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Clonal Analysis of Myogenesis

Its relevance to the general problem of the stability of cell-type in cultured animal cells is discussed.

Irwin R. Konigsberg

Ancient problems in biology, like the cities of antiquity, are frequently covered by many strata deposited during successive epochs. Knowing the scientific fashions of a given period, we can often classify the strata and date the artifacts therein, but unless we have a new way of examining the old ruins it is frequently better to leave them undisturbed than to deposit another layer of sediment.

One of the venerable controversies of developmental biology that has been repeatedly excavated is the problem of the stability of the differentiated state. The two experimental situations which have been employed to explore this question are regeneration, most popularly of the amphibian limb, and the behavior of cells in tissue culture.

From the facts emerging from recent studies of regeneration in which the newer ways of examining the phenomenon were utilized (*1*), we may draw three conclusions.

1) Differentiated cells need not be irreversibly fixed "postmitotics."

2) If differentiated cells are appropriately stimulated in some manner by the trauma of amputation, they may dedifferentiate, in the sense of losing some of their characteristic structures, regaining some of the cytological features typical of an earlier state of differentiation.

3) Such dedifferentiated cells may proliferate and finally redifferentiate. Whether they redifferentiate, invariably, into cells of the same type as the progenitor cell is a question difficult to resolve.

The tissue-culture approach, thus far, has been less fruitful. One observes events which superficially resemble the dedifferentiative and proliferative stages of regeneration (*2*). However, redifferentiation or even retention of some aspects of the differentiated state are rarer events (*3*). We might reasonably question the extent, then, to which the two situations are analogous. Are we indeed, in tissue culture, dealing with a set of conditions which favor dedifferentiation but are insufficient for initiating or supporting redifferentiation? Alternatively, the phenomenon observed in culture may be a completely atypical response unrelated to any process occurring in vivo. The failure of culture techniques to yield results conforming to what might be predicted may indicate either that the techniques are, as yet, imperfect or that they place demands on the cell which, because of its intrinsic limitations, it cannot fully meet. These are by no means novel postulates (see *4*). They are worthy of reexamination only because we do have new ways of examining the old problems.

The observed loss of overt indices of differentiation of cells in culture could arise by way of either of two general mechanisms. Culture conditions might select ubiquitous cell types whose identity in culture might be difficult to establish; or the progeny of differentiated cells might indeed become altered in response to some aspect of the artificial environment.

Two different approaches to the question have been reported recently. One approach exploits the two tissues, cartilage (*5*) and pigmented retina (*6*), which can reasonably be assumed to be homogeneous with respect to cell type. In both cases a rapid alteration in the biosynthetic capacity of the monolayered cells can be demonstrated, which cannot be ascribed to selection.

Another attack on the same question, in which both enzymological and immunochemical criteria are employed, demonstrates with equal clarity the operation of selection in the establishment of cell populations derived from the livers of newborn rats (*7*).

These two sets of results are not in conflict. They do illustrate, however, the need for circumspection and the danger of premature generalization. At the present time we can only evaluate data in relation to a given cell type or population, at a specific time in its ontogenetic history, and under a particular set of conditions.

Selection of cell types, then, cannot explain every case of morphological simplification (*8*). However, the criteria used in establishing culture conditions may select not only between different cell types but among the various metabolic capabilities of a single type of cell. In establishing conditions for the survival and growth of a particular cell we would be selecting for cellular activities common to all cells rather than for those peculiar to the type in question.

The author is a member of the department of embryology of the Carnegie Institution of Washington, Baltimore, Maryland.

Cell and Organ Culture Contrasted

Culture conditions have been devised, however, which do promote the maintenance of the differentiated state and, in fact, the reasonably normal continuance of developmental sequences. Developed largely by the group working at the Strangeways Research Laboratory in Cambridge, these techniques comprise the methodology of organ culture (*9*). They differ considerably from tissue and cell culture in several respects.

1) The normal tissue architecture is preserved.

2) The explant consists of a relatively compact mass of cells cultured under conditions which discourage cell migration and spreading of the explant.

3) Cell proliferation, it is generally thought, is minimal.

We have, then, two contrasting approaches; one favors cellular differentiation whereas the other promotes the morphological and biochemical simplification of the cell population. The obvious conclusion is that somewhere among the several points of difference between these techniques lie the clues to the problem of morphological simplification in vitro.

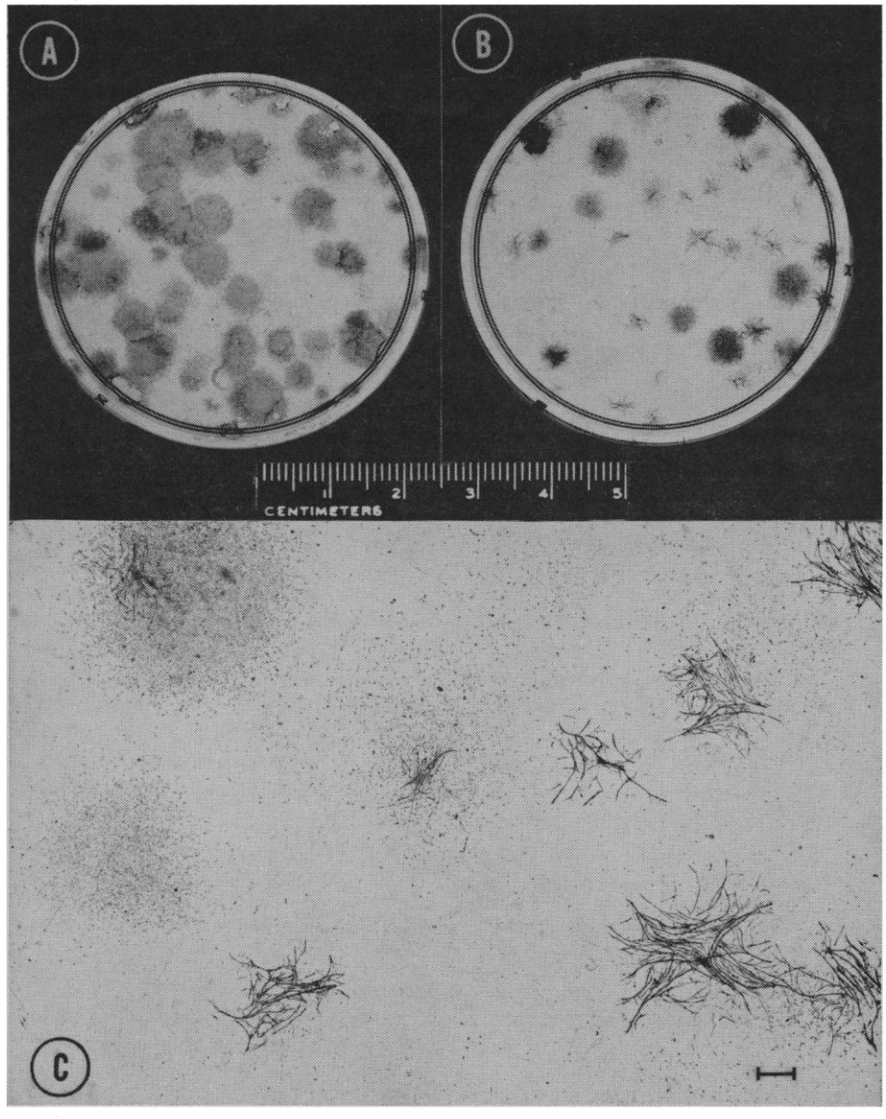


Fig. 1. Replicate cultures established with identical aliquots of the same cell suspension plated into equal volumes of conditioned medium prepared by two different methods. For *A*, fresh medium contained 5 percent embryo extract; the period of "conditioning" was limited to 3 days. For *B*, fresh medium contained 10 percent embryo extract; the period of conditioning was 6 days. The apparent colony density is deceptive, due to the large number of the smaller muscle colonies in *B*. (*C*) An area of *B* at higher magnification; the scale marker represents 1.0 mm.

Tissue and Cell Interactions

Preservation of normal tissue relationships is undoubtedly significant in many instances. Many modes of differentiation depend upon the interaction of populations of *dissimilar* cells.

However, simplification frequently involves cytological features established after such interactions have occurred. Therefore, it seems improbable that cell-type instability is in all cases a result of disruption of precisely this type of "inductive" interaction.

Tissue and cell interactions are by no means limited to early embryogenesis, nor are they exclusively developmental phenomena. Endocrine regulation, trophic effects of nerve upon muscle, regulation of the internal milieu by the specialized functions of various organs are all forms of cell interactions.

One class of interactions whose significance is now being recognized is the interaction of embryonic cells of *similar* ontogenetic fate (*10*). This form of interaction is facilitated by the use, in organ culture, of compact masses of cells and the retardation of their dispersion through migration. A number of observations have shown a correlation between the degree of cell dispersion and the loss of developmental expression (*11*).

Conversely, either more diversified or more complete differentiation has been promoted by fusing and culturing progressively larger amounts of the same embryonic region (*12*) or by crowding embryos (*13*) or embryonic primordia (*14*) into relatively small volumes of medium. Such observations have been classified as "tissue mass effects."

A body of comparable observations can be assembled which relate "mass effects" not specifically to cellular differentiation but to survival and multiplication in cell culture. The use of "conditioned medium" by Earle's group (*15*) to bring about cloning of single cells is one excellent example.

Another example is the ingenious use by Puck and Marcus (see *16*) of irradiated "feeder layers" to support single-cell plating. From Puck's group, also, has come the demonstration that at least one of the roles of the feeder layer is the supplementation of an initially inadequate medium through the metabolic activities of the irradiated cells (see *17*).

Role of Population Density

The role of cell density in augmenting or "conditioning" the medium is nowhere more explicitly (and beautifully) demonstrated than in the work of Eagle and Piez (*18*). Thirteen amino acids and eight vitamins have been shown (*19*) to be "essential" for a broad spectrum of mammalian cells. In addition, three amino acids are required by specific cell lines or under specific culture conditions. At least six of these amino acids and one vitamin can, in fact, be synthesized by most of the cells which show the apparent requirement. The requirement for each, however, exists only at low cell density. As cell density is increased, a level is reached above which exogenous amino acid is no longer essential. Confirmation of the equilibration of the intercellular metabolic pool with the external culture medium is evident in the data presented on the gradual accumulation of amino acids as a function of both time and initial cell density. Within a given cell strain, the critical cell density differs for each amino acid. Thus, varying the size of the inoculum might result in the establishment of qualitatively different milieus invoking, perhaps, different expressions of the cell's biosynthetic capacities.

The two sets of phenomena, the one group dealing with activities (such as survival and growth) that are common to all cells and the other dealing with cell-specific properties, may have more than a superficial relationship (see *20*). Both maintenance and proliferation, on the one hand, and cytodifferentiation, on the other, depend on the maintenance of an adequate milieu, although the specific requirements for the two may be different.

Indeed, many disparate experimental situations have demonstrated that the component processes of development—growth, maintenance, differentiation, and morphogenesis—are separable (*21*). Thus, culture conditions may be adequate for maintenance and proliferation but may fail to support cytodifferentiation. With increasing cell mass relative to the volume of medium, a more nearly adequate environment for supporting differentiation might be established either by limiting leaching of cell components or by permitting cross-feeding within the population, either directly or by way of the medium. One might predict, on the basis of these considerations, that factors which limit diffusion or replace molecular species lost by diffusion might permit the attainment of a higher degree of differentiation.

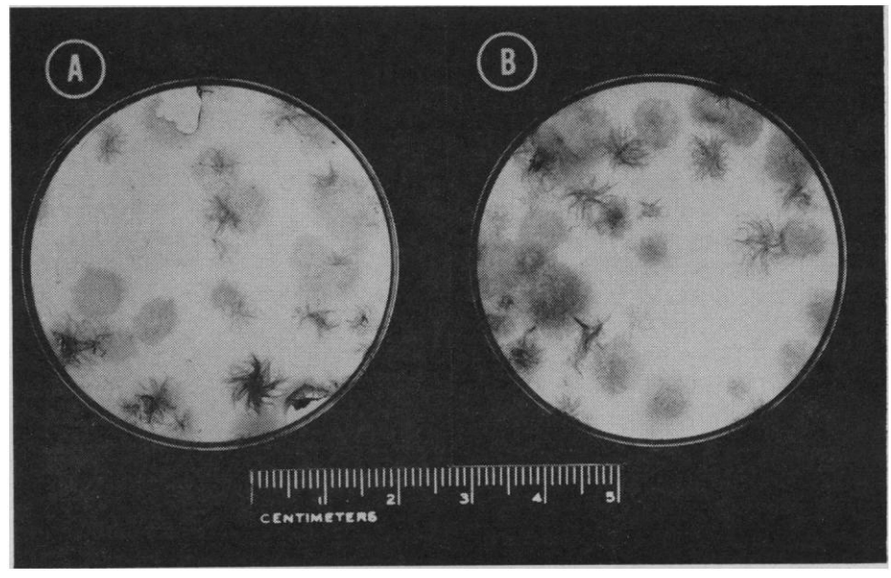


Fig. 2. Plating cultures established with inocula of 200 cells, freshly isolated from the leg musculature of 12-day chick embryos. Cultures were fixed on day 13 (*A*) and on day 16 (*B*). Staining was in Ehrlich's hematoxylin (no counterstain).

The examples, cited earlier, of the effects of tissue mass on subsequent developmental expression are all amenable to such an interpretation. All involve a reduction either of the surface-volume ratio of the tissue mass or of the ratio of the volume of the medium to that of the cells. In a sense, they may represent the transitional states between organ and cell culture. Modifications of cell- and tissue-culture techniques which restrict losses by the cells to the extracellular compartment also seem to promote cytodifferentiation.

For example, recent experiments of Rose and his collaborators (*22*), in which cells were cultivated between sheets of dialysis membrane, do in fact demonstrate that cellular differentiation occurs in that portion of the culture which is confined. In like manner, an agar overlay has been employed in differentiating monolayer cultures, yielding comparable results (*23*).

Similarly, my associates and I have demonstrated that when conditioned medium is used in single-cell platings of skeletal muscle there is a higher incidence of differentiated colonies than there is when freshly prepared medium is used (*24*).

Since frequent change of medium could negate the role of the cell mass, it is of interest that an optimal frequency of replacement has been demonstrated with respect to myogenesis (see *25*).

Table 1. Plating efficiency and frequency of muscle differentiation in 20 experiments, carried out from June through December 1962, in which conditioned medium was used (see *60*). The means, standard deviations $\{(s[x - \bar{x}]^2/[n - 1])^{1/2}\}$, and ranges were derived from the pooled sample of 69 petri plates.

Total colonies (*N*)	Total muscle colonies (*N*)	Plating efficiency* colonies/(cells plated) × 100		Frequency of muscle differentiation [(muscle colonies)/(total colonies) × 100]	
		Mean (± standard deviation, %)	Range (%)	Mean (± standard deviation, %)	Range (%)
3104		17.6 ± 5.9	5.5 — 37.0		
	1313			41.3 ± 12.1	14.8 — 64.6

* Inoculum sizes (number of cells per petri plate) were as follows: 100 in 1 experiment, 200 in 11 experiments; 400 in 8 experiments.

The Synthetic Environment

Failure to obtain normal differentiation of a particular cell type in a synthetic environment might merely reflect inadequate reproduction of in vivo conditions. The criteria normally applied to appraise a particular set of culture conditions—survival, growth, and cytological appearance—are of limited value. Although one would not expect normal cell function in moribund cells, the healthy appearance of a cell is no guarantee that culture conditions are adequate for its differentiation. The uncertainty introduced by a limitless array of possible variables precludes the assumption that it is the inherent capacity of the cell that is being tested rather than the contrived milieu (see *26*).

A variety of culture media have been devised, representing an impressive application of skill and energy. Since they were developed primarily to maintain viable proliferating cells, their adequacy in supporting any specific differentiation would be fortuitous. There is, however, no a priori reason to believe that media which adequately support specific differentiation are unattainable.

One might expect to be confronted with the necessity not only of supplying and adjusting the amounts of known nutrients but also of providing more complex molecular entities whose concentration may normally be small and whose structures, properties, and mechanism of action are unknown (see *27*). Practical considerations, at present, may dictate the use of the less readily definable approaches cited earlier. This may seem a retrograde step in view of the important strides made in the elaboration of defined media; however, for the developmental biologist the choice between ill-defined cells cultivated in well-defined media and well-defined cells cultivated in ill-defined media is no choice at all (see also *28*).

Disaggregation Damage

An important distinction between organ and cell culture arises out of the procedures employed in the latter to render organized tissues into cell suspensions. It is often difficult to assess the cellular damage effected by me-

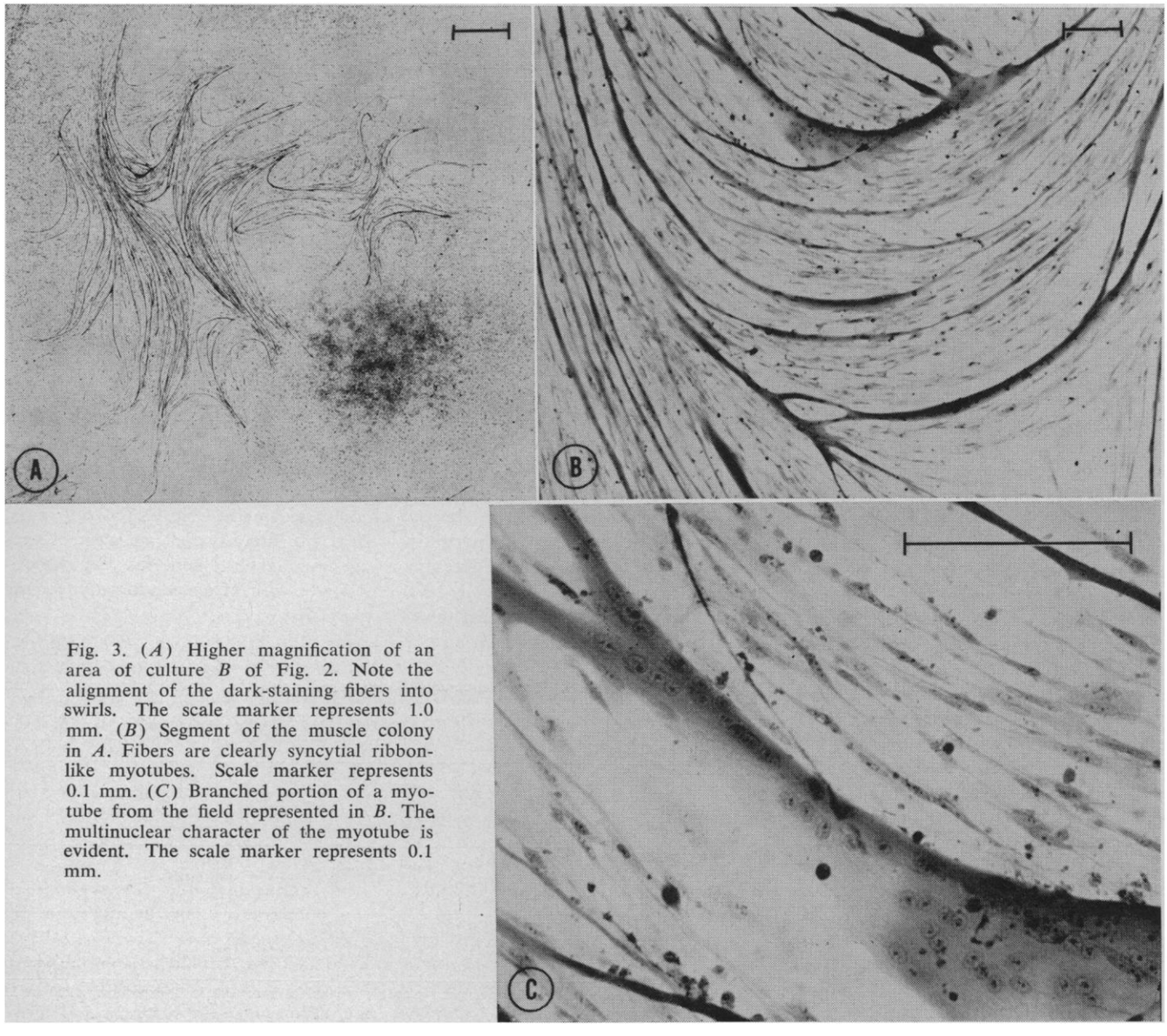


Fig. 3. (*A*) Higher magnification of an area of culture *B* of Fig. 2. Note the alignment of the dark-staining fibers into swirls. The scale marker represents 1.0 mm. (*B*) Segment of the muscle colony in *A*. Fibers are clearly syncytial ribbon-like myotubes. Scale marker represents 0.1 mm. (*C*) Branched portion of a myotube from the field represented in *B*. The multinuclear character of the myotube is evident. The scale marker represents 0.1 mm.

chanical factors, enzymatic digestion, and exposure to solutions which are frequently unphysiological (*29*). Even when no gross lesion is discernible (*30*), we cannot assume that cell function is unimpaired.

The damage may be sublethal, merely accelerating losses due to diffusion. Also, eventual expression of this damage may vary with the culture technique subsequently employed. For example, single-cell plating was feasible, at first, only in the presence of a feeder layer (*16*). The use of more rapid and gentle trypsinization procedures, as well as the development of a more complete medium, made it possible to eliminate the feeder layer (*31*). Similarly, dispersed cells which are subsequently reaggregated exhibit quite normal differentiation (*32*), despite prior exposure to conditions which, one would judge, would not facilitate single-cell plating. (It is doubtful whether such cells could develop as normally in monolayer culture as they do in a reaggregate.)

Perhaps an even more insidious form of damage, insofar as studies of morphological simplification are concerned, is that affecting the genetic apparatus itself. Threefold increases in mitotic and chromosomal abnormalities above control levels have been demonstrated in primary cultures prepared from embryonic mouse tissues subjected to prolonged (3 hours) trypsinization (*33*). Aneuploidy in 5 to 10 percent of the population of trypsinized embryonic cells has been reported after only 24 hours in culture (*34*).

That the judicious use of trypsin does not invariably produce such defects is attested to by the work of Hayflick and Moorhead (*35*), who maintained true diploid strains derived from human fetal tissues through as many as 50 passages. Although most strains were established from minced tissue, serial transfer was affected after brief trypsinization (0.25 percent Difco trypsin 1:250; digestion time, 15 minutes). Unfortunately, trypsin has generally been used rather indiscriminately. In preparing cell suspensions from tissue for primary explanation as monolayers, concentrations ranging from 0.05 to 1.00 percent trypsin (Difco 1:250 or the equivalent) have been used, with digestion times varying from 5 minutes to 3 hours. That cells subjected to the extremes of such treatment survive and multiply is surprising; that they may fail to retain all of their normal functions is not.

Proliferation versus Differentiation

Organ culture, again as opposed to cell and tissue culture, is generally thought to be attended by little proliferative activity. Fell, however, has cited observations of her own which are diametrically opposed to this generalization. Further, she points out that those factors which promote proliferation also promote cell migration. It is the latter phenomenon, she suggests, which may prevent differentiation (*36*).

The assumption that cell division and the accumulation of specific cell products are mutually antagonistic processes is based primarily on the observation that the two processes are not concurrent in vivo. It is assumed that the same relationship holds for cultured cells. It may very well be that it does. However, there is reason to doubt that morphological simplification is invariably the result of this postulated antagonism alone, since the phenomenon in culture is not strictly parallel to the observations made in vivo. After proliferative activity has ceased in vivo, specific cell products can be detected, both during embryogenesis and in regeneration. Although cell cultures (unless they are subdivided) eventually reach a "stationary" phase (*37*), they do not then necessarily differentiate or redifferentiate. (See *26* and *38* for critical reviews of the reversibility of simplification in culture.) The best-documented case for reversibility concerns the loss and reaccumulation of pigment in cultures of iris and pigmented retina (*6, 38, 39*). The reappearance of pigment occurs under conditions in which the cells are crowded but in which proliferation is also reduced.

A recent re-examination of the pigment-cell problem indicates, however, that the primary event in depigmentation is not related to cell division (*6*). Dopa oxidase activity (but not cytochrome oxidase) drops sharply shortly after plating of pure suspensions of retinal pigment cells, reaching minimal levels before any cell division has occurred in some cultures, or after one division, at most, in others. Tyrosinase-dependent incorporation of tyrosine-C^{14} declines in similar fashion, and secondarily, cell division causes dilution of the pre-existing pigment.

Apparently healthy, dispersed embryonic thyroid cells also lose hormone and hormone precursors within the first 3 to 5 days of culture, despite the absence of significant proliferative activity (*40*). Similarly, bovine mammary gland cells lose the ability to synthesize lactose within 24 hours after plating (*41*).

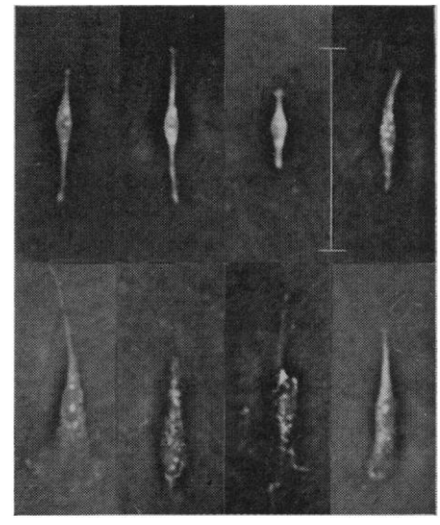


Fig. 4. Photomicrographs of living cells photographed 18 to 24 hours after plating. (The photomicrographs were taken at an initial magnification of 200; bright-medium phase-contrast optics were used.) The cells in the top row produced muscle colonies; those in the bottom row produced colonies of fibroblast-like cells. The scale marker represents 0.1 mm.

A few other rough approximations of the rate of alteration are as follows.

1) Loss of a specific kidney antigen, 3½ days, 2.6 divisions (*42*).

2) Loss of the capacity to incorporate sulfur-35 into chondroitin sulfate, 4 days, maximum of 3 divisions (*5*).

3) Loss of the ability to reaggregate, 2 to 3 days (*43, 44*).

Other cell-specific properties have been reported to persist for longer periods (*3, 45, 46*), and in a few serially propagated cell strains the capacity to synthesize specific cell products is retained through many generations (*3, 38*). Even within the same cell type the various aspects of functional specificity are lost at different rates (*41*) or show varying degrees of stability (*40*). Moreover, alteration is not restricted to cell-specific proteins (*47*). Indeed, all cellular activities may be altered in culture, to greater or lesser extent.

It is clear that the primary event in cell alteration is not, in all cases, related to proliferation. There is, in fact, no conclusive demonstration that proliferation is directly responsible for cell alteration in any specific case.

Utility of Clonal Analysis

The problem of simplification in vitro has currency not merely because, like Everest, "it is there," but because it represents a serious impediment to analysis.

Developmental phenomena in animal cells have, of necessity, been studied in cell populations of varying, and frequently great, heterogeneity. This limitation imposes two types of uncertainty. The first type is generated by the lack of precision inherent in dealing with mixed populations. In investigations of the molecular basis of cytodifferentiation we are all too frequently in the position of applying exact biochemical analysis to imprecisely defined biological material.

Secondly, we are unable to discriminate between cellular properties and properties which are consequences of supracellular organization. The growing body of evidence related to tissue mass effects in cytodifferentiation has prompted a re-examination of some of the classical concepts of the cellular basis of development (*11*). Indeed, the conventional approaches of developmental biology cannot specifically exclude the possibility that what we assume in many cases to be properties of the cells are in fact properties of the aggregate. The significant aspect of the dependence on mass may very well be simply an expression of the elementary physical laws governing diffusion, either between cells or into an extracellular compartment. However, the real impact of the observations relating to mass effects is that they delineate an important area of conceptual ambiguity.

We have, for these reasons, been engaged in analyzing myogenesis in colonial derivatives of freshly isolated individual cells from the leg muscu-

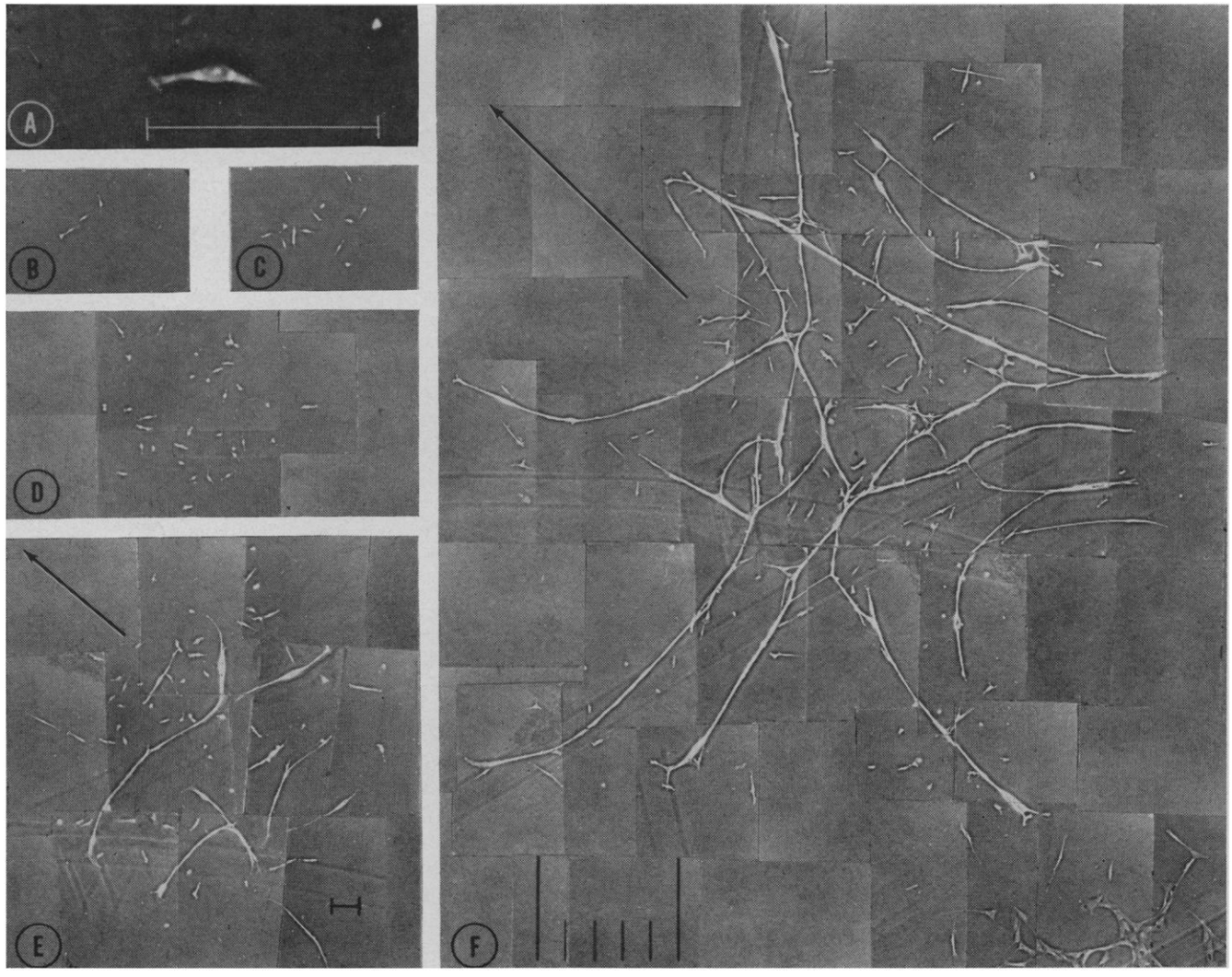


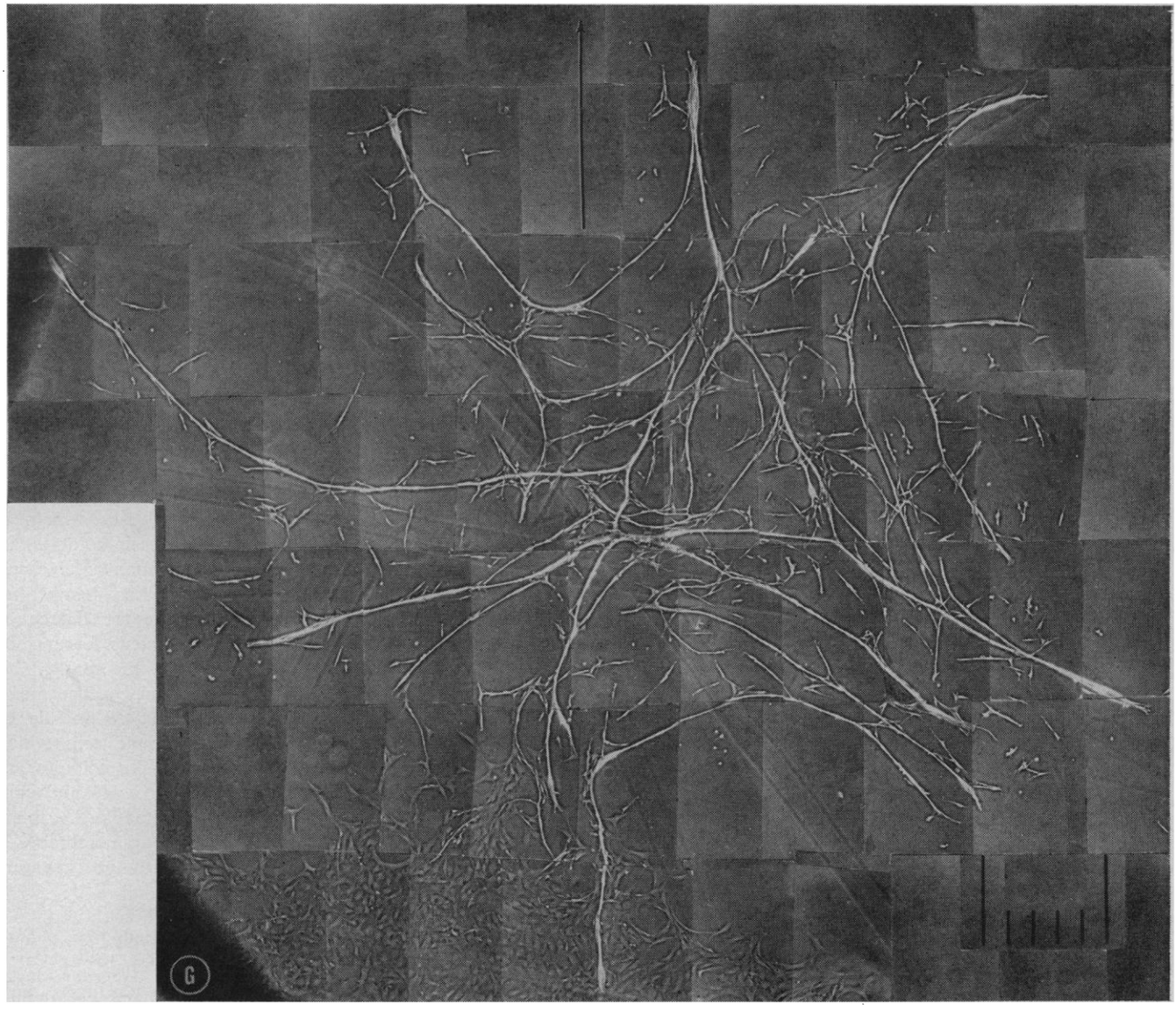
Fig. 5. Photomicrographic record of the development of a muscle colony from a single bipolar cell. The living cells were photographed at an initial magnification of 200 (*A*) or 100 (*B* through *G*); bright-medium phase-contrast optics were used. (*D–G*) Composites of several successive overlapping frames covering the progressively greater expanse of the colony on succeeding days. (*A*) The single cell photographed some time between 18 and 24 hours (day 1) after plating. The nucleus contains one prominent nucleolus. A cluster of highly refractile granules is present in each zone next to the nucleus. Note the ruffled membrane at the tip of the process at left. The scale marker represents 0.1 mm. (*B*) A colony produced by the cell in *A* during the first 24 hours of recording (day 2). The scale marker in *E* pertains to photomicrographs *B* through *E* and represents 0.1 mm. (*C*) The colony on day 3. Three of the cells in the field are rounded and are presumably in an early stage of division. (*D*) The colony on day 4. (*E*) The colony on day 6. Long multinuclear myotubes have formed (these were first observed 24 hours earlier). The arrows in photomicrographs *E*, *F*, and *G* indicate orientation of the colony with respect to the orientation in *G* (the orientation was changed to facilitate photographing). The orientation is confirmed by the matching pattern of strain marks in the

lature of 11- to 12-day chick embryos.

Our earlier studies of such cell populations grown as monolayers from mass inocula (*48–50*) indicated that dispersed cell culture was not, in itself, incompatible with the attainment of reasonably normal differentiation. The developmental sequence in such cultures can be summarized briefly as follows. Initially these cultures appear to be composed of fibroblast-like cells which multiply with a mean generation time of approximately 24 hours. As confluency is attained, long multinuclear cells form, probably by a process of successive cell fusion (see *48, 50–54*). The syncytia are further distinguished from the interspersed mononucleated cells by their intense cytoplasmic basophilia [presumably ribonucleic acid (*51, 55*)] and by the appearance of large numbers of mitochondria (*56*) as well as histochemically demonstrable mitochondrial enzymes (*50, 57*). Shortly after their formation, vigorous spontaneous contractions can be observed in the elongated muscle cells. Concomitantly, the progressive accumulation of cross-striated myofibrils has been observed (*49*), along with a progressive increase in creatine-phosphokinase activity (*58*).

Clonal Analysis of Myogenesis

Although monolayer culturing permitted a degree of control and sample replication far more favorable than that offered by conditions in the egg, it did not obviate the problem of cellular heterogeneity. To circumvent this limitation, the plating technique of Puck and his associates (*35*) was applied in a study of the myogenic differentiation of clones derived from single muscle cells (*24*). After culture for a sufficient length of time (10 to 13 days), the unmistakable indices of myogenic differentiation were observed in a small num-



plastic petri plate. (*F*) The colony on day 9. The network of myotubes has expanded considerably, but note the presence of single cells among the myotubes. Cells of the leading edge of an invading colony of fibroblast-like cells can be seen at lower right. Each division of the scale represents 0.1 mm. (*G*) The colony on day 13. The myotubes are longer and more numerous than they are in *F*. Single cells are still present. Continued proliferation of these single cells is suggested by the rounded appearance of some of them and by associations typical of late anaphase. The invading fibroblast-like cells observed in *F* are now quite obviously the periphery of a contiguous colony. Compare the cells of the impinging colony with the single cells of the muscle colony. (Scale, same as in *F*.)

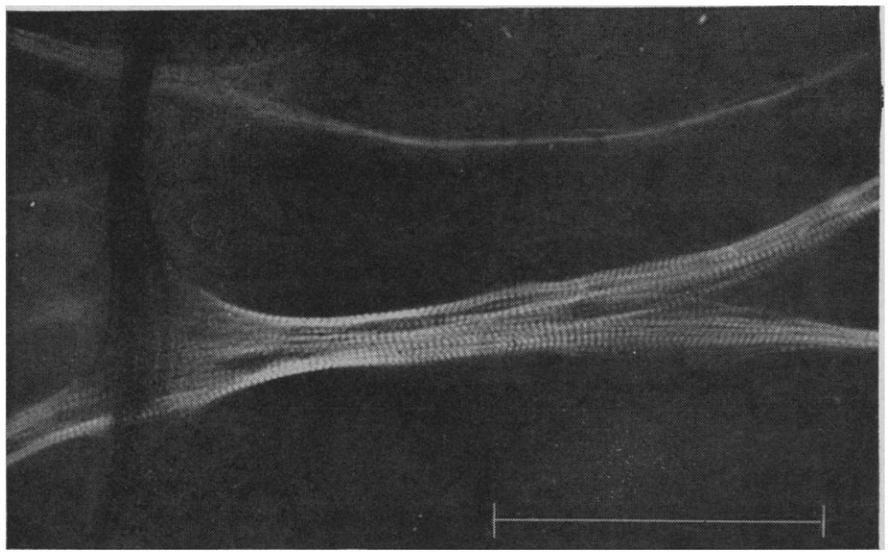


Fig. 6. An area of the colony shown in Fig. 5*G* (roughly, the area at the lower edge of the sixth frame from the right in the fourth row from the bottom). The use of polarizing optics demonstrates the presence of cross-striated myofibrils typical of striated muscle. Fixation: osmium vapor after storage at −20°C in 50 percent glycerol. Scale marker represents 0.1 mm.

ber (approximately 10 percent) of the colonies which had developed. We have since been able to increase the frequency of occurrence of differentiated colonies to more practical levels through the use of conditioned medium.

This approach was suggested by a parallel investigation directed toward exploring the relationship, observed earlier (*50*), between cell density and myotube formation. In the course of our study we found that medium recovered from confluent monolayer cultures induced myotube formation in newly initiated mass cultures some 24 hours earlier than in sister cultures in freshly prepared medium (*59*). Using an empirically determined technique (*60*), we are now able to obtain conditioned medium which consistently gives a higher frequency of differentiated colonies than was observed earlier.

Of all the factors tested thus far, the type of medium used seems to have the greatest influence on the frequency of formation of differentiated colonies. Figure 1, for example, shows a comparison of two plates established with identical aliquots of the same cell suspension in conditioned medium prepared by two different methods. Although the plating efficiency is similar in the two cases, 49 percent of the colonies in group B are differentiated muscle colonies, in contrast to 1.8 percent in group A. Whether the effect operates by selection or by the promotion of differentiation is not known.

In obtaining the data presented below, the conditioning technique described (*60*) was used, and the data apply only to cultures grown in that medium. The variability in plating efficiency and the frequency of differentiation can be judged from Table 1, which summarizes all of the experiments performed with this medium from June to December of 1962.

Identification of Muscle Colonies

In addition to their higher frequency of differentiation, individual colonies of both muscle and nonmuscle cells grown in conditioned medium tend to be larger (at comparable times) than similar colonies grown in unconditioned medium, and they are readily identified by macroscopic features alone.

In the cultures shown in Fig. 2, two distinctly different colonial morphologies can be observed: (i) colonies with a more or less regular circular outline, with little internal structural detail discernible with the unaided eye, and (ii) colonies which have an irregular, frequently elongated shape and which appear to be composed of darkly stained fibers which are often aligned, forming large swirls.

These "fibers," at higher magnification, are revealed to be elongated multinuclear cells (see Fig. 3), comparable to the myotubes observed in monolayer culture. They can be demonstrated, by appropriate techniques, to contain the cross-striated myofibrillar pattern typical of striated muscle. The disk-like colonies, on the other hand, are composed of greatly flattened cells, generally fibroblastic in appearance.

Diagnostic Features of Single Cells

We have, up to this point, been considering observations made by sampling our material at regular intervals without attempting sequential analysis of individual clones. The higher incidence of differentiated colonies obtained with conditioned medium made the latter approach practical.

Petri plates seeded with 200 cells each were examined 18 to 24 hours after plating; phase-contrast optics were used at a magnification of 100 diameters. Cells were selected which had neither divided nor started to divide and which were separated by at least 4 millimeters from the nearest neighbor. The position of the cell was marked on the bottom of the petri plate by means of an inking slide marker (Leitz). Photographic records were made at regular intervals.

The two main objectives of these studies were to determine whether the muscle colonies have a unicellular origin and to establish the temporal sequence of proliferation and myotube formation. During the preliminary trials it became apparent that we could recognize those single cells which, if viable, would yield muscle colonies. In Fig. 4 myogenic and nonmyogenic cell types are compared. An attempt has been made to indicate the degree of variability. The consistent features of the myoblast class are the marked bipolar shape and the small ruffled membrane, usually restricted to one tip of the cell. In contrast, the cells which give rise to fibroblast-like colonies are extremely flattened (see *44*) and consequently are less refractile. A much more extensive ruffled membrane is generally present, with no apparent localization.

The reliability of these criteria has been evaluated. The data are summarized in Table 2. Single cells were located, and their positions were marked in accordance with a color code as a means of recording our prediction of colony type directly on the petri plate. The results were scored after fixation and staining on the 13th day of culture.

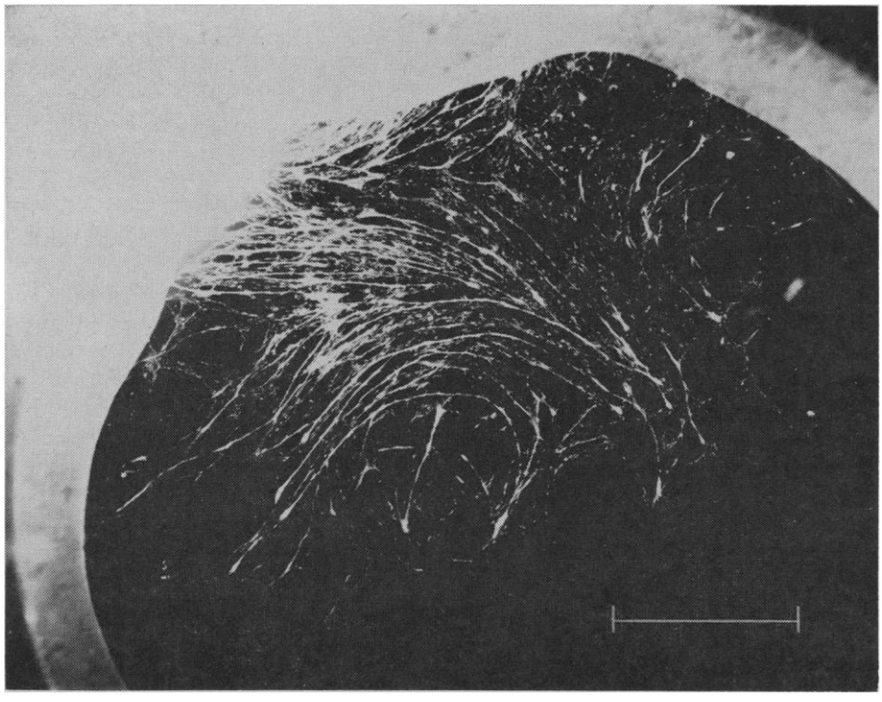


Fig. 7. A colony derived from a single cell isolated by a glass cylinder (see text). The colony was photographed *in situ* in darkfield illumination after fixation with Bouin's fixative. The bright semicircle is not the glass cylinder itself but a continuous bead of silicone grease extruded by pressing the cylinder into position. Scale marker represents 1.0 mm.

The data in Table 2 indicate good correlation between the morphology of single cells and the type of differentiation observed in the clones derived from them. The error involved may be indicative of the observer's error in resolving differences between individual cells. The area of morphological overlap is indicated by the cells at the extreme right, top and bottom rows, in Fig. 4.

Cell shape, in vitro, can be modified both through change in the texture of the substratum (*61*) and through change in the composition of the medium (*31, 62*). In plating experiments these factors are, at least initially, uniform throughout the culture. The differences observed can only be ascribed to intrinsic differences between cells.

Table 2 also indicates approximately equal viability of the two types. If we assume that one group represents cells which have undergone some process of simplification we must also assume that (i) simplification has not affected viability, and (ii) the process of simplification has occurred during the 18 to 24 hours that have elapsed between dissection of the muscle and observation of the single, isolated cell.

Although we can assume, with the degree of confidence warranted by the data in Table 2, that the bipolar cell is a myoblast, we can make no appraisal of its previous developmental history. Cytodifferentiation is markedly asynchronous in the leg musculature of the chick embryo (*55*). Despite this developmental heterogeneity, our cell suspensions consist largely of mononucleated cells (*50*), from which finding we conclude that the cell suspension is not representative of the tissue of origin. We cannot as yet exclude the possibility, however, that the single, mononucleated cells which we observed and followed were secondarily liberated from syncytia by the trauma involved in disaggregating the tissue.

The identity of the nonmyogenic cells is uncertain. They may be (i) prospective myoblasts whose instability reflects different intermediate stages of differentiation, or biosynthetic variation from cell to cell, or alteration through trauma, or (ii) discrete cell types other than myoblasts (for example, fibroblasts).

Table 2. Distribution of colony type (muscle or "fibroblastic") from single cells that were scored on the basis of morphological criteria (see text).

Class of cell	Total number of cells	Total number of colonies	Distribution of colony types	
			"Fibroblastic"	Muscle
"Fibroblastic"	99	61	52	9
Bipolar	72	53	2	51

Moreover, we cannot be certain that we are dealing with a single class of nonmyogenic cells. Consistent, distinguishable differences can be discerned between the cells of different colonies of fibroblast-like cells. These differences would probably not be obvious in mixed populations of these same cells.

In the tempo of modern research, the brilliant work of earlier eras is often overlooked. It seems appropriate here to recall the pioneering studies of the Lewises (*63*), who observed, in the outgrowth area of muscle explants, cells similar to the bipolar cells described here. They noted the striking resemblance of these cells to the myoblast in vivo, and they correctly interpreted their significance. To their remarks we can add only that the spindle shape is an intrinsic property of the cell itself and is not dependent upon intercellular associations.

The distinctive bipolar shape of the myoblast is maintained through many generations. Compare, for example, the single cells scattered among the muscle syncytia in Fig. 5*G* with the cells of the invading colony of fibroblast-like cells at the bottom of the photomicrograph.

Pattern of Muscle Colony Formation

Of the two recognizable cell types, the myoblast thus far has understandably occupied our attention. Applying the criteria discussed earlier, we have been able to select with a high degree of accuracy for those cells which, if viable, will yield colonies of differentiated muscle cells. Figure 5 is a sequence of photomicrographs taken of the living cells, showing the development of such a macroscopic colony (Fig. 5*G*) from the single cell seen in Fig. 5*A*. The colony in Fig. 5*G*, photographed on the 13th day of culture, measures 3.9 by 4.8 millimeters. It arose from a cell roughly 0.05 millimeter long.

Examination of the first four photomicrographs in this sequence indicates that, during this initial period at least,

the cells are dividing with a generation time of the order of 12 to 18 hours. This is by no means unusual in these cloning experiments in conditioned medium, although the doubling time for DNA in the tissue of origin is approximately 48 hours (*64*) and the generation time previously determined for monolayer cultures (*50*) is 24 hours.

The first striking evidence of further differentiation can be observed in Fig. 5*E*, which is a photomicrograph of the colony on the 6th day of culture [when unconditioned medium was used, differentiation was first observed considerably later than day 6 (*24*)]. The greatly elongated structures are multinucleated myotubes, which presumably formed through a process of successive cell fusion. Various experimental approaches (*48*, *50–54*) indicate that cell fusion is the predominant, if not the sole, mechanism of the origin of multinuclearity in skeletal muscle. In this colony, mononucleated myoblasts can be found scattered throughout the enlarging colony (Fig. 5*F*, day 9; Fig. 5*G*, day 13). It is our view that these mononucleated cells constitute a proliferating "stem cell" line which contributes to the subsequent enlargement of the network of muscle cells. A comparison of Figs. 5*E*, 5*F*, and 5*G* indicates the pattern of enlargement of this muscle-cell network. Not only do pre-existing muscle fibers grow longer but new ones are formed.

The individual cells of the colony, in the early stages of its development, do not remain in intimate contact after cell division. This is true of the early stages of nonmyogenic colonies as well. This type of colony, which is composed of single mononucleated cells, does not form a coherent, tightly packed colony until 4 to 6 days have elapsed, and even at late stages some free cells can generally be observed at the advancing edge of the colony (see Fig. 5, *F* and *G*). If we assume that, under the conditions of our experiments, the cells adhere preferentially to the substratum rather than to each other, these observations are in harmony with current concepts of cell locomotion and adhesion (*65*).

The pattern of growth of this colony is consistent with its origin from a single cell. Although all of the colonies which we have followed (irrespective of their developmental type) are, in their early stages, composed of dispersed cells, the degree of dispersion is limited, and the colonial foci remain discrete.

As the population of the colony increases, a more cohesive pattern is established; the network of muscle fibers of myogenic colonies and the circular colonies of contiguous nonmyogenic cells (see Fig. 5). These two types of colonies, as they enlarge, frequently impinge upon each other, as do the muscle colony and the invading edge of the fibroblast-like colony in Fig. 5.

Physical Isolation of Single Myoblasts

The conclusive proof, however, that a single cell can give rise to a macroscopic colony of differentiated muscle cells rests on a modification of the described procedures that excludes any possible contribution of cells other than the progeny of the single cell originally isolated. After cells had been located and their positions had been marked, a small pyrex cylinder (inside diameter, 6 mm; height, 5 mm), bearing a continuous bead of silicone grease on its lower rim, was pressed down in the petri plate to encompass the selected cell. [This is essentially an adaptation of the technique used by Puck's group (*31*) to transfer single colonies.] The cylinder was filled with medium, and a cover slip was laid across the top to create two parallel surfaces which would permit examination, by means of phase optics, of the floor of the chamber thus created. Provision was made for the relief of pressure and for gas exchange by grinding two wide, shallow grooves into the upper surface of the cylinder. After positioning of the cylinder and cover slip, the floor of the chamber was scanned carefully to make sure that only one cell was included. The medium was then drained from the petri dish; this resulted in the destruction of all cells not protected by cylinders. The medium in the cylinder was replaced every 3rd day, and the cultures were termi-

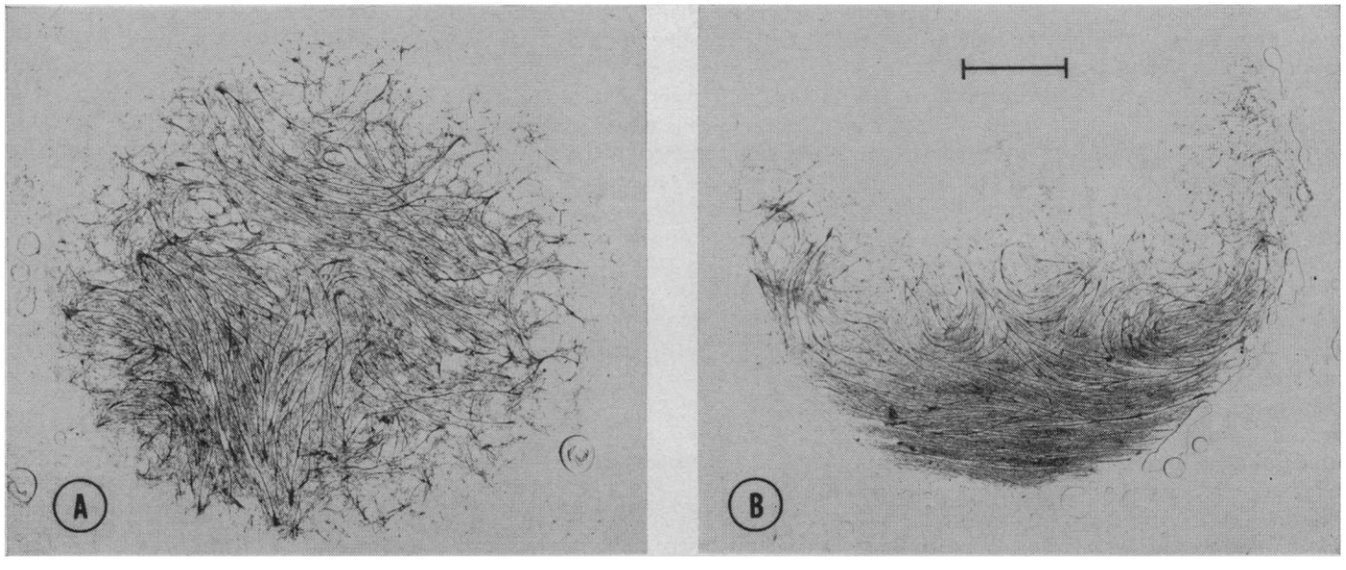


Fig. 8. Two examples of colonies which arose from single cells isolated by glass cylinders. The darkly stained "fibers" are multinucleated myotubes (see Fig. 2). See text for an explanation of the shape of the colony. Fixation, in Bouin's fixative, was followed by staining in Ehrlich's hematoxylin (no counterstain). Scale marker represents 1.0 mm.

nated on day 13 or 16. Figure 7 depicts such a colony after fixation but prior to the removal of the cylinder. Figure 8 shows two colonies which developed from such single, isolated cells. The cylinder was placed directly over the cell from which the colony in Fig. 8*A* was derived. The cylinder was eccentrically positioned over the cell which gave rise to the colony in Fig. 8*B*, to avoid inclusion of a neighboring cell; hence its crescent shape.

From these studies, in which single cells were either followed sequentially or physically isolated, it is clear that single myogenic cells drawn from the population that we sampled did indeed give rise to a clone that formed a colony, of macroscopic dimensions, which clearly contained large numbers of differentiated muscle cells. It is our impression that the vast majority of colonies which we see are, in fact, clones.

Number of Cell Generations

To establish the degree of stability of myogenic properties with reference to the number of antecedent cell generations is of considerable importance. The data presently available permit only a rough approximation of the lower limits, since the modes of growth and differentiation of skeletal muscle cells make accurate estimation impossible. There is good evidence that most, if not all, of the nuclei in muscle syncytia are nonproliferative (*48, 51, 52*). Thus, the nuclei of cells which had been incorporated into syncytia at some early time would have passed through fewer divisions than nuclei added later. If our interpretation of the mode of growth of muscle networks is correct, then nuclei added between day 9 (Fig. 5*F*) and day 13 (Fig. 5*G*) could have passed through 9 to 12, 15 to 19, or 20 to 26 divisions, depending upon whether one assumes a generation time of 24, 18, or 12 hours for the complement of mononucleated cells. In contradistinction, the nuclei of the *earliest* myotube (in this colony it was observed on day 5) would have passed through only 4, 6, or 8 divisions. We cannot, under these circumstances, derive the number of divisions by a simple tally of the number of nuclei in syncytial association. We also must know the rate at which potentially proliferating nuclei are removed from the population of mononucleated cells relative to their rate of division. One can envision three different patterns of colony development.

1) Myotube formation occurs late, after considerable proliferative activity has occurred.

2) Myotube formation occurs early and involves all of the single cells of which the colony is composed.

3) Myotube formation occurs early and involves a variable fraction of the single cells.

Actually, all three modes have been observed, under one or another set of conditions. The majority of the cases studied, however, fall into class 3, with considerable variability of the fraction of single cells initially involved.

If there *is* an intrinsic limitation to the number of times a cell may divide in culture and retain its capacity to differentiate, we have found no supporting evidence for it thus far.

Cellular differentiation in culture has previously been achieved only through the use of an explant, considerably larger than a single cell. In the studies reported here, single, isolated cells were observed to proliferate and to form macroscopic colonies of differentiated muscle.

The use of conditioned medium (that is, medium harvested from mass cultures of muscle cells) was found to be the most effective means of achieving differentiation in a high proportion of the colonies. Conditioned-medium and feeder-layer techniques, both of which simulate the effects of a large mass of cells, have been used effectively in supporting the proliferation of single cells. Our studies suggest that such techniques may, in addition, be useful in reproducing an environment which will permit differentiation as well.

It has been argued that high rates of proliferation are implicated in the loss of cell-type specificity in culture. The argument needs clarification. The premise itself is largely untested and remains unproved. In our experiments certain aspects of differentiation (for example, myofibril formation) are detected only after proliferation has ceased. However, the characteristic shape of the progenitor cell of the muscle clone is itself a differentiated feature, and it is retained by the proliferating cells of the muscle colony. Neither the observed rates of cell division nor the total number of divisions seem to affect the capacity of these cells to differentiate (see also *46*) (*66*).

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60. The technique finally adopted for preparing conditioned medium is as follows. Growth medium (10 cm^3) in petri plates 150 mm in diameter is inoculated with 6×10^6 cells [prepared as described in I. R. Konigsberg *et al.* (*48*)]. The medium is replaced with 15 cm^3 of fresh medium on the 2nd and 5th day of culture, and the old medium is discarded. On day 11 the medium is removed, under conditions of sterility, and passed through a Millipore filter (type HA) to insure removal of any possible cellular or microbial contamination. This medium is our standard conditioned medium. It is prepared each week; unused portions are stored at 5°C and discarded after 2 weeks.
The growth medium used is that described in I. R. Konigsberg *et al.* (*48*), modified as described in I. R. Konigsberg (*24*), except for the following changes. (i) The inorganic salt and glucose concentrations of both the Hanks solution and the amino acid-vitamin supplement have been altered and are now the same for the two solutions (the concentrations, in millimoles per liter, are as follows: NaCl, 128.6; KCl, 4.1; $CaCl_2$, 1.12; $MgCl_2$, 0.49; Na_2HPO_4, 0.93; KH_2PO_4, 0.45; $NaHCO_3$, 15.3; $MgSO_4$, 0.21; and glucose, 6.10). (ii) The growth medium now contains 50-percent embryo extract to a concentration of 10 percent. The only other innovation in our recent techniques is the use of polystyrene petri plates (obtained from Falcon Plastics).
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66. I acknowledge my indebtedness to Mrs. Wilma Gabbay and Mr. Francis J. Kupres for their invaluable and unstinting assistance during the progress of the original work reported. I wish to thank Drs. D. W. Bishop, D. D. Brown, J. D. Ebert, M. S. Steinberg, and J. P. Trinkaus for their valuable criticisms during the preparation of the manuscript.

Can the Direction of Flow of Time Be Determined?

No method has been found, but properties of strange particles offer a new chance to explore the question.

Robert G. Sachs

The notion that the direction of flow of time cannot be determined by any physical experiment has been deeply ingrained in the thought and theories of physicists until very recent years. In all cases in which they have been adequately tested, the laws of physics satisfy this condition and, in unexplored areas, the condition is usually taken as a starting principle for formulating a theory. Violation of this principle would affect the results obtained from laboratory experiments, thereby making it subject to direct experimental verification. It may appear that the notable distinction between past and future from the cosmological, biological, and psychological points of view settles the issue without further experimentation, but even if the laws of physics are assumed to underlie these phenomena, we shall find that they do not stand in contradiction to the principle of the reversibility of time.

"Time reversal" is the term used in physics to describe the procedure of going from one direction of time flow to the other in writing equations describing the physical behavior of a system; the principle responsible for the belief that there is no distinction between the two directions of flow is known as "invariance [of physical laws] under time reversal" or "*T*-invariance." Although recent discussion of *T*-invariance has been associated with quantum phenomena, application of the principle to classical physics is not without interest. The most important application in classical physics was made by Loschmidt (*1*) to show that Boltzmann's proof of the H-theorem contained a fallacy; Loschmidt's work raised a question concerning the connection between the second law of thermodynamics and the mechanical theories of physics. Resolution of this very compelling question, which goes to the very foundations of statistical mechanics, is not my particular concern and is not discussed in this article. I refer the interested reader to the basic exposition of the subject by the Ehrenfests (*2*). I shall consider the application of the principle to a very simple classical mechanical system which provides an insight into the nature of the question being raised here.

The important role of time-reversal invariance in quantum mechanics was

The author is professor of physics at the University of Wisconsin, Madison.