[10] Hartman, T. F., D. A. Grant, and L. E. Ross, *Psychol. Rep.*, **7,** 305 (1960).
[11] Reynolds, W. F., *J. Exp. Psychol.*, **55,** 335 (1958).
[12] Ross, L. E., *J. Exp. Psychol.*, **57,** 74 (1959).
[13] Ross, L. E., and K. W. Spence, *J. Exp. Psychol.*, **59,** 379 (1960).
[14] Spence, K. W., D. F. Haggard, and L. E. Ross, *J. Exp. Psychol.*, **55,** 404 (1958).
[15] Spence, K. W., and J. A. Taylor, *J. Exp. Psychol.*, **42,** 183 (1951).
[16] Spence K. W., and L. E. Ross, *J. Exp. Psychol.*, **58,** 376 (1959).
[17] Spence, K. W., *J. Exp. Psychol.*, **45,** 57 (1953).
[18] McAllister, W. R., *J. Exp. Psychol.*, **45,** 417 (1953).
[19] Spence, K. W., and I. E. Farber, *J. Exp. Psychol.*, **45,** 116 (1953).
[20] Estes, W. K., "The statistical approach to learning theory," in *Psychology: A Study of a Science*, Study 1, vol. 2, ed. S. Koch (New York: McGraw-Hill Co., 1959).

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# *CELLULAR DIFFERENTIATION IN COLONIES DERIVED FROM SINGLE CELL PLATINGS OF FRESHLY ISOLATED CHICK EMBRYO MUSCLE CELLS*

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The loss of cell-type specific characteristics which frequently accompanies growth *in vitro* was recognized almost from the inception of the tissue culture technique.[1] This phenomenon even more acutely attends the application of the newer methods of cell culture in which trypsin-dispersed cells are serially cultivated on glass substrata as rapidly proliferating cell monolayers. The properties of cell strains so cultivated, with remarkably few exceptions, do not resemble those of the predominant cell type of the tissue of origin. The precise nature of this loss of identifiable characteristics remains obscure. Alterations in members of the culture population[2] as well as selection of a rapidly growing contaminant or a variant arising in culture[3] could very well be responsible for the observed loss. Alternatively, it has recently been suggested by Sato and his co-workers[4, 5] that only a small fraction of the cells present in normal tissues can propagate under the conditions of cell and tissue culture techniques. Further, it is assumed that this small fraction represents a class of cells present in all tissues which can be cultivated and which does not share the properties of the parenchymal cells of these various tissues.

Analysis of the problem of the loss of differentiative character is complicated by the disparity between cell culture and the culture techniques generally employed to promote cellular differentiation. Indeed, cultivation of dispersed cells rather than a compact aggregate would be precisely the condition judged to promote such loss. In a previous publication, it was demonstrated that a high degree of differentiation was attained by embryonic chick skeletal muscle cells grown in monolayer cultures.[6] These cultures were initiated with $10^6$ cells, and the first indices of differentiation coincided with the attainment of confluency. The present report extends this finding to cultures initiated with small numbers of cells (200

to 400 per culture) in which differentiation can be observed in a number of the discrete colonies subsequent to their formation.

The present results thus indicate that freshly isolated embryonic muscle cells can survive and proliferate under the conditions imposed by cloning techniques. Further, such cells can retain and express their potency at least through the period of colony formation in primary culture.

*Methods.*—Skeletal leg muscle of 11-day White Leghorn embryos, incubated at 37°C, was employed as the tissue source. The techniques used to prepare cell suspensions as well as the composition of the culture medium have been previously published[7] and were altered only to the extent of substituting saline G (see ref. 8) for saline A and reducing the concentration of streptomycin to half that previously used.

The cloning technique employed was that developed by Puck, Marcus, and Cieciura[9] except that the volume of medium was reduced to 2 cc and the cells were plated on circular coverslips, 46.5 mm in diameter, fastened to glass rings with silicone stopcock grease.

The medium was changed every 3 to 4 days. At various intervals, cultures were fixed in cold calcium-formol, stained with Ehrlich's hematoxylin, dehydrated, and mounted in permount. Cultures prepared for microscopy in polarized light were unstained and mounted in methacrylate.

*Results.*—The plating of small numbers of cells on a relatively large surface area results, after a growth period of sufficient duration, in the formation of discrete colonies. Figure 1 is a photograph of a culture inoculated with 200 cells and fixed after 10 days of growth. Fifty-six individual colonies were counted. The present series of experiments is not extensive enough to critically establish the plating efficiency for this cell type under these experimental conditions. On the basis of the data available it ranges roughly from 8 to 28 per cent.

Colony size, it will be noted, varies widely from those barely visible to the naked eye to colonies fully 3 mm in diameter. Puck and his associates observed a similar variability in uncloned populations of strain Hela which was strikingly reduced in clonal populations of the same strain.[9]

Examination of colonial morphology, at even the relatively low magnification of the dissecting microscope (10×), reveals the presence in approximately 10 per cent of the colonies of greatly elongated fibers (see Fig. 2). At higher magnification (Figs. 3 and 4), these fibers can be resolved into elongated multinuclear myotubes identical to those observed and identified in mass culture.[6, 7]

Conclusive evidence that the multinuclear cells observed within the colonies are indeed myogenic elements is presented in Figure 5. When colonies containing multinuclear "ribbons" are examined with polarizing optics, the presence of birefringent fibrils, frequently exhibiting the pattern of cross-striation characteristic of voluntary muscle, can be readily detected. A weaker birefringence is evident also in the smaller cellular elements of such colonies. Those colonies composed purely of "fibroblast-like" cells, on the other hand, are devoid of such fibrils.

Myotube formation occurs sometime between the sixth and tenth day of culture. Of the 287 colonies examined at 6 days, none show multinuclear cell formation, whereas approximately one-tenth of the colonies examined at 10 days contain indisputably myogenic cells.

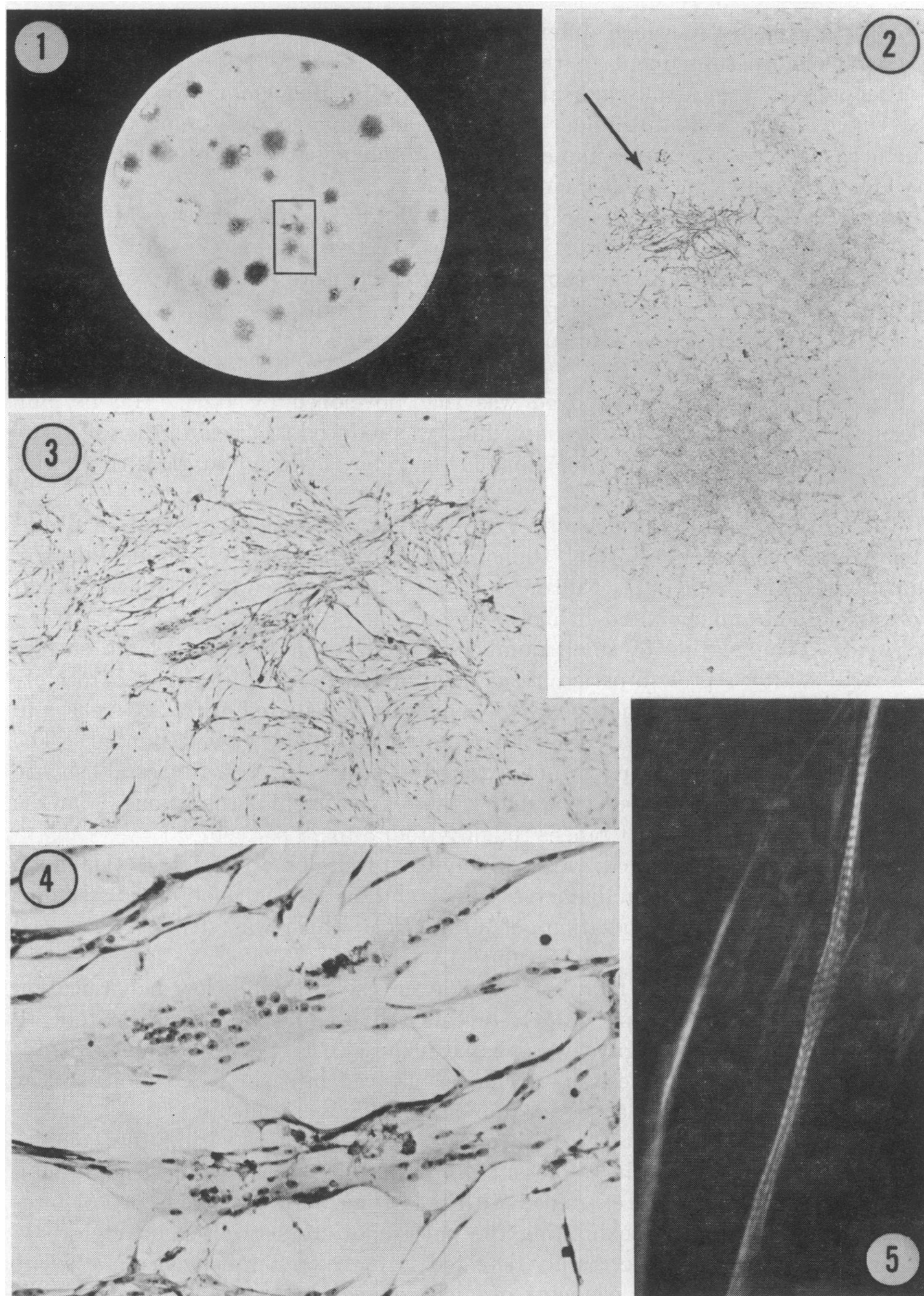


FIG. 1.—Culture initiated with 200 cells, fixed and stained after 10 days (1 ×). Area outlined is enlarged in Figure 2.

FIG. 2.—Several colonies of culture described above. Arrow indicates muscle colony. Note striking difference in appearance of muscle colony as compared to surrounding colonies of "fibroblast-like" cells (11 ×).

FIG. 3.—Muscle colony above at higher magnification. Note the extremely long "ribbon-like" cells which comprise the greater portion of the colony (60 ×).

FIG. 4.—Cells of muscle colony demonstrating high degree of multinuclearity (210 ×).

FIG. 5.—Segment of multinuclear cell of colony in culture fixed at 10 days and examined in polarized light. Note pattern of cross-striation (558 ×).

*Discussion.*—It is quite clear from these experiments that freshly isolated cells of myogenic potency can survive both cell culturing and at least those phases of cloning techniques utilized. Moreover, this potency can be maintained during a period of rapid and extensive proliferation to express itself within the spatial limits of a discrete colony. Whether this is a general property of all myogenic cells or of only certain individuals of the cell type cannot be ascertained as yet, in view of the relatively low plating efficiency encountered. However, it should now be feasible to rigorously examine the stability of developmental potency through subsequent cloning starting with a colony of cells whose origin and identity are known.

The presence of colonies of differentiated muscle cells in these cultures indicates that the conclusions drawn by Sato and his associates are not generally applicable. Their argument against embryological studies in which differentiation of cultured cells is described (ref. 5, p. 970) cannot apply to the present report. The muscle colonies are unquestionably the products of initially proliferating cells and not derived from "non-dividing but surviving portions of the inoculum." Relatively brief, mild trypsinization of embryonic tissues, which are generally more loosely structured than the corresponding adult tissue, yields cell suspensions which are, by and large, monodisperse. What groups of cells do exist exhibit a low multiplicity of from 2 to 6 cells. No aggregate large enough to produce a muscle colony without proliferation has ever been observed, nor would such an aggregate be expected to pass through the 200-mesh bolting silk we routinely use to filter cell suspensions. Whether the muscle colony is derived from a single cell or a group of two or three cells would not alter the necessity for assuming extensive proliferation. Previous investigations in this laboratory and elsewhere[7, 11] indicate that the multinuclear muscle cell is a product of cell fusion and is post-mitotic in nature. One would expect proliferation to occur prior to myotube formation, which is consistent with the absence of colonies containing myotubes on the sixth day of culture.

The present experimental design differs in several specific details from that adopted by Sato's group.[5] For example, the media employed are quite different, tissue disaggregation was achieved with trypsin in the present study rather than with mechanical methods, embryonic chick tissues were used rather than neonatal rat, and of course the tissues tested were not the same in both cases. The difference in results cannot be resolved without testing these variables; however, the developmental stage of the donor organism may very well prove to be the significant factor.

Several studies exist in the older tissue culture literature which demonstrate that with increasing developmental age there is a progressive extension of the lag period before outgrowth from explants.[10] Admittedly, the exact nature of the lag period is still unknown. It might represent the time required for a small number of cells to produce a progeny of sufficient size to produce outgrowth. This would be compatible with Sato's thesis. On the other hand, the lag might be due to some type of adaptation to the tissue culture environment, the necessity for which is imposed by some developmental event or events. If such were the case, one might expect cell culture techniques, which expose individual cells to the new environment immediately, to give poorer yields of viable cells from progressively older donors. Thus, cell culture applied to the tissues of postnatal animals may

very well be selective for a small hardy population of ubiquitous cells unrelated to the parenchymal elements of the source tissue.

In this context, it should be pointed out that the range of plating efficiencies of chick embryo muscle cells observed thus far is 2 to 5 orders of magnitude greater than those reported for neonatal rat tissues.[5] Moreover, inocula used in the latter study ($10^5$ to $10^7$ cells, depending on the tissue source) are, in most cases, larger than those commonly employed by us ($5 \times 10^5$ and $10^6$) in establishing mass cultures of muscle cells. Such mass cultures of embryonic cells grow to confluency within 3 to 4 days.[6, 7, 11] There is obviously a marked difference in the percentage of viable cells in the two types of cell suspensions, which may be due to donor age primarily rather than differences in technique.

[1] Bloom, W., *Physiol. Rev.*, **17,** 589 (1937).

[2] Holtzer, H., J. Abbott, J. Lash, and S. Holtzer, these PROCEEDINGS, **46,** 1533 (1960).

[3] Hsu, T., in *Developmental Cytology,* 16th Symposium of the Society for the Study and Development of Growth, ed. D. Rudnick (New York: Ronald, 1959).

[4] Sato, G., L. Zaroff, and S. E. Mills, these PROCEEDINGS, **46,** 963 (1960).

[5] Zaroff, L., G. Sato, and S. E. Mills, *Exptl. Cell Res.*, **23,** 565 (1961).

[6] Konigsberg, I. R., *Exptl. Cell Res.*, **21,** 414 (1960).

[7] Konigsberg, I. R., N. M. McElvain, M. Tootle, and H. Herrmann, *J. Biophys. Biochem. Cytol.*, **8,** 333 (1960).

[8] Puck, T. T., S. J. Cieciura, and A. Robinson, *J. Exptl. Med.*, **108,** 945 (1958).

[9] Puck, T. T., P. I. Marcus, and S. J. Cieciura, *J. Exptl. Med.*, **103,** 273 (1956).

[10] Medawar, P. B., *Proc. Roy. Soc.*, **B129,** 332 (1940).

[11] Konigsberg, I. R., *Circulation*, **24,** 447–457 (1961).