine incorporation in low-density cultures. Cultures represented by (-●-●-) received a change of medium at 23 hr. The medium was not changed on sister cultures represented by (-O-O-O-). At the indicated times, two cultures from each group were incubated for 1 hr in MEM containing 5 μCi/ml thymidine-^{3}H and then harvested immediately.

1969; de Turenne and Daillie, 1969). The following experiments were performed in order to determine the accuracy with which changes in thymidine incorporation mirror DNA synthesis. The correlation between thymidine incorporation data and the radioautographic labeling index can be seen in Fig. 8. Fig. 8 C shows that tritiated thymidine incorporation during a pulse, when normalized to total DNA, accurately reproduces both the low- and high-density labeling index curves. This indicates that the normalized incorporation rate reflects the proportion of cells in the S phase.

If cell fusion is the only process that reduces the proportion of cells entering the S phase and if the thymidine pools do not fluctuate, the incorporation of thymidine should be an accurate index of the degree of fusion as well. Fig. 10 shows that this is true for low-density cultures. The drop in thymidine incorporation agrees very well with visually scored fusion plotted on the same scale. This is not true for the high-density cultures in which thymidine incorporation drops off disproportionately, probably from a reduction of the growth fraction as well as from cell fusion.

Thymidine incorporation can also be used to confirm the effects of medium change on fusion in low-density cultures discussed earlier. Fig. 11

shows the difference in thymidine incorporation on a per plate basis between cultures which did and those which did not receive a medium change. The cells in the former cultures continue to synthesize DNA, before fusing, for approximately another generation after the cells in the latter cultures have begun to fuse. This result is in good agreement with the doubling in cell count after the change in medium seen in Fig. 9.

Taking advantage of the kinetics of fusion described in this paper, we reexamined a question from the earlier literature on muscle culture: what is the shortest interval between the end of a cell's last S period and the appearance of the nucleus in a myotube?

To answer this question, we used the same type of experiment employed by Okazaki and Holtzer (1966). Muscle cells were exposed to tritiated thymidine and fixed at various intervals, and the time of the first appearance of a labeled nucleus within a myotube was determined by radioautography. This experiment only provides an upper limit in the time required for DNA synthesis and cell fusion because of the difficulty in detecting when a nucleus is definitely in a myotube. In addition, the result is dependent on the rate of cell fusion at the time of the experiment. We, therefore, used low-density cultures, fed at
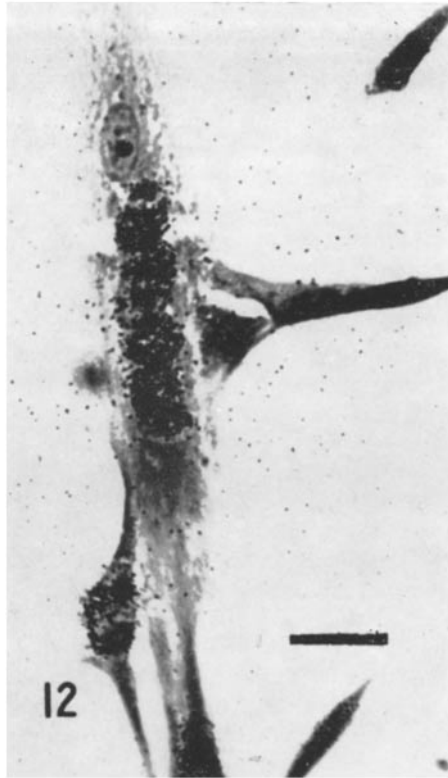
FIGURE 12 A radioautograph of a culture which was labeled with thymidine-^{3}H, 5 μCi/ml, between the 41st and 44th hr and then fixed immediately. Four of the five myotube nuclei are heavily labeled. The nominal plating density was 5 × 10^5 cells per 60 mm dish. The medium was changed a 24 hr. Marker is 15 μ. × 885.

24 hr, and we timed the labeling to fall early in the rapid fusion period shown in Fig. 1. Cultures were pulsed with tritiated thymidine at 40 hr and fixed for radioautographs at 41, 42, 43, 45, and 47 hr. By the end of the 3rd hr of incubation, labeled nuclei began to appear in myotubes (Fig. 12). Cells which had been in the S period only 3 hr before were now fused. This means that these cells must have begun to fuse in the early G$_1$ period since the G$_2$ plus M period is about 2– 2½ hr long (Bischoff and Holtzer, 1969).

DISCUSSION

We conclude from these results that cultures of preplated cells grown on collagen-coated dishes at nominal plating densities of 2–8 × 10^5 cells/dish and fed 24 hr after plating yield the best fusion kinetics. Under these conditions, fusion occurs

largely in a discrete burst of 10 hr. The maximum degree of fusion achieved is high enough that overgrowth by mononucleated cells is slow— cultures fed at the 24 hr point are still 65% fused at 70 hr after plating. The sharply defined beginning and end of the rapid fusion period allows the precise measurement, relative to fusion, of differentiation.

It is unlikely that the fusion rate we observe, which increases the net fusion an average of 7%/hr over a 10 hr period, can be substantially exceeded in asynchronously growing cultures. This follows from the fact that for logarithmically growing cells with a generation time of 12 hr and a G$_1$ period of 4 hr, a 9 hr period is required to guarantee every cell at least 1 hr in G$_1$. Since Okazaki and Holtzer (1966) have shown that the capability of fusion is limited to the G$_1$ period, the ratio of the G$_1$ period to the total generation time should determine the practical minimum amount of time for fusion to occur in asynchronous cultures.

Whereas the length of the fusion period may seem reasonable, the fluctuations in the rate of fusion in fed cultures are not understood. Despite the rapid average rate of fusion, this rate often drops sharply in the middle of the fusion period. Frequently, maximal rates of 10% fusion per hour are separated by rates of as low as 3%. Since the interval between maximal rates is the time required for a cell to go from the end of the G$_1$ period to the beginning of the next G$_1$ period, the fluctuations in rate could be due to fusion of synchronous waves of cells. However, measurements of rates of thymidine incorporation and DNA accumulation lack these fluctuations and, therefore, do not support this explanation. Fusion itself acts to deplete the number of dividing cells, and thus boosts the apparent rate of fusion, since this is calculated as a net fusion increment over and above the increase in cell number. While our calculations indicate that a biphasic rate could result in this way, the magnitude of the fluctuation predicted is not nearly the size observed.

Control over the initiation of cell fusion appears to be dependent upon environmental factors. When the medium is changed in cultures at low density, the onset of fusion is delayed. The initiation of the fusion period is unaffected by changing the medium on the same cells plated at high concentrations. It is as yet unclear whether fusion is delayed by the removal of a conditioning factor with the old medium or, perhaps, by the replace-

ment of a depleted nutritional or growth factor with the fresh medium. The fact that medium effects are very much diminished at high densities is equally consistent with either hypothesis. In preliminary experiments in which cultures are fed with medium previously depleted by another type of differentiated cell, fusion is still delayed, suggesting that the delay is caused by the removal of a conditioning factor rather than by the introduction of a nutritional factor in the medium.

One might expect cell density to have a direct effect on the timing and rate of fusion, independent of conditioning and/or depletion effects of the medium, since cells must make contact to fuse. All else being equal, a higher cell density will result in more frequent contact between cells, and, therefore, cells might fuse sooner. It is shown here as well as in previously published data (O'Neill and Strohman, 1969) that, for a given set of conditions, high-density cultures do fuse earlier than low-density cultures.

One can attempt to compute a lower limit of cell density for rapid fusion from a collision theory (see Kennard, 1938) that is based on the requirement that the fusing cells have at least one collision during the 12 hr period of rapid fusion. Using an observed average mobility of 20 μ/hour and assuming a random distribution of cells, one finds that the mean time between collisions is 4.6 hr for the case of 5×10^5 cells/plate at the beginning of fusion. If it is required that one of the cells be in the G_1 period (as would be necessary for a myoblast to fuse with a myotube), the mean time for such a collision becomes 10.5 hr; and if both cells are required to be in the G_1 period, the mean time between effective collisions becomes 23.5 hr. These calculations ignore the geometrical restrictions imposed on fusion as myotubes come to predominate. Even so, this density appears to be marginal for rapid fusion. Calculations notwithstanding, fusion proceeds very well at this density. This discrepancy between what one might predict and what is observed may be due to several factors. The assumption of random distribution and movement, usually so dependable, may be quite inappropriate here. Every cell which is dividing contradicts the assumption since, after mitosis, daughter cells are nearer than average and both are in the G_1 period. Or, the often observed phenomenon of aligning of cells may abridge random motion to the point that fusion occurs rapidly. There are other possibilities too: cell mobility may increase before fusion, or random

movement may be abridged by chemotaxic or other influences. Whatever the factor, the empirical lower limit for rapid fusion appears to be about 3×10^5 cells/plate at the time fusion begins. Even allowing for one or more of the above factors, it seems very unlikely that a G_1 cell will collide more than once or twice with another G_1 cell within a 10 hr period at this density. Since most of the myoblasts do fuse in this period, the fusion efficiency of permissive collisions must be, at least, 50% but more probably close to 100%.

It has been reported that myoblast nuclei do not appear in myotubes sooner than 8 hr after the end of their final S period (Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1969; Bischoff, 1970). This result was first thought to mean that fusion capability is limited to the end of the G_1 period since it had been shown earlier by these workers that cells do not fuse in the S, G_2, or M periods. Under the conditions prevailing in the earlier experiment, a cell 8 hr past the S period would be in the last third of the G_1 period. Bischoff and Holtzer, using conditions which considerably shortened the G_1 period, found that cells still did not fuse sooner than 8 hr after DNA synthesis. The interpretation of this finding was that the cells withdraw from the cell cycle before fusion or enter a postmitotic period.

Radioautographs from three different experiments reported here show that cells fuse as early as 3 hr after the S period. This indicates that fusion can occur at the beginning of the G_1 period. If there were a period of withdrawal from the proliferative cell cycle or a prolonged G_1, extending 4 or 5 hr beyond the time at which the cell would have normally passed from G_1 into S, the index of normalized thymidine incorporation should begin to fall 4 or 5 hr before fusion and in proportion to the number of cells which have withdrawn. Under conditions employed in our experiments, 4 hr of fusion represents a loss of 25% of the cells from the dividing population. Therefore, the normalized thymidine incorporation should drop about 25% before the beginning of fusion and should continue to lead fusion throughout the fusion period. As shown in Results, the drop in normalized thymidine incorporation agrees very well with the fusion scored in low-density cultures. This experiment has been repeated and the two indices generally agree to within 5%. In more than 15 other studies of thymidine incorporation that employed determinations every 1 or 2 hr throughout the fusion period, we found no instances in

which the drop in thymidine incorporation substantially preceded fusion. The difference between these results and those of other investigators most likely stems from the very large difference in the fusion rates that prevailed. The slower the fusion proceeds, the harder it is to find a clear-cut example of a cell which has fused at the earliest possible time after division.

The short time from DNA synthesis to cell fusion reported here is of some importance. Our understanding of the early literature, in which Holtzer and his collaborators introduced and discussed the concept of a "critical" or "quantal" mitosis, is that the experimental basis for the concept as it specifically applies to myogenesis rests primarily, though not exclusively, on the length of time it takes for a cell labeled in the last cell cycle to fuse. They found that this time greatly exceeded the total time of G_2, mitosis, and G_1, indicating a greatly prolonged time period (a prolonged G_1) following the mitosis of the last cell cycle. This observation suggested that the last cell cycle during myogenesis was quantitatively, if not qualitatively, different than other cell cycles. (See Bischoff, 1970, for a discussion of those data.) Our results indicate there is not a prolonged G_1 period before cell fusion and suggest that the rate of cell fusion that prevails probably determines the length of time it takes a cell to divide and fuse. This is not to dismiss the idea that cell cycle events may play a crucial regulative role during differentiation, as has been demonstrated in some differentiating systems, but to leave open the question as it applies to myogenesis.

Holtzer (1970) has subsequently elaborated and extended the concept of the "critical mitosis" to that of a "quantal cell cycle" and has raised a number of interesting questions, one of which is whether a cell could undergo another cell cycle if it had undergone the putative "quantal cell cycle." The experiments depicted in Figs. 8 B, 9, and 11 suggest that many muscle cells which otherwise would fuse can undergo another round of DNA synthesis before fusion if one manipulates the cell environment. If there is a "quantal cell cycle" in myogenesis, the kinetic consideration reported here suggests that it is characterized neither by marked temporal differences from other cell cycles nor by prevention of cells from undergoing another cell cycle.

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