Production of Malignancy in Vitro.

IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells

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INTRODUCTION

Earle and Voegtlin (*1*) showed that 20-methylcholanthrene added to culture fluid as a fine suspension in concentrations ranging from 1,000γ to 0.2γ per cubic centimeter, has a definitely injurious effect on cultures of subcutaneous rat and mouse fibroblasts grown in chicken fibrin clot with a supernatant-fluid culture medium of horse serum and chick-embryo extract diluted with saline solution. The carcinogen caused partial or complete necrosis, particularly at higher concentrations, and always caused a retardation in the rate of growth. The study was carried to 54 days after the first addition of the carcinogen.

Using the same culture medium and fibroblasts from the strain C3H mouse, Earle and Voegtlin (*2*) continued the study with a concentration of 10γ of carcinogen per cubic centimeter of culture fluid for periods up to 250 days and found less degeneration than was shown at the higher concentrations (*1*), but there was the same severe initial retardation of the increase in size of the clump. Cultures that had received the agent as long as 114 days showed a change in the architecture of the clump to a distinctly more epitheliallike type, which seemed to result from marked changes in the cells, at least some of which was in the cell surfaces. The cells became laterally coherent; this change was gradual, progressing with prolonged exposure to the carcinogen and affecting the culture as a whole rather than a few isolated cells.

Unfortunately, the type cultures did not allow an accurate analysis of the cell changes in the early stages. Changes after 265 days in methylcholanthrene were also not investigated, owing to loss of the series from a bacterial contamination. This loss cut short studies on the behavior of the carcinogen-treated cells on reinjection. Only 12 C3H mice were injected with 1 culture each of cells that had been subjected to the carcinogen about 114 days and then carried 83 days longer without addition of the carcinogen. The results of these injections were negative.

Just before the series was lost, it was noted that the control cultures were showing a trace of the same morphologic change as that in cultures of the treated cells. This was ascribed to trace contamination with methylcholanthrene, which could possibly have occurred with the technique used.

The studies were later extended, with four general aims:

(1) To confirm the previously observed cell changes with a more accurate control of the concentration of methylcholanthrene.

(2) To follow with greater accuracy the progression of changes in the cells, both in the carcinogen and after removal from it, with particular attention to the sequence of changes during the first 60 days after exposure to the carcinogen

(this interval corresponding approximately to the latent interval for production of tumors by injections of the carcinogen in vivo) and the cell changes after exposure for more than 260 days.

(3) To attempt to demonstrate conclusively a change from the normal to the malignant cell by the action of the carcinogen in vitro. This of necessity involved the production of tumors by injection of cells treated with the carcinogen in vitro.

(4) To correlate the relative tumor-producing ability of the cells with morphologic changes observed in them.

Although these studies are as yet incomplete, the present paper reports the technical methods used and the data accumulated on a primary strain of mouse fibroblasts, cultures of which were carried in methylcholanthrene for various intervals up to 406 days. While the general sequence of morphologic changes as observed in the living culture is given, this paper does not include any work with stained preparations. A description of the production of the tumors obtained by inoculation is given in the next paper (*3*), while the pathology of the tumors will be presented in the paper following (*3a*). Other studies in the series will appear in later issues of this Journal. These will cover (1) observations on the mitochondria and Golgi apparatus, and (2) metabolism of the tumors produced. Data from other stained preparations, from motion picture reels, and analysis of the growth rates of the tumors are as yet incomplete and will be presented at a later date. The equipment and method used in cleaning the glassware and the photomicrographic and the microcinematographic equipment designed for use in these experiments are described in the three preceding papers (*4, 5, 6*).

MATERIALS AND METHODS

In addition to the acid cleaning equipment and method already described (*4*), this investigation has necessitated the construction of considerable special apparatus and the elaboration of specialized procedures. Sufficient details are given to make clear the course of the experiment, as well as the various precautions taken.

Cleaning of Equipment

All glassware on hand was first cleaned by using the acid-cleaning method (*4*). Then the glassware, used with normal-tissue-culture solutions only and not contaminated with heavy metallic salts or with carcinogenic substances, was washed with soap and water as usual, rinsed with distilled water, and drained.

With the single exception of the cultures that were fixed in situ in Carrel flasks with Orth's formalin-dichromate solution, the glassware used with tissue cultures was never employed with any solution for fixation or staining. After fixation of cultures in the Carrel flasks, the flasks were given a preliminary cleaning in a mixture of equal parts of concentrated nitric and sulfuric acids at 80° C. for 24 hours and then, after being drained at a sink reserved for waste acids, were rerun through the regular acid cleaning bath. In this way, there was no contamination of the large acid cleaning baths with chromium, and yet the carcinogen was destroyed. In flasks thus cleaned, cultures have grown satisfactorily and have shown no sign of the irregular and poor growth so common in those grown in glassware cleaned with dichromate-sulfuric acid.

All waste carcinogenic solutions were destroyed by sulfuric acid in the acid bath if exceedingly concentrated; but if the concentration was not more than 1γ per cubic centimeter, the solutions were flushed down a small covered sink used for that purpose only.

After being cleaned, all glassware and other materials were wrapped in paper and autoclaved at 20 to 22 pounds steam pressure for 1½ hours, or dry sterilized at about 170° to 175° C. for 3 to 4 hours, after the desired temperature was reached.

Rubber stoppers and rubber equipment that might come in contact with the culture fluid were boiled repeatedly with an approximately 5-percent solution of sodium hydroxide, then washed

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free from alkali, and rinsed repeatedly with distilled water. An attempt was made to have as little rubber as possible come in contact with culture solutions. All rubber equipment was sterilized by autoclaving.

Until about June 8, 1942, all rubber items, including stoppers, tubing, etc., employed with solutions containing carcinogen or with carcinogen-treated cultures, were used once and then destroyed. Since then because of the critical shortage of rubber, stoppers used with cultures removed from the carcinogen for at least 60 days, are cleaned with sodium hydroxide solution as described and used over for such cultures. They are never used with untreated cultures.

The dissecting knives and needles used in transferring carcinogen-treated cultures to fresh flasks were cleaned by soaking them in five to six consecutive changes of acetone and benzene. They were then sterilized with dry heat at 170° C.

Distilled Water

All distilled water used in rinsing glassware was from the general distilled water system of the laboratory. All solutions used with the cultures were prepared from this distilled water, which had been carried through two additional distillations in a double all-glass still and collected and stored in 5-gallon pyrex bottles. The rubber stoppers used with the bottles were cleaned by repeated boiling in sodium hydroxide solution. Care was taken that the distilled water did not touch the rubber.

Isotonic Saline Solution

The saline solution used for washing cultures and for diluting culture media consisted of the following ingredients:

	Grams
Sodium chloride	6. 80
Potassium chloride	. 40
Calcium chloride	. 20
Magnesium sulfate	. 10
Sodium dihydrogen phosphate	. 125
Sodium bicarbonate	2. 20
Dextrose	1. 00
Glass-distilled water to 1,000 cc.	

All concentrations were calculated in terms of anhydrous salts, which were of reagent quality and were purchased in relatively large lots so that there was no change in lot number for most of the salts used. With the exception of the calcium chloride, the salts were dissolved, in the order given, in about 600 cc. of water for each liter of solution. The calcium chloride was kept in the form of an analyzed stock solution containing 0.1 gm. anhydrous salt per cubic centimeter. The correct amount of this solution was added to 100 to 200 cc. of water for each liter of final solution, and this in turn poured into the rest of the solution with agitation. The pH of the solution was set at 7.4; if necessary, a little carbon dioxide was run through it from a cylinder of compressed gas. The solution was then filtered. Inasmuch as no such physiologic saline relying on bicarbonate for its buffer action can be filtered under vacuum without a severe shift in pH to the alkaline side, all filtrations were carried out by using air pressure at 3 to 5 pounds. The filter candles employed were never used for any other purpose. During the early part of the experiment the solution was made up and filtered in lots of 4 l. through a 1-by-5-inch Mandler candle of normal porosity designed to handle 7 to 9 pounds of air pressure; later the solution was made up in lots of 32 l. and was simultaneously filtered through two 2-by-10-inch Mandler candles to give two final lots of 16 l. each. The filtering equipment is shown in figure 1, *A* and *B*.

In autoclaving such large units as the 32-l. saline apparatus, the stopcock permitting access of air to the apparatus was wrapped but left open, while in each of the two large containers about 100 cc. of glass-distilled water was placed, thus insuring the formation of enough steam within each container to act as a sterilizing agent. These units were autoclaved at 20 pounds for 2 hours and then allowed to dry out. After filtration, the saline solution was run into flasks which were sealed with rubber stoppers and stored at 3° C.

During the whole interval of preparation and storage the pH of the saline solution would rise about 0.1 unit. Since the solution was desired at pH 7.5, it was originally set at about pH 7.4. The pH of the other solutions was similarly controlled so that the pH of the final culture media was about 7.5.

Horse Serum

While no particular selection was made as to the age and sex of the horses from which the serum was obtained, records show that all the horses were adult geldings ranging up to 33 years of age. Most of the serum was from horses estimated to be about 16 years old. All serums were clear, and no trouble was ever experienced from hemolysis.

The jugular vein was punctured with a 4-mm. (outside diameter) Graefe trocar, with stopcock, connected with a rubber tube, and the blood collected in 3-1, lots in sterile glass cylinders about

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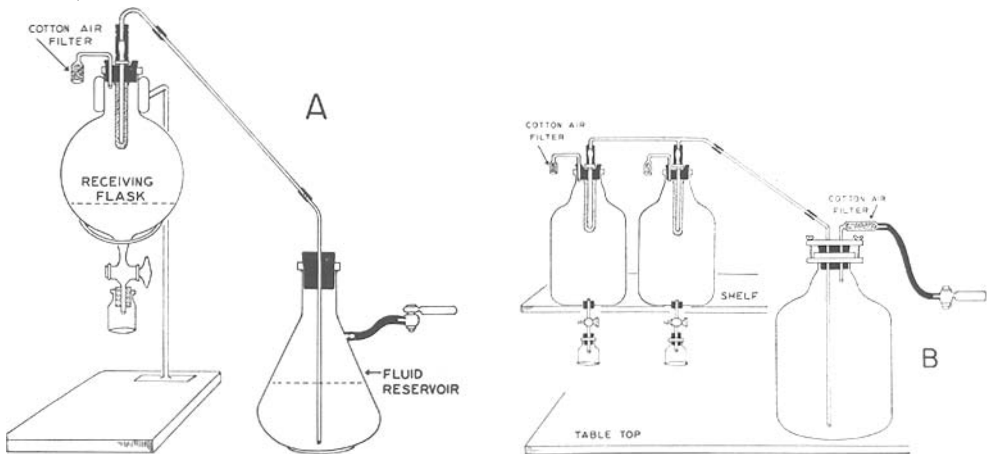

FIGURE 1.—*A*, Equipment used to filter saline solution and horse serum in lots of 4 l. or less; *B*, Equipment used to filter saline solution in lots of 32 l. The small bottles are of 5-gallon capacity, the larger one is of 12-gallon capacity. All are made of pyrex.

100 mm. in diameter and 450 mm. high, which were kept cool in crushed ice to reduce changes in the blood. Usually 9 l. was obtained from one horse. When horses were bled on Saturday and allowed to rest over the week end, the bleeding did not interfere with their regular work. The blood was allowed to clot, and the clots were loosened from the cylinders which were held at 3° C. overnight. During this handling sterility was not maintained although an attempt was made to keep the blood reasonably clean. Next day, the serum was drawn off with a 200-cc. bulb pipette. The clots were minced with scissors, the fragments were centrifuged, and the expressed serum was removed. The total lot of serum was recentrifuged to eliminate any remaining red cells which would be disrupted by freezing, then pooled, and stored in 1,000-cc., flat-bottomed, boiling flasks sealed with rubber stoppers. The flasks were filled not more than half full to prevent their breaking on freezing, then stored at —10°, and held frozen until needed. When needed, the serum was thawed out, recentrifuged to remove any fine precipitate that might (very rapidly) clog the filter, and filtered under pressure through a 1-by-5-inch Mandler candle. The smaller equipment and the method for filtering saline solution were used. The filtered serum was stored at 3° in small flasks sealed with rubber stoppers. Since the filter candles were used repeatedly, they were carefully washed with a dilute sodium chloride-sodium bicarbonate solution, then with glass-distilled water. Filters used for horse serum were reserved for that solution only. The serum when used was usually less than 60 days old. In other work, serums up to 9 months old have been used without any observed deleterious action.

PLASMA

Plasma was obtained from hens weighing 5 to 8 pounds and not more than 1½ years old. It was necessary to watch carefully the age of the hens, as the use of plasma from older hens has been found to result in poor growth of the cultures. A simple glass cannula sterilized in heavy mineral oil at about 140° C. was inserted in the carotid, and the blood collected in a narrow-mouthed, heavy-walled, iced, paraffined, 100-cc. centrifuge flask, which was stoppered with cotton. The surplus melted wax from the tube had been drained into the cotton. Great care was taken not to overheat the mineral oil and wax. The blood was centrifuged in ice for 5 minutes at 3,000 r. p. m.; the plasma was drawn off with a 5-cc. pipette and stored at 3° in a paraffined, cotton-stoppered, 50-cc. centrifuge flask. This plasma was used within 30 days. No anticoagulant was used. While some tubes were lost because of spontaneous clotting, the loss was never so severe as to necessitate the use of heparin.

EMBRYO EXTRACT

Embryo extract was prepared from 9-day chick embryos. The whole unwashed embryo was minced by running it through a 30-cc. Luer syringe in the lower end of the barrel of which was a 28-mesh monel-metal wire gauze. The

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minced tissue was received in a 100-cc. narrow-throated centrifuge flask wrapped integrally with the barrel of the syringe. Approximately one volume of saline was added and the whole agitated briefly by sucking it in and out of a 25-cc. wide-mouthed bulb pipette. The flask was rubber stoppered and centrifuged at 3,000 r. p. m. for 20 minutes. The supernatant fluid was pipetted into 50-cc. centrifuge flasks, rubber stoppered, and frozen at once with carbon dioxide snow to kill any living cells. The solution was kept frozen until needed, at which time it was thawed out and recentrifuged at 3,000 r. p. m. for 20 minutes to clarify it directly before use.

Embryo extract was prepared two, or more usually three times a week. An attempt was made to use it when less than 5 days old, but in a few instances as a result of some accident older extract was used for a single change of fluid on the cultures.

Of the various culture media used, the embryo extract was probably subject to the greatest variation, both from lot to lot of eggs and possibly from season to season. In some earlier work in which refractometer readings were made on different lots of embryo extract, a wide difference was found in lots prepared on different days. Because of these variations, particular care was taken to use the same lot of embryo extract for each experimental culture and its control.

Preparation of Methylcholanthrene

The 20-methylcholanthrene used was all of the same lot, which was different from the lots used in earlier work (*1*, *2*). It was purified by Dr. J. L. Hartwell, of this Institute, and had a melting point of 179.3°–180.0° C., corrected.

Whereas in the previous work methylcholanthrene had been used in the form of a fine suspension, in this experiment it was dissolved in the serum. A lot of 2 l. of serum was placed in a glass-stoppered 4-l. pyrex bottle; about 40 mg. of the crystalline methylcholanthrene was weighed out into a small agate mortar, one or two drops of serum were added, and the mixture was rubbed to an extremely fine, creamy paste; more serum was added and the suspension transferred to the serum in the bottle quantitatively. The serum was held at 3° C. for 24 hours and shaken occasionally, and then shaken in a mechanical shaker for 24 hours. It was next run through filter paper to get out any particles that might clog the filter, and then filtered, through a Mandler candle into flasks that were sealed with rubber stoppers. The Mandler candle was used once, then destroyed.

This method of solution of the carcinogen was suggested by Dr. Egon Lorenz, of this Institute. Dr. Lorenz also analyzed spectroscopically the first lot of solution. The determination showed a concentration of between 2.0γ and 2.5γ of methylcholanthrene per cubic centimeter. Later lots were not analyzed but were prepared with the same technique and were considered to have approximately the same concentration.

Although an attempt was always made to have the relative size of the control and carcinogen-containing lots such that they would last for an equal period, it was rare that the two lots ran out at exactly the same time; usually one was exhausted a few days ahead of the other. Since no differences have been detected in the action of different lots of serum prepared by the method used, no hesitancy was felt in using up the last few flasks of any lot. To avoid any hazard of accidentally using a carcinogen-containing solution in normal cultures, the carcinogen-containing solution was always segregated. As a further precaution, the container of the normal solution carried a blue or black label, that of the carcinogen-containing solution a red label.

Duplication of Cultures

To prevent the loss of a whole strain of cultures through bacterial contamination of a stock solution, duplicate independent lots of solution were used, and all culture series were so divided that contamination of any single solution would result in the loss of only half of any one series or strain of cultures. No such contamination of a stock solution has occurred.

Carrel Flasks

All cultures were prepared and carried in modified Carrel type D flasks made of pyrex and having an outside diameter of 33 to 34 mm., a height through the flat area of approximately 10 mm., and floor and roof thickness of about 0.4 to 0.6 mm. This thickness gave good optical definition with a 16-mm. objective, yet the flask was not so fragile as to be hazardous when used with carcinogenic solutions. The throat length was approximately 33 mm., measured from the juncture of the throat with the top of the flask; the throat had a wall thickness of 1.0 to 1.5 mm. and an inside diameter of 10.0 to 10.5 mm. These changes in the throat were necessary to permit the sealing of the flasks with standard size 00 rubber stoppers.[1]

[1] *Flasks of this type may be obtained from E. Machlett & Son, 220 East 23d St., New York, and from Hopf Glass Apparatus Co., 192 3d Ave., New York.*

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Incubation of Cultures

All cultures were maintained at a temperature of approximately 38.2° to 38.8° C. in an incubator having an ample water jacket. The heaters in the incubator warmed the water jacket rather than the internal air space, while the two thermoregulators, which were wired in series, were immersed in the water bath. In this way sudden overheating of the incubator for a short period after the doors had been opened and closed again was eliminated. One thermoregulator was adjusted for a temperature 0.3° higher than the other so that it did not function at all but merely served as a reserve regulator. An alarm bell was wired into the circuit of this res rve regulator. The air in the incubator was subjected to forced circulation to insure even heating. In the incubator, the culture flasks were arranged in racks of 10 (fig. 2, *A*) and placed on shelves hinged at the left end, while a motor mechanism mounted on the roof of the incubator alternately raised and lowered the right end of the shelves through a cycle of about ±3½° from the horizontal at a rate of six cycles per hour. This caused the culture fluid in the flasks to be washed over the surface of the clots and helped to eliminate local changes in the culture medium within the flasks. The racks held the flasks level. If the racks were ever soiled with carcinogen, they were destroyed.

A temperature of about 38.5° C. was maintained during the visual examination of the cultures by having the microscopes surrounded with a thermoregulated air bath sufficiently large to take several racks of cultures. The photographic equipment

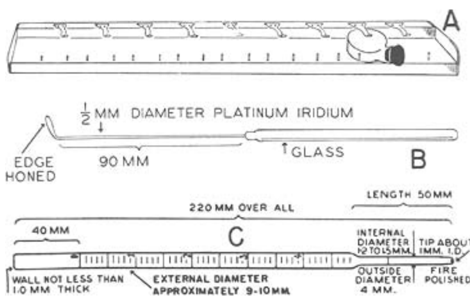

FIGURE 2.—*A*, Flask racks, made of wood with metal ends, and springs of sheet phosphor bronze 0.25 mm. thick. The pins are brass escutcheon pins; *B*, Platinum-iridium spatula used to transfer cultures. The curved portion of the spatula is flattened by hammering and then honed to a sharp edge; *C*, Pipette for handling small lots of solution like plasma.

was similarly held to constant temperature. With these precautions, the only serious intervals of lowering the temperature were when the cultures were reduced to room temperature during the three weekly changes of culture fluid. These intervals of cooling averaged about 4 hours each time and were constant for the whole series. It has as yet been technically impracticable to eliminate them.

Light

To eliminate complications that might result from the action of short wavelengths of light on cells photosensitized by methylcholanthrene, all cultures were handled, examined, and photographed by orange light. All light sources of general illumination in the rooms were shielded with five layers of No. 300 Tango shade of cellophane. The microscope lights were 6-volt, 108-watt, ribbon-filament, incandescent bulbs shielded with water cells and deep orange filters (Corning No. 351), cutting off at about 500 mμ. For photomicrography where the light was concentrated on the culture by a condenser, the light was further shielded with a Corning No. 397 heat-absorbing glass filter. While it cannot be said that no white light ever reached the cultures, the total amount reaching them during the whole course of the experiment was extremely low.

Preparation of the Original Explant

The original explant tissue was dissected out, cut up rapidly into strips about 2.2 x 6.0 mm., and placed in saline in depression spot plates. The cultures were then planted as described in the following section.

Transfer of Cultures

Cultures were transferred to fresh flasks about every 27 to 35 days. The series of flasks containing the cultures were arranged on the table in order of transfer; each flask was flamed very hot around the throat, the stopper removed, a sterile cork inserted lightly, and the flask set aside to allow the throat to cool. By the time the last of the group of flasks had been flamed, the throat of the first one had cooled sufficiently to allow handling. The clot was loosened from the floor of the flask by means of the spatula (fig. 2, *B*); and after a very light flaming of the throat which did not heat the glass through, the clot was slid onto the platform of the dissecting dish assembly, which consisted of a covered pyrex petri dish, 15 mm. high and 100 mm. in diameter, with half of a

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75-by-10-mm. dish (either top or bottom) placed in it to form a platform just below the surface of the lid of the pyrex dish. Dissections were made on this platform, and unnecessary fragments of clot and waste fluid were pushed over the edge into the surrounding moat. Each individual culture was dissected separately, a heavy dissecting needle and a No. 3 or 5 Graefe cataract knife being used. Separate dissecting dishes and instruments were used with each culture. The heavy ridge at the edge of the clot and all parts of the clot containing no cells or too few cells for satisfactory transfer were cut away. From the remainder, as many strips as desired were cut.

The size of the explants varied somewhat. In the first few generations an attempt was made to get strips about 3 mm. wide and 15 mm. long. However, better results were obtained with strips having a somewhat greater width, and in the later work an attempt was made to get them 4 to 5 mm. wide and approximately 20 mm. long. Usually three or four strips were cut at right angles across the original explant. In some instances only two strips, lying parallel, and just lateral to the old explant, were cut, and in others diagonally cut strips were used. The exact type of explant was so chosen as to give what seemed the best possible chance of growth from the old sheet of cells.

The explants were handled rapidly to avoid their drying out; as soon as they were cut, they were placed in approximately 0.7 cc. of saline solution in one of the depressions of a spot plate (Corning No. 7220) set in a 150-by-20-mm. flat pyrex preparation dish. The adhesive label of the flask was transferred to the top of the dish to mark the culture, while the order of explants was identical with that on the work sheet. Each strain of cultures was kept in a separate depression plate so that there was no chance of confusing individual cultures or different strains. Each culture was labeled and records were kept on the origin of each subculture.

Preparation of the Clot

From 0.4 to 0.5 cc. of chicken plasma was inserted in each Carrel flask from a 5-cc. pipette (figs. 2, *C*, and 3). In the early part of the experiment 0.4 cc. was used, but this gave a clot which in some instances was so soft that holes developed near the end of the transfer interval. When the amount was increased to 0.5 cc., no further trouble was experienced. The plasma was shaken over the entire surface of the floor of each flask, and 0.7 to 0.8 cc. of a mixture of 40 percent horse serum, 20 percent chick-embryo extract, and

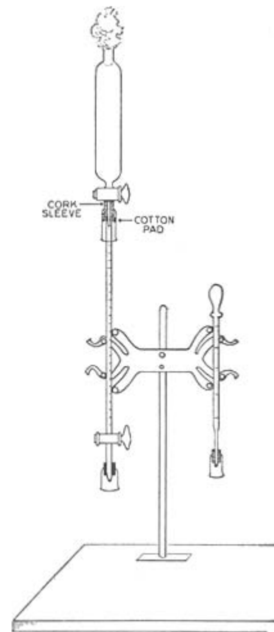

FIGURE 3.—Pipette and burette unit for planting cultures. The rubber bulb on this pipette is of 5-cc. capacity. For washing cultures and adding fresh culture fluid, a second burette unit was substituted for the pipette.

40 percent saline was then added. The explant was inserted in the flask at once by means of the spatula, flattened out, and centered. Each flask was closed with a rubber stopper, marked with a previously prepared adhesive label, then leveled until the mixture had formed a firm clot, and racked. Later all flasks were transferred to the rocking shelves in the incubator. Twenty-four hours later, 1 cc. of a mixture of the horse serum, embryo extract, and saline in the foregoing proportions was added. For cultures that were to receive carcinogen, it was contained in the horse serum in such concentration that each cubic centimeter of the final supernatant culture fluid contained approximately 1γ of carcinogen.

Washing and Renewal of Fluid Culture Medium

The fluid culture medium was changed three times weekly, usually on Monday, Wednesday, and Friday. The only serious deviation from this was near the end of December 1941 and in early January 1942, when the change was made only every 3 or 4 days. The resultant injury to the

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normal cultures was severe and was reflected in the noticeably poorer growth for nearly three generations.

In changing the fluid medium, the flasks were wiped off with a towel moistened with absolute alcohol and the cultures arranged on the table in numerical sequence within each set; the order of the sets was as follows: (1) Cultures which had never been subjected to carcinogen (all flasks carried white labels); (2) cultures which had at some time been subjected to carcinogen but were no longer (flasks carried green labels), the various sets being arranged in order so that those most recently removed from the carcinogen were always handled last; and (3) cultures which were being carried in carcinogen (flasks carried red label) and which were always handled last of all.

Aseptic precautions were taken and all normal solutions made up before any carcinogen-containing solutions were opened, and the culture fluids were correctly mixed in the separatory funnels of the burette units (fig. 3). Each funnel was then mounted on its burette which was locked in the burette rack. The other half of the rack carried a similar unit filled with saline solution.

The throat of each flask was flamed, the rubber stopper slipped out, and the end of the throat again flamed very hot. The old fluid was sucked out with a sterile glass needle attached to a fluid reservoir and a source of vacuum (fig. 4). With the burette arrangement, 3 cc. of saline solution was run into the flask, which was lightly stoppered with a sterile cork, and set aside, until each flask in the set had received saline. The suction needle was sterilized with heat after each use. After the last flask in the set had received saline, the first one was uncorked, the neck lightly flamed, the saline sucked out, 1 cc. of culture fluid added, and the flask at once sealed.

The burettes (fig. 3) were of 25-cc. capacity, graduated to 0.1 cc., and sturdily built to take relatively rough handling. The tip of each was protected, while in use, with a short glass sleeve held on by a cork collar. The open end of the sleeve was flared slightly for easy insertion of the throat of the flask which could be slipped in a distance of 7 to 8 mm., while the burette tip projected through the cork collar about 3 or 4 mm. The sleeve fit rather snugly around the throat of the flask. The freshly flamed throat inserted into the sleeve was free from bacteria, shielded so that bacteria could not fall into it, and so centered around the burette tip that the latter never touched the inside of the throat. In this way the likelihood of spreading bacterial contamination through consecutive flasks was obviated.

A wad of cotton in the upper end of the sleeve permitted air to flow into the burette as fluid was run in and withdrawn. All stopcocks were lubricated with sterile (autoclaved) heavy petroleum jelly. At the end of each run the equipment was disassembled, the cork collars were destroyed, and the glass parts of the apparatus were cleaned (with

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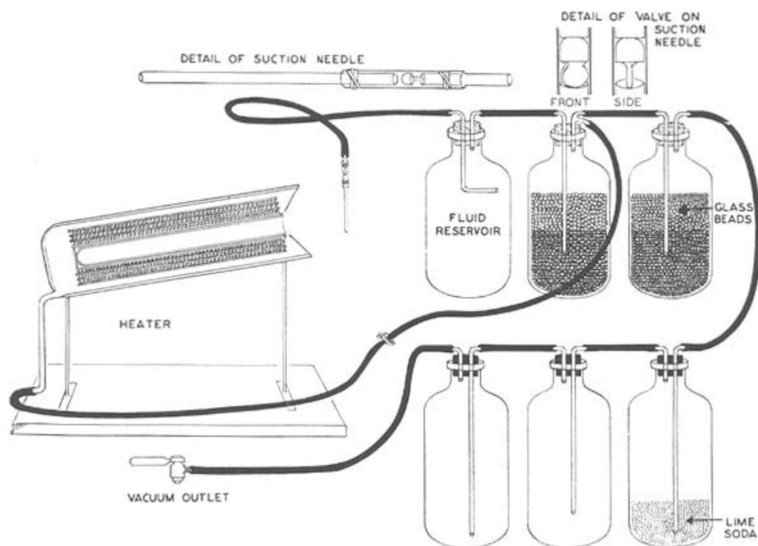

FIGURE 4.—Suction needle unit with heater. The heater diagram is drawn disproportionately large to show detail. Details of suction needle and valve assemblage are also enlarged.

acid if the unit had been used with culture flasks that contained or had ever contained carcinogen).

The suction needle (fig. 4), a piece of standard wall pyrex tubing 6 mm. in outside diameter and 150 mm. long, was attached to a length of gum rubber tubing 60 mm. long, with 6-mm. bore (1.5-mm. wall thickness). A glass bead of special design was inserted midway the length of the tubing to serve as a valve. When the outside of the rubber tube was squeezed gently with the fingers, the resulting deformation caused a leakage of air around the bead. The tubing was sufficiently soft and pliable that a relatively gentle pressure near the bead gave a vigorous suction at the tip of the glass needle. Figure 4 shows in detail the setup. The bottles containing the glass beads each had a 1½-inch layer of sulfuric acid in the bottom, which served to trap any possible spray containing traces of carcinogen that might be carried over into the house vacuum line. The sulfuric acid was replaced about every 6 to 8 weeks. The first of the three bottles connecting with the vacuum outlet contained lime-soda to neutralize any acid spray carried to it.

A small electric heater to sterilize the suction needle between uses is shown in figure 4. The heating element was wound around a 15-mm. (inner diameter) metal tube about 6 inches long and insulated from it with several layers of asbestos paper. The tube was sloped so that the needle could be rapidly inserted without danger of its sliding out. A small glass test tube was used as a lining to protect the metal tube from corrosion and to prevent the carrying over of metal oxides into the culture flasks. It was changed at the end of each series of cultures.

The winding was so designed that, within a few minutes after being switched on, the heater reached and maintained a temperature of about 340° C. within the lumen of the glass tube, as measured by a thermocouple, while a suction needle containing several drops of water boiled dry within 7 seconds after insertion into the heater tube. Inasmuch as the interval between using the suction needle on two consecutive flasks was about 20 to 30 seconds, the hazard of carrying a bacterial infection from one flask to the next was avoided.

Figure 4 shows the metal casing projecting over the open end of the heater, and also the hose connection with the acid shield on the vacuum line. Through this line a slight current of air was kept flowing over the front outlet of the heater in order that any traces of fumes liberated from the heated needle might be carried into the sulfuric acid trap and not released in the room.

When this unit was originally designed, the question arose whether it could be used safely on the cultures lest the hot needle injure them by its own heat or by fluid heated to boiling and expelled from it onto the sheet of cells. The system finally worked out has now been used for more than 2 years and has given satisfactory results. To remove fluid from a culture flask, the needle was removed from the heater and at once inserted into the open mouth of the flask. Before it entered, however, the operator had already exerted pressure on the rubber tubing around the glass-bead valve and so established a rapid flow of air through the glass tube. The tube was lowered along the inner bottom surface of the flask throat; meanwhile an uninterrupted flow of air was maintained through the needle. As the needle tip reached the floor of the flask, the latter was tilted very slightly but not so much that fluid ran into the open end of the throat. The fluid was sucked out cleanly and in a fraction of a second. The needle was removed, the pressure on the valve released, and the needle returned to the heater. In this way a needle could be used for evacuating a series of 150 flasks without clogging. If the needle clogged after a short time or if it did not suck out the fluid cleanly and almost instantaneously, it was not being correctly manipulated. Usually in such cases the valve was not open sufficiently. Once an operator was trained to use this device, the average time necessary to handle each flask was approximately 60 seconds. Cultures used with this unit have never shown local injury to cells near the throat of the flask, and only a very few have had to be discarded because of some incorrect behavior of the suction device.

After use, the suction-needle equipment and its fluid reservoir were disassembled. Rubber units were destroyed, and glass units given the usual acid treatment. The tip of the glass inlet tube of the first acid bottle on the suction unit was flamed very hot to eliminate any traces of carcinogen that might have adhered from spray passing through it, and it was capped until the next change of fluid. The aluminum front of the electric heater was heated with an open flame as hot as possible without fusing to destroy any traces of methylcholanthrene that might have been left on it by the needle.

No culture that was receiving carcinogen or that had ever received it was ever opened until all normal cultures had been washed, fresh fluid added, and the cultures sealed. Similarly, no culture that was receiving carcinogen was opened until those that had once had carcinogen but were no longer receiving it were resealed. There was no exception to this rule. Careful checking and

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the different colored labels on the three series precluded any chance disarrangement in the order of handling.

In a few instances when one or two drops of a carcinogen-containing solution were accidentally spilled on the table or the floor during the handling of the cultures, the solution was cleaned up at once by wiping the area repeatedly with a succession of cotton swabs soaked in acetone or benzene.

Procedure for Recording Changes in Size of Cultures

One of the usual techniques, and the simplest, for estimating the enlargement of a culture is to determine and plot the area at different times after planting. Parker (7) and Cunningham and Kirk (*8*), among others, have pointed out that this is not a true index of growth but that it is complicated by other factors. When cells are treated with methylcholanthrene, the number of cells in each unit area of the treated cultures is different from that in the controls; the curves obtained are, therefore, certainly not a reliable index of growth.

Even though this be true, the need was felt for some simple and easily applied index which could be used to keep some record, other than that of cell description, of the deviation of the carcinogen-treated cultures from the normal. Since the earlier work had shown that the action of the carcinogen could be detected within a very short time by the lagging of the rate of increase in area or width of the treated cultures and since this retardation seemed to be associated with the action of the carcinogen, it was felt that this index was worth using as a working guide. The index, as in the previous work with strip-shaped explants (*2*), consisted of a record of the changes in the average width of a culture, the length of the strip being left out of consideration. In all instances the width was determined by measuring the culture under a compound binocular dissecting microscope fitted with paired 1.0 × objectives and 9 × oculars, one ocular carrying a special micrometer disk divided, as shown in figure 5, to read numbered intervals of 1.0 mm. actual size of the object, with ruled subdivisions of 0.2 mm. This gave a simple and rapid means of estimating the width of the cultures to 0.1 mm. without subjecting them to intense light. The data obtained by this method of measuring were collected on all cultures and plotted to give curves for each generation of each culture strain. These curves are not presented in this paper on account of the difficulties in reproducing them for publication.

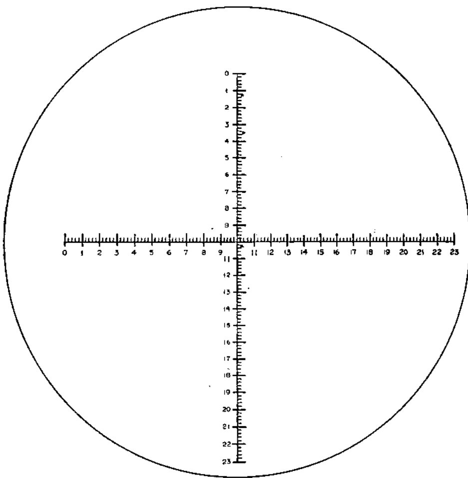

Figure 5.—Ruling of ocular micrometer disk for recording changes in the width of cultures. The ruling is displaced slightly from the center to facilitate the measuring of small cultures.

The interruption of these curves because of periodic transplantation of the cultures into fresh flasks offered a serious difficulty to obtaining a satisfactorily connected picture in the culture strains under experiment. To show this progressive change, the width of the zone of new growth was taken at 5, 10, and 15 days after explantation. These three readings were averaged and plotted as an average width at a time 10 days after explantation (fig. 6). In a few instances where the width curves ran only 13 or 14 days, the value for 15 days was obtained by extrapolation. These various average points on any one strain of cultures were then joined by a continuous line. In this way, for any one carcinogen-treated strain one continuous line was obtained, and for its normal control cultures another line was similarly obtained. The number of cultures averaged to give each point on the curve is shown by the number opposite the point, while the letter adjacent indicates the cell strain used to give that point. This notation is necessary since often one set of controls was used for more than one experimental series. To show the relationship of control and experimental culture when this occurred, the curve from the control was repeated for each series for which it was used. Similarly, where strain N, for instance, originated from strain O, the early part of the curve from strain O has been drawn in for the strain N curve to complete the picture of the curve. The time each experimental strain was left in the carcinogen is

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FIGURE 6.—Continuous width curves for all strains. The carcinogen-treated cultures are shown by the solid line; the control is shown by the broken line. The time the treated cultures were left in carcinogen is shown by the heavy black line. Each curve is identified according to its strain of cells by the large letters opposite it.

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shown by the length of the heavy black line below the respective curves. These continuous width curves are presented through May 18, 1943.

This type of curve was plotted for each of the strains studied. The curves (fig. 6) are designated continuous width curves.

RESULTS

Origin and Description of the Primary Normal-Cell Strain

The strain of fibroblastlike cells used was originally taken from a 100-day-old male mouse of the C3H strain, Andervont substrain. On October 18, 1940, the mouse was decapitated. Without including muscle tissue and the regional lymph node, the connective and fatty tissue pad along the side and just in front of one of the hind legs was removed and cut into six strips each about 2.2 mm. wide and 6.0 mm. long. Each strip was planted in a separate flask. When examined just after planting, much of the tissue was seen to consist of fat cells. Five days later, the only sign of migration was an occasional projecting cell. At 10 days, the width of the strips had increased from 2.2 to 10.2 mm., and the growth was recorded as luxuriant and even but somewhat loose; there were numerous cells in the growth zone which contained large fat droplets, and it appeared certain that the fat cells had contributed materially to this zone. From the reduction in size of the fat droplets, it also appeared that many of these cells were losing their fat droplets. Growth continued until 42 days after explantation. At this time, one of the cultures had been lost through accident. The remaining cultures showed an average width of 18.0 mm. The fringe of cells showed even less fat than earlier, because of a reduction both in the number of cells containing fat droplets and in the size of the contained droplets.

The cultures were transplanted into from 5 to 8 flasks each, the explants being selected so as to include none of the original explant. All grew luxuriantly, and all except 1 were carried on for a number of generations and then closed out. This 1 was transferred to 3 flasks 63 days after planting. In this third generation growth was excellent and even. One selected culture was transferred to 6 flasks 43 days later. One of these was again transferred to 10 flasks 39 days later, and of these 1 was transferred to 5 flasks 30 days later (May 22, 1941). Of these 5 cultures, 2 were selected and built up into a series which was used for the experiments reported herein. All cultures used in this work originated from 1 of these 2 cultures.

The amount of fat droplets seen in the cultures had rapidly lessened until by the third generation often whole cultures were entirely free from all except the few very small fat droplets normal to growing fibroblasts in this culture medium. When the cultures were carried in the same flask so long that the density of the cell population caused overcrowding, particularly among cells deep within the clot, some cultures showed a rapid temporary formation of large fat droplets within the cells, but this formation subsided at once when the cultures were transferred to fresh flasks. In the later history of the strain, large fat droplets showed up sporadically under these conditions, but the number of cells showing such droplets was small in relation to the number of cells in the culture. The droplets disappeared as soon as the culture was shifted into a fresh flask with fresh culture medium.

The structure of these cultures was normal for cultures of fibroblasts previously described (*2*), the architecture was loose, and the cells were of characteristic shape, adhering to each other usually by terminal processes. Figures 8, *B*, 9, *B*, and 11, *B*, show typical culture structure. The refractive index of the cells was low,

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and particularly in very dense cultures the cells were often hard to see. There was no clear relationship between relative growth on the surface of the clot closest to the fluid and that nearest the glass in the early life of the cultures. At both surfaces the cells seemed to be rather similar, although at each they were usually more flattened than were those within the body of the fibrin clot.

The rate of growth of the cells was comparable with that of earlier strains of normal fibroblasts carried in this culture fluid. There was variation from flask to flask, and growth during some generations was better than during others. Frequently these variations could be traced to minor differences in handling; too frequent shifting of the cultures to a new generation resulted in smaller explants and in low cell density within the new explants, and these poorer explants in turn gave rise to even poorer growth. Similarly the holding of cultures too long before transfer resulted in diffuse central necrosis and poorer growth when the cultures were transferred. The best interval for transfer with the range of explants used was probably about 35 to 40 days.

Sequence of Changes in Freshly Transplanted Normal Cultures

During any one generation the sequence of changes in these normal cultures was approximately as follows:

For about 8 to 12 hours after explantation there was no appreciable sign of migration of the cells. At 24 hours there were often a few cells or cell processes out, which lay at all levels within the thickness of the clot and often resembled spikes. This fringe got wider and denser; at several days there was a luxuriant growth, the cells of which usually showed a definite tendency to concentrate along the interfaces of the clot, that is, in the layer of clot closely adherent to the glass and the one next to the overlying fluid. These layers will be referred to later as glass and fluid interface layers, respectively. The cells at the edge of the migrated zone were typically fibroblastlike, with characteristic spindle, flattened-spindle, or less frequently triangular shapes, and with frequent, long, terminal, threadlike processes. At this stage the culture was often troublesome to photograph with higher power lenses for several reasons: (1) There were irregularities in the thickness of the clot very close to the explant; (2) in reaching the glass and fluid interfaces, the cells within the clot sometimes lay along a slope so that when examined with higher lenses the whole length of the cell could not be focused at once; and (3) the layer of cells at this stage was frequently very thick, often with a lack of areas of cell density suitable for photographic records. Nor was the comparison of the increase in size of the clump with that of carcinogen-treated cultures as satisfactory during the first few days as it was a little later, since the experimental and control cultures had not grown sufficiently long to indicate clearly divergent rates of growth.

As the growth of the culture continued, there was a stage at about 8 to 10 days which was probably the most useful for comparing different cultures. The cells were not laterally coherent as the growth of the cultures was typically loose, mitoses were frequent, and there was no sign of degeneration. At this time no regularity was observed, whether the glass or fluid layer was the larger, although as a rule the edges of the fluid layer were somewhat looser. The edge of the normal culture was usually not too loose to permit the estimating of the culture diameter. At this stage also, experimental cultures had had time for their width curves to develop any divergences.

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In the later growth of the culture, a number of complications interfered with the determination of the rate of increase of the size of the clump. Measurements of the width of the normal culture were often complicated by the extreme looseness of the sheet of cells. In some instances, there was no real edge to the culture, loose cells reaching the edge of the flask at 18 days of culture growth. This condition was worse at the fluid interface where sometimes the lack of a definite culture edge was caused by the fact that the cells had been washed loose from the culture and had reanchored themselves in the more peripheral parts of the clot. Often, too, little clumps of cells that had broken loose from the culture at its planting proliferated and at this stage reached such a size as to interfere with determinations of the size of the central clump. Consequently, width curves were irregular in this later period. Frequently the cell sheet at the fluid interface had grown so large and dense that study of the cells at the glass interface was obscured. With a very dense and rapidly growing clump, the study was further confused by traces of diffuse necrosis in the central area of the glass layer.

Once the loose cells reached the edge of the flask, growth could obviously not increase the diameter of the culture. Instead, from about 21 to 30 days on, the growth of the culture was manifested as increased cell density in the more peripheral areas. This increase tended to fill in the body of the clot. The culture was more difficult to examine and to photograph. In its last stages diffuse necrosis began to appear, first along the central area of the glass interface. When a large strip explant was used, the culture in this interval usually thickened to a heavy sheet of cells that reached the edge of the flask. With smaller explants, this migration to the edge of the flask was slower.

The foregoing general description held approximately for normal stock cultures and for controls up to the time lateral cohesion of the cells was observed. Owing to the various complications that occur during the very early and the very late life

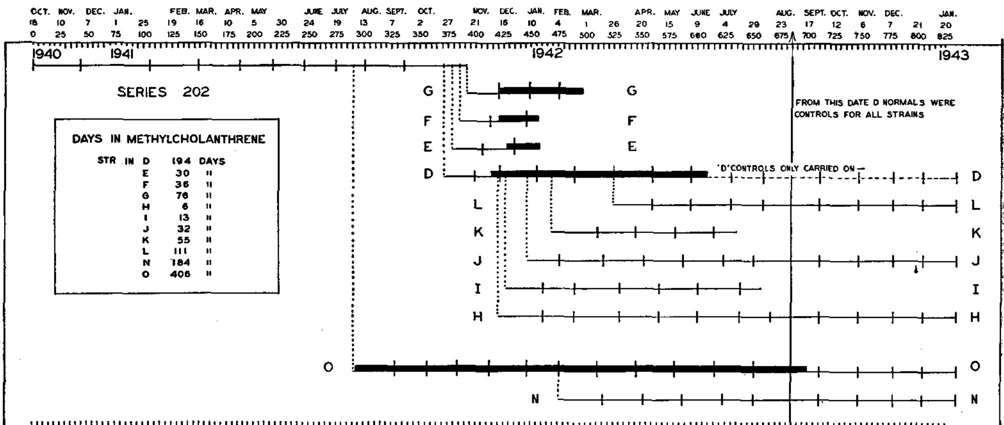

FIGURE 7.—General relationship of the culture strains. The interval during which any strain of cells received carcinogen is shown by the very heavy lines, while the time at which the strain was removed from the carcinogen is shown by the ending of the heavy line or by the vertical dotted lines which indicate when the culture was lifted out as a separate strain. Approximate times of changes of the cultures into new flasks, with corresponding changes of the fibrin clot, are shown by the short upright lines arranged along the horizontal lines. The letters denoting the different cell strains correspond to the strain labels in our records and are used to identify them.

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of the cultures, in these experiments an attempt was made to correlate visual, photographic, cinematographic, and stained-slide records of the cells within the interval from 8 to 15 days after the culture was planted, preferably 8 to 12 days after. At this time, there were usually satisfactory areas of cells clearly defined along the glass and fluid interfaces, the sheets were not too dense for examination, and there was no necrosis of the normal cultures. Furthermore, as will be seen later, this period was probably the most suitable one for study of carcinogen-treated cultures.

Relationship of the Experimental Cell Strains

The relationship of the different strains of the carcinogen-treated cultures may be seen from figure 7. Until August 1942 each strain of cells was carried with its own control. All cultures were in the same culture fluid medium; but since not all the cultures could be transferred to fresh flasks simultaneously, different strains with their individual controls were transferred on different days. In order to compare the different strains more accurately, in August and early September 1942 the strains were regrouped. Since all control cultures had a common origin and since recognized morphologic differences between them were extremely slight, the controls of strain D were carried on as the control on all cultures and all other control strains were closed out. The carcinogen-treated cultures were then regrouped into several sets, so that the cultures of each set were transferred on the same day with the same solutions and so that each set contained cultures from strain D control, and H, J, L, N, and O carcinogen-treated strains. This reorganization allowed close comparison of the different strains under identical conditions.

In the following description of the changes in the cells of the different strains, the progress of the continuous width curves is given to the end of May 1943 and the cell changes observed in the living cultures to September 1942, whereas the cell changes from October 1942 through May 1943 are given in another section, in which the cells of the different strains are more closely compared.

Comparison of Continuous Width Curves for the Different Strains Through April 1943, and Description of Cell Changes Observed in the Cultures Through September 1942

Strains N and O (Series 208)

Strain O, the first of the experimental strains, was started August 5, 1941, after the cells had been carried 291 days in vitro. Four cultures were selected, and from each, two strips were cut lengthwise along the explants. Each was in turn halved transversely. Two explants from each original culture were maintained as controls; 24 hours later the other two explants from each culture were started in the methylcholanthrene solution. Two of these carcinogen-treated explants were damaged with a hot spatula and were discarded shortly after planting. The strain has been carried on by consecutive transfers up to the present time. The continuous width curve of this series is shown in figure 6, section O.

During the early days of this strain, there was no evidence that the carcinogen caused any accelerated rate of increase of width of the cultures. Six to eleven days after the addition of the carcinogen, the width curve of the treated cultures showed a definite depression relative to that of the controls. At 14 days, the depression was more severe; in the second generation it was even more so.

A visual examination of the cells during

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the first few days after addition of the carcinogen showed no clearly defined change. There was no sign of lateral cohesion or of ribbon or sheet formation. If there was any degeneration in the cultures, it was so slight that its existence was uncertain. The only suggestion of an injurious influence was some slight lateral shrinkage of some of the cells.

At 45 to 48 days after addition of the carcinogen and 11 to 14 days after transfer, the treated cells were less spindle-shaped, more sheetlike, with definite shortening of their slender terminal processes. The cultures were more compact than normally, and their edges appeared less reticular and loose and showed fewer loose cells. There was a definitely increased irregularity of cell size. Numerous mitoses were seen in both experimental and control cultures.

At 52 days, 17 days after transfer, the changes described had progressed. The cells were very granular; some cells were up to eight times their normal size; numerous cells appeared in mitosis in both experimental and control cultures.

At 73 to 76 days, 9 to 12 days after transfer, the terminal processes of the cells were shorter, more blunted, and showed definite lateral irregularities suggestive of amoeboid rippling or frothing. The cells were still spindle-shaped and adherent laterally so that the edge of the culture appeared even more compact and less reticular or lacelike in architecture (compare fig. 8, *A* and *B*). There were few, if any, loose cells. The glass interface layer was dominant, and there was little or no fluid layer at this stage.

At 97 days, 7 days after planting, the glass interface layer was extremely compact, with few or no loose cells at its edges. The fluid interface layer was less pronounced and less characteristically coherent than was the glass layer. The culture edge showed numerous short cell processes, and there were only occasional epitheliallike lobes at the edges. The terminal processes of the cells were usually very short, and the amoeboid structure of the lateral edge of these processes had extended farther toward the mid region of the cell. The whole lateral edge of the processes often had an amoeboid appearance (cf. fig. 9, *A* and *B*), which was so pronounced that in some instances it was hard to delimit the exact edge of the cell. The cells seemed more granular than the controls, and there was more local cell disintegration.

At 106 days after addition of the methylcholanthrene and 16 days after planting, the cultures were very dense, and there was a large area of central necrosis. The cells were very closely coherent into sheets over extensive areas, and the culture edge was abrupt (figs. 10, *A* and *B*, and 11, *A*). The cells shown in figure 11, *B*, are the control for those in figure 11, *A*.

In some instances the edge was made up of short projecting spikes of cells, in others the cells were flattened, often epitheliallike. The cell processes were extremely short, while the amoeboid rippling or bubbling along the edges of the cells often extended the whole length of the cells. There was extensive cytoplasmic granulation.

During the subsequent interval up to 184 days after the first addition of carcinogen, there was no feature of note except a gradual progression of the processes already described. Often in this period the cultures showed lobed edges typical of epithelium in culture.

On February 6, 1942, after 184 days in the carcinogen and 31 days after planting, the cultures had gone through six generations of transplantation. At this time a group of four cultures was removed from the carcinogen and set up as strain N (fig. 7). From this time on the cultures of

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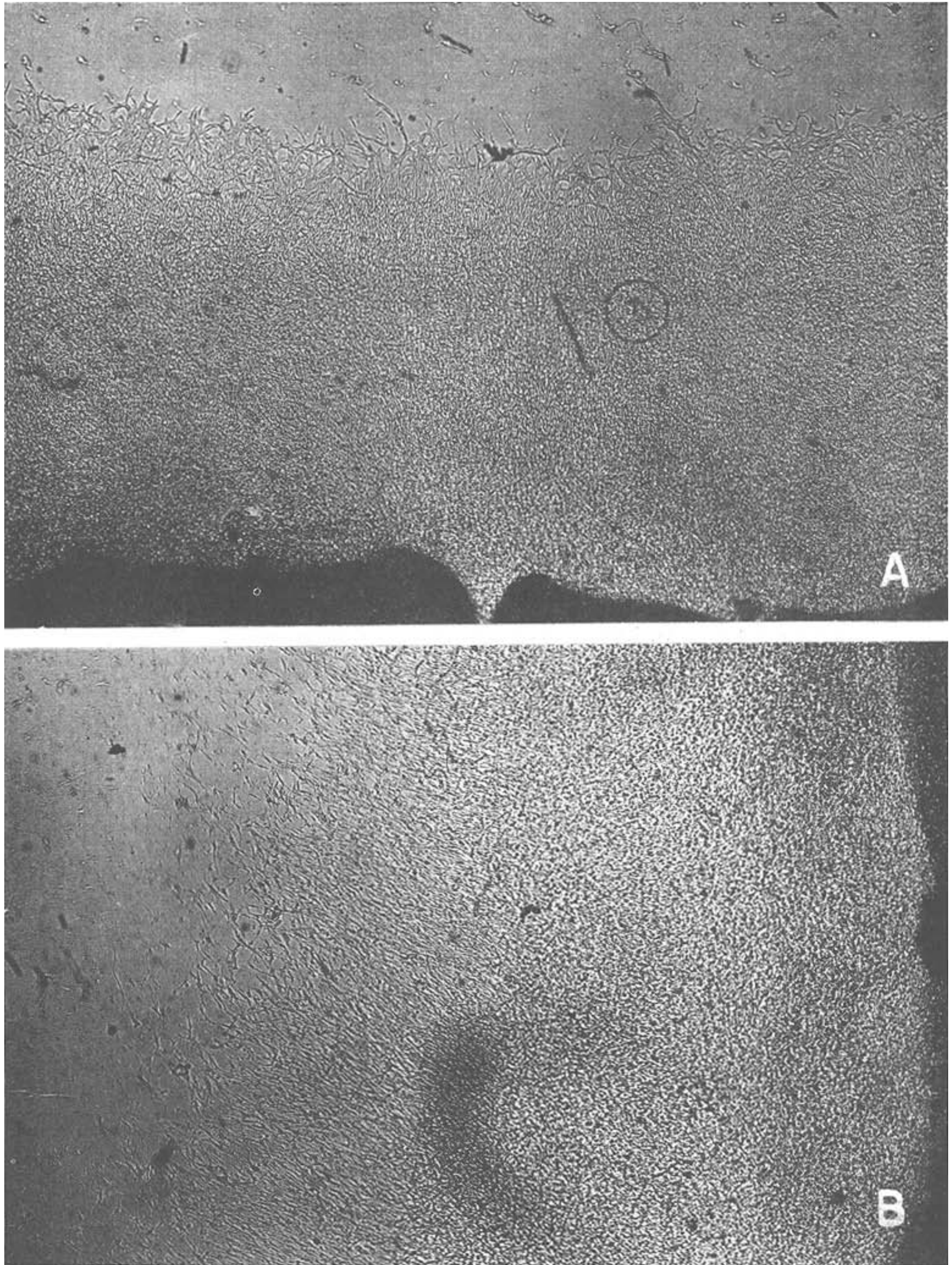

FIGURE 8.—*A*, Culture from strain O, 76 days after first adding carcinogen and 12 days after planting. The black circle is an optical imperfection; *B*, Normal control cells. Both *A* and *B* are cells at the glass interface. × 20.

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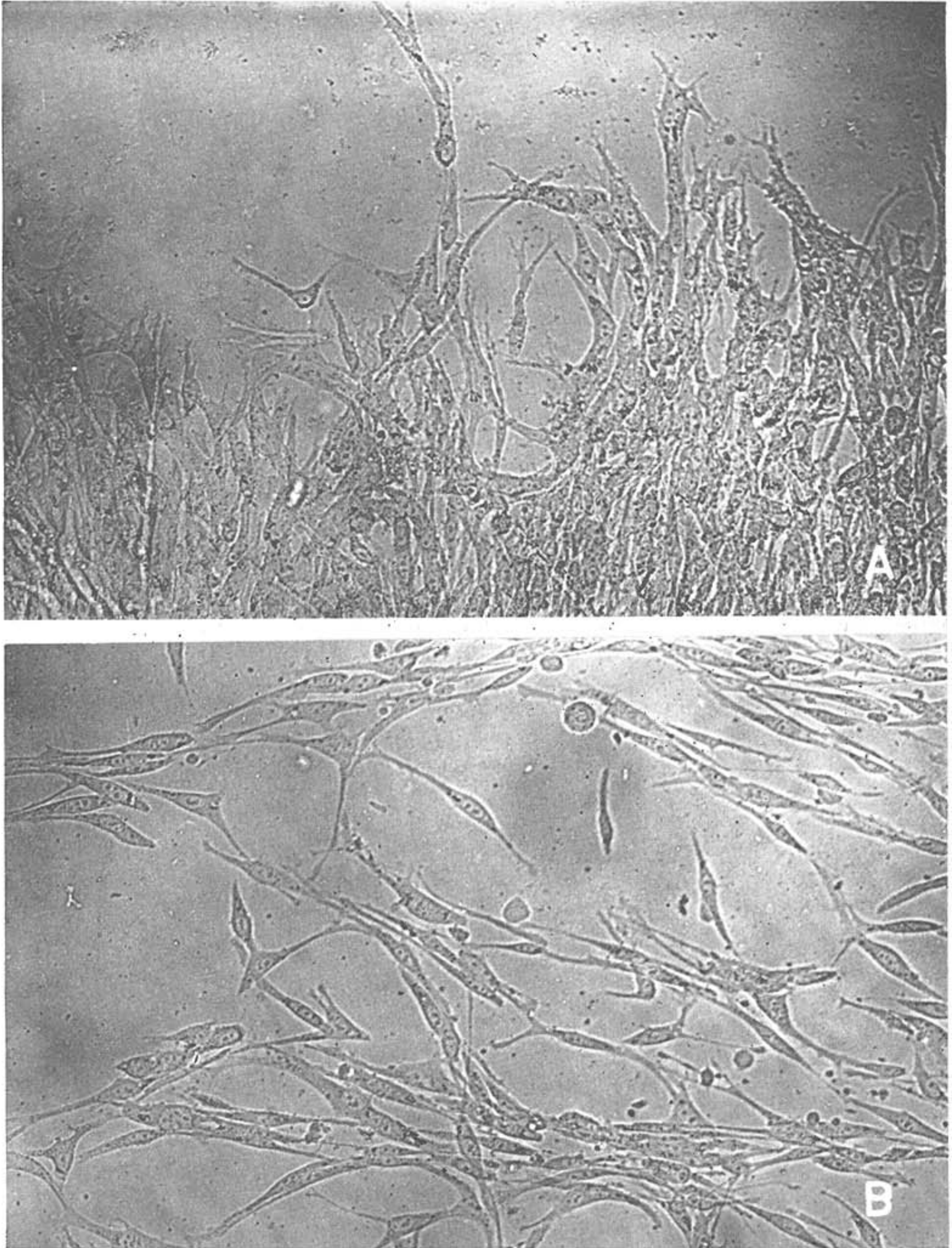

FIGURE 9.—*A*, Edge of culture of strain O, 97 days after addition of carcinogen and 7 days after planting. Note shortening of cell processes and occurrence of lateral frothing or rippling; *B*, Normal control cells. Both *A* and *B* are cells at the glass interface. × 200.

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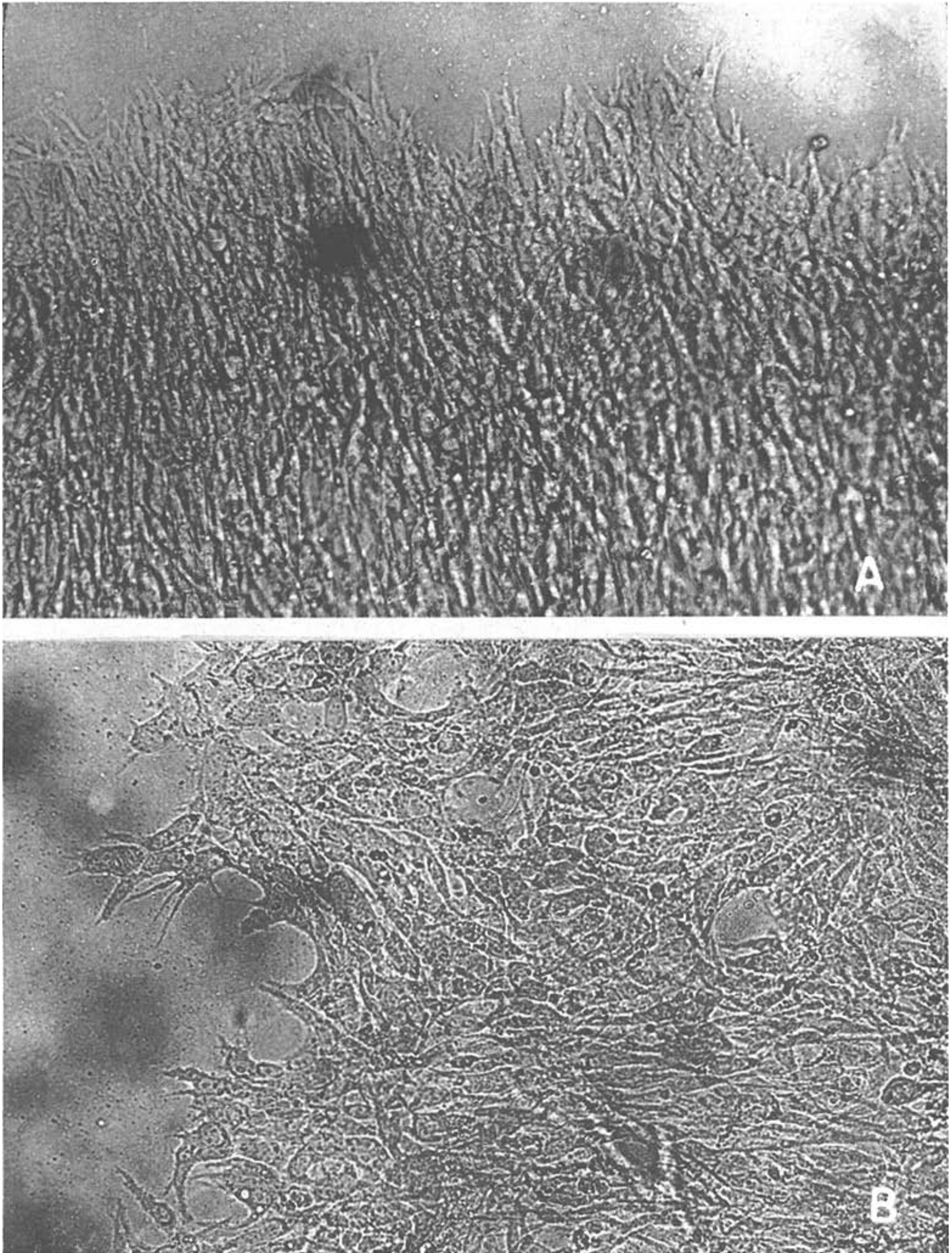

FIGURE 10, *A* and *B*.—Edge of culture of strain O, 106 days after addition of carcinogen and 16 days after planting. Note extremely short cell processes and definitely amoeboid appearing edges. Figure 9, *B*, can be taken as representative of the normal culture at this time. The dark areas of shadow in the print arise from droplets of fluid on the roof of the flask. × 200.

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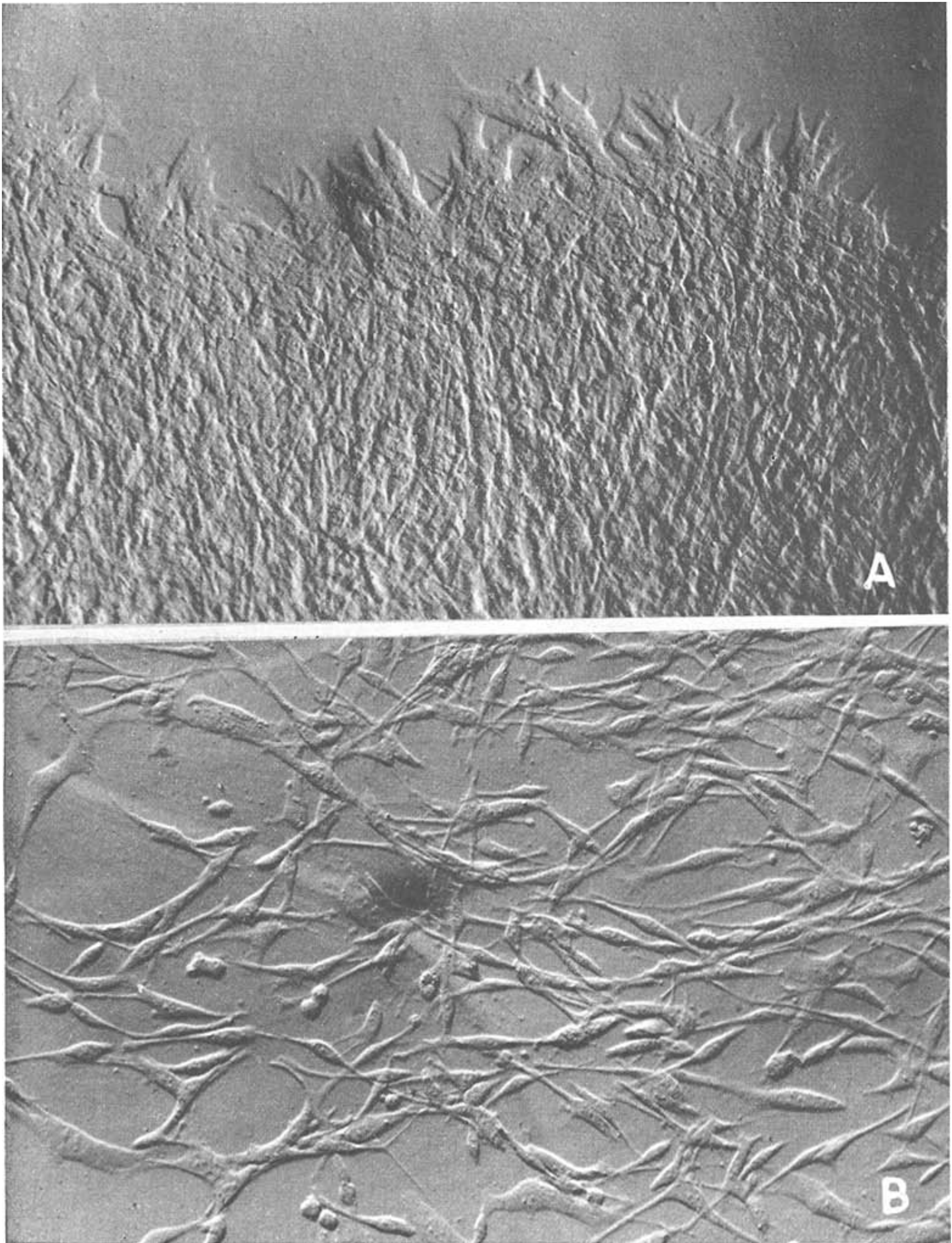

FIGURE 11.—*A*, Edge of culture of strain O, 132 days after addition of carcinogen and 12 days after planting. Note epitheliallike cell shape, the suggestion of granularity, the less clearly defined cell axis, and the shortened processes; *B*, Control cells. Both *A* and *B* are cells at the glass interface. Oblique lighting. × 200.

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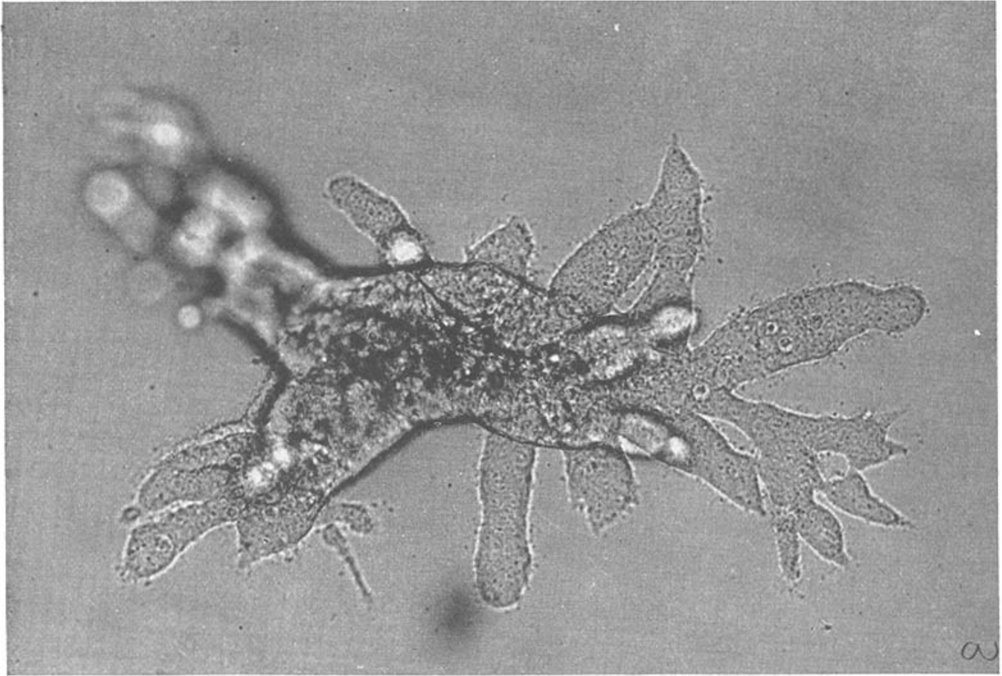

FIGURE 12.—An extremely small cell clump which arose from a fragment in a flask with a larger size clump of strain N, 57 days after removal from carcinogen and 18 days after planting. The clump is situated near and at the glass interface. One end of the clump is at a lower level and is out of focus. Note the close cell cohesion and the irregularity of the cell surface, also the complete absence of necrosis at a time when the central area of the larger clump showed extensive necrosis. × 200.

strain N were carried without further addition of carcinogen. Through the end of May 1943, the strain had been carried a total of 663 days since the original addition of carcinogen and 479 days since removal from it.

During the four transfers following the removal of this strain from the carcinogen, the continuous width curve (fig. 6) held to the same even, depressed level it had shown while the cells were in the carcinogen. With the omission of the first reading in the curve following the original addition of carcinogen, at which time the curve had not reached this low level, the average value for the curve through the fourth generation following removal from carcinogen was 4.6 mm. Beginning with the fifth generation after removal from carcinogen and including the 11 transfer generations through May 1943, the curve showed much greater fluctuation, while the average level of the curve for these 11 generations rose to 6.0 mm.

No great change in the appearance of the cells was noted in the period immediately following removal from the carcinogen. There was no evidence suggesting a further progression of the cell changes noted. The close coherence of the cells and the altered character of the cell surface at this stage are particularly well shown in figure 12, 57 days after removal from methylcholanthrene and 18 days after planting.

The strain of cells continued in the carcinogen after 184 days (strain O) was carried on uninterruptedly until September

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16, 1942, or 406 days after the first addition of carcinogen. Following the initial depression in the first generation after addition of carcinogen, the continuous width curve of this strain held to a relatively constant depressed level through the eleventh transfer generation (fig. 6, sect. O). The average level of the curve during this time was 5.0 mm. Beginning with the twelfth transfer generation, there was a further depression of the curve which held to a relatively constant lower level for two generations.

At the end of that time so much difficulty was being experienced in transferring the strain (the average fringe of migrated cells was only 2.7 mm. wide at the end of 16 days, and even the more central cells of this fringe were already necrotic) that there seemed to be no doubt but that continuation of the cultures in the carcinogen would result in loss of the strain within the next two or three transfers. The carcinogen was therefore discontinued September 16, 1942, 406 days after it was first added to the cultures.

For the next three generations following removal of the culture from the carcinogen, the continuous width curve held to the same low level, the average level for these three generations being 3.0 mm. Beginning with the fourth generation after removal of the culture from the carcinogen, the curve showed increased fluctuation, while the average height of the curve during the next five generations (ending in May) was 5.0 mm.

The progression of cell changes in strain O during the period 184 through 406 days was a continuation of those shown earlier (fig. 13, *A* and *B*, and fig. 14). The adhesion became increasingly severe, with a further increase in production of dense sheets and masses of cells, the sheets at their edges often showing lobe-shaped projections similar to those seen in cultures of epithelial cells. The cells were short, often more or less rounded, and adhered laterally to those around them. Cell structure in such massive sheets was very hard to study; but where more isolated cells were found on the glass interface, the cells no longer showed the characteristic spindle shape of the fibroblast. The individual cells were usually irregular in shape, and there were often short lateral processes. The edges were often frothy and ruffled along the whole length of the cell (fig. 14). During the same period there was a further increase in the granulation of the cytoplasm so that the cells were extremely grandular. No well-defined alterations in the nuclei were observed in the living cells, although this point has not as yet been critically studied.

Series 202

Series 202 was planned to obtain more detailed data on the earlier stages of the action of methylcholanthrene, particularly the influence of graded doses. Strains D, E, F, and G were, therefore, started November 20 and 27 and December 4 and 11, 1941, respectively, while the carcinogen was added December 5, 19, 12, and 12. The addition of carcinogen to the series was so planned that in each of the strains it was made at a different time after planting, at 15, 22, 8, and 1 day, respectively.

Because of circumstances that had no connection with the behavior of the cultures, it was necessary to discontinue strains E, F, and G at 29, 36, and 75 days after the original addition of the carcinogen. For that reason, these strains contribute little to the data. The continuous width curve of strain G is shown in figure 6, section G. The early cellular description of these strains is similar to that of strain O.

Strain D, however, was continued and

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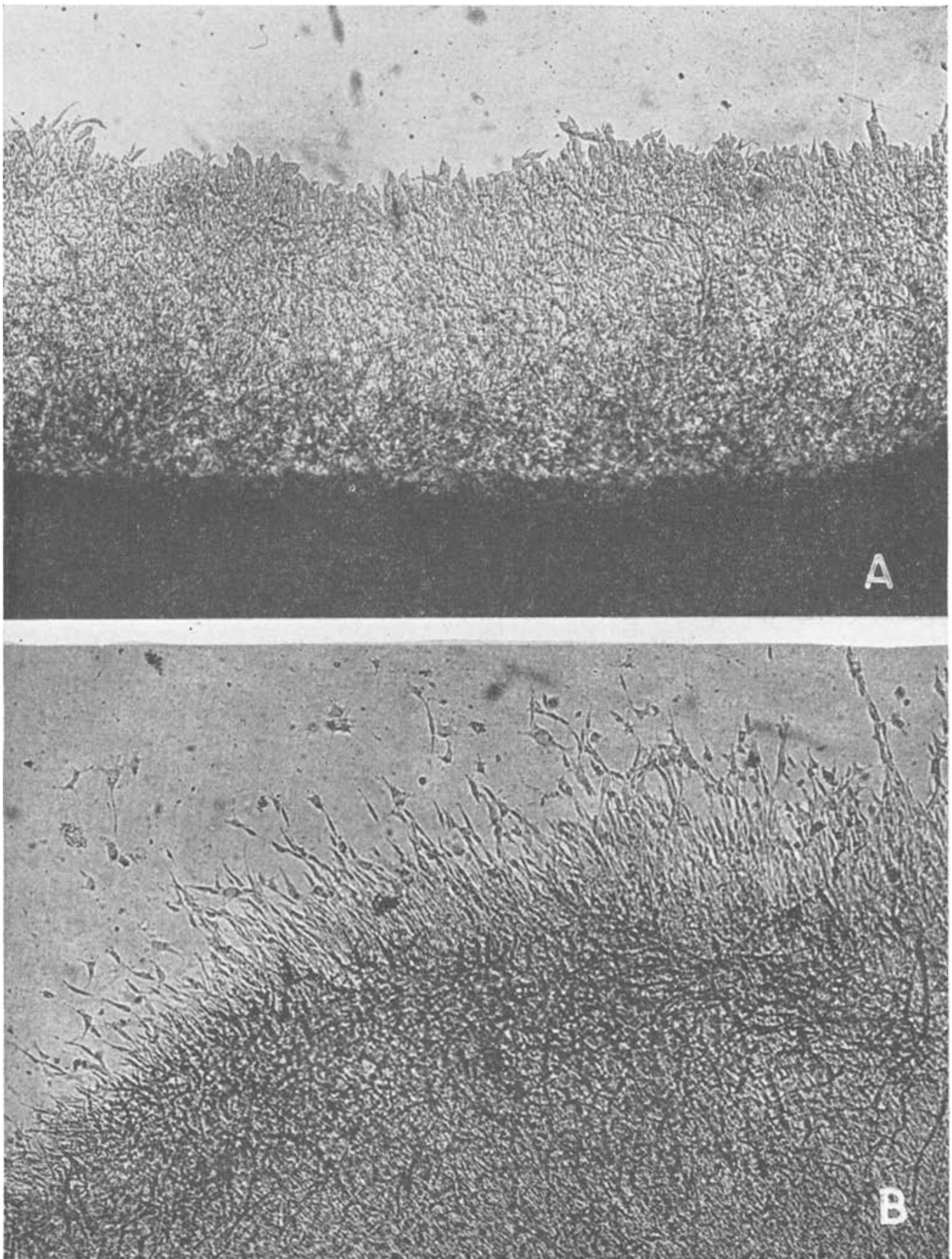

FIGURE 13.—Edge of culture of strain O, 403 days after addition of carcinogen and 11days after planting. *A*, Cells of glass interface. Note the massive sheet formation and the very limited width of the zone of growth; *B*, Cells of the fluid interface. Note the relatively loose structure of the culture and the greater width of the zone of migrated cells Both *A* and *B*. × 40.

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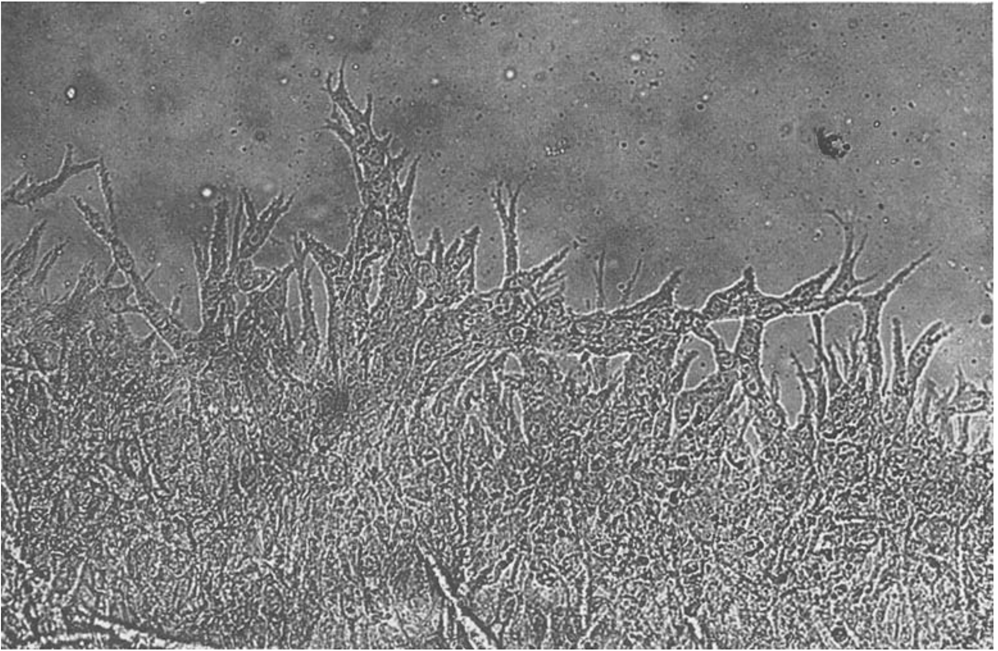

FIGURE 14.—Edge of culture strain O, 403 days after addition of carcinogen and 11 days after planting. Cells of glass interface, just before removal from carcinogen and at a time when growth was so slow that it seemed the series would be lost. × 200.

was studied in greater detail. The cultures of this strain were removed from the carcinogen at different intervals and were carried along with their respective controls. Strain H was removed at 6 days, strain I at 13, strain J at 32, strain K at 55, and strain L at 111 days after the first addition of carcinogen. With the removal of strain L from carcinogen, strain D experimental cultures were closed out, while the controls of this strain were run under their original label (D) as the control cultures for strain L.

Strain D.—The continuous width curves of this strain are shown in figure 6, section D. To get an uninterrupted record, these curves have also been included in the early part of the curves of strains H, I, J, K, and L.

The continuous width curve of strain D was depressed sharply in the second generation at 19 days after addition of the carcinogen. It is most likely that this depression could have been detected even earlier if the time of adding the carcinogen to the cultures had been different in relation to their last planting. In the four subsequent generations the strain was carried, the depression did not increase but growth was held to a level uniformly much lower than that of the control cultures. The average level of the curve for the second through the sixth generation was 6.3 mm.

In a group of four photographs each of controls and treated cells at 25 days after the first addition of carcinogen, one treated culture showed noticeable cell cohesion. There were very few loose cells. The cells were more sheetlike, with shorter and more amoeboid appearing terminal processes than the controls. In instances the cells adhered terminally to form strands and short ribbons of relatively uniform diameter. These formed loops and twisted into arches, occasionally joining other loops laterally. At 47 days, five of nine cultures photographed showed this cohesion. The cells at this time showed definite lateral amoeboid outlines of their terminal processes. The cohesion progressed at what appeared to be a rate approximately comparable with that in series 208, until the strain was closed at 194 days.

Strain H.—Strain H was removed from strain D after 6 days in the carcinogen, and its control was removed from the control of strain D at the same

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time. When examined at this time and shortly thereafter, the carcinogen-treated cells looked entirely normal. There was certainly no sign of change of culture architecture, cell shape, or any increase in cell cohesion. In spite of this, the continuous width curves (fig. 6, sect. H) showed a sharp drop in the generation directly following the removal of the cells from the carcinogen. In the next generation, however, growth was practically equal to that of the controls; and in the generation following, the average width and continuous width curves were slightly higher than those of the controls. During the last part of the second generation after removal from the carcinogen, that is, 64 days after removal, only one culture of eight examined showed recognizable cohesion of the cells.

In the fourth generation following removal from the carcinogen, the continuous width curve of this strain showed a sharp drop, and at this same time nearly all the cultures of the strain showed definite cohesion of the cells. This general cohesion was at 131 days after the carcinogen was originally added and 125 days after it was discontinued. The edges of the cultures were abrupt, and there were few loose cells. The cells were spindle-shaped, shorter, more sheetlike than normal cells, and had short amoeboid-appearing terminal processes. Short cell strands arborescing and re-fusing were observed. There was an increased amoeboid appearance of the lateral edges of the cell, particularly along the terminal edges of the cell processes.

In the interval from the drop of the continuous width curve in the fourth generation to October 1, 1942, the series continued to show a consistent but limited cohesion of the cells with consequent alteration of the architecture of the clump. This cohesion never reached as severe a degree as that shown in either strain N or strain O.

While the continuous width curve of this strain has fluctuated probably more than any other experimental curve, subsequent to the depression in the fourth generation at the time of cellular cohesion and through May 1943, it showed no definite, recognizable trend of change. The average level of the curve after the removal of the cells from carcinogen and through May 1943 was 7.6 mm.

Strain I.—Strain I was removed from strain D after 13 days in the carcinogen, at which time a control was started from the control of strain D. As may be seen from the continuous width curve (fig. 6, sect. I), in the first generation after the removal from the carcinogen there was no depression noted relative to the controls. Since, however, this determination was made on only three flasks for the control and two for the carcinogen-treated cultures, it cannot be relied upon. Only in the third generation were there enough cultures to give a reliable average. At that time the continuous width curve was somewhat lower than that of its controls and continued so until the series was closed out July 28, 1942, 235 days after the original addition of carcinogen. The average level of the curve from the second generation on was 7.3 mm.

The typical cohesion noted for the other series appeared in strain I sometime during the second generation after removal from the carcinogen. An exact record of its first recognized occurrence was not obtained. The degree of cell cohesion seen in strain I was in general comparable with that observed in strain H and was less than that observed in strain J. A detailed description, however, was not recorded.

Strain J.—Strain J was removed from strain D after 32 days in the carcinogen. The controls of this series were removed from the strain D controls at the same time. Even before these cultures were removed from strain D, the continuous width curve of the latter had shown a noticeable depression relative to its controls (fig. 6, sect. J). This depression continued uninterruptedly and at relatively constant level for 12 transfer generations following removal from the carcinogen. The average level during this time was 5.3 mm. Beginning about February 1943, however, the level of the curve became more erratic. The average level during this last period, through May 1943, was 7.4 mm.

The first record of a recognizable alteration in the cells of this strain was at 45 days after the first addition of carcinogen. At this time, of 10 cultures examined 4 showed definite cell cohesion; shortly thereafter all showed cohesion. Cohesion appeared earlier and was more severe than in strains H or I but was less severe than that in strains N or O, and continued to increase for possibly 60 days after removal of the cells from carcinogen, after which further change was less noticeable.

Strain K.—Strain K was removed from strain D after 55 days in carcinogen and after strain D had already shown a severe depression of the continuous width curve (fig. 6, sect. K). This depression continued at a relatively constant level as long as the strain was carried, that is, for five generations following removal from carcinogen. The average level of the curve from the second generation on was 6.6 mm. At 64 days after first

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addition of the carcinogen, one flask of eight examined showed the cells of the culture to be typically coherent. This cohesion became increasingly severe, until at 139 days nearly all flasks showed typical cohesion and the formation of short strands, ribbons, and loose, fenestrated sheets. Later changes in the cells were progressive for a short interval after their removal from the carcinogen, but after this they showed no further recognizable progressive changes, nor did the cells show any recognizable loss of the changes already induced. The strain was closed at 237 days after the first addition of carcinogen. The controls of this strain appeared entirely normal as long as they were carried.

Strain L.—Strain L was removed from strain D, together with its controls, 111 days after the first addition of carcinogen. At this time its continuous width curve was showing the usual depression (fig.6, sect. L). The depression was much more severe than that for either strain H or strain I and was comparable with that shown by strains J and N. The curve continued at this low level through the ninth generation after removal from the carcinogen, the average level being 6.2 mm. During the next five generations (through May 1943) the curve showed erratic fluctuations, while the average level during this period was 9.5 mm.

The progression of cell changes observed in the cultures was similar to that described for the cells of strain N at comparable ages, up to the time of removal from the carcinogen, while after that time the cell changes were similar to but less severe than those of strain N.

Control Cultures

During the early part of the experiment, that is, until May 1942, the control cultures had been watched very closely and had appeared entirely normal. During the interval from May to July they were examined regularly in determining the width curves, but the cellular structure was watched somewhat less closely, more attention being given to increasing the number of cultures in the experimental series in order to study the behavior of the cells on reinjection into mice.

The first evidence of any abnormality in the morphology of the control cultures dated from about August 5, 1942, at which time three cultures in the strain H controls had definitely coherent architecture, typical of the cultures treated with methylcholanthrene. These three cultures were discarded. The other controls were normal at this time. On June 18 a series of normal cultures was reinjected into C3H mice, and some of them showed tumors after a latent period of about 60 days, that is, about August 18. It was only when the tumors appeared that a re-examination revealed a general slight cohesive alteration in the architecture of the control cultures. These alterations were of the same nature as those observed earlier in methylcholanthrene-treated cultures.

From August 18 to September 9, 1942, the normal continuous width curve for the control cultures remained at a relatively high level. About October 9, 1942, however, it showed a sharp depression which became increasingly severe until the middle of December and reached a level as low as that of strain H carcinogen-treated cells. The carcinogen-treated strains may also have shown some depression, but this was not conclusive. A critical visual examination of all control cultures was made in the latter part of August 1942. The findings may be summarized as follows:

(1) Of the various strains of control cultures, not one showed the normal loose structure that the controls had shown up to May 1942. In each strain a large fraction of the cultures showed recognizable but slight cell cohesion of the type which had been considered characteristic of the action of methylcholanthrene on these cells.

(2) The degree of alteration in some cultures was so slight that it could be considered only as probable rather than as clearly recognizable. In other instances there was no question but that the cells were cohering in the usual carcinogen-type architecture. In no instance was this

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cohesion nearly so severe as that seen in the carcinogen-treated flasks of the H strain, which was the least altered of any of the experimental strains.

(3) No unaltered strain or line of cultures could be segregated from the rest of the controls.

(4) Not all strains were equally altered. The controls on strain H were probably the least altered of any, and they were so slightly altered that many of them seemed practically normal. In numerous instances, cultures which had arisen from the same explant at the last transfer showed distinctly different degrees of alteration.

Cultures From September 15, 1942, to May 1, 1943

A repeated detailed examination was made of all living cultures in late September and early October 1942, the cultures being studied at 2, 4, 11, and 16 days after planting. The conditions prevailing in cultures of the different strains are summarized herein, whereas the series of photographs are from a similar study made about December 17, 1942.

The descriptions of these various strains may be considered as characteristic. In all strains there was some overlap of the different cultures of different strains. For instance, in strain D an occasional culture was seen which was changed to a degree characteristic of strain H; strain H showed some cultures typical of those of strain D and some typical of strain J; similarly strain J had some characteristics of strain H and of strain L. Probably the closest overlap was with strains J and L, but even in these the average change for cultures of strain L was somewhat greater than that for strain J.

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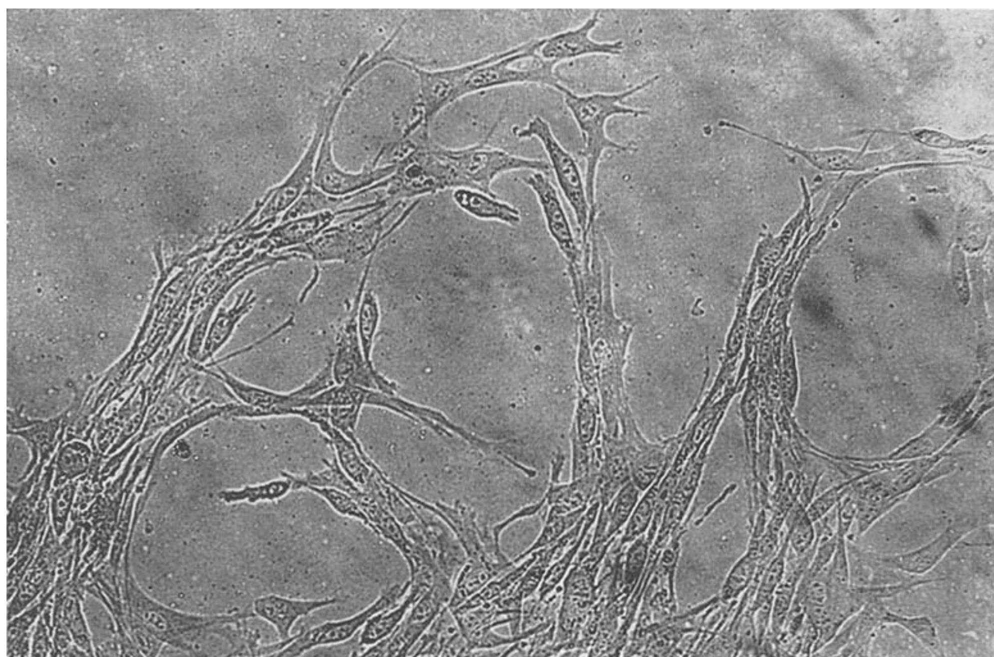

Figure 15.—Culture from strain D, photographed December 17, 1942, 9 days after planting. Note slight shortening of the cell processes and slight increase in lateral cohesion, with loss of the loose reticular structure of the normal fibroblast cultures and with the formation of loose cell strands. Compare with figures 9, *B*, and 11, *B*. × 200.

Strain D controls.—The architecture of the strain D controls was loose, although more coherent than that of the normal cultures at the beginning of this series of experiments in 1941. There were relatively few loose cells at the edges of the cultures, and there was a luxuriant growth at the glass interface. Growth at the fluid interface was irregular but in some instances was even greater than that at the glass interface. The characteristic growth at the latter is shown in figure 15. The cells were loosely coherent laterally and formed no definite ribbons but gave an architecture that lacked the characteristic reticular structure of the normal fibroblast cultures. The terminal processes of the cells were very slightly shortened and were amoeboid-appearing. There was usually no appreciable rippling or frothing of the lateral edges of the body of the cell, although the extreme tips and lateral edges of the terminal processes sometimes showed a slightly amoeboid pattern. The long axis of the cell was well-defined; only occasionally was a cell seen which departed from a more or less definite spindle or related shape; cytoplasmic granulation was not recognizably increased; nuclei were grossly normal; many mitoses were seen. Cell size seemed grossly normal although this estimate was extremely rough.

Strain H.—The growth of the cultures was luxuriant, practically indistinguishable from that of strain D controls; the structure was somewhat less loose (fig. 16), and the cells had a greater tendency to adhere laterally, forming more of a sheetlike structure in the culture. There were no loose cells at the edge of the culture, and the edge was sharp. The cells in general showed a spindle shape, although their terminal processes were shorter than those in strain D. The lateral edges of the terminal processes were often amoeboid along their whole length, down to where they joined with the body of the cell. The cytoplasmic granulation was not recognizably increased. Nuclei appeared grossly normal, many mitoses were seen, and the cell size seemed roughly normal.

Strain J.—There was a noticeable average reduction in the width of the growing zone of the cultures of strain J. The structure of the clump was altered from that of either of the preceding strains; the cell layer at the glass interface was often made up of characteristically slender interconnected loop

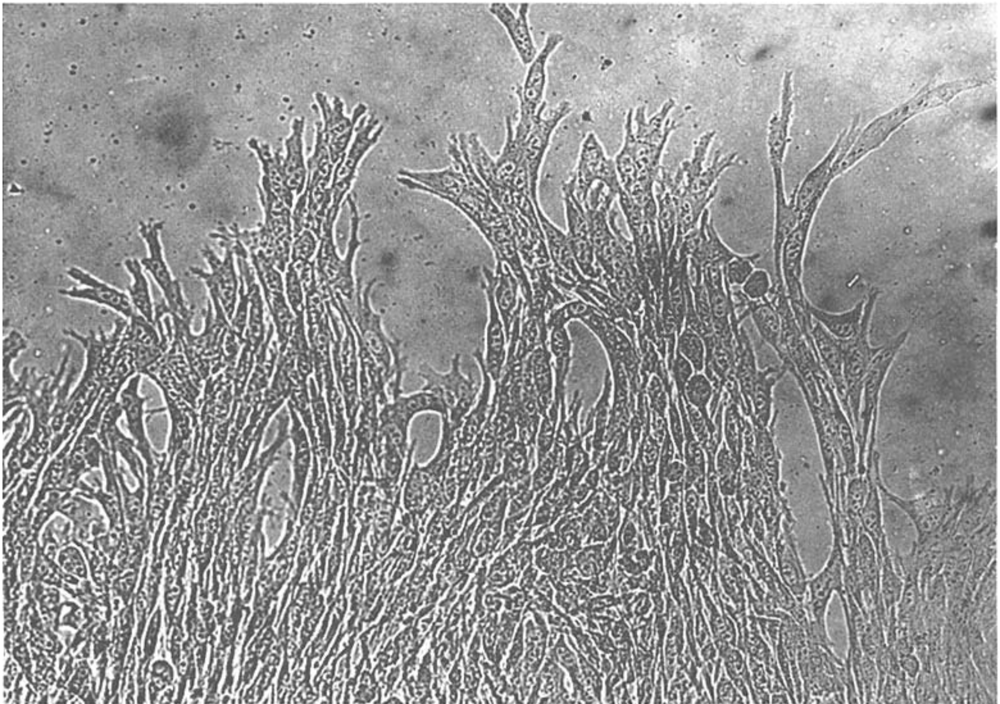

FIGURE 16.—Culture from strain H, 371 days after removal from the carcinogen and 9 days after planting. Note the progressive cohesion, shortening of cell processes, and amoeboid pattern of cell edges as compared with figures 11, *B*, and 15. × 200.

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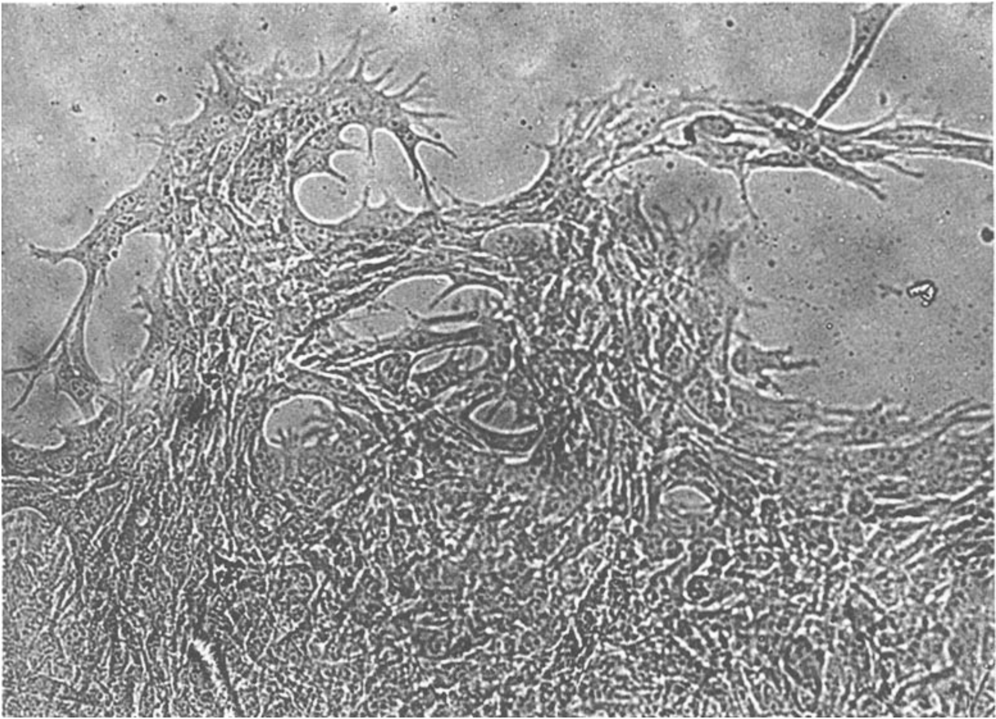

FIGURE 17.—Culture from strain J, 345 days after removal from the carcinogen and 9 days after planting. Note the progressive alteration from strain H (fig. 15). The clump of cells at the upper left illustrates the tendency of the cells at this stage to send off lateral processes, with the result that the cell axis is far less clear. × 200.

of cells. In other areas the cells were even more closely coherent laterally than in D or H. Such cells were no longer spindle-shaped but were more sheetlike, often with a number of lateral processes which gave them the appearance of having a far less definite axis (figs. 17 and 18). Cell processes were shorter, and their lateral and distal margins were frothy or irregular. They presented a pattern suggestive of amoeboid activity. The free edges of the body of the cell were also rippled or amoeboid in appearance. The growth at the edge of the culture was very much less clearly radial, the radial structure being distorted by the irregular loops and strands or ribbons of cells, which often were arched, interlaced, and fused, enclosing islands of clot. The granulation of the cells was only slightly greater than normal. Nuclei looked normal, and a few mitoses were observed, possibly fewer than in strains D and H.

Strain L.—In strain L the cells were even more sheetlike and more closely laterally coherent, tending to form sheeted ribbons and solid sheets, with fewer fenestrations and irregularities (fig. 19). At this stage they often resembled epithelial cells. The cell processes were even shorter than in the preceding stage, and the design of all free edges of the cell was frequently or usually suggestive of amoeboid rippling. Cell size, granulation, and nuclei did not seem greatly altered.

Strain N.—This strain was progressively altered from the preceding strains, and there was an even closer cohesion of the cells. Many cultures showed ribboning or combination of ribboning and sheet formation as the chief form of growth. There was a much greater tendency to sheet formation, and the sheets appeared thicker and denser (fig. 20). The edge was more regular than in strains J and L, and there was a tendency to form epitheliallike projecting lobes along the edges of the cultures. Cell processes were even shorter than in the preceding strain, but the amoeboid or frothy structure of the whole free edge of the cell was more accentuated, often so much so that the exact edge of the cell could not be clearly defined. There was a recognizable increase in the cytoplasmic granulation. Mitoses were fewer in number,

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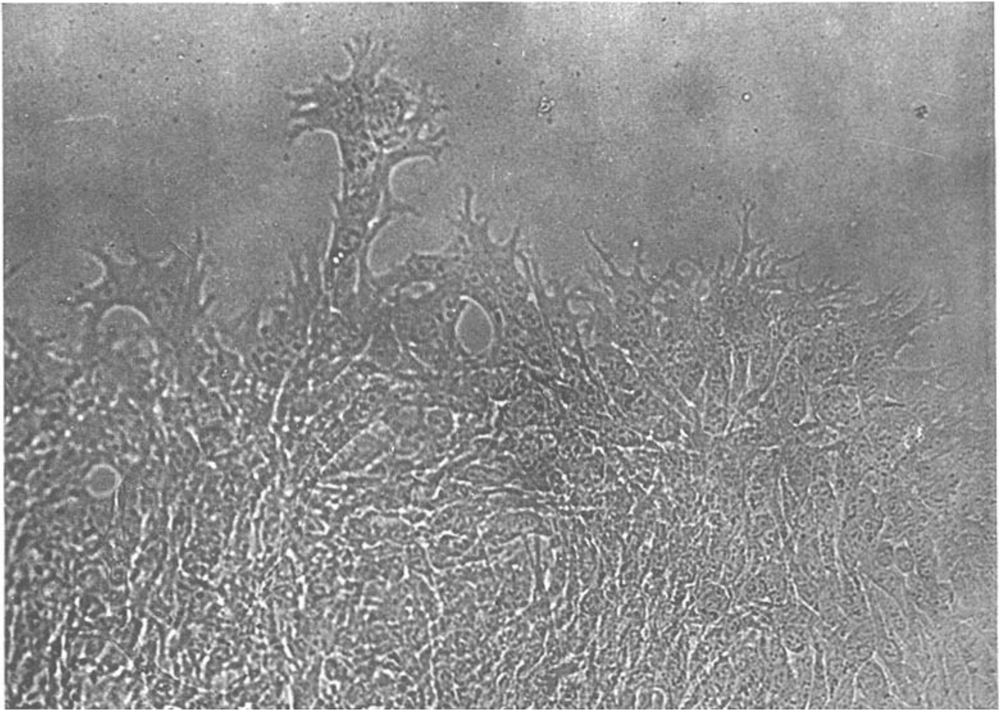

FIGURE 18.—Another culture from strain J, 345 days after removal from the carcinogen and 13 days after planting. This culture shows the tendency of the cells at this stage to send off lateral processes, with resultant obscuring of the cell axis. The photographic contrast is less as this was taken on a softer film. Approximately × 206.

and cells in mitosis were less rounded up and less separated from those around them.

Strain O.—The growing fringe of the culture in strain O was even narrower than in strain N, and the cells were more altered. Individual ribbons of cells or ribbons closely united into sheetlike structures were seen. The culture was often a mass of cells so closely united that it appeared almost as a syncytium (fig. 21), which terminated in an amoeboid-appearing edge which in some instances was lobose and epitheliallike, and in others broken only by very short amoeboid-appearing processes. (In stained preparations there was no evidence of a true syncytium.) Mitoses were often most difficult to recognize, as the cells showed little or no rounding up or separation from the surrounding cells of the sheet. Only in areas of less cell density could they be recognized. A careful examination of such areas revealed almost no cells in mitosis. In areas where the individual cells could be observed, nuclei were grossly normal. One, two, and three nucleoli were visible in each nucleus, and they appeared grossly normal. Nuclei were otherwise free from gross granulation. The cytoplasm was extremely granular, the granules being irregular in shape and size and easily visible with a 16-mm. lens. They often gave the cytoplasm a peculiarly moth-eaten and characteristic appearance. These granules were not to be confused with the occasional, small, highly refractile oil droplets seen in the cells, or with the slightly increased granularity seen in the early stages.

Examination of the cultures in December showed no recognizable progression of the later stages (L, N, O), but at that time there was thought to be possibly a slight progressive alteration of cultures in the earlier stages. For instance, numerous strain D cultures showed structure typical of strain H and in one instance of strain J, while strain H culture often showed structure typical of strain J.

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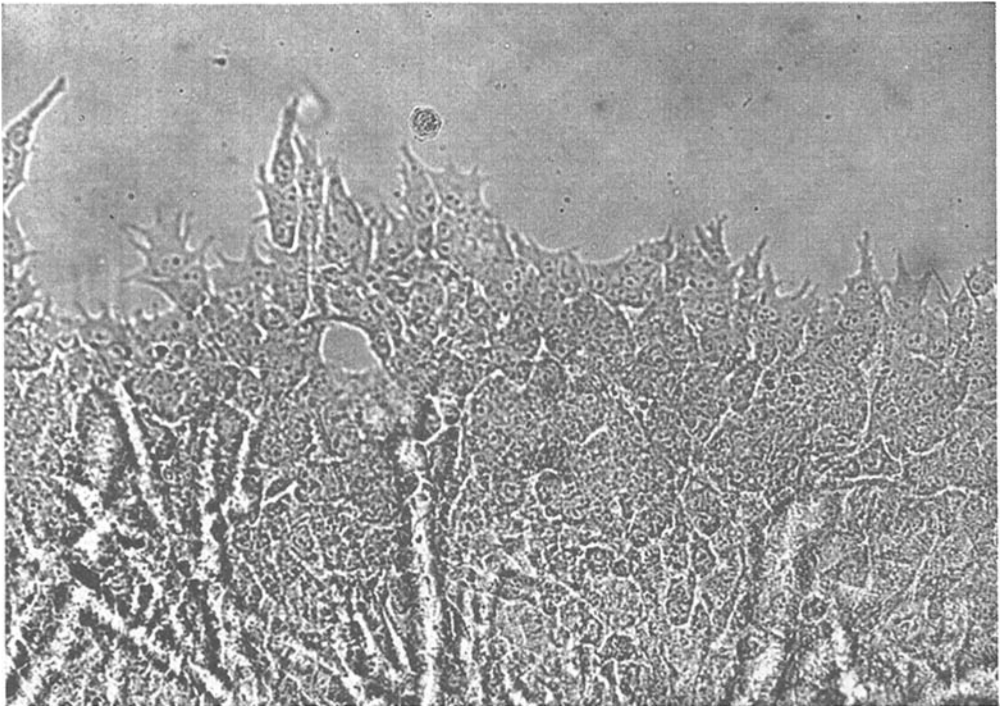

FIGURE 19.—Edge of culture from strain L, 266 days after removal from the carcinogen and 9 days after planting. Note progressive alteration from strain J, with formation of sheets and further shortening of cell processes. × 200.

Examination of the series May 15, 1943, showed the following results for the glass interface layers:

Strain D cultures were characterized by figure 15, a photograph of cells of strain D taken December 17, 1942. A few cultures were characterized by figure 16 (strain H).

Strain H cultures were characterized by figure 16, and a few by figure 15 (strain D control). None were observed showing the structure of figure 17 (strain J).

Strain J cultures showed a cell structure like those in figures 17 and 18 (strain J), and a few cultures were like figure 16 (strain H), while some showed coherent sheets of cells although the cell sheets were not so closely coherent as in figure 19 (strain L).

Most cultures of strain L presented a structure like that in figure 19 (strain L), and some like those in figures 17 and 18 (strain J). No cultures observed resembled figure 18 (strain N).

The degree of cohesion in strain N was definitely greater than in strain L. The cells were coherent, as in figure 20 (strain N), and often showed the same type of terminal edge. Cells were definitely more granular than the cells of strain L.

Strain O cultures were plainly more altered on an average than those of strain N. Culture edges similar to those in figures 20 and 21 were observed; the cells showed greater granulation than in strain N.

In all these cultures the fluid interface cells were looser and showed less coherence than did those at the glass interface. In strain N, two cultures were seen in which the retraction of the terminal and lateral cell processes had gone so far that the fluid layer cells had often lost all connection with each other and were separated and rounded up.

DISCUSSION

The cultures described by Earle and Voegtlin (7) were transferred at various

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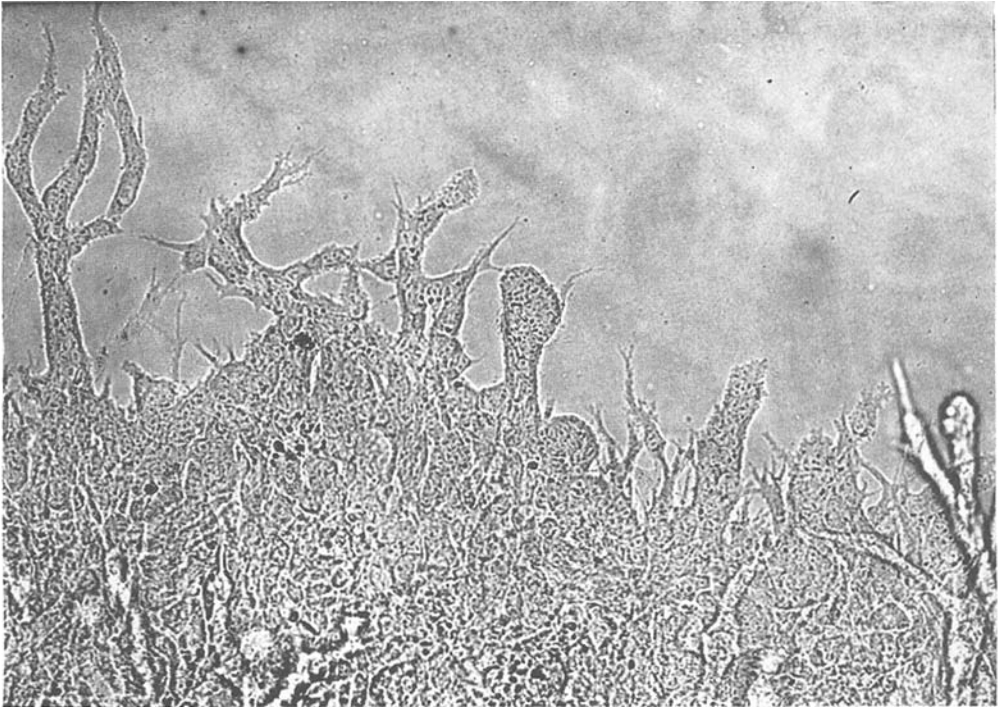

FIGURE 20.—Culture from strain N, 314 days after removal from the carcinogen and 9 days after planting. Note the extreme lateral frothing and rippling of the cell edges and the close cohesion of the cells into a sheet with definitely lobar edges. × 200.

intervals up to about 18 days. It was later felt that the frequency of transfer had interfered with the life of the cultures, and in the later series (*2*) length of the transfer interval was increased to as much as 112 days in some instances. This increase allowed the cultures to survive; but later work with these series and with other tumor strains indicated that the interval had been extended too long for the best life of the carcinogen-treated cells, while detail of the culture was obscured by the clouding of the fibrin clot. Accordingly, in the present studies the interval between transfers was reduced to 28 to 36 days. This period gave much better results than did either of the other two. About 30 days is probably the optimal common period for the strains of cells studied. Under the conditions used, even this period must be considered a compromise, since the normal fibroblast culture would probably give better results with transfer intervals of 36 days; while, because of central necrosis, carcinogen-treated cultures of very coherent architecture probably give better results with somewhat more frequent transfers.

In considering the results, the most outstanding question is that concerned with the behavior of the control cultures. In the earlier work (*1*, *2*), great care was taken in the handling of the dibenzanthracene and methylcholanthrene used. In spite of the precautions taken, just at the last of the experiments the control cultures showed a slight, though unquestionable, alteration in architecture. This alteration was similar to that observed in cultures

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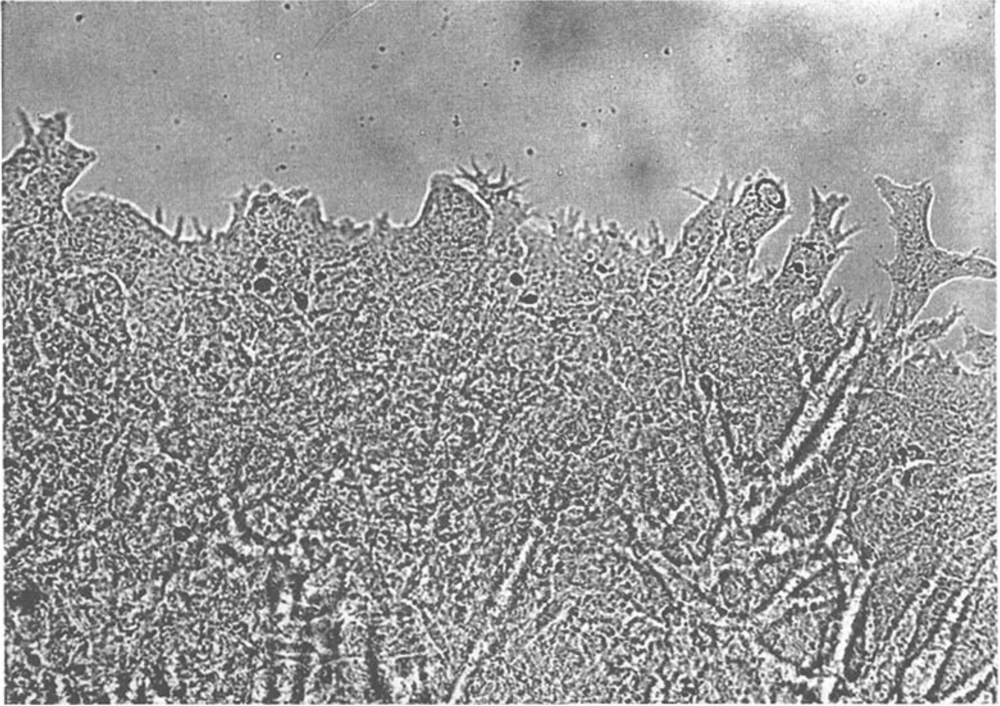

FIGURE 21.—Edge of culture from strain O, 92 days after removal from the carcinogen and 9 days after planting. Note the formation of a dense sheetlike edge often entirely devoid of cell processes for extensive lengths. × 200.

deliberately treated with methylcholanthrene, and the conclusion was reached that in spite of the care taken some trace of contamination of the controls with methylcholanthrene had occurred. In planning the present experiments an attempt was made to eliminate any possibility of contamination of the cultures with the carcinogen. The alteration of the control cultures must, therefore, be interpreted in the light of the care taken to avoid such contamination.

The possibility that the cells of the original parent strain used in this work were not normal cells of the fibroblast type but were malignant cells is most improbable because of the extreme rarity of subcutaneous tumors in this strain of mouse at the age at which it was used (100 days). It also seems improbable, since the appearance of the control cultures, certainly up to May 1942 (559 days after original planting), was entirely normal for fibroblasts from the subcutaneous tissue of mice and rats; and, when development of tumors in mice injected with control cultures led to careful examination of the cultures in August, there was no difficulty in recognizing that many of these control cultures had undergone a definite though slight change in morphology since their last detailed examination. The remainder of the control cultures showed suggestive but less conclusive morphologic changes, which in all instances became conclusive in those cultures that were carried on.

This alteration of the cells was of much less extent but was of the same morphologic type as that induced earlier in the strains of cultures treated with methylcholanthrene

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and of the same type as the alterations we had formerly observed in tissues cultures of tumors arising in rats from the injection of methylcholanthrene.[2] It was also the same as that which Jacoby (9) had described for cells of tumors induced in vivo by the action of 1, 2, 5, 6-dibenzanthracene.

From these data it appears probable that the assumption of malignant properties by the control cells was closely associated with the alteration in the morphology of the cells which occurred about June 1942.

In accepting these original parent strain cultures as normal fibroblasts which underwent a morphologic and probably a malignant transformation about June 1942, several possibilities must be considered as to what induced this change. The possibility that the control cultures were contaminated with altered fibroblasts from the experimental cultures is eliminated by the unvarying order in which the cultures were handled, and by the fact that when the alteration in the controls was first observed the distribution of the altered cultures through the series was such that it could have been produced only by general contamination of all sets of the controls. This hazard seems entirely eliminated in view of the technical care employed. When the change in the controls was first observed, there was no sign of foci of greatly altered cells, distributed in relatively unaltered cultures. The appearance was rather that of a very slight, relatively uniform alteration of the cells, most easily recognizable at the glass interface of the culture. The possibility of contamination from any tumor cultures may similarly be dismissed since during the whole course of this experiment up to October 1942 no tumor cultures were being carried.

The possibility must be considered whether apparently normal fibroblasts of this strain of mice, when grown in a culture medium of chicken plasma, horse serum, and chick-embryo extract, can spontaneously go over into the altered form finally seen in the control cultures and give rise to sarcomas on injection.[3]

[2] *Unpublished work.*

[3] *Personal communication from Dr. G. O. Gey, outlining some results previously presented but never published.*

Gey, using a culture technique and culture medium of which the details are not at present available, describes alterations in several strains of cells, all of which arose originally from mesenchyme, of subcutaneous areolar tissue origin, from a young adult rat of pure strain Philadelphia albino stock. This stock of rat, according to Gey, rarely develops spontaneous tumors.

Three cell strains described showed spontaneous cell alterations; and when cultures of these altered cells were injected, they gave rise to tumors. In the first instance the alteration in the cells was first detected 4 months after primary explantation and "consisted of a change to cells varying greatly in size and with a great number of cells showing a typical and unequal mitosis. Inoculation of these greatly altered cells produced a variety of tumors different in cytological and cultural characteristics from their cells of origin and from each other. Transplantation from tissue culture to host and back again to tissue culture showed that each of the transplantable tumors was of a fixed type. Prolonged cultivation for long periods and further animal transfer revealed that they stayed fixed in type."

The third conversion occurred in a strain of cells related to the other two in that it came from the same small pieces of normal tissue. This strain had been under cultivation for several years without showing any changes of fixed type. When the change occurred, it showed up as microscopic areas of transformed cells within the thinly spread out periphery of only a few of the cultures. The transformation then spread through all the cells of the cultures during a period of several transfers and subcultures. There was thus produced a strain of malignant cells which on inoculation produced tumors of similar cell type. Other normal-appearing cultures of the same strain did not show any changes nor did they produce tumors on inoculation.

Gey also records that other normal cell strains of rat mesenchyme derived from another rat of the same stock were maintained for over 4 years without showing any change in type, and that to date he has not been able to reproduce these cell conversions by any deliberate experimental alteration of the cell culture environment. He states:

"The period of cultivation, whether months or years, is apparently unimportant. We have so far been unable to isolate any virus from these new malignant cell strains, Cell-free filtrates do not produce tumors on inoculations nor does the filtrate alter normal strains maintained in continuous culture. We were at one time inclined to believe that stray gamma radiation had some effect.

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We know that the conditions in the tissue cultures used were different from those in vivo. The culture media and other culture conditions were such that the fibroblast continued to proliferate at a rate far greater than that in the adult animal. That this alone induced such a change in the cells seems unlikely in view of the extended work of Carrel and his associates (*10*, *11*, *7*), who carried fibroblasts from the embryo chick heart in cultures, in which they showed a rapid rate of growth for more than 25 years, apparently without any change in morphology. If these cells had undergone any such drastic morphologic changes as that seen in strains J, L, N, or O, they could hardly have been overlooked. It is emphasized, however, that whereas the Carrel strain of fibroblasts was from the heart of an embryo chick and was carried in solutions obtained from chicken blood and chicken eggs, in the present series of studies the cells were from subcutaneous tissue of a mouse and were carried during their whole period of culture in an entirely heterologous culture fluid. Obviously, in a number of respects the two sets of culture conditions were dissimilar, and the behavior of cells under the one might not hold for the other.

With the limited data available, no conclusion can be reached whether or not normal mouse subcutaneous fibroblasts by simple culturing in the heterologous horse serum-chick embryo extract can be induced to change over into sarcoma cells. This question is fundamental, and in answering it much important information concerning the mechanism of carcinogenesis may be obtained. Obviously, experiments designed to settle the issue should be carried out as soon as practicable.

The second possibility is that in spite of the precautions taken, some active amount of methylcholanthrene got into the control cultures and effected the transformation. The change noted in the control cells was more limited in degree but was of the same type as that induced through the action of methylcholanthrene. This fact, however, cannot be considered as evidence that the tumors arose from the action of methylcholanthrene, since Jacoby (*9*) noted a similar coherent architecture in cultures of malignant cells from a tumor induced in vivo by means of an entirely different carcinogen, 1, 2, 5, 6-dibenzanthracene, and the presumption is that a similar change could be effected with other carcinogens.

The evaluation of the possibility that the controls were contaminated is complicated by the fact that as yet no data are available on how little methylcholanthrene is necessary to change a strain of normal fibroblasts in tissue culture into one with such altered morphology and physiology. Bryan and Shimkin (*12*) have estimated that injection in vivo of about 2.4γ of methylcholanthrene is necessary to produce a sarcoma in strain C3H mice, in only about 1 percent of the injections, whereas 4.5γ produces tumors in about 5 percent of the injections. But conditions in vivo are different from those in vitro. It seems likely that a clump of fibroblasts in vitro is far more reactive or is under conditions far more apt to cause reaction with the carcinogen than are fibroblasts within the mouse. Hollaender, Cole, and Brackett (*13*) showed a definite photosensitizing ac-

Our observations to date on continuous weak irradiation do not confirm such a conclusion. Other factors are being investigated which may have some bearing on these unusual normal to tumor cell conversions which occurred in our cultures."

While Gey described a malignant alteration in several strains of mesenchymal cells, all from one rat, no conclusions are presented as to what induced such changes, nor do the data available indicate whether these transformations could have arisen from some trace contamination of the cultures with a recognized carcinogen. Attention is called to this work; but until more data are available, no attempt will be made to correlate it in detail with the results reported in the present paper.

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tion of methylcholanthrene on yeast in the concentration of 10^{-9}. This reaction occurred within a few hours after addition of the carcinogen to the cells. In the absence of any data on the amount of methylcholanthrene necessary to alter a clump of mouse fibroblasts under the culture conditions in the present experiments, the possibility exists that when allowed to act on the cells for such an extended interval as a year or more, a concentration comparable with or even lower than that which these authors found active for yeast may be adequate. It is obvious that if such minute traces are active, the problem of treating strains of cells with the carcinogen for long intervals and of keeping other control strains entirely normal becomes most difficult.

While careful reconsideration of the technique used has given no definite reason to suspect any step in the handling of the cultures, the solutions, or the soiled glassware, less positive assurance is felt with reference to the re-use of rubber stoppers which were possibly contaminated with traces of methylcholanthrene. Although stoppers from carcinogen-treated cultures were never used on control cultures, the recleaning, sorting, wrapping, sterilizing, and other handling of these rubber stoppers may have introduced a trace-contamination hazard.

The laboratories in which this work was carried out are in the same building with other laboratories and animal rooms where large amounts of methylcholanthrene and similar carcinogens were handled. The design of the air conditioning and heating system of the building is such that there has been some trace recirculation of used air from many rooms, in some of which the carcinogen was certainly handled. Because of insufficient knowledge of the activity of low concentrations of methylcholanthrene and other carcinogens in tissue culture, the hazard of such trace contamination from this source also cannot be evaluated at present, although it is felt that its possible significance must not be overlooked.

The evaluation of another possibility, that the changes were induced by some unrecognized agent of unknown source, must await further work.

As the most probable explanation of the alterations in the control cultures and as a working basis for further study, the hypothesis is advanced that a trace contamination of methylcholanthrene occurred in spite of all precautions taken. If this is the correct explanation, the amount of carcinogen needed to effect such a change in the cultures is probably extremely small. It is suggested that, until more data are available, the most scrupulous care should be taken in all laboratories doing work with long-term tissue cultures so that such trace contamination of the cultures with methylcholanthrene or similar carcinogens does not lead to complications difficult to eliminate, and to erroneous conclusions.

With reference to the other observations reported in this paper, it seems clear that the changes in the control cultures arose at a substantially later date than the changes shown in cultures of even strains H and I, and more than 8 months after recognizable morphologic changes were observed in strains N and O. Furthermore, while of the same type, the changes in the controls have at all times been less accentuated than those seen even in strain H cultures. Regardless of whether the changes finally noted in the controls were spontaneous or arose from some contamination of the controls by the carcinogen, there seems no doubt that the cell changes observed in the experimental cultures are directly correlated with the action of the carcinogen that was delib-

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erately added. This correlation is also shown by the orderly nature of the changes reported for different intentional exposures of the cultures to the carcinogen.

The retardation of growth and the degeneration of rat and mouse fibroblasts under the action of methylcholanthrene in various concentrations from (roughly) 0.2γ to 100γ have already been described by Earle and Voegtlin (*1*, *2*). These papers should be consulted for earlier references to the literature. Lebenson and Magat (*14*) carried out a further extensive series of studies on this point. They cultivated the skin of mouse embryos in chicken plasma and mouse and chick-embryo extract, in hanging-drop cultures, later shifting the skin to Carrel flasks. To these cultures was added 1, 2, 5, 6-dibenzanthracene, which was dissolved by letting the fluid plasma stand 2 days, with occasional shaking, in contact with an excess of the carcinogen. The cultures were left in contact with the carcinogen for intervals of 3 days and were then retransferred to normal medium and carried on. Some of the cultures were passed through dibenzanthracene once (from the fourth to the sixth day), whereas some received an additional exposure from the eighteenth to the twenty-first day. Cultures were apparently carried as long as 54 days.

During the first 3 weeks of growth no manifestation of the action of the carcinogen was noted. Most of the cells of the cultures were fibroblasts with a little epithelium. About the fourth week, culture growth improved considerably, and there was an intense liquefaction of the plasma in most of the cultures. Explantation of fragments of muscle onto the cultures gave rapid invasion of the muscle by the culture cells, destruction of the muscle, and a great acceleration of culture growth, the growth zone reaching 20 to 40 mm.^{2} in 3 days. There was also noted a greatly increased ability of very small clumps of cells to live and to grow rapidly.

Cultures were inoculated into mice from the twenty-second or the thirty-eighth to the fiftieth day after addition of the carcinogen. No mice showed tumors.

Lebenson and Magat concluded that while there was no clear demonstration of production of malignancy in their experiments, it was evident that the treated cells had acquired certain new properties under the influence of the carcinogen, and that the properties had appeared after a considerable latent period following treatment with the carcinogen. Furthermore, they considered that the induced changes were in a direction corresponding to an approach to the blastoma cell.

Larinov, Chertkova, and Samokhvalova (*15*), in an extensive series of studies, grew fibroblasts from the skeletal (femoral) muscles of new-born mice, in Carrel flasks, in a medium of chicken and rabbit plasma and dilute chick-embryo extract. To this medium benzpyrene or dibenzanthracene was added in the form of a fine suspension. Benzpyrene in a concentration of 10γ to 50γ per cubic centimeter gave a considerable inhibition of the growth of the fibroblasts, while dibenzanthracene displayed no such toxic action. For longer term tissue cultures, this carcinogen was used in a concentration of several gamma per cubic centimeter of culture fluid.

Cultures were carried up to 6½ months. There was nothing particularly distinctive in those exposed to the dibenzanthracene 17 days. When they were carried on without further addition of carcinogen, some of them began to grow far more rapidly at 40 days. Benzpyrene had no toxic action on them. In the following 3½ months the rapid growth continued, and

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during this time many cultures were implanted in young mice. Of 55 mice inoculated, none developed a tumor.

In these treated cultures, frequently at the periphery of the culture zone, a culture would arise from a very small, isolated, clump of daughter cells. These daughter clumps grew very rapidly, and their cells showed a greater mobility, sometimes a larger size, and more disordered cell arrangement. In all cases the cells of these daughter cultures showed much more fat than did the parent cultures.

From our own experience with small cell clumps in culture, it is difficult to evaluate the significance of some of these findings of Larinov, Chertkova, and Samokhvalova. The increased storage of fat droplets within the cells is often seen in small clumps of normal fibroblasts (*16*), and in the present author's experience the cells of these clumps are often markedly less orderly in arrangement than are those in large clumps. The acceleration of growth of small clumps of cells treated with carcinogen agrees with the observations of Lebenson and Magat; and in the series of studies reported herein, the same phenomenon was frequently observed, at a stage of carcinogen treatment after definite cohesion of the cells had appeared. For instance, the cell clump shown in figure 12 continued to proliferate rapidly, while substantially larger clumps of control cells died.

The observations of Lebenson and Magat (*14*) and of Larinov, Chertkova, and Samokhvalova (*15*) and the observations reported in the present paper are in entire agreement that the cell transformations appeared after a definite latent interval of several weeks following initial exposure to the carcinogen.

Benevolenskaya (*17*) used cultures of embryonic chick heart and mesenchyme grown in hanging-drop cultures and in Carrel D3.5 flasks in a mixture of chicken plasma and chick-embryo extract, to which methylcholanthrene or 3, 4-benzpyrene was added, usually as a suspension. Concentrations of carcinogen ranged from 50γ to 1γ per cubic centimeter of culture medium, and cultures were carried in the carcinogen to 88 days. Frequently the cultures were transferred to normal culture media and carried for a time after this exposure to carcinogen.

Both carcinogens were toxic and induced stoppage of growth after 3 to 7 days in a concentration of 50γ per cubic centimeter. When treated cells were transplanted into a normal medium after such treatment, degeneration and death still ensued. As lower concentrations were tried the toxic action was less marked, until at 2γ per cubic centimeter the cultures were kept living in methylcholanthrene for 85 days, after which they were carried in normal media 65 days. Such concentrations proved slowly toxic. No changes in the cells were observed to suggest that they had undergone a malignant alteration; the changes were those of toxicity and degeneration.

Eight fowls were each injected with from 6 to 12 cultures that had been carried in a concentration of 2γ of methylcholanthrene per cubic centimeter from 37 to 62 days, and then carried without carcinogen 11 to 42 days, while 6 were each injected with 8 to 9 cultures that had been subjected to a concentration of 1γ of methylcholanthrene per cubic centimeter from 22 to 30 days and then carried 17 to 18 days in normal media. Nine chickens were injected with cultures that had been subjected to 2γ or 3γ of benzpyrene per cubic centimeter from 12 to 19 days, then carried from 6 to 37 days in normal culture medium, and injected. All injections were uniformly negative.

Cooper and Reller (*18*) treated the ears of mice twice weekly with a 0.6-percent

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solution of methylcholanthrene in benzene. They found an increase in the frequency of mitosis of the epithelial cells from a normal average of 0.11 percent to 1.0 percent at 23 to 37 days after first treatment. This count subsided to 0.49 at 65 days and rose to 1.5 percent at 93 days, at which time the experiment was terminated. From 16 days on the ears became definitely hyperplastic and showed a fairly uniform thickening of the entire epidermis, and from the sixty-fifth day the ears of some of the mice began to show "precancerous hyperplasia." No definite carcinomas were observed within the duration of the experiment.

Creech (*19*), working with fibroblasts from connective tissue surrounding the rib of embryonic mice of pure strain, grew the cells for short periods as cover-glass preparations in a medium of fowl plasma and chick-embryo extract containing 20-methylcholanthrene-choleic acid or acenaphthene-choleic acid in concentrations of 100γ, 10γ, and 1γ per cubic centimeter. 1,2,5,6-Dibenzanthracene-choleic acid and phenanthrene-choleic acid were used in concentrations of 100γ and 10γ per cubic centimeter. The outgrowth of the cultures was measured at 45 and 70 hours, and relative areas of outgrowth were determined by camera lucida drawings and planimetric measurements. The cultures were finally fixed and stained and the cells studied. Nearly 1,700 cultures were studied. Methylcholanthrene-choleic acid in a concentration of 1γ per cubic centimeter (equivalent to 0.15γ of methylcholanthrene per cubic centimeter) and dibenzanthracene-choleic acid in a concentration of 10γ per cubic centimeter (equivalent to 1.5γ of dibenzanthracene per cubic centimeter) caused a significant increase in cell proliferation as indicated by measurements of outgrowth and by counts of mitoses, while the same carcinogens in a tenfold or greater concentration caused a retardation. Desoxycholic acid (10γ per cubic centimeter), phenanthrene-choleic acid (10γ and 100γ per cubic centimeter), and acenaphthene-choleic acid (1γ, 10γ, and 100γ per cubic centimeter) all caused a decrease in cell proliferation. With the methylcholanthrene and dibenzanthracene, a premature separation of the chromosomes in the prophase and metaphase was observed. This did not occur with the other substances tried.

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The observation of Creech that methylcholanthrene-choleic acid retarded cell growth in a concentration equivalent to 1.5γ of the carcinogen per cubic centimeter accords closely with the data of Earle and Voegtlin for methylcholanthrene. The observation that a concentration equal to 0.15γ causes a significant increase in the rate of cell cleavage emphasizes further the necessity of investigating, over longer intervals, lower concentrations of the carcinogen than any yet used.

In the present work a concentration of 1γ of methylcholanthrene per cubic centimeter of fluid culture medium was used. With this concentration, after the first addition of the carcinogen to the cultures there was a sharp depression of the rate of increase in width of the cultures in every strain examined. The continuous width curves of the different strains showed that a great part of the depression of the curve was manifest by the end of the first 20 to 35 days after addition of the carcinogen. In strain N, which was continued in the carcinogen for as long as 184 days, the further depression of the continuous width curve was slight, while even in strain O there was no further depression recognizable until after 350 days in carcinogen, at which time there was a further sharp depression of the curve. This brought the rate of increase of width of the cul-

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tures to such low levels that further maintenance of the strain was most difficult. The strain would almost certainly have been lost during the next several generations if it had not been removed from the carcinogen.

Examination of the cells just after the onset of the initial sharp depression following the first addition of carcinogen showed no evidence of an increase in cohesiveness, nor was there any increase in cell density of the cultures. If there was any change in density, the cultures seemed somewhat less densely cellular than normally. Morphologic evidence of injury to the cells of young cultures, if it existed at all, was extremely slight. Certainly, there was no sign of widespread degeneration or disintegration of the cells. The depression of the curves, then, was not caused by any of these factors. While no determination has as yet been made of the actual rate of mitosis in the cultures, the data indicate a definite retardation in the frequency of mitosis in the cells. This would agree with Creech's data (*19*). Whether this retardation continued after cohesion of the cells set in, and up to 325 days after the first addition of carcinogen, was not determined and cannot be inferred from the data to date since the retarded rate of increase of the diameter of the culture was at least partially offset by an increase in cell density.

The data suggest, however, that such a retardation of mitosis did occur in cultures subjected to the carcinogen about 400 days (strain O) since the rate of increase of width of these cultures was greatly retarded, and few cells were observed in mitosis in the living cultures. The study of the action of the carcinogen on the rate of mitosis of the fibroblast for various intervals in long-term cultures under the conditions used must be left for future work. The conclusion is reached that, under the conditions of culture and of concentration of carcinogen used, the changes observed in the cells in the early stages (D, H, J, L) of action of the carcinogen are not recog-

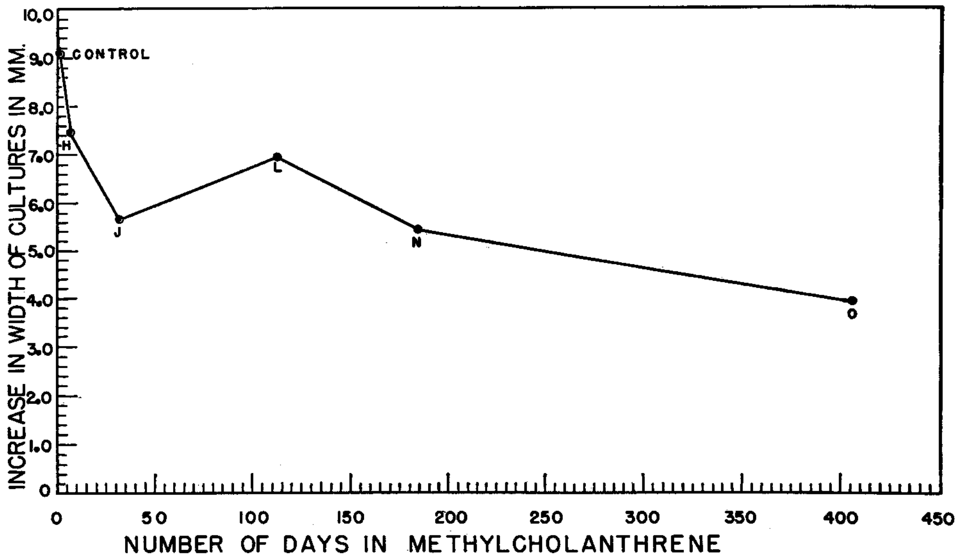

FIGURE 22.—Continued average level of the continuous width curves after removal of culture from carcinogen and through May 1943. The number of days the cultures were in the carcinogen is shown along the abscissa, the average culture width along the ordinate.

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nizably such as to lead to the death of the cell. However, the cumulative, progressive action of the carcinogen for longer intervals and in the concentration used is not only clearly injurious but is almost certainly lethal.

In order to demonstrate more clearly the continued depression of the continuous width curves after removal of the different strains from carcinogen, the average level of the respective curves after removal of the strains from methylcholanthrene and through May 1943 is plotted against the interval of time each strain was subjected to the carcinogen (fig. 22). As a normal control point on these curves, the average of points on the continuous width curves, of the control cultures of strains D, H, I, J, K, L, N, and O through September 11, 1942, have been used. Values later than that have been discarded because of the depression in the curve after that date and the possibility that this depression resulted from trace contamination with the carcinogen.

The general trend of the curve is regular with the exceptions of point J (which is low) and point L (which is high). The longer the cells were left in the carcinogen, the less was their continued rate of increase of culture width after removal therefrom. The general morphologic appearance of the cells of strain J shows that they were more altered than those of strain H, but less altered than those of strain L, while the cells of strain L were less altered than those of strain N. This intermediate morphology confirms the correctness of the lateral position of points J and L along the curve. Other data (*3*) also indicate that this dip in the curve at point J and the rise at point L are real, although no explanation can be offered for this irregularity at the present time.

Leaving out of consideration the depression at point J and the rise at point L, the conclusion is obvious that, as far as is indicated by the continuous width curves, the degree of change induced in the cells with methylcholanthrene under the experimental conditions was a direct function of the duration of exposure (dose) to the carcinogen, and that even after the cell was removed therefrom, the induced changes persisted. In the instance of the cells of strains H, J, L, and N, this alteration has now persisted more than 1 year since their removal from carcinogen.

The question comes up whether or not there has been any rise at all in the continuous width curves since the cells were removed from carcinogen. The curve for strain H fluctuated over a wide range and showed no clearly defined rise. The curves of all the other strains maintained their low level for some time after removal from the carcinogen. This time included, for instance, 3 generations in strain O and 12 generations in strain J. From these times on, the curves lost their level, which had as a rule characterized them during the interval of carcinogen treatment and directly thereafter, and began to show erratic variations, and in some strains of cells, a rise. The significance of these variations is not yet clear, and the interpretation will be left to a later date.

From the various data presented, it seems evident that the cell changes previously described by Earle and Voegtlin have been confirmed, while the accumulation of additional data makes possible a clearer description of the sequence of the cell changes and of the later stages of the cells. As shown herein, in those strains left in the carcinogen for an extended period, at about 25 to 50 days after first addition of the carcinogen, there was a progressive shortening of the slender terminal processes of the cell, and an increased amoeboid appearance of the

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lateral edges of these processes, an increase that extended farther and farther along the surface of the cell toward a zone midway of its length. Meanwhile the cells became more coherent, particularly laterally, and tended to form loose sheets and ribbons, then sheets, and finally massive, thick sheets, particularly prominent at the glass interface of the culture. The individual cells showed a progressively less clearly defined major axis, and there was a definite tendency (at about stage J, as shown in figures 17 and 18 (to send off amoeboid-appearing lateral processes. In later stages the cells were so closely coherent that the extremely short, amoeboid-appearing processes at the edge of the massive cell sheet were the only ones observed. In one or two instances in strain O, it seemed that the process of cell rounding had gone so far that the massive cell sheet or the heavy ribbons of cells had apparently disintegrated into living, isolated, rounded cells. Unfortunately, no photographs were made of this phenomenon. It is interesting to speculate whether such rounding and cell separation might increase the tendency of a tumor to metastasize in vivo.

This progression of changes, as described previously (*2*), was gradual and appeared to affect the whole culture rather than an isolated cell or a small clump of cells. There was no suggestion whatsoever of a sudden drastic change. The gradual change may be seen from a comparison of figures 15 to 21, inclusive. Furthermore, the changes, particularly those of cell cohesiveness and rippling of the surface, and changes in cell processes, seemed to be such as would arise from changes in the cell cytoplasm and more particularly in the cell surface.

The cells of strains H, J, L, and N, over 1 year after their removal from the 1γ concentration of methylcholanthrene, seem to have lost none of the typical cohesion and other morphologic changes induced with the carcinogen. While strain O has been carried for a shorter time since removal from the carcinogen, it, too, has preserved its structure. At eight generations after the removal from carcinogen, the cells of strain O were more altered than those of strain N, but the architecture of the cultures appeared possibly a trifle looser than it had directly after removal. This change was so slight, however, that at present it must be considered uncertain.

The conclusion is reached that over the extended period these cultures have been studied, once these morphologic changes have been induced in the cells by the action of the carcinogen, the cells to a very great degree seem to stabilize at that level or degree of alteration induced in their morphology.

While occasional instances of very large nuclei, or of very large cells, or of cells containing as many as eight very small nuclei were seen, usually these aberrations occurred in cultures more than 10 days old and were far more prevalent in those at about 25 to 30 days after planting. The impression was obtained from the living cultures that in the greater number of instances such aberrations occurred under culture conditions of cell overcrowding and degeneration. A more detailed study of nuclear changes will be made when the stained slides are studied.

Some carcinogen certainly persisted in the cultures for some time following their removal from it. However, in the more than 1 year that strains H, J, L, and N have grown since they were removed from methylcholanthrene at the concentration of 1.0γ per cubic centimeter, each has undergone more than 160 washings, more than 160 additions of fresh fluid, and 14 or more consecutive replantings. In each of the replantings it may be conservatively estimated that not in excess of one-fifth

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of the original plasma (and cell) mass was transferred to the new culture. If any of the original carcinogen used on the culture still remains, it must be an extremely slight trace.

From the changes undergone by the control cultures, the presence of carcinogen in active though trace concentrations must be assumed as probable in the experimental cultures as well as the controls sometime in June 1942.

While a trace concentration of carcinogen might conceivably have had an effect on a normal cell, or on one which had had only a very low concentration of carcinogen for an extremely short interval, it appears most unlikely that such a trace contamination, if it occurred at all, has substantially altered the course of experimental cultures of strains H, J, L, N, and O. In spite of this, these strains and the altered controls have to a most remarkable degree stabilized at levels of alteration of the cells which are proportional to the time of their exposure to carcinogen. Minor changes which have occurred in the last few months or which may occur in the future find their most probable explanation in cell selection and in the overgrowth of more rapidly growing cell types within the cultures.

Bryan and Shimkin (*12*) injected methylcholanthrene in tricaprylin into the axillae of male mice of strain C3H and determined the final tumor incidence, as well as the time-frequency relationship, for various doses of the hydrocarbon. Table 1 is derived from the equations to their estimated relationships. The recognition of a tumor was determined when the cell mass reached the size of a palpable nodule which continued to increase in size.

Obviously these latent periods must each be considered as consisting of an initial transformative latent period during which the cells were changed from normal to malignant, and of a latent period of

TABLE 1.—*Estimated dose-response relationships for 20-methylcholanthrene*

Mice with tumors (percent)	Estimated dose	Estimated latent periods		
		Extreme lower limit [1]	Extreme upper limit [2]	Average
	Gamma	*Days*	*Days*	*Days*
99	182	39	163	91
95	96	40	205	110
50	21	43	306	154
5	4.5	46	407	197
1	2.4	48	449	217

Derived from the data of Bryan and Shimkin (*12*).

[1] Time at which not more than 1 percent of the mice in the tumor population would be expected to have developed tumors.

[2] Time at which 99 percent of the mice in the tumor population would be expected to have developed tumors.

growth, an interval necessary for the cell mass to attain a palpable size. The data in another paper (*3*) show that the time required for subinoculated tumors, which were originally induced by the inoculation of cells treated with methylcholanthrene in vitro, to reach a palpable size is roughly 7 days. If this interval is accepted as being a rough index of the latent period of growth of such tumors and is subtracted from the latent periods given by Bryan and Shimkin, the time remaining may be considered a rough guide to the duration of the latent period of transformation.

The comparison of these results with those obtained from the treatment of cells in vitro with methylcholanthrene is obviously complicated by differences in concentration of the carcinogen and by the solvent used. In the results of Bryan and Shimkin (*12*), the minimal latent period for all doses of carcinogen ranges from 39 to 48 days, which would indicate a minimal transformative latent period of from 32 to 41 days. This period coincides with the period necessary to effect general characteristic morphologic alterations of the cells treated in vitro. The correlation, however, is incomplete since with Bryan and Shimkin's data the period from 32 to 41 days was the minimal, from

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156 to 442 days the maximal, and from 84 to 210 days the average transformative latent period. The closer correlation of these results must await further work.

Grady and Stewart (*20*), working with pulmonary tumors induced with methylcholanthrene and dibenzanthracene in strain A mice, showed by section of the lungs that definite tumors appear about 5 weeks after injection. This latent interval for production of malignant tumors is in close correlation with the general period required to induce cell cohesion through the action of methylcholanthrene in vitro under the conditions of the present experiment.

Furthermore, confirmation that cell cohesiveness is intimately linked with the assumption of malignancy by the fibroblast is shown by some unpublished studies made on tumors that arose in vivo through injection of methylcholanthrene into rats. Several of these tumors were cultured for short intervals and showed definite cell cohesion. In a recent short study of a strain of tumor cells that originated from a subcutaneous injection of methylcholanthrene into a C3H mouse, cultures of the thirty-third passage of the tumors in mice showed a degree of cohesion roughly comparable with or slightly greater than that shown in strain D (control) cells (fig. 15).

Jacoby (*9*), using a pure strain of fibroblastic sarcoma cells derived from a spindle-cell sarcoma originally induced in mice with dibenzanthracene, described a similar cohesion of the cells. Jacoby's description, a part of which is quoted(*9, p. 301*), agrees with description of cell cohesion by Earle and Voegtlin and the changes in vitro reported herein.

When grown in Carrel flasks in a hen plasma coagulum and fed with either Heparin hen plasma or a hen serum-chick embryo juice-Tyrode mixture, in which the embryo juice concentration is kept low, the cells regularly show a tendency to grow out in close association with one another, and to form ribbon-like strands or even broad sheets which resemble very much the epithelial type of growth *in vitro*. These ribbons frequently arborize and their branches often join up with one another, forming loops and bridges enclosing the coagulum, as shown in the accompanying illustration. The sheet-like growth is found especially at the interface coagulum-glass, whereas the cell ribbons occur also within the clot. This architecture seems to be very characteristic, and has been maintained through frequent passages. It permits the diagnosis of such a sarcomatous colony with the naked eye, being vastly different from the architecture of normal fibroblast colonies; aided by the more highly refractive cytoplasm of the tumour cells it brings the whole colony much more into relief against the background of the coagulum so that, even for the naked eye or under low power, the entire edge of the colony is sharply outlined and clearly defined.

The culture architecture shown in Jacoby's illustration is typically similar to some shown by Earle and Voegtlin (*2*) and comparable with a type of ribbon growth seen at about stage J or L of the present paper. It should be noted that at the time Jacoby's article was written, he had not seen the second one (*2*) by Earle and Voegtlin in which this cell cohesion was described.

These data all confirm the concept that the change in culture architecture is closely associated with the assumption of malignancy by the cells. This concept is further strengthened by the demonstration (*3*) that fibroblasts that have undergone a scarcely recognizable cohesion are capable of inducing typical sarcomas in a small percentage of injections into mice. The point cannot yet be considered as conclusively demonstrated, however, because, owing to the transformation of the controls, no study has as yet been possible to determine whether or not fibroblasts possessing the normal loose radial growth pattern can be made to produce sarcomas on injection.

Earle and Voegtlin (*2*) stated that the cohesiveness of cells under the action of

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methylcholanthrene regularly occurred at both glass and fluid interfaces of the culture. Later data from all carcinogen-treated strains necessitated modification of this statement. Jacoby's recognition (*9*) that the cohesion was especially prominent on the glass interface of the culture is entirely in accord with later observations.

No definite relationship has been worked out for the relative growth of the cells at the glass and fluid interfaces, although the impression has been that in the carcinogen-treated culture up to about 12 days of age, the cells were particularly prominent at the glass interface, and often there was no fluid interface cell layer. The cells of the glass interface showed conclusively the increase in the cohesiveness of the cells from the action of the carcinogen. In some cultures, about 10 days old, the prominent layer of cells was at the fluid interface. When this occurred the cohesion was almost without exception less marked and particularly in cultures subjected to carcinogen for shorter intervals was sometimes unrecognizable. In cultures of an older age group the layer of cells at the glass interface of carcinogen-treated cultures was often overgrown or had become entirely necrotic; and in these cultures, too, the cells at the fluid interface were often not clearly cohesive, nor did they show so plainly such extreme shortening of the terminal cell processes in the early stages of carcinogenic alteration. They did show the granulation observed in the later stages. Because of these differences in the cells at the two interfaces, care must be taken to determine correctly the positions of the cells within the depth of the culture before reaching any conclusion as to the degree of carcinogen-induced morphologic alteration they have undergone.

The difference in the structure of glass and fluid layers is shown in figure 13, *A* and *B*, from the glass and fluid interfaces, respectively, of companion cultures of strain O at 403 days after addition of carcinogen and 11 days after planting. While these photomicrographs are entirely typical and illustrate the point, they are unfortunately not nearly so remarkable in contrast with each other as the two layers have often appeared, sometimes in the same culture in directly overlying regions.

The question arises why this increase in cohesiveness of the cells has not been commented on by other workers who have grown in vitro carcinogen-induced sarcomas which presumably arose from fibroblasts. The possibility is that this cohesiveness is prominent or occurs only in tumors that have arisen from certain strains of fibroblasts or from tumors that have arisen from a very limited group of carcinogens, or in a very limited group of culture media. The fact, however, that the cohesiveness is so much more prominent at the glass interface suggests another explanation which seems more likely. In tissue-culture preparations of the type used (in Carrel flasks) the thickness of the fibrin clot is substantially greater than the thickness of the clot in either "roller tube" cultures (*21*) or in many, if not all, hanging- or lying-drop slide preparations. This would give a greater separation of glass and fluid interface cell layers. Since the glass interface layer is the one closer to the microscope objective, in the flask culture it naturally assumes a prominence, definition, and isolation which are probably not so accentuated in other types of cultures. With many slide cultures the extremely short interval during which the cells may be carried undisturbed, usually just a few days, would itself interfere greatly with the appearance of this cohesion architecture as it is most easily recognized at about 8 to 10 days of culture.

A further question is why this change in cell cohesiveness should be more promi-

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nent at the glass than at the fluid interface. While with the data available differences in such factors as oxygen tension or relative nutrition at the two levels in the plasma clot cannot be ruled out, it seems more probable that the difference lies in the reaction of the surface of the cell to interfacial forces, which would be different at the plasma-fluid interface from those at the plasma-glass interface.

Central necrosis in carcinogen-treated cultures appeared regularly at an earlier date than in the controls. It usually appeared first at the glass interface. This central necrosis seemed to result from the extremely compact architecture of the culture with consequent crowding and interference with the metabolism of the deepest and most central cells. Young cultures, where the cells were not overcrowded, appeared to be free of necrosis.

During the whole course of the experiment, there was no detectable increase in the rate of liquefaction of the clot, nor did any cell strain show an increase. A similar absence of unusual liquefaction has also been noted in other malignant cells grown in this chicken plasma-horse serum- chick embryo extract medium.

In considering the nature of the cell changes observed, it is desired to call attention to certain points. These are: (1) The drastic morphologic changes in cells treated with carcinogen resulted in the production of cell types greatly altered from the original cell type used; (2) the morphologic changes were gradual; (3) they could be considered as changes that might arise from alteration in the cell surface; (4) these changes stabilized after the removal of the cells from carcinogen, and different cell strains treated for different periods stabilized at different levels of morphologic alteration; (5) the morphologic changes were correlated with the action of a definite, highly purified, crystalline chemical substance in extremely low concentration; and (6) the changes were induced in cells in an entirely heterologous culture medium quite removed from the systemic influence of the parent host.

With our present lack of knowledge these considerations cannot lead to any definite conclusion. Even with the recognition of this fact, the suggestion is made that the cell changes observed may well be of the same category as those seen in the process of cell differentiation of the organism, and it is emphasized that these changes are considered by some workers to result from definite chemical organizers acting in extremely low concentrations. It is felt that this similarity is too close to be overlooked or to be dismissed without most careful consideration and that the data call for further evidence either to confirm the similarity or to reveal dissimilarities.

SUMMARY

A primary strain of fibroblasts from the subcutaneous and adipose tissue of a 100-day-old male mouse of the C3H strain, Andervont substrain, was started in vitro in horse serum-chick embryo extract-saline solution on October 18, 1940. Cultures were grown in Carrel D3.4 flasks, slightly modified. When the strain reached an age of 291 days in vitro, the first addition of purified 20-methylcholanthrene was made to selected groups of the cultures in a solution of such strength that the final concentration of carcinogen in the fluid culture medium was 1γ per cubic centimeter.

Various selected groups of cultures were carried in the carcinogen for different intervals. Particular attention is directed to cell strains designated H, J, L, N, and O, which were subjected to the carcinogen for 6, 32, 111, 184, and 406 days, respectively, at the end of which time they were carried on without further addition of

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carcinogen. To June 1, 1943, these strains had been carried 542, 542, 542, 663, and 663 days, respectively, since first addition of carcinogen, and 536, 510, 431, 479, and 257 days since their removal from it.

The first noticeable effect of the carcinogen was a definite decline in the rate of increase in width of the cultures. This initial reaction appeared after a very few days in the carcinogen and in its preliminary stages at least seemed to arise from a retardation of cell proliferation. This depression of the rate of increase of size of the cell clump continued even after the cells were removed from methylcholanthrene. It appeared that the depresssion of the rate of cell proliferation was also present at about 400 days in the carcinogen, but whether the depression of the rate of increase in width of the cell clump arose from this factor alone was not determined. The conclusion is that the action of the carcinogen on the cells was progressive and gradual, and in the course of time almost certainly lethal.

The earliest recognizable morphologic change in the cultures appeared about 40 days after first exposure to the carcinogen. In general, the cells showed a gradual diminution in the length of the terminal cell processes. The lateral edges of the processes became increasingly amoeboid in appearance, and this change extended progressively from the tips of the cell processes toward the middle part of the cell as time in the carcinogen went on. The cells gradually tended to send out numerous short lateral processes at their free edges; meanwhile the cells became increasingly coherent, particularly laterally, with the production of cell strands, cell ribbons, and finally of sheets which showed many characteristics of epithelial sheets in tissue culture. As time went on, the sheets became increasingly massive and the cell cytoplasm extremely granular.

The changes induced in different cell strains with different intervals of exposure continued after the carcinogen was discontinued. To date, four cell strains, subjected to the carcinogen in the concentration mentioned for 6, 32, 111, and 184 days, respectively, have been carried for more than a year since the carcinogen was discontinued, without loss of their induced characteristics. One strain has not been carried so long without carcinogen, but it also shows no clearly defined diminution.

Over 8 months after the first recognizable morphologic cell alterations in the earliest group of experimental cultures (strain N) and approximately 2 months after the last experimental strain (H) had shown recognizable cell alterations, the control cultures started to show definite alteration of the same type as but to a lesser degree than the experimental cultures. Of the various control strains examined, strain H control showed least alteration, the change in the architecture of the cultures of this strain being barely discernible morphologically. The possibilities whether this change arose from a spontaneous tendency of the apparently normal fibroblast to undergo a malignant change under cultivation in vitro or whether the change was induced from trace contamination of the control cultures with carcinogen, are discussed. The data available do not permit a definite conclusion, but the working hypothesis is advanced that such trace contamination with carcinogen did occur, although the mechanism by which it occurred is unknown. In view of the precautions taken to guard against contamination, it is emphasized that the contamination, if it occurred, was probably extremely slight. Until further data are available to define more clearly the active concentration of carcinogen necessary to induce changes in cells in long-term tissue cultures, great care should be taken in the

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handling of such cultures with carcinogen to eliminate the possibility of complications that might arise from trace contamination.

After all deliberate treatment of the cells with carcinogen had been discontinued, and after strains H, J, L, and N had been carried for more than a year after discontinuance of the carcinogen whereas the other strains had been carried for shorter periods, the characteristics of the control cultures and cultures subjected to carcinogen for 6, 32, 111, 184, and 406 days were compared. The progression of morphologic changes with longer exposure to the carcinogen was similar to that described previously.

The similarity of the latent period for the earliest in vivo production of tumors with methylcholanthrene and the time necessary for the production of recognizable morphologic alteration of the cells in vitro is pointed out.

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