# DNA SYNTHESIS AND MYOGENESIS[1, 2]

F. E. STOCKDALE[3] and H. HOLTZER[4]

*Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa., U.S.A.*

Received January 30, 1961

THIS paper is concerned with two related aspects of myogenesis. The first is whether nuclei within myotubes synthesize DNA. The second is whether embryonic muscle cells which are synthesizing myosin, the meromyosins, and actin are also synthesizing DNA.

Three theories emerge from the older literature [12, 16, 19, 20, 45] to explain the formation of multinucleated myotubes during embryogenesis and regeneration: (1) mitotic divisions of myoblast and myotube nuclei without cytoplasmic divisions; (2) amitotic divisions of myoblast and myotube nuclei without cytoplasmic divisions; (3) fusion of mononucleated myoblasts with one another and with myotubes. Many recent reports favor amitosis as being largely responsible for the multinucleated myotube [1, 2, 3, 5, 7, 9, 10, 15, 17, 18, 21, 37, 38, 40]. Evidence for fusion of cells may be found in Lash, Holtzer and Swift [30], Holtzer, Marshall and Finck [28], Holtzer, Abbot, and Lash [27], Holtzer [22], Capers [8], and Konigsberg *et al.* [32].

In conventionally embedded and stained material embryonic cell membranes are often difficult to see; consequently it is often difficult to determine whether a dividing nucleus lies within a myotube or within a mononucleated cell adhering to the surface of a myotube. To avoid these difficulties, monolayers of myotubes in tissue culture or glycerinated squashes of developing muscle from embryos were examined. DNA synthesis was followed by tracing the incorporation of tritiated thymidine by means of radioautographs and by a study of mitotic figures.

It was found that myotubes form by fusion of cells. Nuclei within myotubes do not synthesize DNA, hence they cannot divide mitotically or amitotically.

[1] This investigation was supported in part by Research Grants B-493 and B-1629 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service, and NSF G-14123 from the National Science Foundation.

[2] An abstract of these experiments appeared in *Anat. Record* **138**: 384 (1960).

[3] Post Sophomore Medical Student Research Fellow of the Public Health Service.

[4] Scholar in Cancer Research of the American Cancer Society.

In addition, evidence will be presented suggesting that when embryonic muscle cells begin to synthesize contractile proteins they do not replicate their DNA.

## MATERIAL AND METHODS

Thigh muscle from 10 day and somites from 3 day chick embryos were used for tissue culture. Ten day thigh muscle consists of long, multinucleated myotubes, connective tissue cells and presumptive myoblasts; the somites consist of a small number of mononucleated myoblasts, connective tissue cells, presumptive cartilage cells and presumptive myoblasts. The muscle was removed, cut into small pieces (1 $mm^3$), and incubated for 1 hr at 37°C in a 0.5 per cent trypsin (Difco 250) solution (Ca-, Mg-free Simms' solution) after the procedure of Moscona [36]. The tissue was washed in Simms' balanced salt solution (BSS) and nutrient medium (2 parts horse serum: 2 parts BSS: 1 part 11 day embryo extract) and dissociated into a suspension of mononucleated cells by repeated pipetting [41]. The suspension was filtered through a Swinny Filter (Millipore Co.) containing lens paper, and diluted with nutrient medium. The suspension was then plated in Leighton tubes on coverslips covered with thin chicken plasma clots, or on Gey slides, at concentrations of $5 \times 10^5$ to $2 \times 10^6$ cells per milliliter. They were incubated at 37° for periods up to three weeks. Leighton tube cultures were fed every third or fourth day. After two to five days, depending upon the concentration of the original suspension, a monolayer of multinucleated myotubes interspersed with mononucleated cells was obtained.

Living cells on Gey slides were inspected under the phase microscope. For studies of mitotic figures Leighton tube cultures were fixed in Bouin's solution or acetic alcohol and stained with hematoxylin. Other cultures were fixed with 1 per cent neutral formalin or extracted in 50 per cent glycerol at 0°C for 2 days and then treated with fluorescein-labelled antibodies to myosin, the meromyosins or actin as described in Holtzer, Marshall and Finck [28] and Holtzer [23].

To detect DNA synthesis Leighton tube cultures were exposed to tritiated thymidine (Schwarz BioResearch, Inc., Mt. Vernon, N.Y., 1.9 C/m*M*) at various times during the formation of the myotubes. Individual experiments will be described in context. Radioautographs were made by covering the tissue on the coverslips with Kodak NTB-3 emulsion [29] or stripping film [13].[1] After exposures of 2 to 7 days the radioautographs were developed, followed by staining with Delafield's hematoxylin. When radioautographs were to be studied in conjunction with the binding of labelled antibodies, the cells on coverslips were first stained with the antisera and then radioautographed. Gelatin was used to bind the coverslips to the slides, since it was found that HSR mounting medium was autofluorescent and albumin absorbed too much of the exciting UV light. The grains produced by the tritium were readily observed under the fluorescence microscope with antibody-stained material.

In this paper a "myotube" is a bi-, tri- or higher multinucleated cell containing

[1] We are indebted to Dr. L. Goldstein and Dr. M. Mendelsohn of the University of Pennsylvania for advice on radioautographic procedures.

myofibrils; a "myoblast" is a mononucleated cell containing a single myofibril; and a "presumptive myoblast" refers to a theoretical mononucleated cell which will differentiate into a myoblast but which currently cannot be chemically or histochemically distinguished from any other embryonic mesenchymal cell.

## RESULTS

*Mitotic activity in cultures of 10 day thigh muscle and myotubes* in vivo.—Within 3 hr many of the suspended cells settle on and attach to the coverslips. Mitotic figures are rare before the 16th hour (Table I). With concentrated cell suspensions bi- and trinucleated cells appear at the end of the first day and increase thereafter. Multinucleated myotubes become abundant by the 4th day. Myotubes may exceed 3 mm in length and may contain over 200 nuclei by the 3rd day. The distance between nuclei within a myotube may be over 100 $\mu$, whereas in other regions of the same myotube the nuclei may be so packed as to deform one another's surface (Fig. 1).

Nuclei within the myotubes move longitudinally. Shifts of nuclei over a

TABLE I. *Cultures of suspended 11 day thigh muscle fixed at different times and stained* in toto *with iron hematoxylin.*

Only metaphase plates were scored as dividing cells: the mitotic activity is given in terms of numbers of metaphase plates/1000 cells. The increase in numbers of myotubes and the increase in numbers of nuclei/myotube with time is shown in the second part of the table.

| | 16 hr | 25 hr | 40 hr | 66 hr |
|---|---|---|---|---|
| | *Mitotic Figures* | | | |
| Metaphase figures in mononucleated cells | 10/1000 | 54/1000 | 28/1000 | 11/1000 |
| Metaphase figures in multinucleated myotubes | 0 | 0 | 0 | 0 |
| | *Formation of Myotubes* | | | |
| Number of multinucleated myotubes per 10 fields | 5 | 8 | 18 | 23 |
| Median number of nuclei per myotube (range) | 2.19 (2–10) | 2.65 (2–10) | 3.33 (2–19) | 8.25 (2–198) |
| Total number of nuclei in 100 myotubes | 322 | 376 | 519 | 1620 |

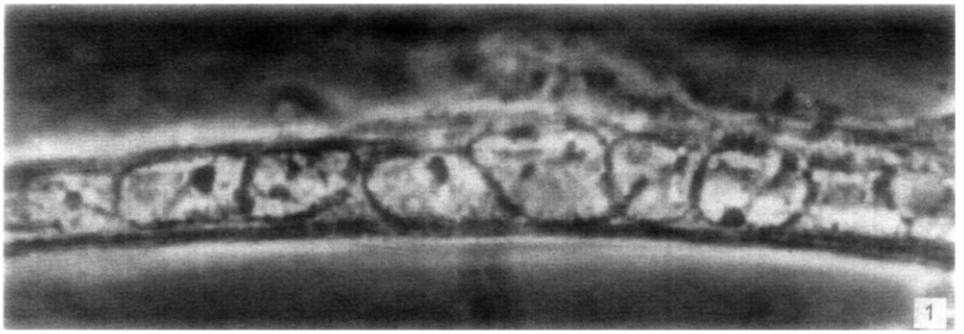
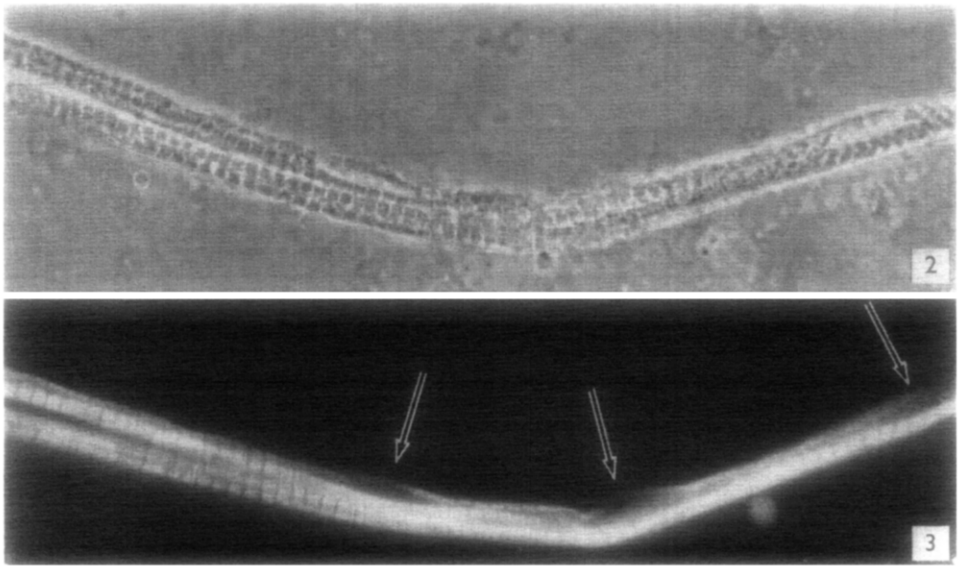


Fig. 1.—A phase photomicrograph of a living unstained myotube from a 5 day old Gey slide culture. The irregular shape of the individual nuclei is due to their being crowded together. Several hours after this photograph was taken the nuclei separated and they exhibited their more common oval configuration. In the past photographs of this type have been cited as evidence of amitosis.



Figs. 2 and 3.—Fluorescence and phase photomicrographs of an isolated myotube from a 7 day chick embryo treated with fluorescein-labelled antimyosin. The arrows indicate individual nuclei. Many thousands of such myotubes have been inspected under the phase and fluorescence microscopes and in not a single case has a mitotic figure been observed.

distance of 80 $\mu$ in one hour have been observed. Nuclear profiles change due to the appearance and disappearance of deep clefts in the nuclear membranes. Where living nuclei have been observed over several hours, we have never

seen these invaginations result in nuclear division. It is believed these transitory invaginations of the nuclear membranes and the deformation of the nuclei due to crowding are responsible for claims of amitosis in fixed and stained myotubes.

TABLE II. *See text for explanation.*

| Labelled nuclei present in: | Cultures exposed to tritiated thymidine as: Mononucleated cells (Series I) | Mononucleated cells followed by 4 day culture (Series II) | Myotubes (Series III) |
|---|---|---|---|
| Mononucleated cells | + | + | + |
| Myotubes | 0 | + | 0 |

Table I summarizes observations on metaphase plate figures and nuclear counts of stained cultures from a typical experiment. Inspection of living and stained cultures from the time cells adhere to the coverslips, until a week or more following myotube formation reveals mitotic figures only in mononucleated cells.

To determine whether nuclei within myotubes *in vivo* divide mitotically, squashes of 7 day trunk muscle were prepared. These myotubes were stained with the fluorescein-labelled antibodies and inspected under the phase and fluorescence microscopes.

Fig. 2 is a phase photomicrograph of a single myotube treated with fluorescein-labelled antibodies against myosin. Fig. 3 is a photomicrograph of the same myotube as observed under the fluorescence microscope. Of the several thousand nuclei examined not a single mitotic figure has been observed in myotubes obtained directly from embryos.

These findings clearly eliminate the possibility that conventional mitotic divisions of myotube nuclei lead to the multinucleated conditon.

*Thymidine uptake in cultures of 10 day thigh muscle.*—It is possible that either mitosis or amitosis was occurring but in a fashion not readily observed under the microscope. If myotube nuclei divide mitotically or amitotically, they must synthesize DNA at some stage in the division cycle, for muscle nuclei contain the normal $2n$ complement of DNA. Therefore a study of DNA synthesis *in vitro* using tritiated thymidine was undertaken. Three types of experiments were performed. *Series I*: Labelled thymidine (0.25 $\mu$C/ml of nutrient medium) was added to cultures of mononucleated cells for 30 min 18 hr after the cultures were established, and the cultures

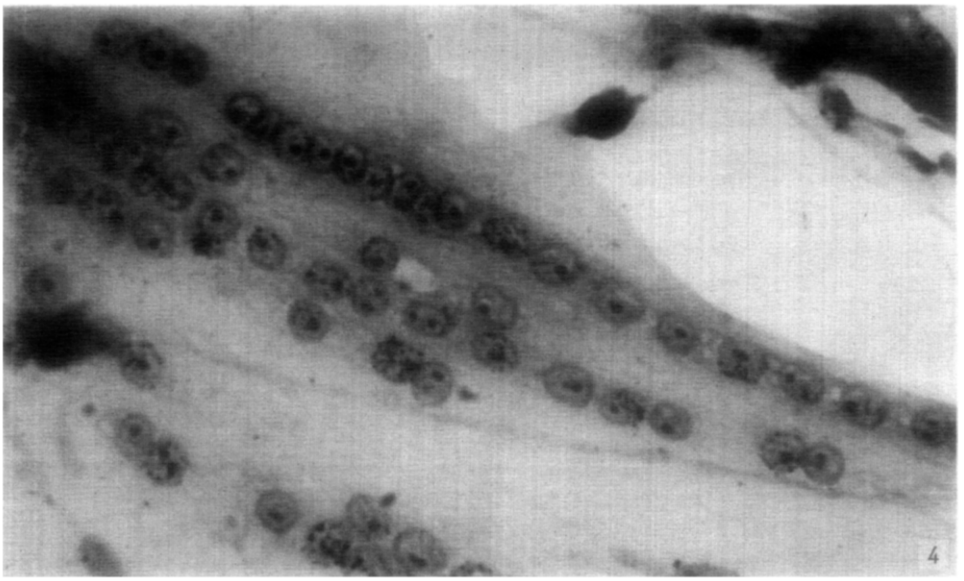


Fig. 4.—A radioautograph of fixed and stained myotubes from a 4 day old culture. The tritiated thymidine was added for 30 min when the culture was 18 hr old. At the time the culture was exposed to the thymidine only mononucleated cells were present. This photomicrograph, which was taken through the emulsion, is focused on the nuclei within the myotubes.

were immediately fixed and radioautographed. *Series II*: The same as in series I, but instead of fixing immediately, the cultures were rinsed 3 times in BSS, placed in fresh nutrient medium, cultured until myotubes formed 3 to 5 days later and then fixed and radioautographed. *Series III*: After myotubes had formed (in cultures 3 to 5 days old) the labelled thymidine was added for 30 minutes and the cultures immediately fixed and radioautographed. The results of these experiments are shown in Table II.

The nuclei of mononucleated cells take up the labelled thymidine in all series, but there is no incorporation of the thymidine by nuclei inside the myotubes in Series III. In Series II, where the labelled mononucleated cells were allowed to form multinucleated myotubes, a random distribution of labelled and unlabelled nuclei are found within the myotubes. Figs. 4 and 5 illustrate myotubes from Series II; Fig. 6 illustrates a myotube from Series III. Clearly nuclei labelled in mononucleated cells can contribute to the formation of multinucleated myotubes, but once within a myotube, nuclei cease to incorporate thymidine. These results are incompatible with theories that myotube nuclei divide mitotically or amitotically, but are consistent with the theory that fusion leads to the multinucleated units.

Cultures from Series II and III were also treated with fluorescein-labelled antibodies. The developing process and the presence of emulsion obscured fine cytological detail under the fluorescence microscope; nevertheless the following observations were made. Grains were found over myotube nuclei in Series II, but not over those from Series III. In both series grains were

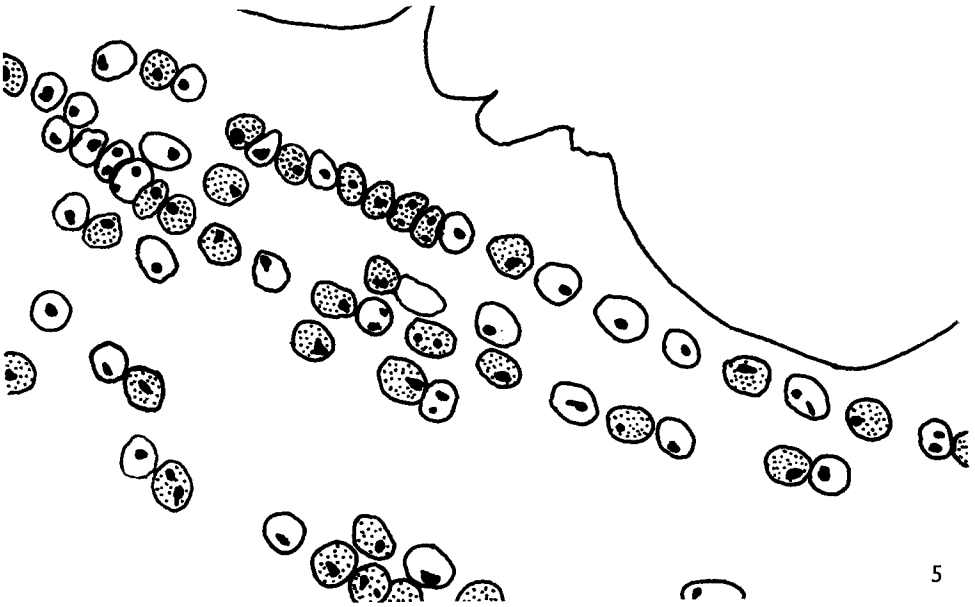



Fig. 5.—A tracing of the above section showing diagrammatically the presence of grains above the individual nuclei. The stippled nuclei were labelled.

found only over mononucleated cells. Mononucleated cells were not fluorescent. Only the longitudinal filaments of the myotubes of both series bound the different antisera. A more detailed description of the fluorescent patterns produced by the different antibodies in developing myotubes has been presented elsewhere [23]. The number of cross-striated myofibrils in myotubes from 5 day cultures was considerably greater than that found in myotubes from 3 day cultures. From these experiments it is concluded that multinucleated myotubes are synthesizing contractile proteins and that the intracellular irradiation due to the tritiated thymidine does not greatly interfere with these synthetic activities.

*Mixed cultures of 3 day somites and 10 day thigh muscle.*—If marked cells which by themselves do not form myotubes are incorporated into myotubes when mixed with cells capable of forming myotubes, fusion would be

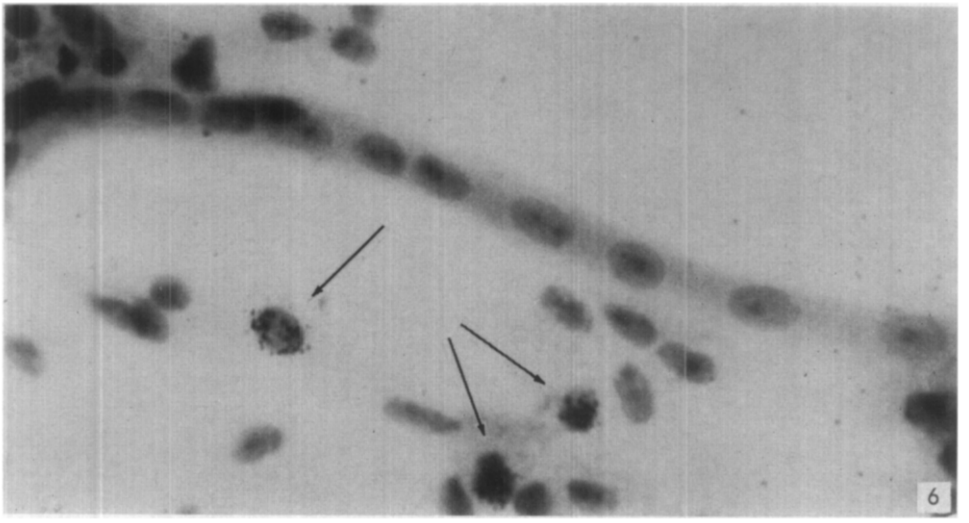


Fig. 6.—A radioautograph of a fixed and stained myotube from a 4 day old culture. The tritiated thymidine was added for 30 min when the culture was 4 days old and the material was immediately fixed. Nuclei within the myotube do not incorporate the labelled thymidine, though many mononucleated cells (arrows) outside the myotube do.

demonstrated in still another manner. In addition, such experiments could yield information as to what kinds of cells are capable of fusing to form a myotube. Accordingly the following experiments were performed. Somites from 3 day chick embryos were cultured on plasma clots until they formed a sheet of cells. These cells were exposed for 24 hr to tritiated thymidine (0.05 $\mu$C/ml of nutrient medium), suspended by means of trypsin, and mixed with freshly suspended 10 day thigh muscle in proportions of 10 or 20 per cent somite cells to 90 or 80 per cent thigh muscle cells. Control cultures consisting of labelled somite cells alone at the same initial cell concentration ($5 \times 10^5$ cells/ml) as that of the mixtures, did not form myotubes. In the mixed cultures of labelled somite cells and unlabelled thigh muscle cells, long myotubes developed containing a random distribution of labelled and unlabelled nuclei. The presence of labelled nuclei within myotubes from such mixtures can only be explained by fusion. These results suggest that fusion may occur between mononucleated and "initiating" or "seeding" cells, in that somite cells which do not form myotubes by themselves can fuse with other cells undergoing myotube formation. It is of interest that 3 day somite cells can fuse with 11 day thigh muscle cells.

*Mitotic divisons and the synthesis of contractile proteins.*—The experiments

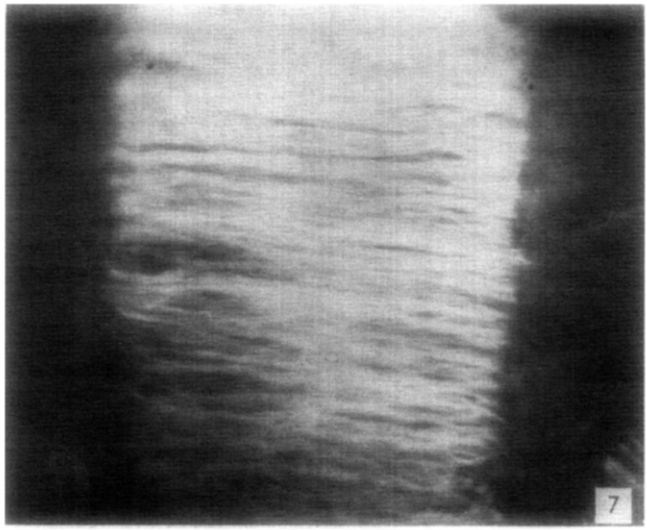


Fig. 7.—A low-power fluorescence photomicrograph of a single 4 day somite. This photograph was taken through the skin and dermis. In the photographic field there are large numbers of non-fluorescing mesenchyme cells, nerve cells, and notochord cells. Only the cross-striated myofibrils (see Fig. 8) within the elongated, mononucleated myoblasts bind the antibody. The fluorescent haze is due to the over-lay of individual myofibrils in different myoblasts.

in which radioautography was combined with fluorescein-labelled antibody staining demonstrate that myotubes formed *in vitro* do not synthesize DNA but do synthesize myosin, the meromyosins and actin. These findings suggest that the synthesis of DNA and the synthesis of contractile proteins may be mutually exclusive events, that is, a cell synthesizing DNA does not synthesize myosin, the meromyosins and actin and vice versa.

There are some uncertainties as to the meaning of the staining patterns obtained with fluorescein-labelled antibodies immediately after cells are trypsinized and cultured [25, 26]. Therefore *in vivo* material was analyzed to test whether an embryonic muscle cell which is just beginning to produce contractile proteins does indeed undergo mitosis. Particularly favorable for this study are the myotomal plates of 3 or 4 day embryos. In this area there are large numbers of dividing cells, and the individual myoblasts can be readily identified.

Somites were removed from 3 to 4 day embryos and treated with labelled antisera to myosin, actin, and H- and L-meromyosin. Fig. 7 is a low-power fluorescence photomicrograph of a single somite and its myotomal plate. At this stage the myotomal plate consists of hundreds of elongated myoblasts

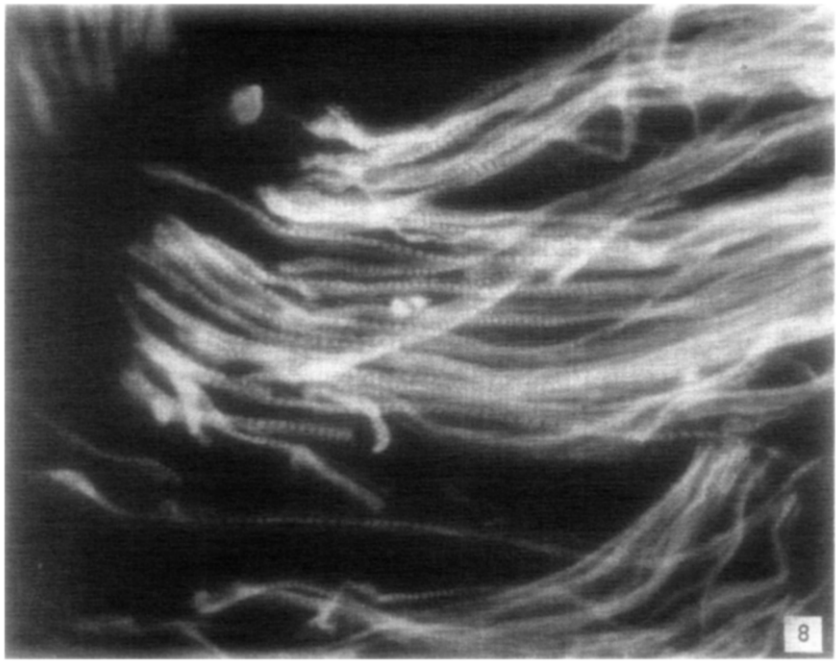


Fig. 8.—A fluorescence photomicrograph of a portion of a squashed 4 day somite taken directly from the embryo and treated with fluorescein-labelled antimyosin. The individual, mononucleated myoblasts are remarkably uniform in length. Only the A bands of the myofibrils bind the antimyosin. The thousands of mononucleated cells in the photographic field not committed to myogenesis cannot be observed for they do not react with the antimyosin. This preparation is over 200 $\mu$ in thickness, hence all of the A bands are not in the same plane of focus. This is why in some areas the fluorescence appears diffuse.

aligned in parallel. As reported previously [23, 33, 43], the myofibrils in these mononucleated cells bind the different antisera in the same pattern as myofibrils found in mature muscle (Fig. 8). To facilitate observation of cytological detail the myotomal plate can be teased and observed under the phase and fluorescence microscopes. The following observations were made with such teased material. (1) Mitotic figures were found in many hundreds of rounded, mononucleated cells, particularly in the myoseptal area. (2) Fluorescein-labelled antisera against myosin, actin and the meromyosins were never bound by rounded, mononucleated cells. (3) Mitotic figures were never found in elongated mononucleated myoblasts which bound the antibodies. From these results it is concluded that myoblasts, like myotubes, which are synthesizing contractile proteins, do not synthesize DNA for mitotic division.

## DISCUSSION

These experiments demonstrate that during myogenesis cells fuse to form multinucleated myotubes and that once nuclei are within myotubes they neither divide mitotically nor do they synthesize DNA.

The absence of mitotic figures in myotubes from *in vitro* and *in vivo* material rules out mitotic divisions as being responsible for myotube formation (see also ref. [31]). If amitosis were responsible for multinucleated myotubes, it should be detected by an increase in the DNA of myotube nuclei before [6, 39, 42, 44] or after the amitotic division—otherwise muscle nuclei could not retain their constant diploid value. Our findings can be reconciled with amitosis if it is assumed that only nuclei with a $4n$ complement of DNA fuse and that such nuclei undergo direct division forming two nuclei with the normal amount of DNA. But if this occurred it must be a rare event or a large number of paired labelled nuclei would have been found. The random distribution of labelled and unlabelled nuclei within the myotubes makes even this assumption unlikely.

The failure of embryonic myotube nuclei to synthesize DNA are in agreement with the observations reported by Bintliff and Walker [4], who followed thymidine uptake in rat muscle during regeneration. They are also in harmony with the finding that of all tissues studied thymidine phosphorylase is lowest in skeletal muscle [46].

With respect to fusion, many challenging problems remain. What types of cells fuse? Is it a selective process occurring only between presumptive myoblasts, myoblasts and myotubes? Can cells not normally associated with myogenesis be swept into myotubes? If fusion is a specialized kind of phagocytosis, what are the mechanisms which selectively destroy the cell membranes at the area of junction, but which leave the rest of the sarcolemma intact?

It is an old observation that cell differentiation and cell division are antagonistic processes. In many tissues differentiation only occurs in cells which have ceased dividing (e.g. nerve cells, keratinizing cells, reproductive cells and others). The finding that myoblasts and myotubes containing myofibrils fail to incorporate thymidine or fail to exhibit mitotic figures may indicate that these cells cannot synthesize DNA and contractile proteins concurrently. Recent work on virus systems also indicates a possible mutual exclusiveness between the synthesis of DNA and of specialized proteins [11, 35]. It should be stressed that it is the production of specialized proteins like myosin and actin which may be linked to the cessation of DNA synthesis and it does not

preclude the resumption of DNA synthesis and the inhibition of contractile protein synthesis under other conditions such as regeneration [24, 30]. It is still another problem whether proteins required for cell division—proteins associated with DNA synthesis [14], the mitotic spindle [34], or energy-yielding systems [47]—can be synthesized concurrently with DNA. It would be of great interest to know if the synthesis of DNA in other embryonic cells is incompatible with the synthesis of specialized macromolecules.

## SUMMARY

Differentiating muscle cells synthesizing myosin, the meromyosins, and actin do not concurrently synthesize DNA. Presumptive myoblasts which synthesize DNA do not concurrently synthesize myosin, the meromyosins or actin. The multinucleated skeletal muscle fiber is the product of cell fusion.

## REFERENCES

1. Adams, R. D., Denny-Brown, D. and Pearson, C. M., Diseases of Muscle. Paul B. Hoeber, Inc., New York, 1954.
2. Altschul, R., *Science* **103**, 566 (1946).
3. —— *Anat. Record* **136**, 153 (1960).
4. Bintliff, S. and Walker, B. E., *Am. J. Anat.* **106**, 233 (1960).
5. Boyd, J. D., *In* Muscle, Vol. I. Ed. G. Bourne, Academic Press, New York, 1960.
6. Brachet, J., Biochemical Cytology. Academic Press, New York, 1957.
7. Bucher, O., *Protoplasmologia* **6**, 1 (1959).
8. Capers, C. R., *J. Biophys. Biochem. Cytol.* **7**, 559 (1960).
9. Chévremont, M., *Arch. Biol.* **51**, 313 (1940).
10. Clark, W. E. and LeGros, *J. Anat.* **80**, 24 (1946).
11. Cohen, S., *Cancer Research* **20**, 698 (1960).
12. D'Ancona, U. and Fosi, V., *Arch. Ital. Anat. Embriol.* **39**, 377 (1937).
13. Doniach, I. and Pelc, S. R., *Brit. J. Radiol.* **23**, 184 (1958).
14. Flaks, J. G. and Cohen, S., *Biochim. et Biophys. Acta* **25**, 667 (1957).
15. Frédéric, J., *Compt. Rend. Soc. Biol.* **148**, 621 (1948).
16. Godlewski, E., *Arch. Mikroskop. Anat.* **60**, 111 (1902).
17. Godman, G. C., *In* Frontiers of Cytology. Ed. S. L. Palay, Yale University Press, New Haven, 1958.
18. —— *J. Morphol.* **100**, 27 (1957).
19. Häggquist, G., Handbuch der mikroskopischen Anatomie des Menschen, Vol. **3**. J. Springer, Berlin, 1931.
20. Heidenhain, M., Handbuch der Anatomie des Menschen. Plasma und Zelle, L. 2. Verlag von G. Fischer, Jena, 1911.
21. Herrmann, H., *in* Cytodifferentiation. Ed. D. Rudnick, University of Chicago Press, Chicago, 1958.
22. Holtzer, H., *in* Cytodifferentiation. Ed. D. Rudnick, University of Chicago Press, Chicago, 1958.
23. —— XIX Growth Symposium. Ed. D. Rudnick, Randall Co., 1961.
24. —— *in* Regeneration. Ed. C. Thornton, University of Chicago Press, Chicago, 1959.
25. —— *Exptl. Cell Research Suppl.* **7**, 234 (1959).
26. Holtzer, H., Abbot, J. and Cavanaugh, M., *Exptl. Cell Research* **16**, 595 (1959).
27. Holtzer, H., Abbot, J. and Lash, J., *Anat. Record* **131**, 567 (1958).

28. HOLTZER, H., MARSHALL, J. and FINCK, H., *J. Biophys. Biochem. Cytol.* **3**, 705 (1957).
29. JOFTES, D. L., *Lab. Investigations* **8**, 131 (1959).
30. LASH, J., HOLTZER, H., and SWIFT, H., *Anat. Record* **128**, 679 (1957).
31. LEWIS, W. R. and LEWIS, M. R., *Am. J. Anat.* **22**, 169 (1917).
32. KONIGSBERG, I. R., MCELVAIN, N., TOOTLE, M. and HERRMANN, H., *J. Biophys. Biochem. Cytol.* **8**, 333 (1960).
33. MARSHALL, J., HOLTZER, H., FINCK, H. and PEPE, F., *Exptl. Cell Research Suppl.* **7**, 219 (1959).
34. MAZIA, D., *in* Harvey Lectures, Series 53, 1959.
35. MELLORS, R. C., *Cancer Research* **20**, 744 (1960).
36. MOSCONA, A., *Exptl. Cell Research* **3**, 535 (1952).
37. —— *in* Cytodifferentiation. Ed. D. RUDNICK, University of Chicago Press, Chicago, 1958.
38. MURRAY, M., MUSCLE, Vol. I. Ed. G. BOURNE, Academic Press, New York, 1960.
39. PAINTER, R. B. and DREW, R. M., *Lab. Investigations* **8**, 278 (1959).
40. POGOGEFF, I. A. and MURRAY, M. R., *Anat. Record* **95**, 321 (1946).
41. STOCKDALE, F., HOLTZER, H. and LASH, J., *Acta Embryol. Morphol. Exptl.* **4**, 40 (1961).
42. SWIFT, H., *Int. Rev. Cytol.* **2**, 1 (1953).
43. SZENT-GYÖRGYI, A. G. and HOLTZER, H., *Biochim. et Biophys. Acta* **41**, 14 (1960).
44. WALKER, P. M. B. and MITCHISON, J. M., *Exptl. Cell Research* **13**, 167 (1957).
45. WEED, I., *Z. Zellforsch. u. Mikroskop. Anat.* **25**, 516 (1936).
46. WEISSMAN, J., PAUL, J., THOMSON, R., SMELLIE, B. and DAVIDSON, J., *Biochem. J.* **76**, 1 (1960).
47. ZEUTHEN, E., *Arch. neerl. Zool.* **10**, *Suppl.* **1**, 31 (1953).