INDUCED MALIGNANCY IN CELLS FROM RAT MYOCARDIUM SUBJECTED TO INTERMITTENT ANAEROBIOSIS DURING LONG PROPAGATION IN VITRO*

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(Received for publication, September 22, 1952)

The experimental production of malignant tumors in animals has now been accomplished many times as a result of the action of a large number and considerable variety of agents, both biological (1) and chemical (2–4). Carcinogenic chemicals, in pure form, and of known structure, some of them synthetic, are now abundant (2), but the exact nature of the activity which brings about the malignant change is not known. Even the successful transformation of normal tissue, growing *in vitro*, into malignant tissue, by the addition to the tissue culture medium of some of the recognized carcinogens, *e.g.* methylcholanthrene, (4–6), and the observation of the accidental transformation in tissue cultures of normal mesenchymal cells from adult rats (7–9) and mice (4–6) into malignant cells, have not resulted in the discovery of the basic cause of the malignant change.

In 1923, Otto Warburg and his associates (10) began a series of reports on the metabolism of various tissues *in vitro*, which led to the discovery that malignant tissue differs from normal tissue surviving in a medium containing bicarbonate and glucose

* This study, aided by a grant from the National Research Council, was begun by one of the authors, H. Goldblatt, with the technical assistance of Charles O. Seward, in 1930, at the Institute of Pathology, School of Medicine, Western Reserve University, Cleveland, but it had to be abandoned after 7 months. The investigation did not even reach the stage of exposure of the pure cultures of fibroblasts to intermittent anaerobiosis. The grant from the National Research Council is acknowledged with sincere thanks.

The experiments were resumed in 1949, with the technical assistance and collaboration of Miss Gladys Cameron, assisted, from the beginning, by Mrs. Virginia Sheehan, and, more recently, by Miss Anne Whitehouse. Since the resumption of this study, at the Institute for Medical Research of Cedars of Lebanon Hospital in 1949, financial support has come entirely from a yearly grant from the L. G. Beaumont Trust Fund, of Cleveland, supplemented by a grant from the Martha Washington Straus–Harry H. Straus Foundation, Inc., Biltmore, North Carolina. The authors give their grateful thanks to the Trustees of both of these Funds for making the resumption and completion of this study possible.

in its ability to produce much larger quantities of lactic acid under both aerobic and anaerobic conditions. From this they concluded that malignant cells can obtain energy from glycolysis even under aerobic conditions, and that the growth of malignant tumors in the living body may be dependent in great part upon this enzymatic activity. They found that embryonic tissues, and even some highly specialized, adult, normal tissues, such as retina, may use the glycolytic mechanism to almost as great an extent as malignant tissues, under anaerobic, but not under aerobic, conditions. When oxygen is available, normal tissues use it for the metabolism of glucose, while malignant tissues still employ the glycolytic mechanism. In a long series of studies, Warburg and collaborators (10–14) investigated this mechanism and the difference between the metabolism of tumors, both benign and malignant, and adult or embryonic normal tissue. They even studied the effect of temporary anaerobiosis on the metabolism of cultures of normal and malignant tissues surviving, or growing temporarily, *in vitro*. Wind (15) was able to show that a culture of chicken fibroblasts growing for several days in an atmosphere of nitrogen may undergo a change which results in virtual abeyance of the respiratory mechanism while the enzymatic glycolytic mechanism is retained. He stated specifically, however, that a transformation to the type of metabolism of sarcoma, that is to say, to glycolysis, even under aerobic conditions, did not occur in his cultures. He did not study the effect of intermittent anaerobiosis over a long period and did not investigate the effect on the morphology of such cultures of long continued or intermittent exposure to anaerobic conditions.

It seems possible that in all embryonic, and even adult, normal tissues there may be scattered cells, or groups of cells of potentially neoplastic sort, which naturally possess the ability to use the fermentative, glycolytic mechanism, at least under anaerobic conditions, and that repeated, brief exposure of cultures of normal tissue containing such cells to an atmosphere deprived of oxygen, alternating with long periods when adequate oxygen is available, thus permitting recovery, might favor their multiplication and even interfere with the growth of the regional, normal cells. The alternative idea is also worth consideration, namely, that repeated exposure to anaerobic conditions might actually render some cells capable of using the glycolytic mechanism as a source of energy. Perhaps eventually this might result in the development of atypical tissue possessing the property of using glycolysis as a source of energy under both aerobic and anaerobic conditions, and even having the morphologic and growth characteristics of true malignant tissue. These ideas have been put to the test in the work here reported, with resulting actual production of malignant change *in vitro*.

Materials and Methods

For the source of the original cultures, the rat was used because in our colony (the Slonaker-Addis strain of albino rat) spontaneous, malignant tumors have not occurred in many years. Fibroblasts were chosen for the first study, because sarcomas originating from them occur spontaneously in some strains of rat, and because pure cultures of mammalian fibroblasts can be obtained with greater ease and be grown continuously for longer periods than epithelium.

Also, normal fibroblastic tissue has been more carefully studied and more thoroughly described than any other tissue grown *in vitro*.

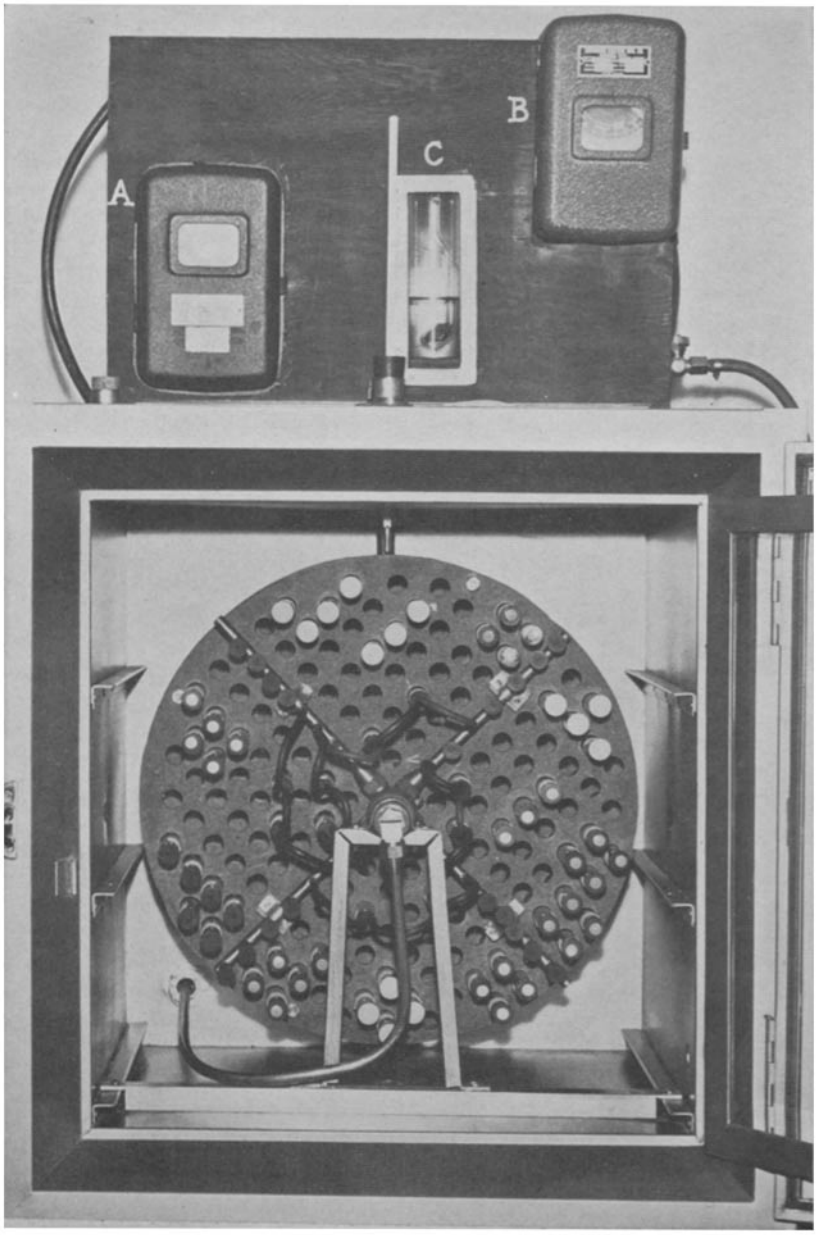
The original cultures were made from the heart of a 5 day old rat. The tissue was first grown under aerobic conditions in roller tubes by the technique of Gey (16, 17), modified to suit the special requirements of this investigation. Strain 15 of normal fibroblasts, from which abnormal strains of fibroblasts 111 and 120 eventually developed, was first subjected to intermittent anaerobiosis early, even before all recognizable cardiac muscle fibers had disappeared from the cultures. These later yielded abnormal strain 111. Other cultures of strain 15 were first subjected to intermittent anaerobiosis after they had consisted of pure fibroblasts for 1 year, during which time they had shown the growth characteristics and appearance of ordinary proliferating connective tissue. For the intermittent anaerobiosis, exposure of the cultures to nitrogen alone was practised, though any other inert gas would have served the same purpose. The length and the frequency of exposure of the different cultures to nitrogen were varied greatly at first, in order to determine the periods that would prove definitely injurious yet fail to kill the cultures and permit recovery after the return to aerobic conditions. It was finally found that circumstances proving injurious in greater or less degree, but from which most of the cultures recovered readily after the return to aerobic conditions were 15 minutes of nitrogen twice, in 24 hours, for 3 successive days, with an interval of 11 ¾ hours between successive exposures. It was found that even after exposure to nitrogen for ½ hour, 3 times in every 24 hours for 7 consecutive days, with an interval of 7 ½ hours between successive exposures, recovery could still occur, although the injury was great; but recovery was slower and less certain after such long periods of anaerobiosis, and some of the cultures did not recover.

During a short period in the early part of the investigation, the change from air to pure nitrogen was effected by hand, but during the greater part of the study the procedure was carried out automatically by means of a device constructed specially for the purpose:—

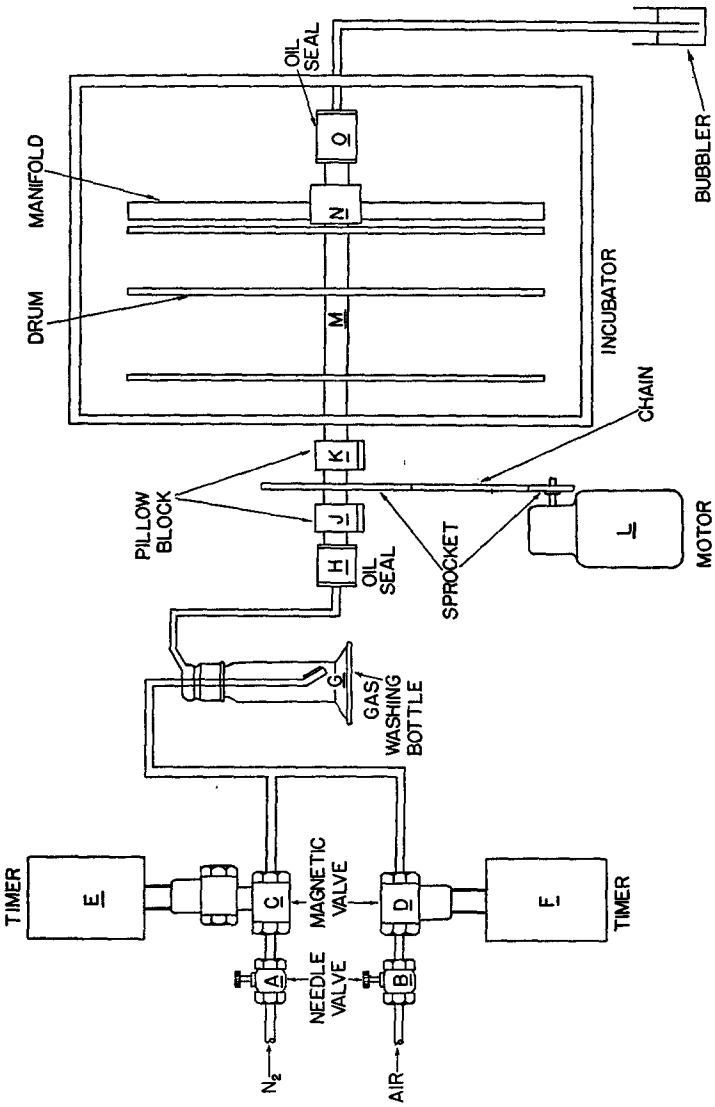
Apparatus for the Automatic Change of the Atmosphere in Culture Tubes Kept in the Rotating Drum of an Incubator.—A photograph of the incubator, with roller tubes in place in the rotating drum, and fitted with inlet and outlet rubber and glass tubes for the flow of the gases through the culture tubes, is given in Text-fig. 1. A schematic drawing of the apparatus by means of which the automatic change of the atmosphere in the culture tubes is effected is given in Text-fig. 2. At a set time, automatically controlled by a clock on top of the incubator, the nitrogen begins to flow in and out of the tightly stoppered culture tubes for a period which can be fixed at will by changing the disc of the clock motor timer. When the nitrogen is automatically shut off air immediately begins to flow in and out of the tubes, and continues for the same length of time as the nitrogen. A complete change of atmosphere occurs in a few minutes. The period for the flow of air is controlled by another clock motor timer. When the flow of air stops, the roller tubes contain air until the nitrogen begins to flow again.

Methods of Tissue Culture.—Test tubes 150 by 15 mm. were first lined with a thin film of non-heparinized chicken blood plasma (2 or 3 drops). 3 or 4 small fragments of tissue, suspended in 2 drops of fluid in a long pipette with bent end, were then introduced into the test tube and deposited on the plasma at intervals in a straight line along the length of the wall of the tube, which was held in a horizontal position. After coagulation of the plasma 0.5 cc. of a supernatant, nutrient medium was added and the tube corked with a hard rubber stopper. Each tube was placed in an incubator, kept at 37°C., in a drum revolving 6 times a minute and tilted at a 5° angle to prevent the fluid medium from touching the stopper (Text-fig. 1).

An investigation was first made of the growth-promoting value of several fluid mixtures containing various balanced solutions (Tyrode's, Hanks's, Gey's, Earle's, and Simms's (18)) and sera from rat, guinea pig, rabbit, horse, and human placental cord blood, with the addi-



TEXT-FIG. 1. Incubator, kept at 37°C., housing a rotating drum with glass culture tubes in place. Some of the culture tubes are attached to the main inlet and outlet for the automatic change of air to nitrogen and back to air. The special inlets and outlets for the gases, which are attached to the individual roller tubes, are made of glass, and the connections are made of rubber. The changes of atmosphere are effected automatically, while the drum is rotating, by means of the attachments situated at the back of this incubator, schematically represented in Text-fig. 2. Timer activator (*A*), attached to the board on top of the incubator, controls the change from air to nitrogen, and timer activator (*B*) controls the change from nitrogen back to air. The gases are washed before they enter the culture tubes by passage through a gas-washing bottle (*C*) with fritted disc, which contains a saturated solution of copper sulfate.



TEXT-FIG. 2. Schematic representation of automatic atmosphere changer. The appropriate gas enters the apparatus through needle valves *A* and *B*. Gas flow through the system is regulated by magnetic valves *C* and *D* which, in turn, are controlled automatically by disc type clock motor timers, *E* and *F*.

After nitrogen flow has proceeded for a predetermined period, the closing of valve *C* actuates the opening of valve *D*, and the operation of timer *F*, and flow of air proceeds for the same length of time as for the preceding flow of nitrogen. Valve *D* then closes, and no flow of air takes place for a chosen interval, during which the culture tubes contain air, after which the cycle is again repeated through timer activation. Thus, a preselected change of atmosphere during the incubation period of the cultures is effected. During flow, the gas is cleansed of foreign material by gas-washing bottle *G*, with fritted disc, containing a saturated solution of copper sulfate, whence it flows through the oil seal *H* and through the shaft *M* into the manifold *N*. The gas passes by way of the oil seal *O*, into the bubbler, containing water, this indicating that gas is actually passing in and out of the culture tubes.

The drum is rotated by means of sprocket and chain drive, impelled by a geared-down fractional horsepower Bodine electric motor, *L*. The shaft is supported by pillow blocks, *J* and *K*, and by a clamp, at oil seal *O*. The oil seal effectively prevents leakage in an apparatus which entails the flow of a gas from a stationary to a rotating system carrying the culture tubes.

tion in every case, of chick embryo extract. The best results were obtained with a mixture of 6 cc. Tyrode's solution, 3 cc. human placental cord blood serum, and 1 cc. chicken embryo extract. To these was added 0.003 per cent phenol red, as pH indicator. The embryo extract was made by adding an equal volume of Tyrode's solution to minced 10 day chick embryos, and the supernatant opalescent fluid was separated from it by centrifugation after thorough mixing, either by pipetting off or by decanting. The fluid mixture just described was adopted as the routine, liquid, nutrient medium for all the cultures. Strangely enough, although the original source of the cultures of fibroblasts used in this study was the rat, the poorest growth occurred when rat's blood serum was used to replace human, placental cord serum in the fluid medium. No antibiotics were added to the cultures at any time. The nutrient fluid was changed twice weekly in all tubes, and the tissues were transferred to fresh tubes, and usually subcultured every 2 or 3 weeks, depending upon the rate of growth and condition of the cells. From March 15, 1950, to September 10, 1952, every continuous culture was transferred, with or without subculture, at least 60 times.

Many cultures were also prepared from which the plasma clot was omitted. In some of these preparations the tissue fragments, placed on the inner wall of the tube, floated off into the supernate and did not grow; in most cases, however, the tissue became attached to the inner surface of the glass tube and grew well, although usually less luxuriantly than those tissue fragments which were fixed in a thin coating of plasma on the inner surface of the tube. As a precautionary measure, and in order to reduce the frequency of transfer, a few cultures were placed at times in a separate rotating drum at reduced room temperature (about 28 or 29°C.). At the reduced temperature, control cultures of normal fibroblasts remained in good condition for about 1 month, with only 2 changes of fluid during that time. Cultures altered by exposure to nitrogen could remain at the lower temperature without obvious detriment for only 7 to 10 days. Although some of the cultures were grown in Carrel flasks, with or without perforated cellophane, or on glass coverslips in Porter flasks, or on coverslips in roller tubes and on perforated cellophane in roller tubes, none of these methods proved as good as the routine roller tube method originally described by Gey (16, 17). In some of the cultures cardiac muscle fibers, still present in the small remnants of myocardium from which the original normal cultures of fibroblasts were obtained, contracted persistently for from 1 to 3 months after explantation. This muscular contraction was temporarily inhibited by exposure of the cultures to nitrogen, but was resumed during a variable period after the exposure to nitrogen was discontinued. Finally, as a result of repeated division and subculture, the obvious myocardial fibers were lost and only what appeared to be pure cultures of fibroblasts remained.

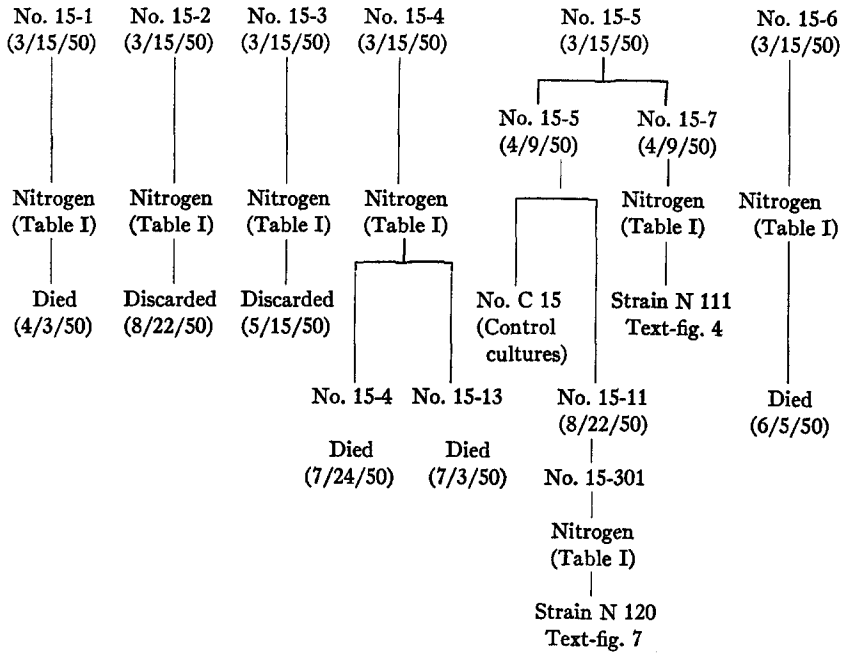
History of the Abnormal Strain of Fibroblasts N 111

On March 15, 1950, the heart of a 5 day old albino rat of the Slonaker-Addis strain was excised aseptically, under ether anesthesia, and cut immediately into fragments about 2 mm. in diameter. Six roller culture tubes, each containing 3 or 4 fragments of myocardium, designated as culture 15, with separate subnumbers from 1 to 6, for the six tubes, were placed in a rotating drum in an incubator kept at 37°C. At various times (Text-fig. 3 and Table I) when the cultures in the six original tubes were growing vigorously and some consisted mainly of fibroblasts, with a few myocardial fibers still remaining in some of the fragments, repeated exposure of the cultures to an atmosphere of nitrogen was begun.

In the case of one of the six tubes just mentioned, tube 15-5, the culture was divided and transferred to three tubes, 15-5, 15-7, and 15-11, before the exposure to nitrogen was begun. The later subcultures of No. 15-5, called No. C 15, eventually developed into a strain of pure fibroblasts which never were

exposed to nitrogen and served as the control cultures for this entire study. Culture 15-7 eventually became abnormal strain N 111, and a subculture of No. 15-11 finally underwent transformation into abnormal strain N 120, in both cases after periods of exposure of the cultures to nitrogen (Text-figs. 3, 4, and 7 and Table I). The cultures in the remaining five original tubes, each containing 3 or 4 myocardial fragments, were all exposed to nitrogen for various lengths of time and for various periods, beginning on different dates (Table I). In these, the tissues either died some time after the last exposure to nitrogen,

TEXT-FIG. 3. Brief history of the six original cultures of myocardium of a 5 day old albino rat of the Slonaker-Addis strain:



or grew so poorly and were subcultured with such difficulty, that they were all finally abandoned (Text-fig. 3 and Table I). This study, therefore, deals entirely with the subsequent experimental history of the tissues in the original culture tube 15-5 (Text-fig. 3).

From April 13 to 16, 1950, inclusive, No. 15-7, a subculture of No. 15-5, (Text-fig. 3) from which abnormal strain of fibroblasts N 111 eventually developed, was first exposed to nitrogen (Text-fig. 4). This was accomplished by hand and the total daily period of effective exposure is uncertain. It is estimated that it was for about 3 hours daily for the 4 days. Growth of the 4 fragments in the tube stopped and, on April 19, 1950, it was recorded that the cultures were "probably dead." Many cells did die and, for 3 days after the return to continuous

aerobic conditions, degenerative changes were evident in the surviving cells, manifested mainly by coarse granularity of the cytoplasm and shrinkage of the nucleus. Then recovery began and, on April 24, 1950, considerable improvement in the appearance of the cells and renewed growth were noted. Mitoses were abundant but of the normal type, and the coarse granularity of the cytoplasm had disappeared. During the next 2 weeks, the cultures con-

TABLE I

Periods and Length of Exposures to Nitrogen of the Six Original Cultures of Myocardium from a 5 Day Old Albino Rat of the Slonaker-Addis Strain

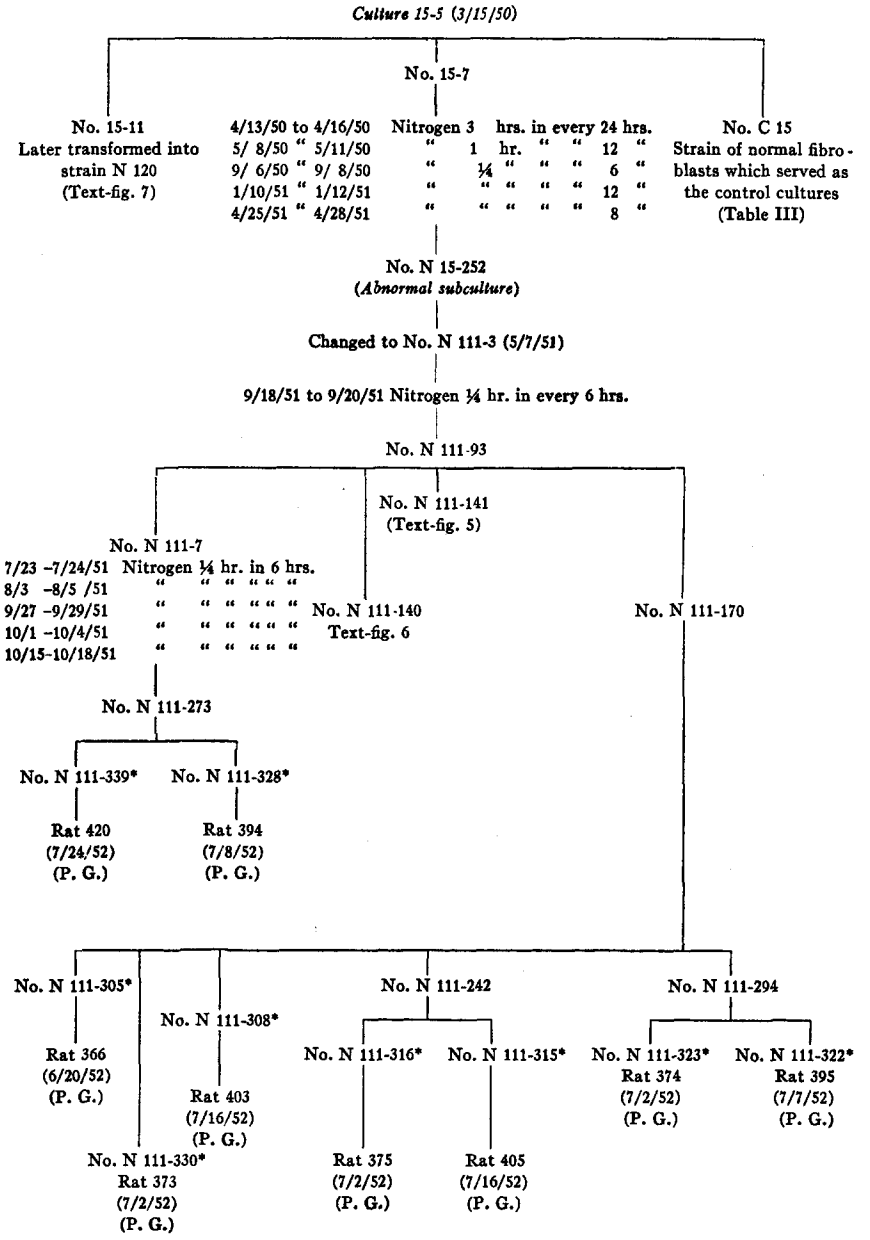
Culture No.	Dates of exposure	Time of exposure	Result
15-1	3/22–3/27/50	3 hrs. in every 24 hrs.	Died 4/3/50
15-2	3/27–4/4/50	1 hr. in every 12 hrs.	Growth poor
	4/6–4/13/50	" " " " " "	Subcultures unsatis-
	4/24–4/25/50	½ " " " " "	factory
	5/15–5/18/50	¼ " " " " "	Discarded (8/22/50)
	5/29–5/31/50	" " " " " "	
15-3	4/14–4/25/50	1 hr. in every 12 hrs.	Poor growth Subcultures unsatis- factory Discarded (5/15/50)
15-4	3/27–4/2/50	1 hr. in every 12 hrs.	Poor growth
	4/17–4/25/50	½ " " " " "	Culture 15-4 died
	5/15–5/21/50	¼ " " " " "	(7/24/50/)
			Subculture 15-13 died
			(7/3/50)
15-5	4/14–4/17/50	3 hrs. in every 24 hrs.	This is the culture
Later became N 111	5/8–5/11/50	1 hr. " " 12 "	from which the con-
(Text-fig. 4)	9/6–9/8/50	¼ " " " 6 "	trol subcultures and
	1/10–1/12/51	" " " " 12 "	the abnormal strains
	4/25–4/28/51	" " " " 8 "	N 111 and N 120
	9/18–9/20/51	" " " " 6 "	were obtained (see
			Text-figs. 4 and 5)
Subculture 15-301	(Text-fig. 7)		
Later became N 120-3			
15-6	3/27–4/4/50	1 hr. in every 12 hrs.	Died 6/5/50
	4/17–4/25/50	" " " " " "	

tinued to improve and, on May 8, 1950, they appeared healthy and normal, except for a small number of cells which were rounder and larger than normal, with cytoplasm somewhat more granular than usual, and with a relatively large nucleus which, in stained sections of some of the subcultures, appeared hyperchromatic.

On May 8, 1950, intermittent, brief exposure of the original culture and subcultures of No. 15-7 to nitrogen, alternating with long periods of exposure to air, was begun again, but now this was accomplished automatically (Text-figs. 1 and 2), and from then on exposure

TEXT-FIG. 4. During the entire study many cultures of normal fibroblasts, subcultures of No. C 15, which originated from the myocardial fragments in tube 15-5, were implanted, with and without the addition of embryonic rat lung, liver, and brain in sites similar to those used for the subcultures of abnormal fibroblasts of No. N 111, but in no case did even slight growth result (Table III).

During the interval between the time (4/28/51) of the fifth exposure to nitrogen, and the time (6/26/52) of the first implantation of abnormal subcultures of strain N 111, which resulted finally in the development of tumors, the subcultures of this strain became more and more abnormal. In this period many of the subcultures not indicated in this text-figure were implanted into the eye, skin, muscle, peritoneum, and brain of many animals (Table III, First and Second periods). Survival and slight, or even moderate, growth did occur in some of these sites, and this occurred more frequently during the later part of the second period; but true progressive growth (P.G.), with the development of fibrosarcoma, did not occur until fragments of embryonic rat lung were implanted with the abnormal cultures (Table III, Third period).



* Plus lung.

to nitrogen and to air was always carried out in that way. From May 8 to May 11 inclusive (Text-fig. 4) exposure to nitrogen was for 1 hour in every 12 hours, with an interval of 11 ¾ hours between the anaerobic periods. From May 11, 1950, to September 6, 1950, no further exposure to nitrogen was carried out on these cultures, for a variety of extraneous reasons; but they remained somewhat altered and, as described above, gradually became more abnormal, so that even at this stage they were already distinguishable from the controls. From September 6 to 8, 1950, inclusive, cultures 15-7, which now appeared to consist only of fibroblasts, were exposed to nitrogen for 15 minutes, 4 times in every 24 hours, with an interval of 5 ¾ hours between successive exposures to nitrogen. The next period of anaerobiosis began on January 10, 1951. During that interval the subcultures still remained sufficiently altered, so that they were easily distinguishable from the controls. The controls were subcultures grown from the original myocardial fragments in tube 15-5, which were never exposed to nitrogen (Text-fig. 3). From January 10 to 12, 1951, inclusive, the anaerobic period of culture 15-7 was for 15 minutes in every 12 hours for the 3 days, with an interval of 11 ¾ hours between exposures. Again the tissues appeared dead, but in about 1 week after their return to a continuous atmosphere of air, recovery with resumption of growth occurred and after that time the number of abnormal cells kept increasing. From April 25 to 28, 1951, inclusive, the abnormally growing tissues were again exposed to nitrogen for 15 minutes in every 8 hours, for the 4 days, with an interval of 7 ¾ hours between exposures. After this most of the cells of No. 15-7 became distinctly abnormal. *Subculture 15-252, which was a direct descendant of No. 15-7 (Text-fig. 4), was now designated as strain N 111.* Subculture N 111-3 (Text-fig. 4) was given only one more period of exposure to nitrogen, from September 18 to 20, 1951, for ¼ hour in every 6 hours for the 3 days with an interval of 5 ¾ hours between exposures. No. N 111-7, a subculture of No. 15-252, was given five more periods of exposure to nitrogen between July 23, 1951, and October 18, 1951 (see Text-fig. 4 for details). In Nos. N 111-3 and N 111-7 and their subcultures, which were never exposed to nitrogen again, the tissues became progressively and almost uniformly altered until all the cells appeared highly abnormal.

After the second period of exposure of No. 15-7 (Text-fig. 4) to nitrogen, the morphologic changes in the tissues consisted in the appearance of abnormal single cells (Figs. 5, 6, and 11) or small clumps of transformed cells (Fig. 12) usually at the periphery of the culture; but the number of abnormal cells gradually increased, and eventually, after the end of the fifth period of anaerobiosis (April 28, 1951) the changes involved the entire culture (Figs. 7 to 9, 13, and 14).

At intervals, the nutrient fluid of the cultures was changed and some of the fragments with their outgrowths were cut out of the plasma clot, transferred, and either subcultured or fixed, sectioned, and stained for microscopic examination (Figs. 11 to 14). Some of the subcultures were transferred to Carrel or Porter flasks, or to coverslips on glass slides, for microscopic examination of the cells in the unstained state while they were still growing (Figs. 9 and 10). Some of the subcultures which were grown for a few days on coverslips were fixed and stained, *in toto*, on the coverslips (Figs. 5 to 8). Mounted on glass slides, they afforded excellent material for microscopic examination under high power.

After September 20, 1951, in the case of culture N 111-3, and after October 17, 1951, in the case of No. N 111-7 (Text-fig. 4), the tissues were not exposed to nitrogen again, but in the abnormal form which they had acquired they and their subcultures continued to grow well, the alterations became progressively

worse, and the subsequent history of both cultures is identical (Text-fig. 4). There was never any sign of a return to the original normal form, despite the growth of the subcultures under continuous aerobic conditions, and, as time went on, their abnormal characteristics became more pronounced. There were some huge cells with one or more relatively large nuclei (Figs. 13 and 14) which, in stained preparations of sections of the cultures, were either hyperchromatic (Fig. 14), or vesicular and reticulated (Fig. 13), or contained abnormal masses of chromatin. Tripolar (Figs. 15 to 17), tetrapolar (Fig. 18), and other abnormal mitoses, or abnormal, sometimes bizarre, nuclear forms of various kinds (Figs. 13 and 14), and multinucleated cells (Fig. 19), with nuclei containing large nucleoli or abnormal masses of chromatin, made their appearance. Anisocytosis (Figs. 13 and 14) and anisonucleosis (Fig. 19) were common characteristics of the abnormal cultures. Even the smaller cells were abnormal and the cellular relationship also became abnormal, in that the loose, reticular structure typical of the cultures of normal fibroblasts (Figs. 1–4 and Fig. 10) was lost, the processes of the cells became shortened and in many cases disappeared, while progressive lateral cohesion of the cells resulted in the transformation of the tissue into sheets having some resemblance to abnormal epithelium growing *in vitro* (Figs. 7 to 9).

In November, 1951, the subcultures of strain N 111-3 (Text-fig. 4) began to liquefy the plasma clot and, even more striking, to produce much earlier and much greater acidity of the fluid medium than did the control cultures, as shown in a change in the indicator (neutral red) and by direct determinations of the pH of the supernatant nutrient fluid. Foci of cytolysis also began to appear in the tissue. These characteristics became more pronounced with time and finally, with the complete morphologic transformation of the cultures to the abnormal type of tissue, the existence of malignant change was considered highly probable. All pathologists who saw stained sections of these cultures, objectively, without knowing the nature and origin of the tissue, invariably made a diagnosis of malignant growth, usually "some form of sarcoma."

During the 2½ years of this study, many of the control subcultures of normal fibroblasts, developed from the same original fragments of myocardium as abnormal cultures N 111 and N 120 (No. 15-5, Text-fig. 3) have continued to grow in normal form and fashion without any signs of significant alterations in the nucleus and cytoplasm of the cells or in the cellular relationships of the tissue (Figs. 1 to 4 and Fig. 10). In these cultures the outgrowth surrounding the fragments of tissue continued to show the normal reticulated structure and was composed of spindle cells with long processes, having nuclei of normal size, shape, and staining properties. These cultures were never exposed to nitrogen, but the culture tubes were kept in the same drum and incubator as those which were exposed to nitrogen. These cultures of fibroblasts have remained practically unaltered. Occasional cells and nuclei slightly larger than the average were

seen in some of the normal cultures throughout this entire period, but this is not unusual, and normal mitoses were usually present in moderate number during the early active growth after transfer or subculture. In September, 1952 (Fig. 4), the control cultures still looked exactly as they had 2 ½ years earlier (Fig. 1).

Results of Implantation into Animals of Cultures of Strain N 111 and of Control Cultures C 15

As soon as enough subcultures of strain N 111 showed the generally altered and highly abnormal state described above, attempts were made to determine the possibly malignant nature of the tissue, by the implantation of some of these cultures into animals. The first attempts at implantation were made into the anterior chamber of one eye of guinea pigs, rabbits, and adult rats of the Slonaker-Addis strain (Table III, First period). Later, subcutaneous, intramuscular, intracerebral, and intraperitoneal implantations were also made in rats (Table III, Second and Third periods).

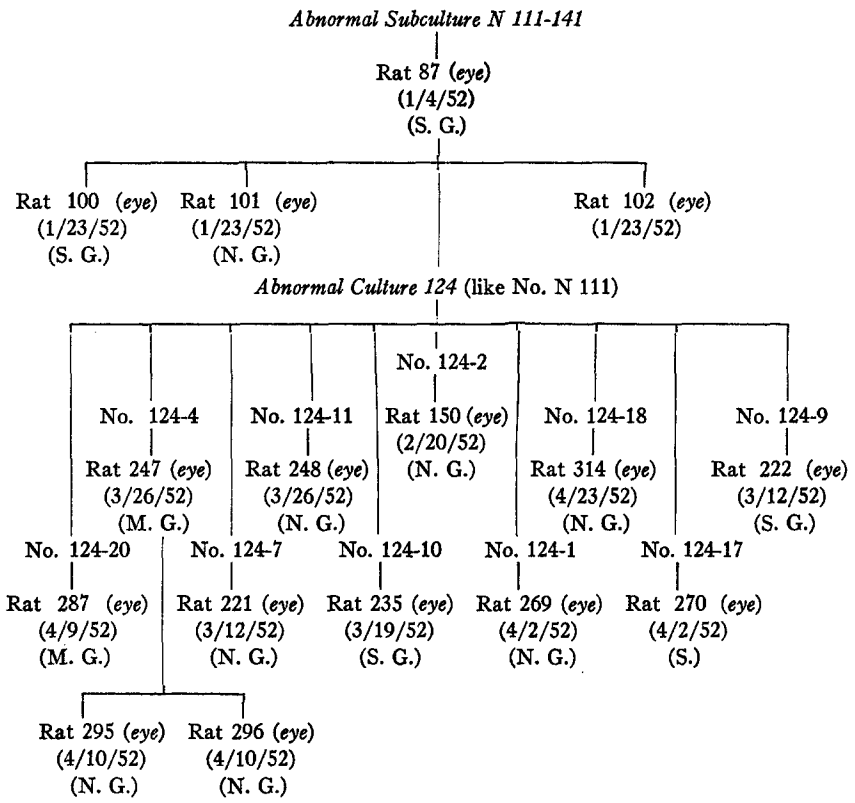
Methods of Implantation:

The procedure for the preparation of the cultures for implantation was to loosen one or more (usually all) of the cultures in the tube by cutting into the plasma around them by means of a pointed, bent pipette. The loosened cultures, in plasma, were then sucked up gently into the pipette and expelled into the depression in a microculture slide containing some of the fluid nutrient medium. The excess plasma around each culture was then cut away with sharp cataract knives and each culture was divided into two or more pieces, depending upon the size of the growth. The number of fragments used for the implantation varied from a single culture to several cultures. In some instances, to make a massive implantation, all the cultures in several tubes were combined. The pieces of tissue were sucked up or shoved into the end of a 16 gauge, bluntly bevelled needle 3 ½ inches long, provided with a tight fitting trocar. A small amount of the nutrient medium was sucked up with the tissue. During all implantations by all methods the animals were kept under anesthesia with ether. Two or three drops of terramycin solution (ophthalmic) were placed in the conjunctival sac for about a minute before the cornea was incised. An incision about 3 mm. long was made in the line of the corneoscleral junction with a sharp keratome, and the end of the needle containing the cultures was inserted through it, with precaution not to injure the iris as the needle was being pushed gently into the anterior chamber. The tissue fragments in the lower end of the needle were then emptied into the anterior chamber by pushing down the trocar to its full length. The needle was then withdrawn, with precaution not to pull the tissue culture fragments out with it. This is essentially the technique used by Greene (21-23).

For the subcutaneous implantation of the cultures, the method of preparation of the fragments was the same as for the implantation into the anterior chamber of the eye. The only difference between the two methods was that the needle containing the tissue fragments was inserted through a small incision in the skin high up in the flank. The skin was first shaved and then cleaned with 1:1000 zephiran solution. An incision about 3 mm. in length was made in the skin with a cataract knife, and the 3 ½ inch, 16 gauge needle was inserted through it, subcutaneously, so that the bevelled end was situated low in the flank. Then the tissue fragments were deposited by pushing in the trocar and withdrawing the needle. A little pressure on the skin in the region immediately above the end of the needle prevented withdrawal of the fragments of tissue with the needle.

For the implantation of the cultures into the brain, muscle, and peritoneum the method was exactly as described for the subcutaneous implantation, except that the needle was inserted into the brain through an opening in the skull made with a small trephine and into the peritoneum and skeletal muscles through a small incision in the skin of the lower part of the abdomen, or over the muscle, respectively.

TEXT-FIG. 5



N. G. = no growth.
S. = survival of culture, but no growth.
S. G. = slight growth (not a tumor).
M. G. = moderate growth (tumor?).
P. G. = progressive growth. Definite tumor (fibrosarcoma).

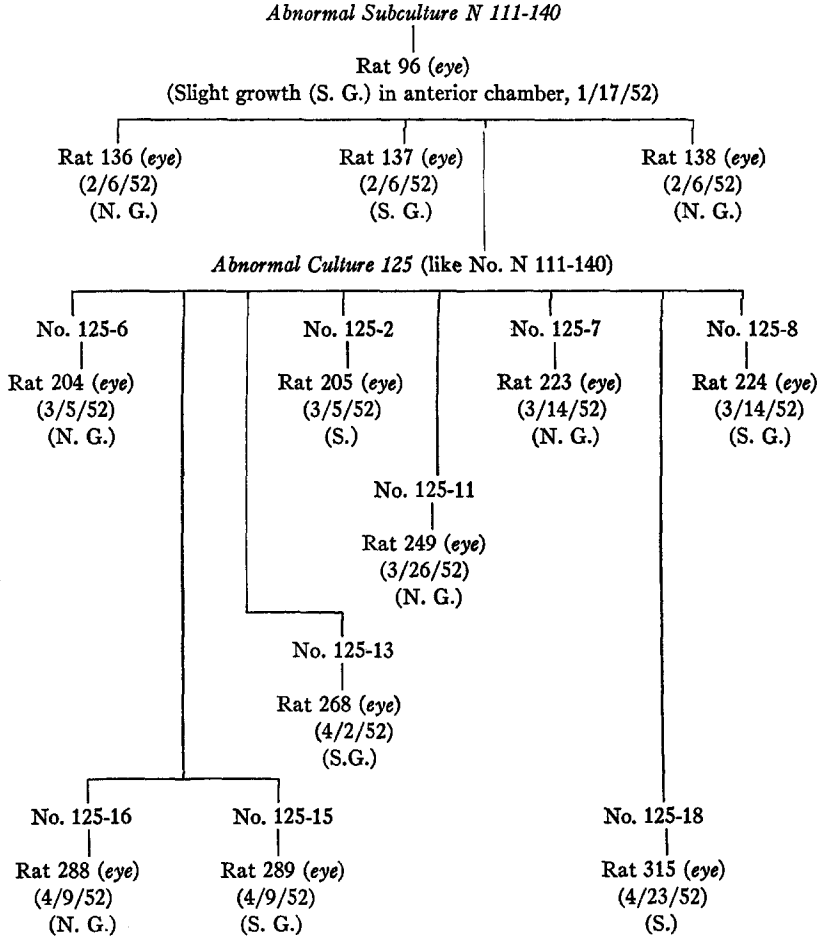
Implantation of Cultures of Strain N 111 as Such

In the case of the subcultures of No. N 111, implanted some time after the completion of the exposures to nitrogen (Table III, Second period), temporary survival of the cultures took place in the anterior chamber of the eyes of rats and guinea pigs, but progressive growth did not occur and finally the fragments disappeared.

In some of the rats slight growth occurred, but regression soon followed. In two rats, No. 87 (Text-fig. 5 and No. 96 (Text-fig. 6), of the Slonaker-Addis strain, the result appeared to

be what is usually called positive in the case of heterologous transplants, but these were rated merely as "slight growth." Sections of a portion of these growths showed that they consisted of highly atypical mesenchymal cells of variable size and shape, with mitoses and

TEXT-FIG. 6



N. G. = no survival or growth of implanted abnormal tissue.

S. = survival of implanted abnormal tissue.

S. G. = survival and slight growth of implanted abnormal tissue. (Not regarded as a progressively growing tumor.)

abnormal nuclear forms in considerable number, and the tissue resembled the original cultures which were implanted. Transplantation of minute fragments of the tissue removed from the eye of rat 87 into the anterior chamber of one eye of three other rats (Nos. 100, 101, and 102) of the Slonaker-Addis strain resulted in slight growth in only one rat, No. 100 (Text-fig. 5).

The same occurred in the case of the transplantation of the tissue into the anterior chamber of the eye of rat 96. A fragment of this growth transplanted into the anterior chamber of one eye of three rats showed slight growth in one (rat 137, Text-fig. 6).

Tissue cultures made of the tissue removed from the anterior chamber of the eye of rat 87 (culture 124, Text-fig. 5), and of rat 96 (culture 125, Text-fig. 6), both of which appeared abnormal and resembled culture N 111-3, from which they originated, were inoculated into the anterior chamber of one eye of 10 rats of the Slonaker-Addis strain. In the case of culture 124, the subcultures survived (S.) or grew slightly (S.G.) in the eyes of 3 of the 10 rats and moderate growth (M.G.) occurred in 2 rats, but further transplantation of the latter failed to show any growth (Text-fig. 5). In the case of the subcultures of abnormal culture 125, made from the growth in the anterior chamber of the eye of rat 96, survival (S.) or slight growth (S.G.) occurred in the anterior chamber of the eyes of 5 of the 9 rats to which fragments were transferred, but progressive growth (P.G.) did not occur (Text-fig. 6). These results were all rated merely as survival of abnormal tissue and not as true growth. Fig. 20 is an illustration of the slight growth in the anterior chamber of the right eye of rat 96.

That the Slonaker-Addis strain of rat is unusually resistant to the progressive growth of transplantable malignant tumors, after implantation in the eye, was shown much later, when a known transplantable fibrosarcoma of the rat, originating in the A × C strain of the Crocker Institute and obtained from Dr. Anna Goldfeder of New York City, was transplanted into the eyes of rats of the Slonaker-Addis strain. In the anterior chamber of the eyes of some rats of the Slonaker-Addis strain, this true tumor did survive and, in some of the rats, seemed to grow for a while, but soon most of the growths underwent complete regression. An occasional implant did grow to fill the anterior chamber and microscopically, had the appearance of the original tumor.

Implantation of subcultures of strain N 111 into the brain, the subarachnoid space, the peritoneal cavity, the muscles of the hind limb, and the subcutaneous tissue of the right loin of rats of the Slonaker-Addis strain resulted in survival, or at most in temporary slight growth, of some of the fragments, with eventual complete regression of those not removed for section. Progressive growth did not occur. In one rat, the brain tissues showed slight growth and seeming invasion by the abnormal cells, but the amount of growth was small (Table III, Second period).

With the aim of reducing the resistance of the animals to the growth of the abnormal tissue, some rats of the Slonaker-Addis strain, about 3 weeks old and weighing 35 to 45 gm., were pretreated with cortisone (2 mg. daily), intraperitoneally, for 2 or 3 days before the subcutaneous implantation of the cultures. A similar dose was given every day, for at least a week after the implantation of the cultures, but even slight growth did not occur in this small group of rats (Table II). The cultures were completely absorbed and left no trace.

Toolan (19) found that irradiation with x-rays favored the growth of transplants of heterologous (human) tumors. Hence young rats of the Slonaker-Addis strain were exposed to Roentgen ray irradiation, according to the method of Toolan (20); namely, 150 r for 2 consecutive days before the transfer of the fragments of the tissue cultures to the anterior chamber of the eye or to the subcutaneous tissue. The implantation was made about 24 hours after the second irradiation period. No true growth occurred subcutaneously, but survival and slight or even

TABLE II

Rat No.	Subculture No.	Site of implantation	Special treatment	Date	Result
327	N 111-275	Subcutaneous	Cortisone*	6/5/52	N. G.
328	N 111-278	"	"	"	"
346	N 111-176	"	"	"	"
347	N 111-277	"	"	"	"
348	N 111-270	"	"	"	"
349	N 111-279	"	"	"	"
350	N 111-281	"	"	"	"
345	N 120-145	"	"	6/19/52	"
351	N 120-172	"	"	"	"
357	N 120-161	"	"	"	"
364	N 120-166	"	"	"	"
329	N 111-248 N 111-254 N 111-263	Subcutaneous	X-ray	5/15/52	"
330	N 111-234	"	"	"	"
338	N 111-267 N 111-271	"	"	5/29/52	"
339	N 111-244 N 111-260	"	"	"	S.
340	N 111-257	"	"	"	S. G.
343	N 111-255	"	"	"	N. G.
362	N 111-301	Eye	"	6/19/52	S. G.
363	N 111-296	"	"	"	N. G.
331	N 120-92 N 120-150	Subcutaneous	"	5/15/52	S. G.

* 2 mg. daily intraperitoneally, for 2 or 3 days before the subcutaneous implantation of the cultures and for at least a week after the implantation.

TABLE II—*Concluded*

Rat No.	Subculture No.	Site of implantation	Special treatment	Date	Result
341	N 120-146	Subcutaneous	X-ray	5/29/52	M. G.
342	N 120-163 N 120-169	"	"	"	N. G.
359‡	N 120-147	Eye	"	6/19/52	M. G.
360	N 120-153	"	"	"	S. G.
361	N 120-180	"	"	"	M. G.
352	N 111-274 N 111-284	Subcutaneous	X-ray and cortisone	6/12/52 "	N. G. "
353	N 111-250	"	" " "	"	"
354	N 111-265	"	" " "	"	"
358	N 111-291	"	" " "	"	S. G.
355	N 120-149 N 120-174	"	" " "	"	N. G.
356	N 120-160	"	" " "	"	S. G.

‡ For an illustration of the gross appearance of the eye of rat 359, which showed what was considered as definitely beginning growth of an implanted fragment, See Fig. 20 which is an illustration of a slight growth (S. G.) in the eye of rat 96. Rats 341 and 361 showed a similar appearance.

moderate growth of the implanted tissue were more common than in the untreated rats or in those treated with cortisone. In a few rats a combination of injections of cortisone and of irradiation with x-rays resulted in slight growth, at most, in a smaller percentage of animals than in those treated with irradiation alone (Table II).

In an effort to obtain favorable conditions for the growth of implants of the abnormal fibroblasts, which were showing a definitely greater growth potential than normal fibroblasts, adult rats of strains known to be less resistant to the transplantation of known tumor tissue were obtained from several sources.[1] Only survival, or temporary growth, was obtained at most, when tissue cultures N 111 and later N 120 were implanted in the animals of these strains.

[1] They were provided by Dr. Anna Goldfeder of the Cancer Research Laboratory, Department of Health, City of New York, New York, Dr. Gey, of Johns Hopkins Hospital, Baltimore, Dr. W. F. Dunning of the University of Miami, Coral Gables, and Dr. Richard Winzler of the University of Southern California, Los Angeles. The great kindness of these investigators is gratefully acknowledged.

Effect of the Addition of Embryonic Tissue to the Implants

Finally, in an attempt to make the abnormal fibroblasts grow progressively after implantation, minute fragments of embryonic rat tissues (liver, brain, or lung of 10 day old rat embryo) were added to the cultures and implanted with them into the anterior chamber of the eye, or subcutaneously, in young rats of the Slonaker-Addis or Dunning's A × C "Irish" strain. Greene (23) used embryonic tissues of various kinds, with success, to favor the growth of fragments of heterologous malignant tumor (Brown-Pearce carcinoma of the rabbit) in various sites in mice, rats, and guinea pigs. In his publication, Greene did not emphasize the value of embryonic lung, although he mentioned it, but in personal communications he has stated that whenever he employs the method he now generally introduces a minute fragment of embryonic lung with the fragments of tumor tissue, to provide "a ready made stroma" for the transplant, and that the "percentage of takes increases a great deal" as a result of this procedure. He has found that even embryonic lung will not promote the growth of any tissue which has not, by itself, some potentiality for growth after transplantation. Greene has not mentioned the transplantation of embryonic lung with tissue cultures of malignant tumors.

The implantation of subcultures of strain N 111 with 1 or more minute fragments of lung from a 10 day old rat embryo into rats of the Slonaker-Addis strain, or of the A × C "Irish" strain, resulted promptly in definite growth both subcutaneously and in the anterior chamber of the eye, the only sites used (Table III, Third period). Subcutaneous nodules became visible and palpable in 3 or 4 days and were as much as 3.5 by 2.5 by 1.5 cm. in diameter after 2 weeks of growth (Fig. 21). Microscopically, the tumors were all moderately differentiated, spindle cell, and pleomorphic cell sarcomas, showing many mitoses of various kinds, hyperchromatic nuclei of abnormal type, and many abnormal nuclear forms (Figs. 22 and 23). Within the growth, or at its periphery, were remnants of the fragments of embryonic lung, showing degenerative changes of the lining epithelium of the respiratory passages. These remnants were surrounded either by tumor or by low grade inflammatory tissue, consisting of well vascularized reactive tissue containing many lymphocytes and large mononuclear cells.

Tissue cultures grown from fragments of one intraocular and three subcutaneous growths resembled in all respects the original cultures of abnormal strain N 111. Serial transplantation of one of the first tumors which developed as a result of the implantation of strain N 111, with the addition of embryonic rat lung, has now been carried out successfully, without the addition of embryonic lung, five successive times in a total of 32 rats of the Slonaker-Addis strain. Fibrosarcoma resulted in all, and seemingly the transfers can be continued indefinitely if desired. A tendency to regression characterized some of the tumors thus obtained, so that it was desirable and better to transfer them

early, but recently, in one rat, a tumor weighing 28 gm. and measuring 5.5 by 5.0 by 4.5 cm. developed in 3 months. At autopsy, there were foci of necrosis in the tumor, but the major part of it was well preserved and fragments of it, transferred subcutaneously to 5 other rats, grew well in all.

Results of the Implantation of Control Cultures:

The subcutaneous or intraocular implantation of cultures of normal fibroblasts (strain C 15), as such, into a total of 75 rats, 14 guinea pigs, and 16 rabbits (Table III) has never resulted in the development of a visible or palpable subcutaneous nodule nor even in the survival of the cultures in the anterior chamber of the eye. In 2 weeks or less the tissue usually disappeared without trace. Subcutaneous and intraocular implantation of cultures of normal fibroblasts with fragments of embryonic rat lung into 14 rats resulted only in the development of low grade inflammatory tissue in which lung could still be recognized for about 2 weeks. No tumors developed. The same occurred when lung tissue alone was implanted.

History of Abnormal Strain N 120

As already made plain, the cultures of fibroblasts which later became transformed into abnormal strain N 120 originated from the same 5 day old rat heart and from the same fragments of myocardium (No. 15-5) as did abnormal strain N 111. The culture had been growing normally for exactly 1 year, during which 22 transfers had been carried out, with subcultures at the time of most of the transfers, before the exposure to nitrogen of subculture C 15-301 changed to N 120-3 was begun (Text-fig. 7). During the preceding year many of the subcultures of normal fibroblasts had been implanted into the eyes of rats, guinea pigs, and rabbits without growth resulting in any of the animals (Table III, First period).

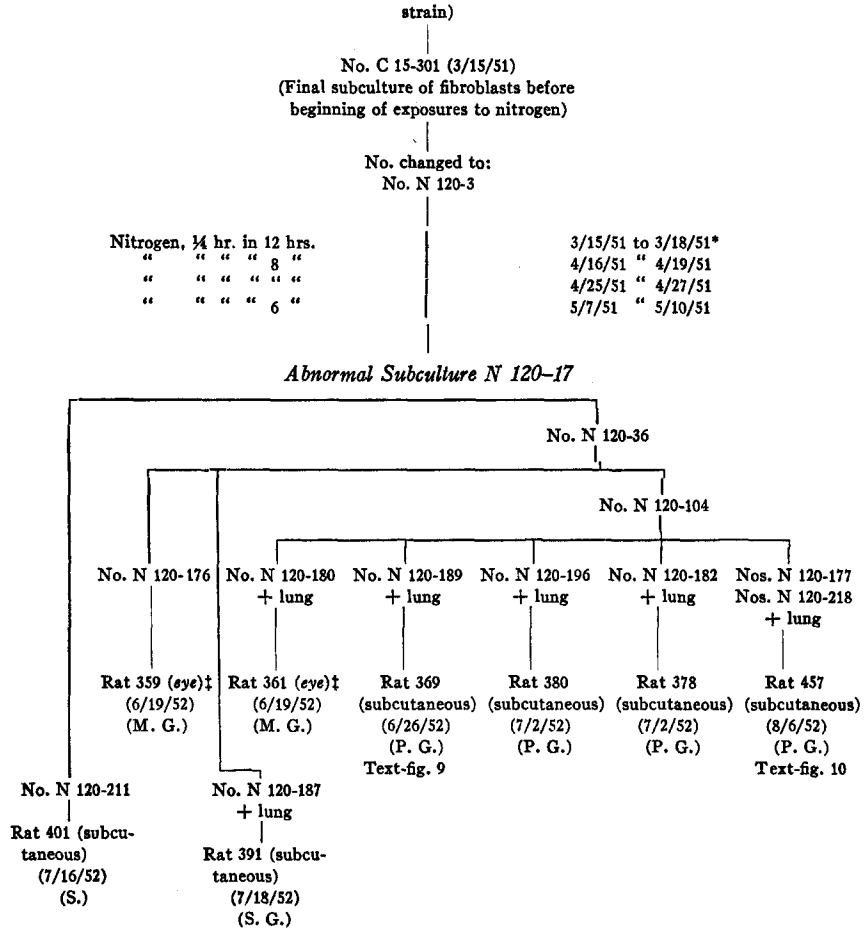
Initially, from March 15 to 18, 1951, inclusive, culture C 15-301 (N 120-3) was automatically exposed to nitrogen for 15 minutes in every 12 hours (Text-fig. 7). As in the case of strain N 111, the flowing nitrogen was replaced by flowing air for an equal period of time, and, after the flow of air was automatically (Text-fig. 2) shut off, air remained in the culture tube until the next exposure to nitrogen. From April 16 to 19, inclusive, and from April 25 to 27, inclusive, the exposures to nitrogen were increased to 15 minutes in every 8 hours, and from May 8 to 10 they were again increased to 15 minutes in every 6 hours (Text-fig. 7). Some of the subcultures of this strain were again exposed to nitrogen from December 7 to 10, 1951, inclusive, but these are not shown in Text-fig. 7. After the second period of exposure (April 19, 1951) some large, irregularly shaped cells, with relatively large nuclei and coarse, granular cytoplasm made their appearance. In stained preparations of subcultures the large nuclei were hyperchromatic. After the third period of exposure to nitrogen (April 27, 1951), the cells appeared in unusually poor condition and it was questionable whether or not they would survive. They remained in that condition for several days and then began to recover. After the end of the fourth exposure to nitrogen (May 10, 1951) multinucleated cells were first noted, but these appeared in good condition although abnormal. There remained a goodly number of

seemingly normal spindle cells with long processes. In various portions of some of the subcultures, however, there was a definite shortening and, in some cases, entire disappearance of

TEXT-FIG. 7

Culture C 15-5 (3/15/50)

(One of the six original cultures of the myocardium, of a 5 day old albino rat of the Slonaker-Addis strain)



S. = survival of culture. M. G. = moderate growth.
S. G. = slight growth. P. G. = progressive growth (fibrosarcoma).

Rat 361: Piebald strain of Dr. Goldfeder—Rat 369: AXC "Irish" strain of Dr. Dunning. All others, Slonaker-Addis strain.

* All dates are inclusive.

‡ These rats were exposed to x-ray irradiation (150 r) for 2 days before the implantation of the cultures (with embryonic lung, where indicated), into the anterior chamber of one eye.

the cellular processes, with a tendency for the cells to develop a cohesive property which resulted in the formation of sheets of cells, such as epithelium forms *in vitro*, and resembling in this respect strain N 111 previously described. By July 26, 1951, however, there was a

great increase in the abnormally large, round, and irregularly shaped cells; abnormal mitoses and abnormal nuclear forms had become more abundant, despite discontinuance of the exposures to nitrogen. Multipolar mitoses, like those of strain N 111 (Figs. 15–18), were not uncommon, and some bizarre forms also were present. Shortening and disappearance of the cellular processes became more pronounced, and the loose, reticular structure of the growth gave place gradually to the diffuse development of sheets of cells, like those of strain N 111

TABLE III

Results of Implantation of Nitrogen-Treated Cultures with or without the Addition of Embryonic Rat Lung, in Various Sites, during the Entire Experiment, Exclusive of Rats Treated with Cortisone or X-Rays (Table II)

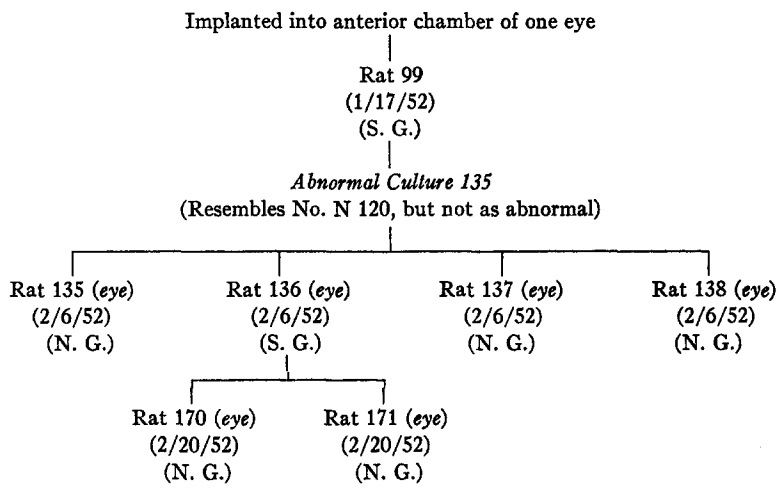
Type of animal	Site of implantation of culture	Result	No. of animals implanted with untreated control cultures	No. of animals implanted with nitrogen-treated cultures		Remarks
Guinea pig	Intraocular	No growth	8	14		*First period.*—From 3/15/50 to 9/16/51, the date of the last exposure of the cultures to nitrogen, before establishment of the abnormal strains
Rabbit	"	" "	4	6		
Rat	"	" "	20	18		
				Strain N 111	Strain N 120	
Guinea pig	Intraocular	No growth	6	7	8	*Second period.*—From 9/17/51 (after the completion of all the exposures of the cultures to nitrogen) to 6/18/52, the day before fragments of embryonic rat lung were first implanted with the cultures
"	"	Survival	0	3	4	
Rabbit	"	No growth	12	23	18	
Rat	"	" "	35	27	42	
"	"	Survival	0	3	2	
"	"	Slight growth	0	2	6	
"	Subcutaneous	No "	12	10	9	
"	Intramuscular	" "	4	6	4	
"	Intracerebral	" "	6	8	5	
"	"	Slight growth	0	1	0	
"	Intraperitoneal	No "	4	5	5	
Rat	Intraocular	Moderate growth	0	0	2	*Third period.*—From 6/19/52 (the date of the first implantation of embryonic lung with the cultures) to 8/15/52
"	Subcutaneous, with fragments of embryonic lung added to the cultures	No growth	14	0	0	
"		Survival	0	0	1	
"		Slight growth	0	2	1	
"		Progressive growth (tumor)	0	9	4	
"						

(Figs. 7–9). During the period from March 15, 1951, to September 10, 1952, this strain was transferred, with or without subculture, 32 times. At the beginning of 1952 the complete transformation of the tissue into sheets had occurred; the cells had become so abnormal that the cultures could not be distinguished from the most abnormal cultures of strain N 111. In this strain, too, the rapid development of pronounced acidity of the nutrient fluid, the foci of cytolysis in the culture, and the early liquefaction of the plasma clot were features that appeared as the tissue became more abnormal. The cultures were therefore considered probably malignant and ready for transfer to animals.

Results of Implantation of Abnormal Strain N 120

As in the case of the cultures of strain N 111, after they had become highly abnormal, implantation of subcultures of strain N 120-3 into various sites, and under similar conditions, but without the addition of embryonic tissue, failed to result in progressive growths. Survival and slight temporary growth did occur in the anterior chamber of the eye of 8 rats (Table III, Second period) but regression soon followed. Text-fig. 8 shows that a culture of tissue removed from a slight growth in the eye of one rat (No. 99), which had been implanted

TEXT-FIG. 8

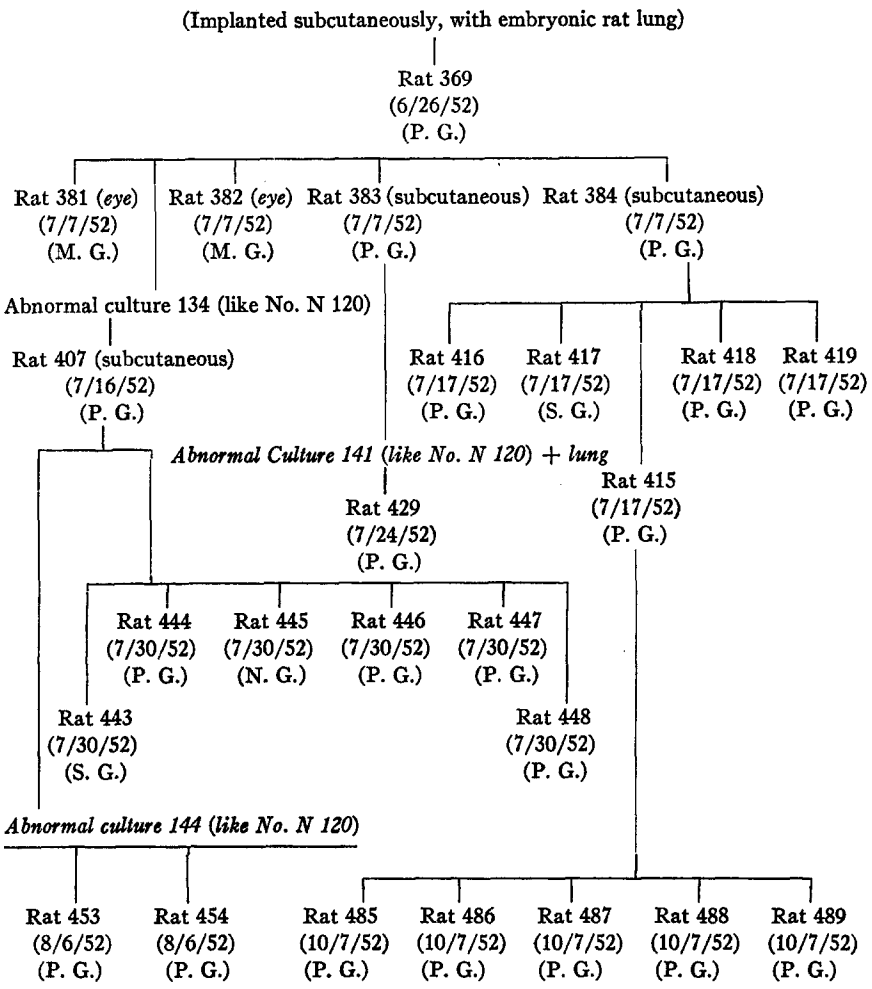


N. G. = no growth. S. G. = slight growth.

with subculture N 120-71, resulted in growth (No. 135) of abnormal fibroblasts which were in all respects similar to N 120. Implantation of culture No. 135 into the anterior chamber of the eye of 4 rats resulted in slight growth in one of the rats (rat 136, Text-fig. 8). Tissue from the small growth in the eye of this rat, when transferred to the anterior chamber of one eye of 2 other rats (Nos. 170 and 171), did not grow at all (Text-fig. 8).

Attempts were made to favor the growth of implants of subcultures of strain N 120 by the administration of cortisone, by x-ray irradiation, and by a combination of the two methods (Table II). The results with this strain were similar to those obtained with strain N 111. As in the case of No. N 111, cortisone appeared to be inhibitory, while a favorable effect of irradiation with x-rays was even more definite than with N 111. Both intraocular and subcutaneous implants resulted in some growth in 6 rats, but in no case was the result such as to characterize it as progressive.

TEXT-FIG. 9



In this text-figure, whenever embryonic lung was added to the culture or fragment of tumor at time of implantation, it is specifically indicated. All implantations of subcultures and of fragments of malignant tumors from rats 383, 384, and 407 and other rats were made subcutaneously.

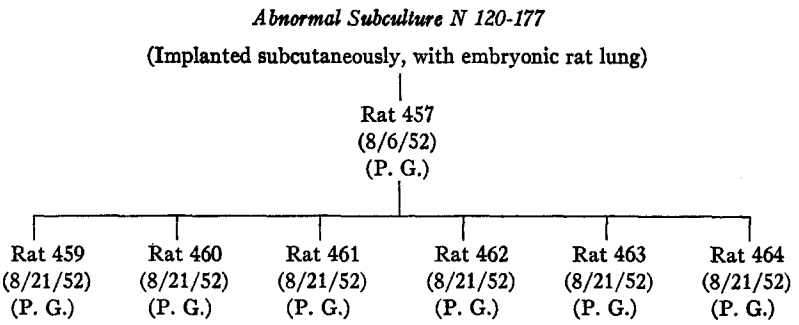
S. G. = slight growth. P. G. = progressive growth (fibrosarcoma).

Effect of the Addition of Embryonic Lung:

At about the same time as in the case of subcultures of strain N 111, the effect of adding fragments of embryonic rat lung to subcultures of No. N 120 was tested.

One or more minute fragments of embryonic lung from a rat embryo about 10 days old of the Slonaker-Addis strain were implanted together with a subculture, either into the anterior chamber of the eye or subcutaneously into the right flank of rats—both the A × C "Irish" and the Slonaker-Addis strains. In rat 369 (Irish strain) a visible, palpable subcutaneous nodule was perceptible in 4 days, and on the 11th day this had become a firm nodule measuring 1.5 by 0.9 by 0.6 cm. (Text-figs. 7 and 9). The animal was killed then. Microscopically, the growth proved to be a typical, moderately differentiated fibrosarcoma, like

TEXT-FIG. 10



P. G. = progressive growth of implant (fibrosarcoma).

Fragments of embryonic rat lung were added only to the original subcutaneous implant of subculture N 120-177, in rat 457. All rats in this text-figure are of the Slonaker-Addis strain, the same strain as the rat from which the original cultures of fragments of myocardium were made (Text-fig. 3) and all implants were placed subcutaneously, low in the right flank (Fig. 21).

that resulting from the subcutaneous implantation of cultures of strain N 111 with minute fragments of embryonic rat lung.

The subcutaneous growth in rat 369 was successfully transferred to other rats by the implantation of fragments of the tumor, without the addition of embryonic rat lung, into the anterior chamber of one eye of rats 381 and 382, and subcutaneously in the right flank of rats 383 and 384, of the Slonaker-Addis strain (Text-fig. 9). Other fragments of the subcutaneous tumor of rat 369, grown in roller tubes, resulted in abnormal culture 134 which closely resembled strain N 120 from which the tumor had originated (Text-fig. 9). The implantation of this culture, with the addition of small fragments of embryonic lung, subcutaneously, into rat 407, of the Slonaker-Addis strain, again resulted in the development of a typical fibrosarcoma. Fragments of this tumor yielded abnormal culture N 144 (Text-fig. 9) which also resembled N 120, and these cultures, transplanted subcutaneously, without the addition of embryonic rat lung in rats 453 and 454 also resulted in the development of typical fibro-

sarcomas in both rats (Text-fig. 9). In less than 2 weeks, some of these tumors measured as much as 3 by 2.0 by 1.5 cm. Similar results were obtained in the case of the tumor nodules which developed in rats 383 and 384 of the Slonaker-Addis strain (Text-fig. 9). Fragments of a subcutaneous tumor in rat 384, transplanted subcutaneously in 5 rats (Nos. 415–419), developed into tumors in all (Text-fig. 9). The subcutaneous transplantation of fragments from a subcutaneous tumor nodule of one of these rats (rat 415) to 5 other rats of the Slonaker-Addis strain, again resulted in the development of fibrosarcomatous nodules in all (Text-fig. 9), rats 485 to 489, inclusive). The subcutaneous implantation of abnormal subculture N 120–177, with the addition of embryonic rat lung, into rat 457 (Text-fig. 10), also resulted in the development of a fibrosarcomatous nodule, and fragments of this tumor implanted subcutaneously, without the addition of embryonic rat lung, in 6 rats of the Slonaker-Addis strain, yielded fibrosarcomas (P.G.) in all the rats.

As in the case of strain N 111, the indications are that it will be possible to continue indefinitely the serial transfer of these tumors which have resulted from the original implantation of subcultures of No. N 120, especially if the transplantation is carried out early.

DISCUSSION

The experiments show that fibroblasts grown *in vitro* and submitted to repeated periods of anaerobiosis, sometimes become neoplastic. Of the five original roller tube cultures with which this experiment was begun, one (No. 15-5) underwent malignant change in two separate subcultures which were first exposed to an atmosphere of nitrogen a year apart, that is to say one a year later than the other (Text-figs. 3, 4, and 7). The same tube of cultures (No. 15-5) also yielded the subcultures of normal fibroblasts which served as controls for this entire study. The remaining five tubes of cultures with which this experiment was begun, although all exposed intermittently to nitrogen, for various periods, either died early (Table I), before implantation of the cultures in animals could be carried out (Text-fig. 3, Nos. 15-1, 15-4, and 15-6), or the cultures failed to grow adequately and could not be subcultured with sufficient success to yield enough tissue for implantation. These cultures were discarded (Text-fig. 3, Nos. 15-2 and 15-3).

Gey (7–9), who followed in tissue cultures the change of normal subcutaneous areolar tissue of an adult rat into malignant connective tissue, was unable to determine during more than 12 years of work just what it was that brought about the separation of the original culture of fibroblasts into "normal" and malignant strains. He has listed as the most possible likely causative agents: (*a*) the heterologous medium used for the cultivation of the tissues, (*b*) a filterable agent, and (*c*) gamma radiation present in the laboratory during the early cultivation of these tissues. He does not believe, however, that the irradiation was significant, because at least one other con-

version of a culture of normal tissue into malignant tissue has since occurred in his laboratory with no abnormal background of radiation.

Earle and his collaborators (4–6) have also been at a loss to explain exactly what were the experimental conditions which brought about the abnormal changes in their cultures of normal, adult mouse fibroblasts. In their experiments the possibility of contamination of some of the normal fibroblasts with methylcholanthrene was not excluded. But they have not shown that minute amounts of methylcholanthrene deliberately added to cultures of fibroblasts will result in the development of fibrosarcoma *in vitro* and they have been obliged to conclude that the changes may have been induced by some "unrecognized agent of unknown source."

Before suggesting a possible causative relationship between the intermittent anaerobiosis of the present experiments and the final transformation of the cultured cells into malignant, fibroblastic tissue, it is necessary, as in the case of Gey's (7–9) and Earle's (4–6) observations, to consider all other possibilities. Gey and Earle made no planned effort to produce malignant alterations in their cultures and their occurrence was unexpected. In the present study, *a priori* reasoning led to a deliberate attempt to bring about such a change. The appearance of abnormal cells and the eventual development of malignant change in two separate cultures of normal fibroblasts originating from the same rat heart, after their intermittent exposure to nitrogen, which, in the case of strain N 111 was begun early and, in the case of strain N 120, after the normal fibroblasts had been growing in pure form for 1 year, is at least an indication that the malignant change may have resulted from the intermittent exposure of the normal fibroblasts to anaerobic conditions.

Possible contamination of the normal fibroblastic tissue with malignant fibroblasts from some other source need not be considered, as it had to be by Gey and Earle, because no kind of malignant growth was being grown in the laboratory during the entire period of the exposure of the cultures to nitrogen and for a long time after the abnormal changes in the cultures of strains N 111 and N 120 had become permanent.

Possible effects of stray, gamma irradiation, which Gey and collaborators (7–9) were obliged to take into consideration in their observations, can be ruled out, because at no time during the entire period of these experiments was there any source of radiation present in the laboratory. The possible contamination of the cultures with a chemical carcinogen, which had to be considered by Earle (4–6), can also be excluded, because at no time during the entire period of our study were chemical carcinogens being used in the Institute.

The one set of conditions common to Gey's, Earle's, and our experiments was the use of a heterologous fluid mixture as the nutrient medium and heterologous plasma for the solid part of the culture medium. Gey used human cord blood serum and so did we, but Earle used horse serum. In Earle's and our studies, chick embryo extract was used and, in all three studies, chicken plasma furnished the solid part of the culture medium. The most substantial reason

which can be offered against the possibility that the heterologous medium was the cause of the development of the malignant changes of our study is the fact that the same heterologous medium was used for 2 ½ years for the control cultures of normal fibroblasts originating from the same rat heart as the abnormal strains, without the development of abnormal changes in the cultures of fibroblasts. Heterologous media have long been used by other investigators who have not reported the development of abnormal changes in their cultures of rat tissue.

The one definite difference between our experiments and those of Gey and Earle is that in ours deliberate use was made of intermittent exposure of the cultures to alternating anaerobic and aerobic conditions on the assumption that this might result in neoplastic changes. A possibility exists that the cultures of Gey and of Earle may have been inadvertently subjected for variable periods to atmospheres with reduced content of oxygen. Gey has stated that he has kept many of the cultures at 28°C., with less frequent changes of fluid medium than in other instances and infrequent subculture or transfer. This means that these cultures remained tightly stoppered for long periods without renewal of the air in the tubes. Since, in our experiments, the total time of intermittent exposure to nitrogen was not great before the morphologic changes began to appear, it is suggested that the cultures of Earle and of Gey may sometimes have been subjected to conditions approximating ours. Conceivably, therefore, in all three sets of experiments, Gey's, Earle's, and our own, the exposure of the cultures to an atmosphere poor in oxygen may have been the basic cause of the transformation of normal fibroblasts into malignant cells. It must nevertheless be recognized that in all three experiments the changes may have been induced, as Earle has stated with regard to his own findings, "by some unrecognized agent of unknown source."

SUMMARY AND CONCLUSIONS

In two strains of fibroblasts originating from fragments of myocardium from a 5 day old rat of the Slonaker-Addis strain, a strain known to develop tumors but rarely, abnormal morphologic alterations took place which eventuated in neoplastic changes. This happened only after repeated exposure of the cultures to an atmosphere of nitrogen (anaerobiosis) and did not occur in the control cultures of fibroblasts originating from the same rat heart, and grown continuously throughout the same period (2 ½ years) without exposure to nitrogen.

BIBLIOGRAPHY

1. Rous, P., *Harvey Lectures*, 1935–36, **31,** 74.
2. Hartwell, J. L., Survey of Compounds Which Have Been Tested for Carcinogenic Activity, Public Health Service Publication, No. 149, Washington, D. C., United States Government Printing Office, 2nd edition, 1951.

3. Cameron, G. R., Pathology of the Cell, Springfield, Illinois, Charles C Thomas, 1951.
4. Earle, W. R., *J. Nat. Cancer Inst.*, 1943–44, **4,** 165.
5. Earle, W. R., and Nettleship, A., *J. Nat. Cancer Inst.*, 1943–44, **4,** 43.
6. Nettleship, A. and Earle, W. R., *J. Nat. Cancer Inst.*, 1943–44, **4,** 229.
7. Gey, G. O., *Cancer Research*, 1941, **1,** 737.
8. Firor, W. M., and Gey, G. O., *Ann. Surg.*, 1945, **121,** 700.
9. Gey, G. O., Gey, M. K., Firor, W. M., and Self, W. O., *Acta Unio Internat. Cancrum*, 1949, **6,** 706.
10. Warburg, O., and Minami, S., *Klin. Woch.*, 1923, **2,** 776.
11. Warburg, O., *J. Cancer Research*, 1925, **9,** 148.
12. Warburg, O., *Strahlentherapie*, 1926, **23,** 1.
13. Warburg, O., Wind, F., and Negelein, E., *Klin. Woch.*, 1926, **5,** 829.
14. Warburg, O., Wind, F., and Negelein, E., *J. Gen. Physiol.*, 1927, **8,** 519.
15. Wind, F., *Biochem. Z.*, 1926, **179,** 384.
16. Gey, G. O., *Am. J. Cancer*, 1933, **17,** 752.
17. Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27,** 45.
18. Cameron, G., Tissue Culture Technique, New York, Academic Press, 1951.
19. Toolan, H. W., *Proc. Soc. Exp. Biol. and Med.*, 1951, **77,** 572.
20. Toolan, H. W., *Proc. Soc. Exp. Biol. and Med.*, 1951, **78,** 540.
21. Greene, H. S. N., *J. Exp. Med.*, 1938, **67,** 691.
22. Greene, H. S. N., *Cancer Research*, 1947, **7,** 491.
23. Greene, H. S. N., *Cancer Research*, 1949, **9,** 728.

EXPLANATION OF PLATES

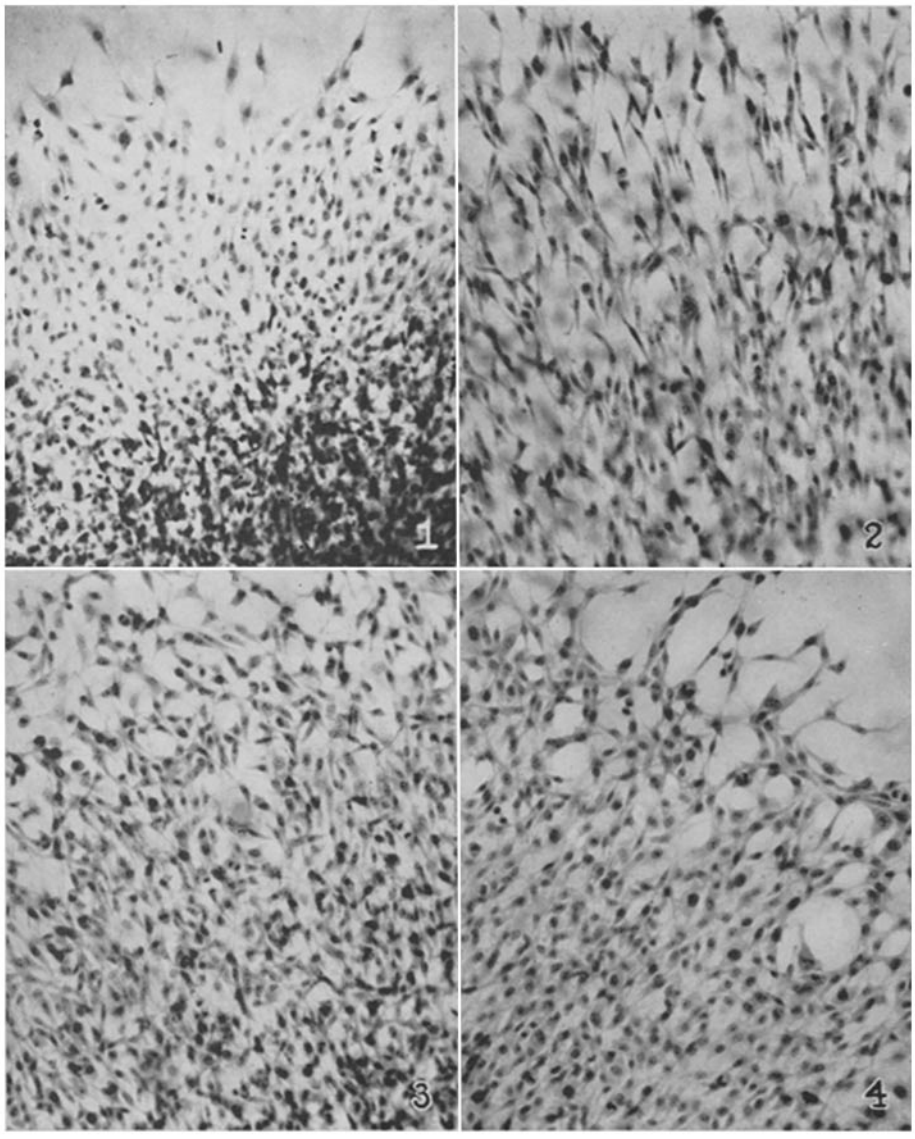
PLATE 21

FIG. 1. Normal culture of fibroblasts, in November, 1950. This was taken about 6 months after the culture had come to consist only of normal fibroblasts. The spindle cells with long processes, the normal mitoses, and the reticular structure are characteristic. Whole culture growing on glass coverslip, stained with hematoxylin and eosin. × 180.

FIG. 2. Culture of the same strain of normal fibroblasts, on glass coverslip, in July, 1951, more than 1 year after the beginning of the strain; to be compared with Fig. 1. Hematoxylin and eosin. × 180.

FIG. 3. Culture of the same strain of normal fibroblasts, on glass coverslip, in January, 1952. The appearance of this culture is still unchanged. Hematoxylin and eosin. × 180.

FIG. 4. Culture of the same strain of normal fibroblasts, growing on glass coverslip, in June, 1952. No significant alteration, compared with Figs. 1 to 3. The spindle cells, the long processes, the occasional normal mitoses, and the reticular structure are still present, as in Figs. 1 to 3. Hematoxylin and eosin. × 180.



(Goldblatt and Cameron: Induced malignancy in cells from myocardium)

PLATE 22

FIG. 5. Strain N 111, early. Part of a culture growing on a glass coverslip. This illustrates the early outgrowth, after subculture, from a culture that had been exposed a number of times to nitrogen. There is one large cell with two nuclei and one with a large nucleus. Some of the remaining cells are slightly larger than ordinary, some have lost their processes, and others have shortened ones. Hematoxylin and eosin. × 180.

FIG. 6. Strain N 111, slightly later stage. Part of a culture growing on a glass coverslip. Early outgrowth, with changes more pronounced than in Fig. 5, showing some unusually large and some multinucleated cells. The nuclei vary in size and shape. There are still some small spindle-shaped nuclei and long processes, and the reticular structure is still evident. Hematoxylin and eosin. × 180.

FIGS. 7 and 8. Late stage of strain N 111; part of culture growing on glass coverslip. The architecture of the tissue is greatly altered. Many of the processes have disappeared and, in Fig. 8, the sheet of cells resembles epithelium growing *in vitro*. Hematoxylin and eosin. × 180.

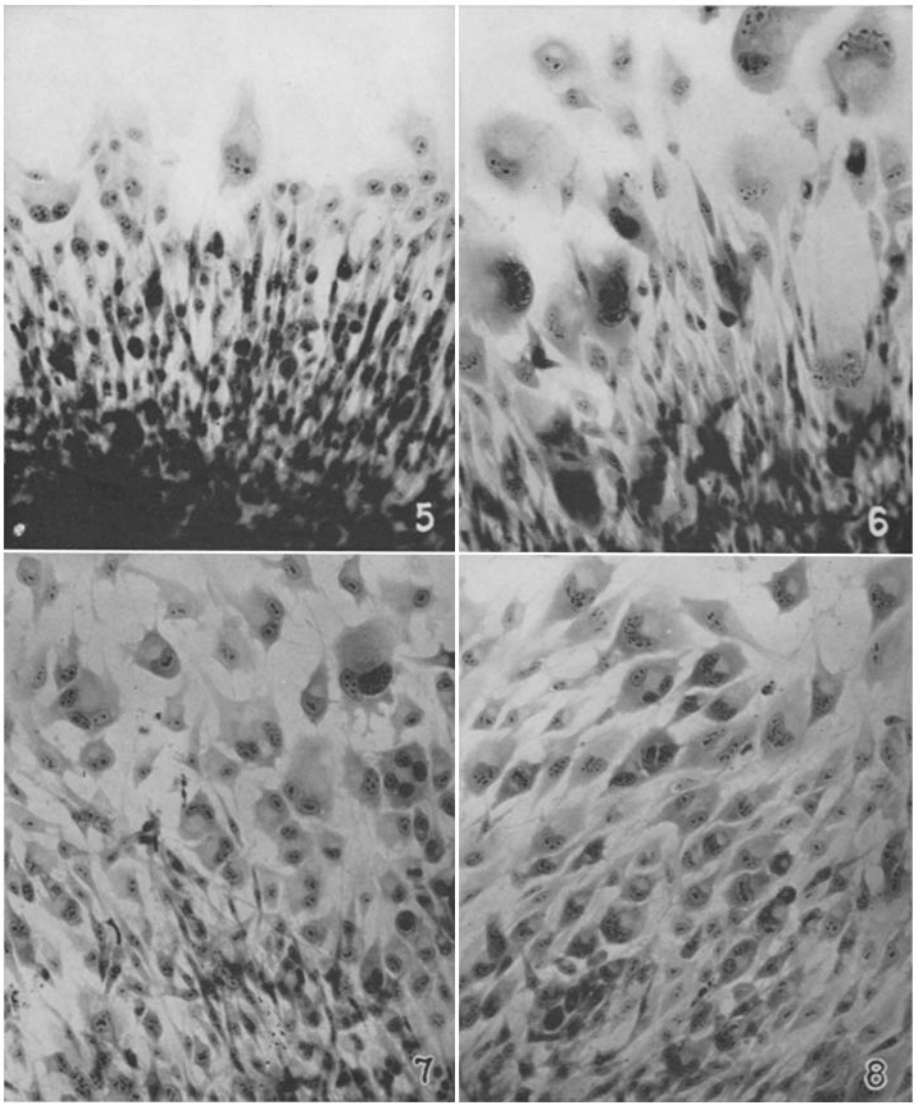


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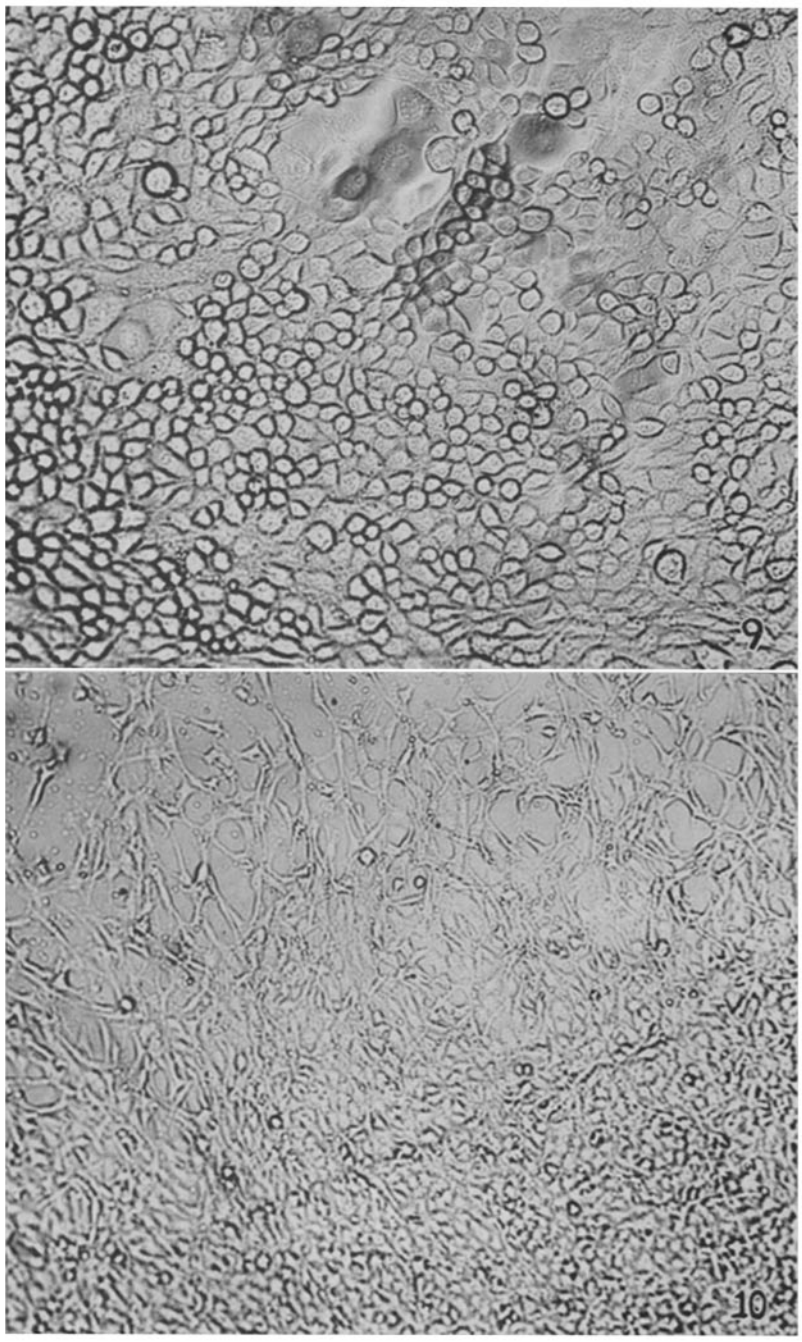
PLATE 23

FIG. 9. Strain N 111, late stage. Part of unstained culture, growing in Porter flask about 15 months after the first exposure to nitrogen. It shows large round or polygonal cells in a sheet. Loss of the processes and cohesion have together resulted in this change, which makes the tissue look like epithelium growing *in vitro*. There is an occasional, unusually large cell with one or more relatively large nuclei not clearly seen in the picture. Mitoses of various, abnormal sorts were also present. × 180.

FIG. 10. Unstained culture of the control strain of fibroblasts, growing in a Porter flask about 1 ½ years after the beginning of culture. This still shows the normal appearance, resembling that of the stained specimens of Figs. 1 to 4 inclusive. × 70.



(Goldblatt and Cameron: Induced malignancy in cells from myocardium)

PLATE 24

FIG. 11. Section of culture of strain N 111, growing on plasma, in roller tube; early stage of alteration, after exposure to nitrogen. The single cells and small groups of cells, growing in a single layer, are larger than ordinary, with relatively large hyperchromatic nuclei. There are some abnormal mitoses, but they are not perceptible in the figure. Hematoxylin and eosin. × 180.

FIG. 12. Sections of culture of strain N 111, growing on plasma, in roller tube; later stage of alteration, after further exposure to nitrogen. Abnormal cells in increasing numbers are growing on and into the plasma. The cells and nuclei vary considerably in size and shape, and many are hyperchromatic. Mitoses and abnormal nuclear forms of various kinds were present but cannot be clearly seen at the magnification given. Hematoxylin and eosin. × 180.

FIGS. 13 and 14. Sections of strain N 111, growing on plasma in roller tube; late stage of alteration, after exposure to nitrogen. There are some large cells with one or more large nuclei, and great variation in size and shape of all the cells and nuclei, many of the latter being deeply hyperchromatic. Mitoses and abnormal nuclear forms, like those illustrated in Figs. 15 to 19, were also abundant. Hematoxylin and eosin. × 480.

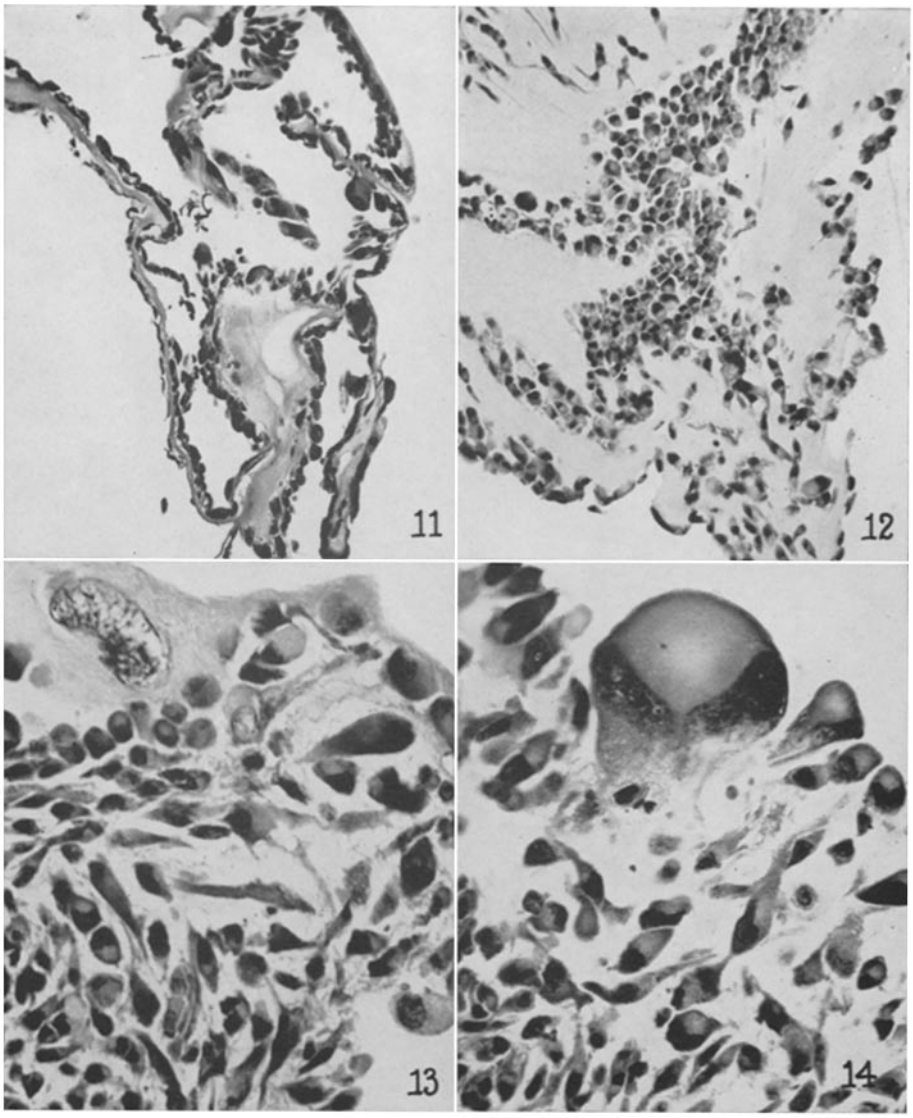


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PLATE 25

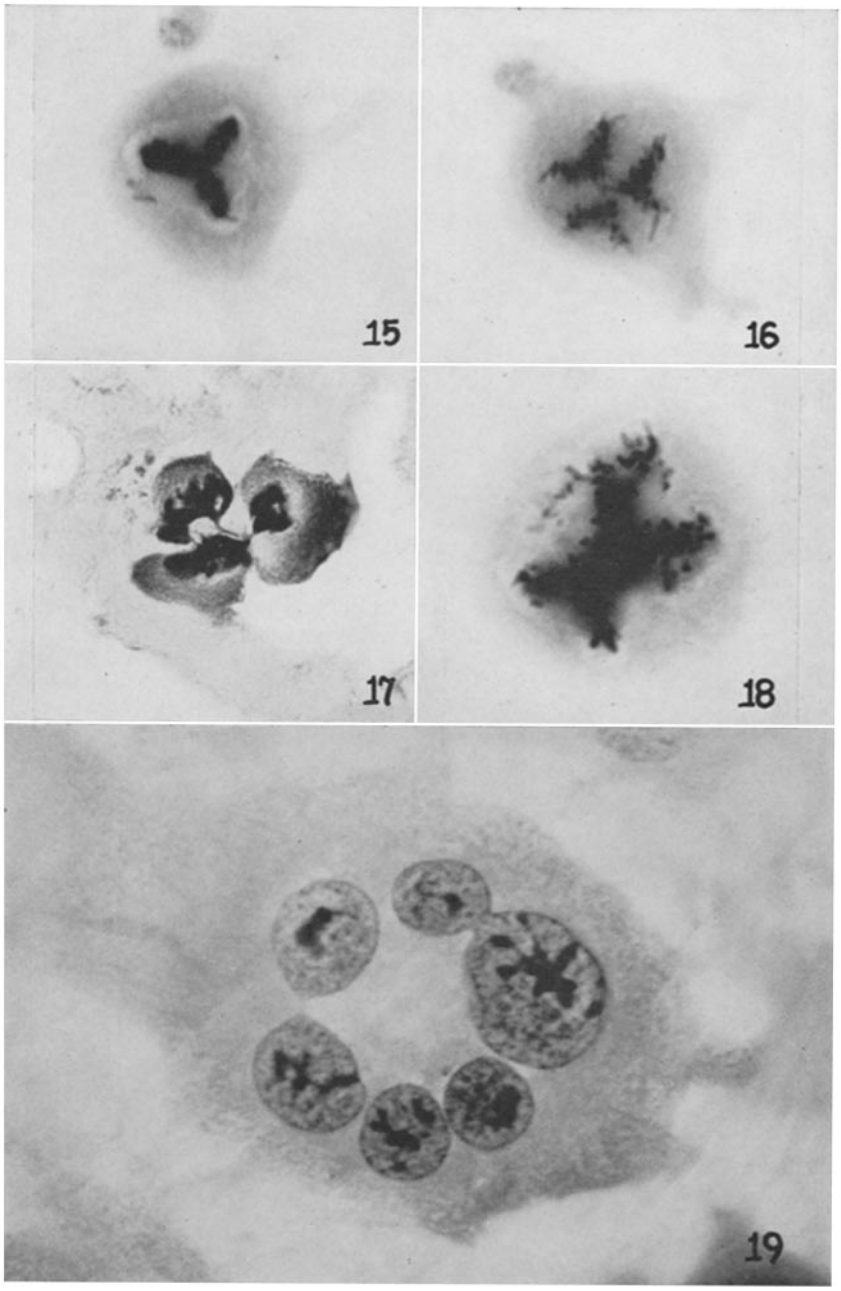
FIG. 15. Cell with tripolar mitosis, early stage. Hematoxylin and eosin. × 900.

FIG. 16. Cell with tripolar mitosis, later stage. Hematoxylin and eosin. × 900.

FIG. 17. Cell with tripolar mitosis in late stage, with beginning division of the cytoplasm. Hematoxylin and eosin. × 900.

FIG. 18. Cell with tetrapolar mitosis, in early stage. Hematoxylin and eosin. × 900.

FIG. 19. Large multinucleated cell exhibiting anisonucleosis, with masses of chromatin of variable size in the nuclei. Hematoxylin and eosin. × 1080.



(Goldblatt and Cameron: Induced malignancy in cells from myocardium)

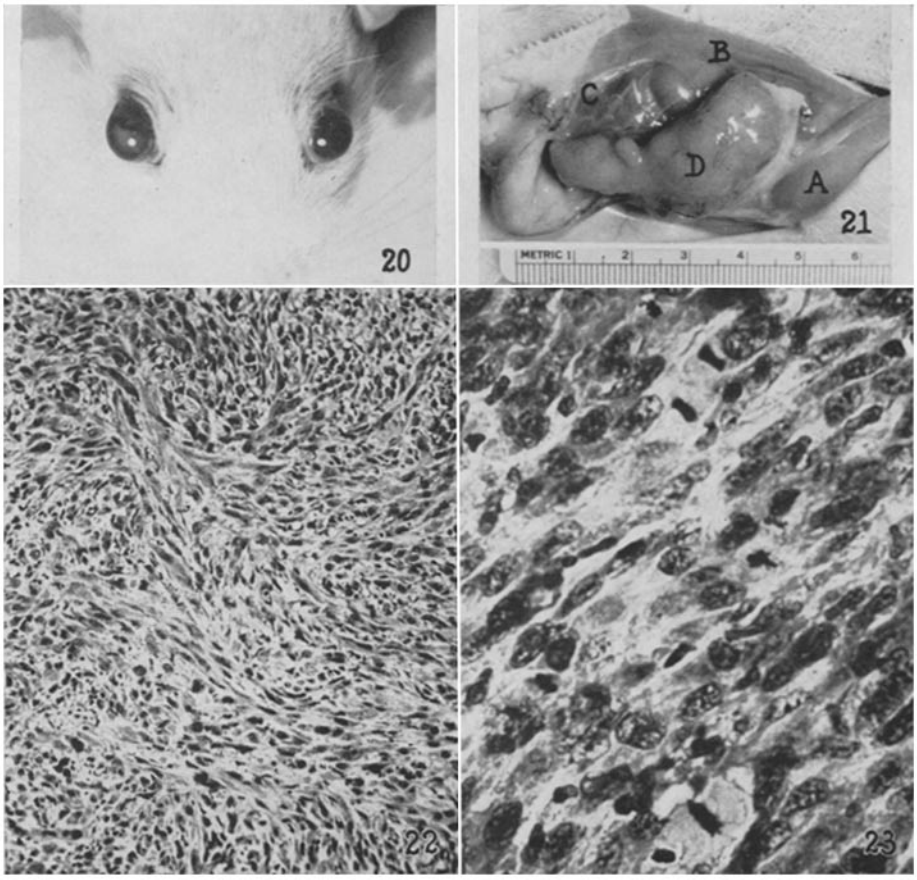
PLATE 26

FIG. 20. Small growth in the anterior chamber of the right eye of rat 96. × 1.5. (See Text-fig. 6.)

FIG. 21. Subcutaneous tumor in the right flank of rat 384, of the Slonaker-Addis strain. The tumor (*D*) resulted from the implantation of several fragments of the subcutaneous tumor of rat 369 of the "Irish" strain, and resembled in all respects the original tumor in rat 369, which was not photographed. The subcutaneous implantation of the fragments of tumor into rat 384 were made without the addition of embryonic rat lung, whereas the subcutaneous implantation of the culture of abnormal fibroblasts of strain N 120 into rat 369 was made with the addition of such fragments from the lung of a 10 day old rat embryo of the Slonaker-Addis strain. *A*, upper part of right thigh; *B*, anterior abdominal wall; *C*, subcostal margin, right side; *D*, tumor in right flank.

FIG. 22. Section of subcutaneous nodule of rat 384. The nuclei vary in size and shape and many are hyperchromatic; typical fibrosarcoma. Hematoxylin and eosin. × 180.

FIG. 23. Same as Fig. 22. Four mitoses, two abnormal, in a small portion of a single high power field. × 480.



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