16. Nalbandov, A., Hockhauser, M., Dugas, M., *Endocrinol.*, 1945, v36, 251.
17. Juhn, M., Harris, P. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 709.
18. Patel, M. D., *Physiol. Zool.*, 1936, v9, 129.
19. Lehrman, D. S., Brody, P., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 373.
20. van Tienhoven, A., *Poultry Sci.*, 1958, v37, 428.

Received October 30, 1958. P.S.E.B.M., 1959, v101.

Mammalian Cell Cultures for Study of Influenza Virus. I. Preparation of Monolayer Cultures with Collagenase.* (24818)

RONALD W. HINZ†‡ AND JEROME T. SYVERTON
Dept. of Bacteriology and Immunology, University of Minnesota, Minneapolis

Enzymatic treatment of cells, developed(1) for routine passage of cells in continuous culture, has been employed widely for primary dispersion of cells from tissue. While parts of some tissues, notably renal cortex, are dispersed readily by application of trypsin, other tissues are more or less refractory. Consequent restriction of cell types that can be dispersed efficiently by trypsin from tissue hampers *in vitro* study of some viruses, comparison of virus susceptibility of cells *in vivo* and in primary culture, and direct clonal derivation of cell lines from tissue. This paper describes the use of collagenase for preparation of primary monolayer cultures of lung, a tissue usually poorly dispersed by trypsin, for study of influenza viruses.

Materials and methods. Solutions included a) GKN (solution of glucose, potassium chloride and sodium chloride in concentrations appropriate to Hanks' solution)(2); b) Hanks' balanced salt solution brought to pH 7.4-7.6 with sodium bicarbonate; and c) nutrient medium containing 20% serum of the same species as the tissue cultivated in YEM (3) with penicillin and streptomycin added to final concentrations of 50 units and 100 μg per ml. *Tissues* were obtained as a) human and swine lungs from fetuses aged approximately 80 (14 cm) and 70 days respectively, and b) lung from an adult American Dutch rabbit about 250 days of age. Fetal swine lungs were obtained from miniature swine developed for research(4), and from sows randomly selected at an abattoir (by kind cooperation of Dr. Eldon G. Hill of the Hormel Institute, Univ. of Minnesota). Animals were opened aseptically and lung or other tissue removed to sterile Petri dishes containing about 2 ml of Hanks' solution. *Enzymes* in GKN were sterilized by passage through Selas #02 filters. Collagenase (Worthington Biochemical Corp., Freehold, N. J.) was prepared in stock concentration of 0.1% for use as 100 μg per ml (0.01%); trypsin as Difco Yeastolate was prepared in stock concentration of 20 mg per ml (2.0%) for use as 2 mg per ml (0.2%). *Preparation of dispersed cell suspensions. Lung* tissue was minced into 1-2 mm fragments cut by crossed scalpel blades handled like scissors; portions of bronchial tree separable from parenchyma were discarded. Fragments were rinsed repeatedly with Hanks' solution to remove erythrocytes, transferred to a screw-capped flask containing a Teflon-covered magnetic stirring bar, covered to an approximate depth of 30 mm with collagenase solution, and incubated for 10 min in a water bath at 37°C. Flask contents then were agitated by magnetic stirring for 2 hours in a 37°C incubator. An essential precautionary measure to prevent cell damage from heat was the insertion of an inverted Petri

* Aided by Grants from Frederica Beard Memorial Grant for Cancer Research, Am. Cancer Soc., Nat. Cancer Inst., N.I.H., and National Fn.

† Predoctoral fellow of the Nat. Cancer Inst., N.I.H., P.H.S.

‡ This work was done in partial fulfillment of requirements for Ph.D. degree in Bacteriology and Immunology, Univ. of Minnesota.

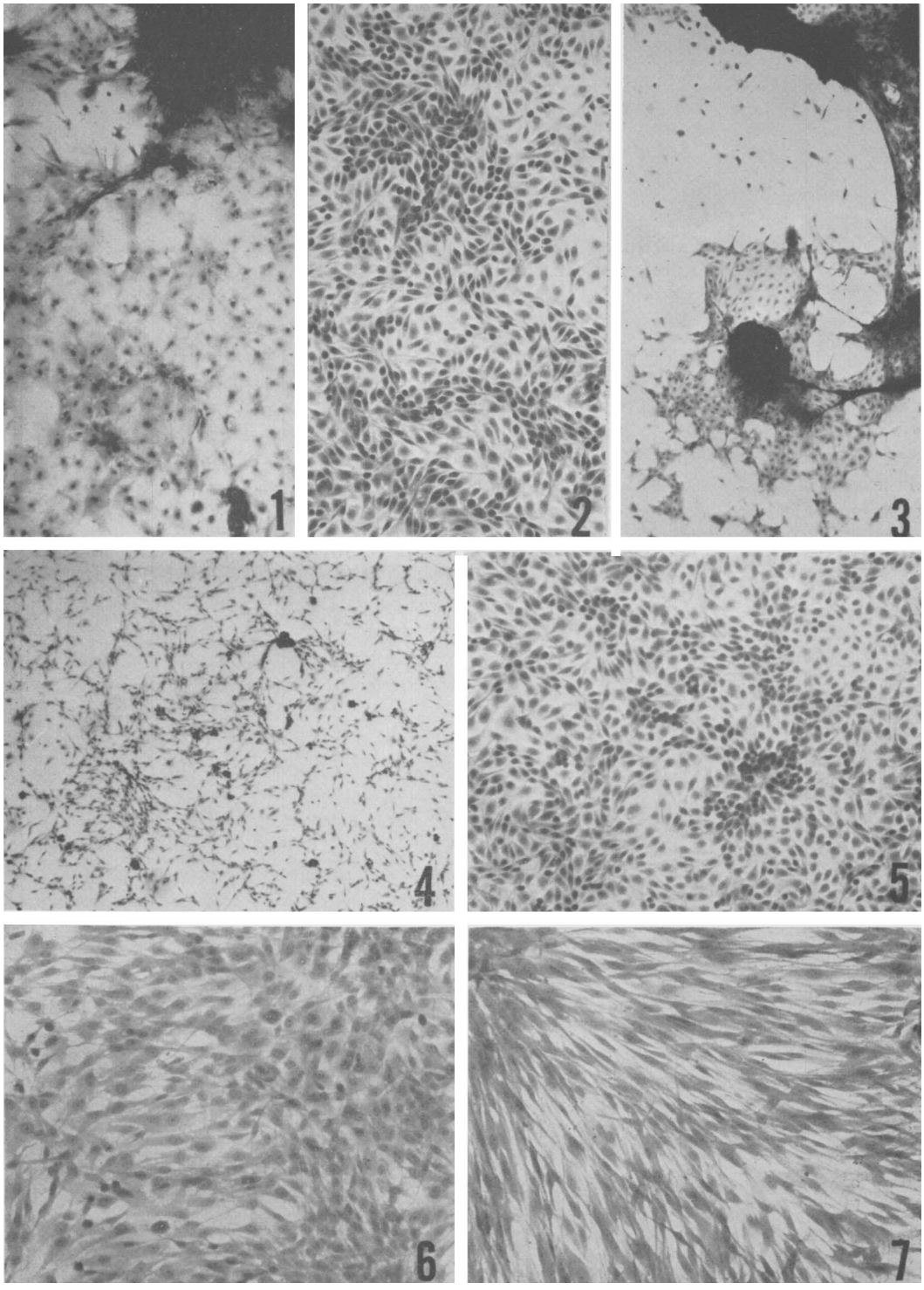

FIG. 1. Seven-day primary culture of porcine lung digested with trypsin; outgrowth extending from tissue fragment on right. ×120.

FIG. 2. Four-day primary culture of porcine lung dispersed with collagenase. ×120.

plate between flask and the Magnestir plate (Labline Inc., Chicago). After this treatment, usually sufficient to disperse the fragments, the resulting cell suspension was filtered through 4 layers of sterile gauze, sedimented at 20 x g for 6 minutes, separated from supernatant fluid and resuspended directly in nutrient medium. *Fetal kidney* cell suspensions were prepared similarly; collagenase dispersed both cortical and medullary tissue. With blood-engorged tissue, collagenase treatment was preceded by agitation of tissue fragments with trypsin for 2-10 hours at 5°C. After trypsin treatment, supernatant fluid was discarded from settled fragments and collagenase treatment carried out as previously described. Initial trypsinization reduced concentration of erythrocytes in the cell suspension, and reduced fragment size to facilitate more efficient collagenase action. *Monolayer primary cell cultures* were prepared from cell suspension in nutrient medium diluted to contain 10^6 cells per ml according to hemocytometer count(5) or diluted 1:150 according to packed cell volume(6). Screw-capped 16 x 125 mm culture tubes or 200 ml rectangular bottles were seeded with 1.0 ml or 10 ml amounts of suspension, respectively. After preliminary incubation at 37°C for 24-48 hrs, cultures were rinsed and the nutrient medium completely renewed. After 1-4 days of additional incubation, cell monolayers were ready for use. Subsequent dispersal of cells for transfer was accomplished by trypsin treatment.

Results. Adult or fetal lung tissue was found refractory to treatment with 0.2% trypsin in GKN or Hanks' solution. Agitation with trypsin at 37°C or overnight at 5°C, commonly sufficient to disperse monkey kidney tissue efficiently, produced fragments of lung about 0.2-2.0 mm in size. These course dispersions yielded short-lived outgrowth (Figs. 1, 3). In contrast, collagenase digested lung completely to liberate cell suspensions generating healthy monolayers in primary culture. After 3-4 days of incubation, monolayers were confluent and ready for use in virus studies (Figs. 2, 4). Unlike primary cultures prepared from trypsinized tissue, cells from collagenase-dispersed suspensions were readily passed by conventional trypsinization. Continuous cultures are shown in the 5th to 9th passages (Figs. 5, 6, 7). Cells in continuous swine lung cultures have the morphological appearance of mesothelial cells, while those of rabbit lung cultures appear more fibroblastic. Human lung in primary culture appeared to contain a mixture of fibroblastic and epithelial cell types; after 9 serial passages, cells appear uniformly epithelial. Effectiveness of trypsin and collagenase for dispersion was compared also with adult and fetal kidney tissue. Medullary tissue proved refractory to trypsin digestion but not to collagenase treatment.

Discussion. Commercially prepared collagenase was found an effective dispersing agent for preparation of monolayer primary cultures from lung and renal medulla, as examples of tissue difficult to disperse efficiently with trypsin. Cultures of lung tissue were of particular interest for study of influenza viruses.

Methods for dispersal of cells from tissue or culture monolayers have been instrumental in advancement of cell culture technics for study of viruses. Trypsin and versene have been widely and successfully employed for dispersion of embryonic or adult soft tissue, or cultures(1,7). Tissues relatively rich in connective tissue, however, have been difficult to disperse with trypsin(8). Since collagen fibers are basic elements of most types of connective tissue, collagenase was attractive as a dispersing agent. Lasfargues(9,10) obtained

FIG. 3. Two-day culture of trypsin-digested porcine lung. ×40.

FIG. 4. One-day culture of collagenase-dispersed porcine lung. ×40.

FIG. 5. Five-day culture of porcine lung cells in 5th serial passage by trypsin dispersal, from primary culture prepared from collagenase-treated lung. ×120.

FIG. 6. Six-day culture of human lung cells in 9th serial passage by trypsin dispersal, from primary culture of collagenase-treated lung. ×120.

FIG. 7. Six-day culture of rabbit lung cells in 6th serial passage by trypsin dispersal, from primary culture of collagenase-treated lung. ×120.

short-lived cultures from parenchymal fragments of mouse mammary gland released by exposure to collagenase. The present studies employed glucose-potassium chloride-sodium chloride solution as diluent for collagenase rather than Hanks' or other such phosphate-buffered solutions, because the enzyme has been reported unstable in phosphate buffer (11). Present experience indicates that the commercial enzyme in stock solution is stored better at 5°C than frozen, and that best results are obtained when enzyme solutions in GKN are prepared shortly before use. Although prior digestion of lung tissue with trypsin often was advantageous to reduce erythrocyte contamination, well dispersed cell suspensions were prepared with collagenase alone. The purified bacterial enzyme as commercially prepared may include sufficient proteolytic enzyme, in addition to collagenase, to separate cells released from collagen-containing stroma. Use of collagenase(12) to release cells in quantity from tissue parenchyma extensively interlaced with connective tissue can be expected to make available new cell types for diagnosis of viral infection, for wider study of virus-host cell relationships, and for new sources of virus vaccine. Use of the enzyme makes available a convenient system for study of swine influenza virus, in particular, since porcine fetal lung together with a supply of serum can be obtained from local abattoirs.

Summary. Primary monolayer cultures of human, rabbit and porcine fetal and adult lung were prepared conveniently by dispersion of tissue with commercially available collagenase in phosphate-free salt solution.

1. Scherer, W. F., Syverton, J. T., Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

2. McLaren, L. C., Holland, J. J., Syverton, J. T., *J. Exp. Med.*, 1959, v109, 475.

3. Syverton, J. T., McLaren, L. C., *Cancer Research*, 1957, v17, 923.

4. England, D. C., Proc. 4th Ann. Meeting Animal Care Panel, U. of Chicago, 1953.

5. Syverton, J. T., Scherer, W. F., Ellwood, P. M., *J. Lab. Clin. Med.*, 1954, v43, 286.

6. Waymouth, C., *J. Nat. Cancer Inst.*, 1956, v17, 305.

7. Zwilling, E., *Science*, 1954, v120, 219.

8. Dresner, E., Schubert, M., *J. Histochem.*, 1955, v3, 360.

9. Lasfargues, E. Y., *Anat. Rec.*, 1957, v127, 117.

10. ———, *Exp. Cell Research*, 1957, v13, 553.

11. Sumner, J. B., Somers, G. F., *Chemistry and Methods of Enzymes*, Academic Press, 3rd Ed., 1953, 179.

12. Hinz, R. W., Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1959,

Received February 10, 1959. P.S.E.B.M., 1959, v101.

Mammalian Cell Cultures for Study of Influenza Virus. II. Virus Propagation.* (24819)

RONALD W. HINZ†‡ AND JEROME T. SYVERTON

Dept. of Bacteriology and Immunology, University of Minnesota, Minneapolis

Cells easily dispersed from continuous cultures or from particular tissue sources such as kidney and amnion are the mainstay of cell culture procedures for detection and assay of viruses and antibodies. For specific study of host-cell relationships, however, it is desirable to work with primary and/or continuous cultures of cell types known to be affected *in vivo*. This paper describes susceptibility to human and swine influenza viruses of primary cultures of cells released from lung parenchyma by treatment with collagenase.

Materials and methods. Cultures of human, rabbit and porcine lung, and whole por-

* Aided by Grants from Frederica Beard Memorial Grant for Cancer Research, Am. Cancer Soc., Nat. Cancer Inst., N.I.H., and The National Foundation.

† Predoctoral fellow of Nat. Cancer Inst., N.I.H., P.H.S.

‡ This work was done in partial fulfillment of requirements for Ph.D. degree in Bacteriology and Immunology, Univ. of Minnesota.