fractory or had narrow susceptibility ranges. The significance of differences in morphology with respect to virus susceptibility was discussed.

1. Committee on ECHO Viruses, *Science,* 1955, v122, 1187.

2. Melnick, J. L., *Ann. N. Y. Acad. Sci.,* 1957, v67, 363.

3. Archetti, I., Weston, J., Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 265.

4. Berman, L., Stulberg, C. S., *ibid.,* 1956, v92, 730.

5. Eagle, H., *Science,* 1955, v122, 501.

6. Barron, A. L., Karzon, D. T., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 393.

7. Ramos-Alvarez, M., personal communication.

8. Scherer, W. F., Syverton, J. T., *Am. J. Path.,* 1955, v31, 31.

9. Stulberg, C. S., Berman, L., Ruddle, F. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 438.

10. Eagle, H., Habel, K., Rowe, W. P., Huebner, R. J., *ibid.,* 1956, v91, 361.

11. Stulberg, C. S., Berman, L., Page, R. H., *Virology,* 1956, v2, 844.

12. Syverton, J. T., McLaren, L. C., *Texas Rep. Biol. and Med.,* 1957, v15, 577.

13. Deinhardt, F., Henle, G., *J. Immunol.,* 1957, v79, 60.

14. Henle, G., Deinhardt, F., *ibid.,* 1957, v79, 54.

15. Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., Huebner, R. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 418.

16. Berman, L., Stulberg, C. S., Ruddle, F. H., *Cancer Res.,* 1957, v17, 668.

17. Puck, T. T., Cieciura, S. J., Fisher, H. W., *J. Exp. Med.,* 1957, v106, 145.

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Fiber Formation in Suspension Cultures of L Strain Fibroblasts.* (23743)

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While numerous workers have reported fiber formation in primary explant cultures of fibroblasts, as indicated in reviews by Bloom and Fell(1,2) there have been no such reports concerning monolayer or suspension cultures of stable cell strains. In this paper, conditions are described under which L strain mouse fibroblasts in agitated cultures give rise to membranous structures containing numerous discrete fibers. A preliminary study of the origin and nature of these fibers is reported herein.

Materials and methods. Strain L 929 fibroblasts(3) were maintained in stationary bottle cultures in synthetic mixture 199† supplemented with 1.0% Bactopeptone‡ (199P). To prepare suspension cultures cells were scraped from the glass, dispersed by vigorous pipetting and diluted to a concentration of 1.5-2.5 x 10^5 cells/ml in medium 199P. One hundred milliliters of the suspension were put into a 250 ml Erlenmeyer flask. Control cultures were prepared by suspending an equal number of cells in medium 199P supplemented with 5% horse serum (199P-HS). Both the experimental flasks and the controls were stoppered and placed on a rotary action shaker§ (100 rpm) at 35°C(4). After several hours incubation delicate membranes appeared on the surface of the serum-free medium. A number of these membranes were pipetted onto cover glasses. Such preparations were fixed either in Zenker-acetic or in 10% formalin, and were stained with Regaud's modification of the Masson trichrome technic or by Foote's modification of the Bielschowsky silver method for argyrophilia (5). Membranes from several flasks were also collected by centrifugation and fixed in 10% formalin. These were minced in a Waring blendor and the resulting material

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‡ Difco Laboratories, Detroit, Mich. Lot No. 431352.

§ Eberbach and Co., Ann Arbor, Mich.

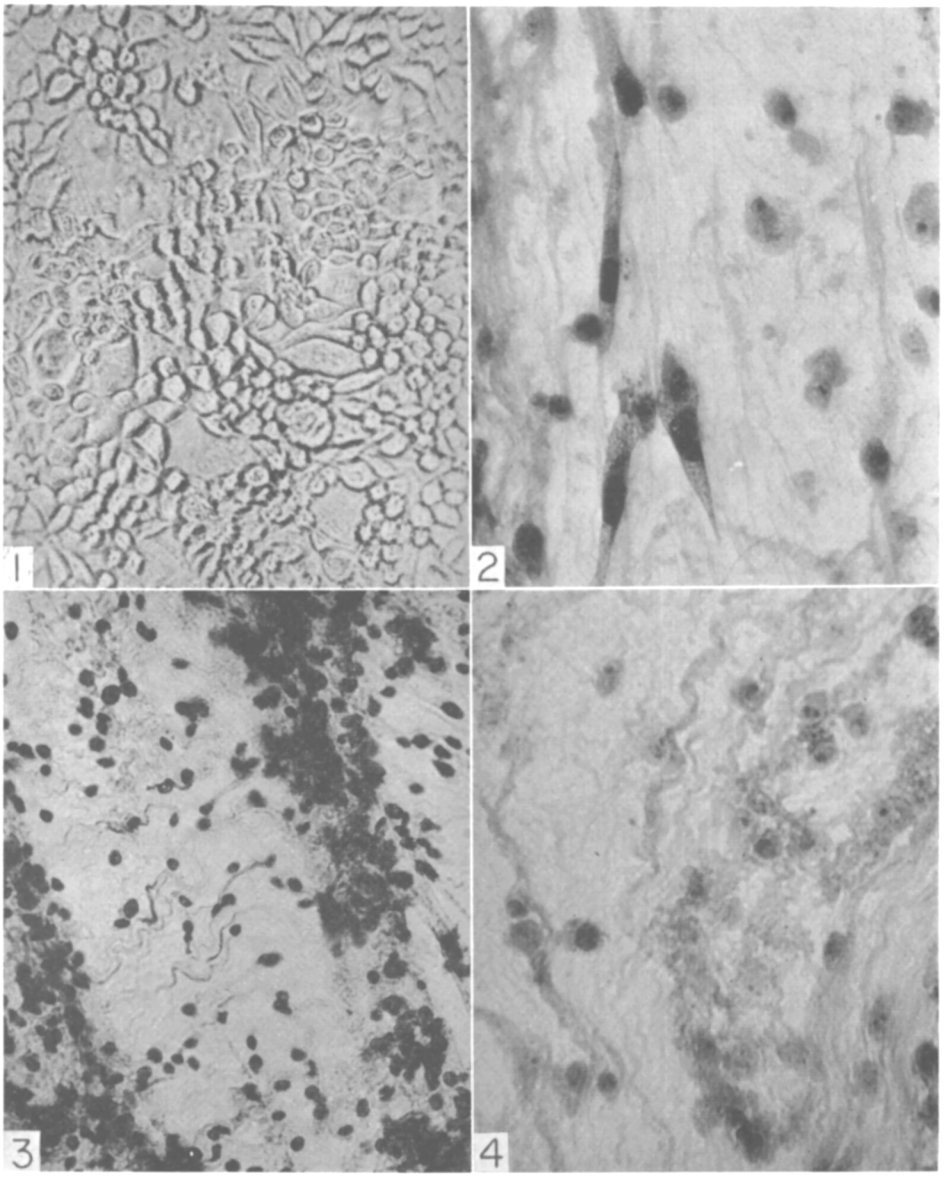

FIG. 1. L strain fibroblasts grown as a monolayer in medium 199 supplemented with 0.5% Bactopeptone. Unstained. × 300.

FIG. 2. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopeptone. Haematoxylin and eosin. × 700.

FIG. 3. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopeptone. Silver stain. × 300.

FIG. 4. L strain fibroblasts grown in suspension culture in medium 199 with 1% Bactopeptone. Masson stain. × 700.

was used to prepare grids for electron microscopy. Such specimens were treated with 5% phosphotungstic acid at pH 5.6 for ½ minute, washed. dried, and treated with 1% phosphotungstic acid at pH 3.5 for 1 minute(6). Control preparations were made by fixing rat-tail tendon in 10% formalin and following through with the preparation of grids for electron miscroscopy as already outlined.

Results. L strain fibroblasts grown on a glass substrate in medium 199P with an initial inoculum of 2 x 10^5 cells/ml yielded a

monolayer of spindle shaped cells in 10-14 days (Fig. 1). The generation time during logarithmic growth was approximately 50 hours and the maximum population density was 1.5-2.0 x 10^6 cells/ml. The addition of serum to the medium (199P-HS) shortened the generation time (35-40 hours) but did not significantly alter either the growth pattern or the maximum cell density. Stationary bottle controls of L strain in medium 199P exhibited cells trapped at the liquid-air interface which grew there much as they did on glass. The cells extended in a spindle-shaped fashion and were attached to one another laterally by long processes. Often these cell masses were quite extensive and floated as fairly rigid units.

In an agitated fluid suspension, the addition of 5% whole horse serum to the medium (199P-HS) permitted the rapid proliferation of L strain fibroblasts. Under these conditions the population developed as an even suspension of cells which were essentially spherical. The generation time in such cultures was 26-30 hours and the maximum population density approached or even exceeded 2.0 x 10^6 cells/ml. When, however, the cells were suspended in medium 199P and agitated on a rotary action shaker, delicate membranes were observed to form at the fluid-air interface within 24-48 hours and continued to float on the surface of the medium. Microscopic examination of such membranes revealed pleomorphic cells dispersed among large numbers of fibers arranged singly and in bundles (Fig. 2, 4).

Continued agitation of the cultures caused the membranes to fold and become submerged as they increased in diameter. However, when flasks were removed from the shaker after membrane formation had begun and incubation was continued under static conditions, the membranes continued to increase in diameter, ranging up to 5 cm. Staining of these older membranes with silver-staining procedures demonstrated argyrophilic fibers (Fig. 3) and when the Masson trichrome technic was used the fibers stained a deep blue. The intensity of argyrophilia could be increased by immersing the membranes in

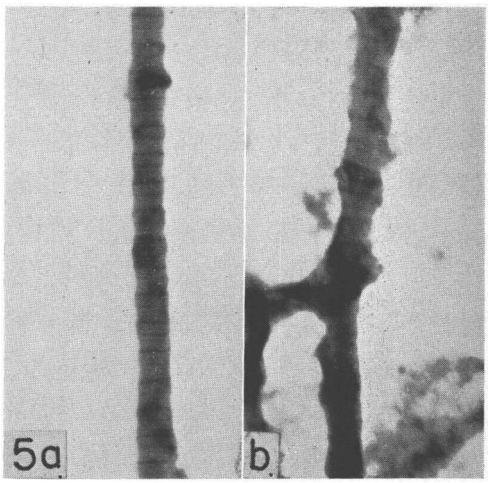

FIG. 5a. Electron micrograph of collagen fibril from rat-tail tendon. Phosphotungstic acid stain. × 55,000.

FIG. 5b. Electron micrograph of fibril from L strain culture grown in medium 199 with 1% Bactopeptone. Phosphotungstic acid stain. × 55,000.

dilute joint fluid‖ prior to staining. This procedure also intensified the staining of fibers in young membranes by the Masson technic. The cells in these membranes demonstrated a gradation of morphology and staining properties. Although the rounded cells in suspension ranged from 12 μ to 18 μ in diameter these spindle-shaped cells found in the membranes ranged from 18 μ to 145 μ in length. With the Masson technic, they varied in tinctorial properties from smaller cells exhibiting a lightly stained nucleus and fine eosinophilic granules in the cytoplasm to the larger cells displaying a deeply stained nucleus and coarse cyanophilic cytoplasmic granules.

In an effort to determine the nature of the fibers, preparations were examined with the aid of an electron microscope. When standard procedures were followed, fibrils were obtained from the membranes which had major periodicity of 640 A (Fig. 5). A control preparation of a fibril from rat-tail tendon is shown for comparison. The fibrils from the membranes were digested by collagenase¶ from *Clostridium perfringens* and were par-

‖ Courtesy of Dr. William Castor. Rackham Arthritis Unit, Univ. of Michigan.

¶ Courtesy Dr. John McLennan, Dept. of Microbiology, Columbia University.

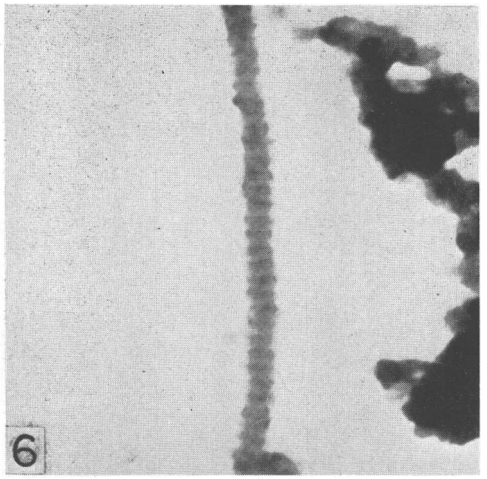

FIG. 6. Electron micrograph of fibril from L strain culture grown in medium 199 with 1% Bactopeptone, treated with dilute acetic acid. Phosphotungstic acid stain. × 55,000.

tially degraded by trypsin, but were resistant to α amylase and hyaluronidase. The fibrils swelled when treated with acetic acid (Fig. 6).

Discussion. At least 3 explanations of the observed facts seem plausible: (a) In a medium which is suboptimal for growth, many cells may degenerate, freeing fibrous material which accumulates at the liquid-air interface and entraps other free cells; (b) under all conditions of growth, the cells may produce soluble proteins which denature at the liquid-air interface contingent upon the methods described here; (c) under all culture conditions, the cells may synthesize a fibrous protein which is intimately associated with the cell surface though not necessarily a part of the cell membrane. When cells aggregate at the liquid-air interface, are bound together laterally and undergo tension as the result of agitation, this fibrous protein may be protracted and entwined in strands. With regard to the latter possibility it is of interest to note that when L-strain cell suspensions are treated with 0.1 M citric acid, a fibrous material is rapidly shed from the cell surface. This shedding of material does not immediately affect the cell membrane which remains intact and only later ruptures suggesting a labile protein surface (unpublished). Further evidence of the existence of a superficial proteinaceous material related to the binding of cells to one another and to a solid substrate is indicated by the fact that trypsin and other proteolytic enzymes are effective in the dispersal of tissue cells *in vitro*.

It is of some interest that the fibrogenic capacities exhibited by the L strain cells are not unique to this strain but have been duplicated in our laboratory using the LLC-Ml strain of mouse fibroblasts isolated by Hull(7).

Summary. The *in vitro* formation of fibrous membranes by the L and LLC-Ml strains of mouse fibroblasts in a protein-free medium has been demonstrated. When cells of either of these strains are suspended in medium 199 plus 0.5-1.0% peptone and incubated at 35°C on a rotary action shaker at 100 rpm, delicate membranes form at the liquid-air interface. Enzymatic digestion, staining reactions and electron micrographs suggest that the fibers are fibrous protein in nature and belong to the collagen class of protein. The possible relationship of this fibrous protein to the cell surface is discussed.

We are indebted to Mrs. Frances Loranger for preparation of the electron micrographs.

1. Bloom, W., *Physiol. Rev.*, 1937, v17, 589.

2. Fell, H. B., Histogenesis in tissue culture. In: *Cytology and Cell Physiology*, A. H. Bourne, Ed., Clarendon Press, Oxford, 1951.

3. Sanford, K. K., Earle, W. R., Likely, G. D., *J. Nat. Cancer Inst.*, 1948, v9, 229.

4. Kuchler, R. J., Merchant, D. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 803.

5. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry*, Blakiston Co., N. Y., 1954.

6. Hall, C. R., *Introduction to Electron Microscopy*, McGraw-Hill, N. Y., 1953.

7. Hull, R. N., *Science*, 1953, v117, 223.

Received December 9, 1957. P.S.E.B.M., 1958, v97.