THE EFFECTS OF X-RAY IRRADIATION ON LIVING CARCINOMA AND SARCOMA CELLS IN TISSUE CULTURES IN VITRO

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During the years which have passed since the method of tissue culture in vitro was commenced by Harrison, and modified by Carrel and Burrows (4), very little attention has been paid the investigation of the biological effects of x-rays upon cells growing in culture in vitro.

Contamin (7) exposed mouse carcinoma to x-rays under various experimental conditions. From the result of a number of his observations, he came to the conclusion that the younger the tumors, the more sensitive they were to x-rays, and that the disappearance of a large tumor under x-rays caused the death of the animal, probably by intoxication. In other experiments made by Contamin (18) with Nogier and Jaubert de Beaujeu, extirpated mouse carcinoma was exposed to x-rays and then inoculated into normal mice. They concluded that the action of x-rays on extirpated tumor cells hinders their subsequent growth in the animal body.

Clunet (5) and Raulot-Lapointe treated malignant tumors in situ with x-rays and studied them histologically at various stages of the treatment. They found that the squamous carcinoma cells in the human subject finally disappeared, passing through five successive stages from the latent phase to the formation of the connective tissue scar. With sarcomatous growths, Clunet and Raulot-Lapointe found that the latent phase was much shorter than in the other types of malignant growths.

1The author has not read the proof of this article.

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Wedd and Russ (31) reported a series of experiments in which a transplantable mouse carcinoma was removed from the animal in which it had grown, kept between mica sheets during the exposure to radium rays, and then inoculated into normal mice. It was found that no growth resulted from grafts which were exposed a sufficient length of time.

Russ and H. Chambers (25) reported an observation made with Jensen's sarcoma. The authors concluded that the tumor cells irradiated by $\beta$-rays of radium (1.63 mgm. per square centimeter for ninety-six minutes) or radium emanation of 0.53 millicuries per cubic centimeter for forty-five minutes, did not produce tumors after inoculation into normal mice, though they showed no histological changes in the cells.

Wassermann (30) exposed extirpated cancerous tissue to radium rays from mesothorium, and then inoculated it into animals, no tumor resulting. He supposed that the multiplication and cell division were affected by the rays while the nutrition of the cells remained uninfluenced, though he did not try cultures in vitro.

C. Price Jones (12) exposed mouse carcinoma and mouse sarcoma to radium rays, made tissue cultures of them, and found that the mitotic division of cells was inhibited, though their spreading growth was not retarded.

Prime (23) has recently worked in the same direction, reporting that a carefully measured amount of radium rays injured the power of mitotic cell division in the tissue, and that the inoculation of a sufficiently irradiated tumor produced no growths in mice.

In all these articles, however, no observations seem to have been made on the effects of x-rays upon living cells growing in plasma outside of the body. The present experiments were undertaken, therefore, to clear up the question of the biological effect of x-rays on tumor cells, and to determine what dose of x-rays would render tumor cells incapable of producing tumors when subsequently inoculated into animals.
PREPARATION OF THE CULTURE MEDIA

A small glass tube (A in text-fig. 1) was coated thickly with paraffin and inserted into an aluminum centrifuge tube (B in text-fig. 1) stopped by means of a cork. The space between tubes A and B was filled with ice and salt mixture, and the whole apparatus kept in a glass with ice mixture until ready for use.
Under ether anesthesia the carotid artery of a guinea-pig was exposed about 2 to 3 cm. The distal part was ligatured by a thread while the proximal part was fixed by a small artery clamp to stop the blood stream. Proximal to the ligature the artery was pinched by a small clamp without narrowing the lumen.

Between this and the ligature the artery was cut off sharply. After the proximal clamp had been released, the blood was allowed to flow directly into the tube A, which was kept cool in ice mixture. When the blood was filled up to one-third the height of the tube A, the artery was clamped again. The tube with blood was promptly and powerfully centrifugalized for three minutes. The supernatant plasma (text-fig. 1) was separated by a small pipette into four or five small tubes, which were coated thickly with paraffin and prepared in ice mixture. It was necessary neither to keep the pipette cool nor to coat it with paraffin. These tubes which held the plasma were plugged with sterilized cotton and covered with tinfoil in order to protect them from contamination or drying, and preserved in a frozen condition in a Universal jar filled with ice mixture. The greatest difficulties encountered were obtaining a sufficient amount of mouse blood and keeping the plasma from coagulating. The mouse was prepared by the removal of the hair from the throat region, and the skin rendered sterile by iodin. The skin over the thyroid region was picked up with a forceps and cut off with scissors. The thyroid gland was picked up and bluntly loosened from the under layer without injuring the tissues. The carotid artery appeared on both sides in the bottom of the space made by picking up the gland. After the artery had been cut, the escaping blood was collected in the paraffin coated tube (text fig. 1) in ice mixture. In the same manner as described already, the tube was immediately centrifugalized for three minutes. The supernatant plasma was drawn up and put into cold tubes in ice mixture.

It was found very easy to obtain chicken plasma. A heavy syringe needle, sterilized by boiling, was inserted directly into the wing vein and the blood was collected drop by drop in an ice cold
paraffin coated tube, and centrifugalized in the same way as above. The supernatant plasma was drawn off into tubes.

The plasma thus obtained from guinea-pigs, mice, and chickens, did not lose its coagulability for about ten to fourteen days as it was kept in ice mixture, and remained fluid for hours during each series of experiments, when it was kept cool in ice, and at no time did coagulation take place before the completion of the experiment. One tube containing 0.2 to 0.3 cc. of plasma was enough for each series of experiments.

MATERIAL AND TECHNIQUE OF CULTURE

The tumors used were mouse carcinoma R.T. 33, which had been propagated for three years at the State Institute for the Study of Malignant Disease, Buffalo, New York. These tumors, for which I am indebted to Dr. Gaylord, showed a type of adenocarcinoma (fig. 2) and with the mice which I had inoculated gave "takes" in about 90 per cent of implants. Another tumor, for which I am indebted to Dr. F. C. Wood of the Crocker Fund, was the Ehrlich mouse sarcoma (fig. 3), which had been under observation for some years in the Crocker Laboratory in New York, and gave approximately 100 per cent of successful inoculations in Chicago mice.

The tumors, after reaching a full growth, were cut out strictly aseptically in Ringer's solution and cut into many pieces of equal size. They were then put into three or four small sterilized glass tubes with a diameter of 1 cc. and 1.5 cc. in depth. Each of these glass tubes was stopped with a cork pushed in over a sheet of sterile Japanese paper in order to prevent any contamination that might occur during the exposure of the x-rays. After the removal of the cork stopper, the pieces of tumor, which were kept in these tubes with Ringer's solution, were exposed to x-rays merely through the layer of Japanese paper at a distance of 2 cm. from the x-ray bulb. In this manner each piece of tumor was exposed for a certain required length of time without other obstacles between the bulb and the tumor pieces. Another factor in favor of this method was the fact that the tissues did
not begin to grow while exposed to the ray as was found to be the case when they were planted first into the plasma and then exposed.

The ray used for this series of experiments was of a moderately soft type, the spark gaps ranging from 4 to 8 cm.; the length of time for exposure varied from five to thirty-six minutes. The amount of the effective ray was measured by Hampson's radiometer. The initial tint is the color of the unexposed pastille and the sixteenth change represents the browner shade of color, equivalent to the maximum or B tint of the Sabouraud's pastille. The terms E.1, E.4, E.8, or E.12 in the following experiments indicate that the Hampson's pastille used showed no. 1, no. 4, no. 8 or no. 12 tint, that is, equivalent to a dose $\frac{1}{16}$, $\frac{1}{4}$, $\frac{1}{2}$, or $\frac{3}{4}$ of Sabouraud's B tint.

The irradiated tissue was removed to a watch glass with Ringer's solution and was cut into fine fragments. Each fragment was transferred to a cover glass by a small pipette provided with suction bulb. The excess Ringer's solution was sucked up with the same pipette. Some small drops of plasma were added immediately to the tissue fragment, then some drops of mouse serum with Ringer's solution were added. The plasma and serum were mixed up and spread around the fragment with a pointed cataract knife. The cover slip, which previously was ringed with vaseline on all its edges, was then inverted over a fairly deep hollow ground slide. The cover slip was then sealed with molten paraffin around its edges. An equal number of control cultures from an irradiated tissue was made in each series. The slide preparations were incubated at $37^\circ$C. and microscopic observation was made every twelve to twenty-four hours.

To obtain the stained specimen the cover slips, mounted with growing culture, were put into 10 per cent formalin for twenty-four hours or more, then they were washed in water for one hour and stained with diluted Delafield's haematoxylin for one to two hours, and decolorized for about thirty minutes in water, to which a few drops of diluted hydrochloric acid was added. The specimens were washed again in water until a violet blue color developed. It is important to decolorize the over-stained plasma and
tissue as much as possible, otherwise no good specimen was obtained.

The tissue piece irradiated with certain required doses of x-ray in Ringer's solution was divided into many fragments, each of about 20 mgm., and these were inoculated into the right axilla of a number of normal mice. An equal number of tissue fragments from the control tissue were inoculated into the other axilla of the same mice. The observations respecting the rate and course of the grafts planted into the animals follows.

The culture media used for the experiments were: Mouse plasma, guinea-pig plasma, chicken plasma, guinea-pig plasma plus mouse serum diluted with Ringer's fluid, chicken plasma plus mouse serum diluted with Ringer's fluid.

In comparing all these media used for the culture of the tumor, it was found that the mouse plasma (homogenous and autogenous) or a mixture of guinea-pig plasma and mouse serum diluted with Ringer's solution were most satisfactory as media for the culture of the tumors. There was no noticeable difference in either case, whether the mouse plasma or the guinea-pig plasma with diluted mouse serum was used. Because the guinea-pig plasma can be obtained much more easily and in greater quantity than mouse plasma, most of the cultures, except a few specimens, were made with guinea-pig plasma, to which was added the mouse serum and Ringer's solution.

RESULTS OF EXPERIMENTS

Mouse carcinoma

Mouse carcinoma, cultivated under the conditions described above in respect to technique, showed but very little growth the first twenty-four hours in most cases of all series, both in the control and in the exposed tissue. After twenty-four hours of incubation, the original fragment of tissue became thinner and somewhat translucent, especially on the edge of the fragments, from which the cells spread out into the plasma media. The cells did not migrate separately into the media, but merely formed cell groups composed of a number of cells. The boundaries of each cell were indistinct.
Many of the cells on the advancing edge formed pseudopodia. There were many cells of different morphological type which supposedly originated from the stroma. Cells of this type were present in some specimens, while others showed no such type of cells.

Most cultures reached the maximum growth in forty-eight to ninety-six hours after incubation. Karyokinetic figures were observed in the cells in the growing zone in stained specimens from the control tissue (fig. 4). The specimen from the tumor piece exposed to E.1 of rays, showed no difference from the control in growth in the plasma media during the whole observation, and mitotic figures of growing cells were seen frequently in the stained preparations in all stages. Both the tissues, control and irradiated, produced good sized tumors after inoculation into normal mice.
In cultures from the tissue exposed to E.4 of the ray, the proliferation of cells was just as extensive as in the specimen made from control tissue (figs. 9, 10), while the spreading-out of the thread-like cells, which supposedly originated from stroma, showed itself a little more active than in the control. The mitotic figures were found as abundant as in control cultures (fig. 5).

No difference was seen in speed and rate of growing tumors, produced by inoculation of either the treated or the control piece, as is shown by the preceding chart (text-fig. 6) and by table 1.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<td>Carcinoma</td>
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<th>NUMBER OF &quot;TAKES&quot;</th>
<th>PER CENT</th>
<th>NUMBER OF &quot;TAKES&quot;</th>
<th>PER CENT</th>
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<tr>
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<td>Control</td>
<td>E.8</td>
<td>Control</td>
<td>E.12</td>
<td>Control</td>
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<td>19</td>
<td>100</td>
<td>3</td>
<td>19</td>
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<tr>
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<td>16</td>
<td>100</td>
<td>8</td>
<td>18</td>
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<td>Third week</td>
<td>10</td>
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<td>100</td>
<td>6</td>
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</table>

In the unstained culture, when E.8 doses of x-ray were given, the proliferation of cells growing in groups showed almost the same extension as did the specimen of control tissue (fig. 11). In some specimens, however, the cells of the stroma type, which spread out like fibrillae into the plasma media, seemed more vigorous than those in the control specimens. But in the stained specimen a few mitotic figures were found in most of the specimens cultivated from the irradiated tissue (fig. 7), while many more mitotic figures appeared in the control specimen (fig. 4). After the inoculation into mice, it was found that the treated tissue gave "takes" of 16 per cent in the first, 44.5 per cent in the second, and 60 per cent in the third week, according to table 1, while the control tissue produced tumors in 100 per cent of the grafts.

All the tumors grown in mice from the treated cells, however, reached only one-third of the size of those from the control inoculations.
In the cultures of the tissue which was exposed to E.12 of the active ray, it was found that after forty-eight hours there was still a marked outgrowth of cells (fig. 12). The same results were found in the control specimen. None of the stained specimens of the exposed tissues showed mitotic figures in growing cells (fig. 8).

The inoculation of the treated tissue into mice produced in the first week no tumor in 27 cases in 6 series. In the second and third weeks two nodules were developed to the size of a rice grain; they, however, did not grow further, but disappeared.

**Mouse sarcoma**

In the cultures made in guinea-pig plasma diluted with mouse serum, the margin of the fragment became gradually sharp and opaque. In five to twelve hours a few round cells had emigrated into the plasma media and a few irregular cells began to grow out radially from the edges of the fragment, which began to have a serrated appearance. The individual cells formed many pseudopodia, which were seen especially in the advancing side of the cell body. The number of cells emigrating into the media increased more rapidly than those in the culture of carcinoma tissue. After forty-eight hours the original fragment became more translucent than it was before, and it was surrounded with thick layers of growing cells, which were rich in protoplasm. When the stained specimens were fixed, at the end of twenty-four to seventy-two hours, they exhibited the presence of numerous mitotic figures in the outgrowing cells (fig. 13).

The specimen from the tissue exposed to E.1 of x-ray showed no difference in respect to the rate of growth from that of the control tissue (fig. 17). The inoculation of the treated piece into mice produced tumors as large as those of the control tissue.

In the culture of the tissue exposed to E.4, the proliferation of cells was found to be more vigorous than that in the control cultures (fig. 18). In the stained preparations, however, the number of mitotic figures of cells was about equal to that in the control specimen (fig. 14). After inoculation into mice, the
tumors produced from irradiated cells in the first week seemed to grow a little faster than those in controls; but in the second and the third week there was no difference either in speed or size, and the inoculation gave "takes" of 100 per cent.

In the unstained cultures from the tissue which was exposed to E.8, the proliferation of cells and the area of growth were similar to the proliferation in the control specimen (fig. 19). But the mitotic figures in the stained specimen diminished greatly in number compared with those in the control specimen (fig. 15). In the first week after inoculation, the grafts gave "takes" of 35 per cent, 62 per cent in the second, and 85.7 per cent in the third week. These tumors, however, developed only to one-third the size of those in the controls (text-fig. 6).

<table>
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<table>
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<th></th>
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<td>100</td>
<td>6</td>
<td>7</td>
<td>85.7</td>
</tr>
</tbody>
</table>

In the cultures made from the tissue which had been irradiated to E.12, it was found that there was still a marked outgrowth of cells as well as in the control specimen (fig. 20). No mitotic figures, however, were found in the stained specimen (fig. 16). None of the inoculated grafts into 23 mice in 5 series produced any tumor the first week. In the second week there were found 4 hardly perceptible nodules in 21 mice. These, however, did not grow further and disappeared entirely in the third week, as is shown in text-fig. 6 and table 2.

**DISCUSSION**

In accordance with the results of the experiments described above, the rate of the cell growth in cultures of mouse carcinoma and mouse sarcoma was, after forty-eight to ninety-six hours'
incubation, nearly equal in both the control and the exposed
tissues which were x-rayed to E.1–E.12 (figs. 9, 10, 11, 12, 17, 18,
19, 20). The sarcoma, as compared with the carcinoma, however,
was always superior in its growth in culture media. After an
exposure of E.8, the number of the mitotic figures in the culture
of growing tumor cells was diminished to a minimum of 2 to 4 in
carcinoma and 2 to 13 in sarcoma cultures. An exposure, how-
ever, of E.12, entirely inhibited the mitotic division of cells, and
they were never found in the stained specimen, either in carci-
noma or in sarcoma (figs. 8, 16). See table 3.

By an exposure of E.4, the sarcoma not only remained without
injury to the power of proliferation by mitotic cell division, but
the exposed tissue produced tumors in the first week after inocu-
lation —i.e., somewhat earlier than the control tissue did (text-
fig. 6). This phenomenon was due to the action of x-rays, to
which the tissue was exposed. It seems that the x-ray in this
dose acted upon the tissue as a stimulation and temporarily
raised the metabolism of the tumor cells. Consequently the
cells in the exposed tissue were stimulated to grow more quickly
the first week after inoculation than those in the control.

In connection with the process of oxidation in the tumor tissue,
I tried some experiments hoping that the effects of x-ray on the
living cells might be explained to some extent. The tumor
pieces were exposed to x-ray of various doses varying from E.4 to
E.12. One piece of control was put in the chamber of one side
of Dr. Tashiro's biometer and an irradiated piece in the other
chamber of the other side.

Observation was made as to the quantitative difference of CO₂
production in both chambers. The results of these experiments
are shown in the record of tables 4 and 5.

In the experiments with the tumor pieces, both in carcinoma
and sarcoma, which were exposed to E.4 of x-ray, carbon dioxide
production began to appear in ten minutes after the arrangement.
The quantity of precipitation by barium hydroxide in the chamber
which contained the exposed tissue was greater than that in the
chamber which had the control tissue.
### Table 3

<table>
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<th>SPECIMEN</th>
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<th>AMOUNT OF X-RAY</th>
<th>LENGTH OF TIME</th>
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<td>Growth in culture</td>
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<td>53</td>
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On the contrary, in the experiments made with the tissue exposed to E.12 of x-ray, the production of carbon dioxide was less than that of the control.

In the unstained specimen cultured from tissue which was exposed to E.4, the outgrowth of cells seemed somewhat more extensive than the outgrowth of the control culture, both in sarcoma and carcinoma. But in the stained specimens no noteworthy difference in number of the mitotic figures was seen. This stimulating effect on the sarcoma tissue was more prominent than the effect shown in the culture of the carcinoma. A some-
what similar difference of the stimulating effect of x-ray E.4 on the tissues appeared prominently in the inoculation experiments of sarcoma, but the carcinoma was not much stimulated by the rays and consequently the power of proliferation was diminished.

**TABLE 5**

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<th>CO₂ compared</th>
<th>E.12</th>
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<th>CO₂ compared</th>
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<td>3.25</td>
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<tr>
<td>22 mgm.</td>
<td>16 mgm.</td>
<td></td>
<td>4.13</td>
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<td>2.36</td>
<td>&lt;</td>
<td></td>
<td>4.23</td>
<td>&gt;</td>
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<tr>
<td>2.46</td>
<td>&lt;</td>
<td></td>
<td>4.33</td>
<td>&gt;</td>
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<tr>
<td>2.56</td>
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<td>5.13</td>
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<td>48 mgm.</td>
<td>47 mgm.</td>
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<td>85 mgm.</td>
<td>85 mgm.</td>
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<td>3.38</td>
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<td>4.08</td>
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<td>7.03</td>
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<tr>
<td>4.18</td>
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<td>4.28</td>
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<td>+</td>
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* The figures are the weights of the pieces of tumor tissue used in each experiment. No quantitative determination of the amount of CO₂ produced was made, but the relative amounts produced by irradiated tumor and control are indicated by the > sign.

gradually without any preliminary stimulation (text-fig. 6 and table 4).

The fact that the number of mitotic figures in culture and the quickness of growth of the grafts inoculated into mice increased to some extent when the tissues previously were exposed to E.4
of rays, and the fact that both decreased gradually when the tissues were irradiated to E.8–E.12, coincide with the results of the experiments on the oxidation of the tumor tissues.

For the explanation of the presence of only a few mitotic figures in the culture and retardation of the growth of the grafts when the tissue was exposed to E.8, the reports made by Bordier and others are to be taken into consideration. We know that the younger the cell generation the greater the radio-sensibility of the living protoplasm; and consequently the younger neoplastic cells are most sensitive, while the cells in the latent stage are less sensitive to the x-ray action. Hence the mitotic figures seen in the tissues that had been exposed to E.8 supposedly originated from some resting cells in the fragments, in which they remained without an intensive effect of x-ray and produced the further division in culture in vitro or developed to a tumor in mice, though their mitotic proliferation was greatly retarded.

On the contrary, all the cells in the specimen exposed to E.12 were sufficiently damaged by the ray action, and consequently the dividing process of the cell chromosomes had ceased, and the fragments did not grow any more to a tumor in mice.

CONCLUSIONS

1. The mouse carcinoma and sarcoma grow as well in guinea-pig plasma to which has been added mouse serum diluted with Ringer’s solution, as in mouse plasma itself.

2. The culture growths of carcinoma and sarcoma from mice showed each the characteristics of the original tissues. Sarcoma produced a radial outgrowth spreading widely into the plasma media, while the carcinoma cells grew continuously into the media, as cell groups, from the edges of the fragments.

3. The outspreading growth of cells in culture, both sarcoma and carcinoma, was not stopped by x-ray action varying from E.4 to E.12. The mitotic figures of cells were limited to a minimum after an exposure of E.8 (one-half dose of Sabouraud’s B tint). After exposure to E.12 (three-fourth dose of Sabouraud’s B tint), however, they disappeared entirely, and the treated tissue produced no tumor when inoculated into mice.
4. The growing power of sarcoma after E.4 exposure was stimulated to some extent, while carcinoma was not appreciably influenced. An exposure of tissues to E.12, both sarcoma and carcinoma, stopped the growing power of these tissues when inoculated into mice, and eliminated the process of mitotic division of cells.

5. The process of oxidation of tissues, both sarcoma and carcinoma, was stimulated by the x-ray action of E.4 and retarded by exposure to E.12 of the ray.

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Fig. 2. Stained section of original carcinoma, many mitotic figures. X 275.
PLATE 2

Fig. 3. Stained section of original sarcoma, many mitotic figures. × 275.
PLATE 3

Fig. 4. Tissue culture, control, carcinoma, 2 mitotic figures. × 275.
PLATE 4

Fig. 5. Tissue culture, experiment 4, carcinoma, 1 mitotic figure. × 275.
PLATE 5

Fig. 7. Tissue culture, experiment 8, carcinoma, 1 mitotic figure. $\times 275$. 
PLATE 6
Fig. 8. Tissue culture, experiment 12, carcinoma, no mitotic figure.  × 275.
PLATE 7

Fig. 9. Tissue culture, control, carcinoma.  $\times 37$.
Fig. 10. Tissue culture, experiment 4, carcinoma.  $\times 37$.
Fig. 11. Tissue culture, experiment 8, carcinoma.  $\times 37$.
Fig. 12. Tissue culture, experiment 12, carcinoma.  $\times 37$. 

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PLATE 7

FIG. 9

FIG. 10

FIG. 11

FIG. 12
PLATE 8

Fig. 13. Tissue culture, control, sarcoma, 2 mitotic figures. \( \times 275 \).
PLATE 9

Fig. 14. Tissue culture, experiment 4, sarcoma, 4 mitotic figures.  × 275.
PLATE 10

Fig. 15. Tissue culture, experiment 8, sarcoma, 1 mitotic figure. $\times 275$. 
PLATE 11

Fig. 16. Tissue culture, experiment 12, sarcoma, no mitotic figure. × 275.
PLATE 12

Fig. 17. Tissue culture, control, sarcoma. × 37.
Fig. 18. Tissue culture, experiment 4, sarcoma. × 37.
Fig. 19. Tissue culture, experiment 8, sarcoma. × 37.
Fig 20. Tissue culture, experiment 12, sarcoma. × 37.
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PLATE 12

Fig. 17

Fig. 18

Fig. 19

Fig. 20

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