THE EFFECTS OF ROENTGEN RAYS ON CELL-VIRUS ASSOCIATIONS

Findings with Virus-Induced Rabbit Papillomas and Fibromas

By WILLIAM F. FRIEDEWALD, M.D., and RUBERT S. ANDERSON, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research and Memorial Hospital, New York)

Plates 8 and 9

(Received for publication, May 12, 1943)

The virus-induced papillomas of domestic rabbits (Shope) (1) rapidly regress after exposure to 3,600 r or more of Roentgen rays (2, 3). Much more irradiation is required to inactivate the virus in saline extracts of the highly infectious papillomas of cottontail rabbits (2, 3), although the amount needed can be greatly reduced by purification or dilution of the virus extracts (4). Because of this difference in the resistance of the papilloma cells of domestic rabbits *in vivo* and the virus of cottontail rabbit papillomas *in vitro*, it has been concluded that the x-rays cause papillomas to regress by affecting the cells, not the virus (2, 3).

In the present experiments we have been primarily concerned with the effect of x-ray irradiation on the virus associated with living papilloma cells. In addition to possible quantitative changes in the amount of virus associated with the cells, it seemed possible that a variant of the virus might be produced by irradiation. In the first part of the paper the effect of x-rays on the course of the papillomas of cottontail rabbits and on the virus associated therewith is described. The recent development of a sensitive method for the detection of papilloma virus (5), whereby it can be demonstrated in extracts of nearly all papillomas of domestic rabbits as was previously not the case, has enabled us to study the fate of the virus in the growths of these animals during regression induced by the irradiation. Finally, some comparable experiments on the effect of irradiation on the virus causing another growth—infectious fibromatosis of rabbits (6)—are reported.

Material and Methods

The Papillomas.—Cottontail rabbits with naturally occurring papillomas were obtained from a dealer in Kansas. The growths were irradiated *in vivo* for test or else utilized as a source of virus to produce papillomas experimentally in normal cottontail or domestic rabbits for irradiation experiments. Virus was obtained from the papillomas by grinding weighed portions in sand, suspending in 10 or 20 volumes of 0.9 per cent saline, and centrifuging the extracts at about 4,000 R.P.M. for 10 minutes in an angle head centrifuge. Extracts of cottontail rabbit papillomas were tested for

virus by rubbing them into scarified skin areas of normal domestic rabbits which were protected against contamination thereafter (7). Extracts of the papillomas of domestic rabbits often cause no growths when inoculated in this way but will do so if rubbed into scarified skin areas which have been rendered hyperplastic by four applications of a mixture of turpentine and acetone in equal parts (5), and this was the procedure adopted with them. The growths which arose are recorded in the tables according to a standard scale: ++++ = confluent papillomas; +++ = semiconfluent papillomas; ++ = many discrete growths; + = 5 to 15 papillomas; ± = 2, 3, or 4 papillomas; ± = 1 papilloma; 0 = negative.

Spontaneous or induced papillomas situated on the chest and abdomen were used almost entirely in the irradiation experiments. The rabbits were given a solution of nembutal intraperitoneally for anesthesia during the irradiation. Ordinarily 50 mg. of nembutal in saline per kilogram body weight sufficed for domestic rabbits, but cottontail rabbits usually required 70 to 100 mg. Occasionally it was necessary to repeat the injection before the irradiation was completed, using one-half of the original amount of nembutal.

Irradiation.—Each papilloma was exposed to radiation through a 2.5 or 3.2 cm. circular portal in a lead shield. Except for the tissue directly under the opening in the lead plate the rabbit was protected from the radiation. In a few experiments papillomas were excised and irradiated in a celluloid container. Unfiltered x-rays, H.V.L., 0.19 mm. copper, produced by two water-cooled tubes at about 185 kv. (peak) and 30 ma., were used. The intensity in air at the site of the papilloma, 20 cm. from the target, was 800 r/min. for irradiations *in vivo* and either 800 r/min. or 5,000 r/min. for the growths irradiated *in vitro*. Only in the latter case was the radiation from both tubes used. The amounts of radiation given are those measured in air. The tissue dose did not differ from this by more than 10 per cent.

The Fibromas.—The OA strain of fibroma virus, which was generously provided by Dr. Shope, was used to produce the growths. It consisted of pieces of testicular fibroma tissue resulting from inoculation of the virus into rabbit testicles, and it had been kept in 50 per cent glycerin. A 5 per cent saline extract of the material was injected intratesticularly into a group of normal domestic rabbits. Six days after inoculation the large, firm, fibromatous growths were removed, cut into small pieces, and kept frozen at about −64°C. Small portions of the material thus preserved were removed as required for the individual experiments and extracted by grinding in sand and suspending in 0.9 per cent saline to make 5 or 10 per cent suspensions by weight. The extracts were centrifuged at about 2,500 R.P.M. for 10 minutes, and 0.1 cc. of the turbid, supernatant fluid was injected intradermally into normal domestic rabbits with non-pigmented skin, eight injections usually, on either side of the abdomen. Swellings at the injection sites ordinarily appeared in 2 to 3 days and had developed into firm, ruddy, sharply defined hemispherical nodules by the 6th to 8th day. Then they ceased to enlarge and frequently became capped with a broad vesicle and underwent central pressure necrosis later. Thereafter the growths gradually regressed and usually had disappeared within 3 weeks of the inoculation.

The intracutaneous fibroma nodules were exposed to radiation under precisely the same conditions as the rabbit papillomas. In most of the experiments, growths of 4 to 6 days' duration were used. Following irradiation they were removed and 5 or 10

per cent saline extracts prepared as described above. These were tested for virus by inoculating 0.1 cc. of serial tenfold dilutions of the extracts intradermally, as indicated in the tables, into each one of a group of four normal domestic rabbits. The inoculations were done in duplicate on each test rabbit and as many as ten different extracts were tested concurrently. The number and size of the resulting growths were recorded at daily intervals until they ceased to enlarge. It was found that the size, incubation period, and the highest dilution of the extract causing growths, were all proportional to the amount of virus in the inoculum. The dilution end-point proved to be most convenient for comparing the virus content of different fibroma extracts. The results of the tests are recorded in the tables, but to conserve space the readings of only 1 day are given, these being representative of the findings as a whole.

Regression of Irradiated Cottontail Rabbit Papillomas

In testing the effects of the Roentgen rays on papillomas previous workers (2, 3) have used growths produced with the virus in domestic rabbits. Cottontail rabbit papillomas have the advantage that virus can be readily recovered from them in large amount, as not in the case of domestic rabbits. Cottontail rabbits, however, are more difficult to handle, and Syverton (3) has reported that their tissues will not withstand the amount of irradiation required to eradicate the papillomas. We found that if the rabbits had been given an anesthetic and small, discrete papillomas on the abdomen were used with the protection above described no complications were encountered.

Some papillomas were exposed to 5,000 r of x-ray irradiation as described under Material and Methods. Animals which carried multiple, naturally occurring growths were chosen by preference and usually several on the same rabbit were x-rayed and removed after different periods for microscopic study. Comparable unirradiated papillomas from the same host were removed at the same time for comparison. Representative blocks of the growths were fixed in acid Zenker fluid and stained with eosin and methylene blue.

Microscopic sections of the irradiated papillomas showed little or no change from the control growths until 2 to 4 days had elapsed. Then there was perceptible a thinning of the basal layer of living cells and a corresponding increase in the keratinized material (Figs. 5 and 6). Apparently there had been a cessation of proliferation, but with continuing differentiation of the cells, accompanied by pathological changes in them. Some of the individual cells became greatly swollen and took on a ballooned appearance, many had giant nuclei or became multinucleated (Figs. 1 and 2), but most of them succeeded in keratinizing and the general architecture of the papillomas was preserved. No reaction occurred around the growths and in the gross it was often difficult to distinguish irradiated from unirradiated papillomas. Within *8 to 10 days* though the irradiated growths, instead of enlarging or remaining stationary like the controls, had decreased to about two-thirds of their original size, and the entire mass was now dry and brittle. Microscopically the growths had still a shallow basal layer of living, highly pathological cells beneath the dead, keratinized material, the proportion of which to living epidermal tissue was greatly increased (Figs. 3 and 4). Round

cells and polymorphonuclear leucocytes were now present in the underlying connective tissue.

After *15 to 18 days* the irradiated papillomas consisted of a much smaller, dry, keratinized mass, with a few highly abnormal cells remaining at the base. At about this time the growths became detached from the skin surface or flaked away, leaving a smooth, pink scar covered with epidermis. They did not recur. Unirradiated papillomas of the same animals remained unchanged both in the gross and histologically. Some of the rabbits which had five or more papillomas irradiated died between the 10th and the 15th day whereas those with fewer irradiated growths survived and showed no sign of irradiation sickness.

The findings show that the virus-induced papillomas of cottontail rabbits will regress completely after exposure to 5,000 r of x-ray irradiation. It has already been reported that this amount of irradiation (2), or even 3,600 r (3), causes the papillomas of domestic rabbits to regress. We have found the histological changes during regression in the papillomas of the two species to be similar, except that the process occurs faster and regression is completed sooner in the domestic rabbit under comparable conditions, as will be shown further on.

Recoverability of Virus during Regression of Irradiated Papillomas

Experiments were now undertaken to see what effect radiation, in amount sufficient to cause virus-induced papillomas to regress, might have on the virus associated with the papilloma cells. In the following experiment the quantity of virus obtainable from papillomas of cottontail rabbits at various stages of regression was determined.

Experiment 2.—A 10 per cent saline extract of glycerolated, naturally occurring cottontail papillomas (W.R. 2-95) was inoculated into twelve spots about 0.5 cm. across on the chest and abdomen of a normal cottontail rabbit (W.R. 54) by means of a tattoo machine. Discrete, characteristic papillomas appeared within 2 weeks. When the growths were about 4 months old, four of them were exposed individually to 5,000 r of x-ray irradiation, and four comparable papillomas were not irradiated. One of each lot was removed 1, 5, 10 and 15 days after irradiation. When this was done a representative block of each was taken for microscopic study and the remainder was kept frozen at −72°C. until all had been assembled. 10 per cent saline extracts of all were prepared, and after dilution to 1:100 with saline (in terms of weight of papilloma tissue extracted) they were rubbed into sacrified areas of four normal domestic rabbits.

Table I shows the results of the experiment. The control papillomas removed at various times all yielded about the same amount of virus, as evidenced by length of the incubation period and number of growths elicited by the extracts. Comparable amounts were obtained from the papillomas taken 5, 10, and 15 days after irradiation. The papilloma removed 24 hours after

irradiation, however, yielded much more virus than did any of the other materials. Microscopic sections of the x-rayed papillomas taken on the 1st day did not differ from the controls whereas on the 5th day the characteristic changes already described were apparent. By the 10th and 15th days regression was far advanced and the growths consisted largely of dead keratinized material (Fig. 4). In another experiment, using papillomas from a different cottontail rabbit, produced by inoculation with the same virus strain, precisely

TABLE I

Recoverability of Virus from Cottontail Rabbit Papillomas during Regression Induced by X-Rays

Time from irradiation to removal of papillomas	X-ray irradiation	Pathogenicity tests											
		14th day				20th day				35th day			
		a	b	c	d	a	b	c	d	a	b	c	d
1 day	Control	0	±	±	0	+	++	+	+±	++	++±	+±	++
	5,000 r	+±	+++	++	±	+++	+++±	+++	+++±	++++	++++	++++	+++±
5 days	Control	0	0	0	0	+	±	+	±	+±	+	+±	+
	5,000 r	0	0	±	0	±	+	+	+	+	+	+±	+±
10 days	Control	±	0	0	±	±	+±	+	+	+	++	+±	+
	5,000 r	0	0	0	0	±	±	+	±	+	+±	++	+
15 days	Control	±	±	0	+	+±	+	+±	+±	++	+±	++	++
	5,000 r	0	0	0	±	±	±	+	+	+	±	+	+±

1 per cent saline extracts of papillomas inoculated into normal scarified skin areas of four domestic rabbits a, b, c, d.

++++ = confluent papillomas.
+++ = semiconfluent papillomas.
++ = many discrete growths.
+ = 5 to 15 papillomas.
± = 2,3,or 4 papillomas.
± = one papilloma.
0 = negative.

similar findings were obtained, but in a third no increased yield of virus was obtained 24 hours after irradiation, the amount recovered remaining the same as in all the controls.

Experiments were now begun to test the recoverability of virus from domestic rabbit papillomas during regression induced by irradiation. The papillomas were exposed to 5,000 r, for comparison with the experiments on cottontail rabbit papillomas (Table I), but they were extracted at somewhat different times thereafter.

Experiment 3.—With a view to obtaining growths of the same size a 10 per cent saline extract of the glycerolated papillomas of W.R. 1-27 was inoculated into sixteen

small abdominal skin areas of three normal domestic rabbits by means of a tattoo machine. Four other domestic rabbits were similarly inoculated with another virus extract (W.R.A). About 7 weeks later one rabbit from each group, carrying multiple growths that were closely alike in size and form, was selected for test. The papillomas were then discrete hassocks about 1 cm. across at the base and 5 to 8 mm. high. Under nembutal anesthesia seven on each rabbit were exposed to 5,000 r of radiation, while seven comparable growths were left unirradiated. Two papillomas from each rabbit were removed just before irradiation, and pooled. Two irradiated papillomas and two control growths were likewise removed and pooled 1 day, 2 days, and 4 days after irradiation, and on each occasion a small representative piece was taken for microscopic study. The remainder of the growths was trimmed free of keratinized material so far as possible and the fleshy, living tissue was kept frozen at about −65°C. until tested. One irradiated papilloma and several control growths were left *in situ* in order to ascertain the subsequent effects of the radiation. When all the frozen papillomas had been assembled 10 per cent saline extracts of them were prepared as usual and rubbed into scarified skin areas of four normal domestic rabbits, prepared by four applications of a mixture of turpentine and acetone at 2 day intervals (6).

The microscopic sections of the irradiated papillomas showed findings similar to those described by Lacassagne (2) for domestic rabbits and, except for the rate of regression, also similar to those described above for the cottontail rabbit papillomas. Within 24 hours they showed a cessation of cellular proliferation, which was soon followed by cellular degeneration. Two days after irradiation the growths were highly abnormal, with a greatly thickened keratinized layer and degenerating, swollen basal cells which stained poorly. After 4 days the irradiated papillomas still retained their structure, but consisted almost entirely of a dead keratinized mass with a few highly pathological cells at the base. Eight days later the irradiated papillomas were much smaller, and after 12 days only small, dry, flaky scabs remained, which soon came away, leaving smooth, pink, skin surfaces. The unirradiated papillomas taken from the same rabbits showed no change either microscopically or in the gross during regression of the x-rayed growths.

Table II summarizes the results of the experiment. It will be seen that all of the control papillomas from D.R. 5-86 yielded about the same amount of virus. The irradiated growths removed on the 2nd and 4th days after irradiation yielded as much virus as the controls, even though regressing and consisting largely of dead keratinized material, as revealed by microscopic sections of the growths. The growths taken 1 day after irradiation by contrast yielded considerably more virus, as evidenced by the increased number and shorter incubation period of the papillomas produced by the extract. No such result was obtained in the case of rabbit D.R. 5-62, the growths all yielding about the same amount of virus.

Manifestly the virus persists in practically undiminished amount in the papillomas of cottontail and domestic rabbits during regression induced by 5,000 r of irradiation (Tables I and II). No decrease in the amount of virus recovered from the growths could be detected even when microscopic sections

TABLE II

Yield of Virus from Domestic Rabbit Papillomas during Regression Induced by Irradiation

Rabbit No. D.R.	Virus strain used to produce papillomas†	Time from x-ray irradiation to removal of papillomas	X-ray irradiation	Microscopic findings	Pathogenicity tests* 18th day a	b	c	d	42nd day a	b	c	d
5-86	A	Before irradiation	Control	Characteristic papilloma	0	0	+	0	±	±	+	±
		1 day	Control	Characteristic papilloma	0	0	0	0	±	±	+	+±
			5,000 r	A few abnormal mitoses	+	+	++	+	+++±	++	+++±	+++±
		2 days	Control	Characteristic papilloma	0	0	0	±	±	±	±	+±
			5,000 r	Increased keratinization, living cells degenerating	0	0	±	0	±	±	+	+±
		4 days	Control	Characteristic papilloma	0	0	0	±	±	±	±	+
			5,000 r	Dead keratinized mass with a few degenerating living cells	0	0	±	0	+	±	±	±
5-62	1-27	Before irradiation	Control	As above	±	±	0	±	+±	++	+±	+
		1 day	Control	" "	±	±	±	+	+±	++	+±	+±
			5,000 r		+	±	±	+	++	+	+	+
		2 days	Control	" "	0	±	±	+	+±	+±	+±	+±
			5,000 r		±	±	±	±	+±	+±	+±	+
		4 days	Control	" "	+±	±	±	+±	++	+±	+±	+±
			5,000 r		±	±	±	+±	++	+±	+±	+±

D.R. = domestic rabbit.

* 10 per cent saline extracts of the control and irradiated papillomas were rubbed into scarified skin areas which had been treated with a mixture of turpentine and acetone four times at 2 day intervals.

† Both virus strains came from cottontail rabbit papillomas.

of the growths showed only dead keratinized material. In some cases considerably more virus was recovered from papillomas extracted 24 hours after irradiation.

Increased Yield of Virus from Irradiated Papillomas

Further tests were now undertaken to learn more about the increased yield just mentioned. The possibility that the virus strain used to produce the papillomas might be a factor was tested in the next experiment.

Experiment 4.—Multiple discrete papillomas were produced on the abdomens of two normal domestic rabbits (D.R. 6-18 and 6-19) by tattoo inoculation of two different virus extracts (W.R. 2-95 and W.R. 1-27) into the skin of each rabbit. It was known from numerous previous tests that papillomas elicited by virus extract W.R. 2-95 usually yielded little or no demonstrable virus, whereas growths produced by virus extract W.R. 1-27 regularly yielded it. The variation in virus yield from domestic rabbit papillomas produced by different virus strains will be described in another report (9). When the papillomas were about 5 weeks old, some of them were

TABLE III

Yield of Virus from X-Rayed Domestic Rabbit Papillomas

Source of papillomas		Time from irradiation (5,000 r) to removal of growths	Pathogenicity tests*											
			15th day				20th day				35th day			
Rabbit No.	Virus strain		a	b	c	d	a	b	c	d	a	b	c	d
D.R.	W.R.													
6-19	2-95	Control	0	0	0	0	0	±	±	+	0	+	+	+
		7 hrs.	0	0	0	0	±	+	+±	+±	±	++	++	+±
		22 "	+±	++±	++	++±	+++±	+++±	+++	+++±	++++	++++	++++	++++
	1-27	Control	±	+	+	+±	+±	++	++	+++	+++	+++	+++	++++
		7 hrs.	0	±	+	++	++	++	++	+++	+++	++±	+++	++++
		22 "	±	+	+	++	++	++	++	+++	++++	+++	+++	+++±
6-18	2-95	Control	0	0	0	0	0	0	0	0	0	0	0	0
		5 hrs.	0	0	0	0	0	0	0	0	0	0	0	0
		20 "	0	0	0	0	0	0	0	0	0	0	0	0
	1-27	Control	0	0	0	0	0	±	±	+	0	±	±	+
		5 hrs.	0	0	0	0	0	±	0	±	0	0	±	+
		20 "	0	0	0	0	0	±	±	0	0	±	±	±

The extracts of D.R. 6-19 papillomas were 20 per cent, those of D.R. 6-18, 10 per cent.

* Saline extracts of papillomas rubbed into scarified skin areas which had been treated with a mixture of turpentine and acetone four times at 2 day intervals.

exposed to 5,000 r of x-ray irradiation, as in Experiment 3. About 7 and 22 hours later irradiated and unirradiated papillomas were removed and 20 per cent saline extracts of the papillomas of D.R. 6-19 and 10 per cent extracts of the growths of D.R. 6-18 were prepared in the usual way. They were then rubbed into scarified skin areas of four domestic rabbits which had been treated with a turpentine-acetone mixture, as already described.

The papillomas which had been produced by virus strain W.R. 2-95 on inoculation into D.R. 6-19 and removed 22 hours after irradiation, yielded much more virus than the control growths (Table III). The difference in incubation period of the papillomas produced by the extracts of the control and irradiated papillomas was about 6 days, which indicates roughly a 100-fold difference in the amount of virus (4, 8). The growths removed 7 hours after irradiation

showed only a slight increase in virus yield. The papillomas produced on the same rabbit by virus strain W.R. 1-27 failed on the other hand to show any such difference. As the table shows, no virus was recovered from the control or irradiated papillomas produced by virus strain W.R. 2-95 in rabbit D.R. 6-18, and x-ray irradiation of the growths due to strain W.R. 1-27 had no effect on the amount of virus obtained.

The experiment suggests that the virus strain used to produce the papillomas is a factor in determining whether x-ray irradiation will cause an increased yield of virus from the growths, and it is possible that host peculiarities also play a rôle. Virus strain W.R. 1-27 had been used in Experiment 3, and in it the irradiation did not increase the yield of virus, nor did this happen in two further tests with the strain. These will not be given in detail. The large yield of virus from the irradiated W.R. 2-95 papillomas of rabbit D.R. 6-19 is striking, because of the difficulty ordinarily encountered in demonstrating any virus in papillomas produced by this virus strain, a fact well illustrated in the case of D.R. 6-18.

The effect of 2,000 r, 5,000 r, and 10,000 r on the yield of virus from domestic rabbit papillomas was tested in the next experiment.

Experiment 5.—Three domestic rabbits (D.R. 6-47, 6-48, 6-53) had multiple discrete papillomas of about 9 weeks' duration which had been produced by tattoo inoculation of virus W.R.A. This strain was selected because papillomas produced by it in Experiment 3 had yielded more virus following irradiation than the controls (Table II). Under nembutal anesthesia one papilloma of each rabbit received 2,000 r, another 5,000 r, and a third 10,000 r, in the usual way. A fourth unirradiated growth of each rabbit served as control. All of the papillomas utilized were comparable in size and character. 10 per cent saline extracts of the papillomas were made in the usual way about 24 hours after the irradiation and rubbed into scarified skin areas of four normal domestic rabbits, previously prepared with turpentine and acetone.

In this experiment the papillomas that received 10,000 r of irradiation regularly yielded more virus than the unirradiated growths (Table IV), the number of growths produced on the inoculation of extracts being about ten times or more greater. In one rabbit (D.R. 6-53) the unirradiated papilloma failed to yield any detectable virus, yet an extract of a comparable growth which had received 10,000 r of x-ray irradiation produced an average of seventeen papillomas. 2,000 r was without effect on the yield of virus from any of the papillomas, and 5,000 r caused only a slight increase in virus from one growth (D.R. 6-48). In another experiment 20,000 r and 30,000 r were not more effective than 10,000 r in causing an increased yield of virus from the papillomas of domestic rabbits.

In subsequent tests it soon became apparent that even 10,000 r of irradiation does not regularly cause an increased yield of virus from the papillomas of either

domestic or cottontail rabbits. A total of sixteen papillomas produced by two different virus strains (W.R.A and W.R. 11-69) have now been tested 24 hours after 10,000 r of irradiation. Eleven of these papillomas yielded considerably more virus than comparable unirradiated growths, whereas the remaining five failed to show any definite difference. Of thirteen papillomas, produced by three virus strains (W.R.A, 1-27, 2-95), which were tested following 5,000 r of

TABLE IV

Effect of Varying Amounts of Irradiation on Yield of Virus from Papillomas

Rabbit No. D.R.	X-ray irradiation of papillomas*	Pathogenicity tests‡												Average No. of papillomas
		18th day				24th day				42nd day				
		a	b	c	d	a	b	c	d	a	b	c	d	
6-53	Control	0	0	0	0	0	0	0	0	0	0	0	0	0
	2,000 r	0	0	0	0	0	0	0	0	0	0	0	0	0
	5,000 "	0	0	0	0	0	0	0	±	0	0	0	±	0.2
	10,000 "	+	0	±	0	++	+	+	±	++	+±	+±	+	17.0
		e	f	g	h	e	f	g	h	e	f	g	h	
6-47	Control	0	0	0	0	±	±	±	0	±	±	±	±	2.5
	2,000 r	0	0	0	0	0	±	0	±	0	±	0	±	1.7
	5,000 "	0	0	0	0	0	+	±	±	0	+	±	±	2.0
	10,000 "	±	±	0	0	++	+±	++	+	++	+±	++	+	21.7
		i	j	k	l	i	j	k	l	i	j	k	l	
6-48	Control	0	0	0	0	±	±	±	±	±	±	±	±	1.5
	2,000 r	0	0	0	0	±	0	±	0	±	0	±	0	0.5
	5,000 "	0	0	0	0	+	±	+	+	+	±	+±	+	7.0
	10,000 "	+	0	±	0	++	+	++	+±	++	+	++	++	22.7

* All papillomas were produced by a single virus extract, W.R. A.

‡ 10 per cent saline extracts rubbed into scarified skin areas which had been prepared with four applications of turpentine and acetone at 2 day intervals.

x-ray irradiation, only four showed an increased yield of virus. In two instances an increased yield of virus was obtained from papillomas removed within an hour after irradiation. Eight growths tested from 2 to 6 days after irradiation failed to show an increase.

Irradiation of Fibroma Extracts

A comparable study was now undertaken with the Shope fibroma of rabbits (6). The fibroma virus elicits rapidly proliferating connective tissue growths from which it can be readily recovered, and preliminary tests revealed that in crude saline extracts it can be inactivated by relatively small amounts of x-ray

irradiation. In the following experiment fibroma extracts were exposed to 10,000 r and 50,000 r of x-ray irradiation.

Experiment 6.—0.1 cc. of a 10 per cent saline extract of frozen testicular fibromatous tissue was inoculated intradermally into eight sites on either side of the abdomen of two normal domestic rabbits (D.R. 8-70, 8-71). Lesions appeared within 2 days and rapidly enlarged into characteristic fibromas 1 to 2 cm. in diameter by the 6th day. At this time several growths from each animal were ground separately with sand and saline to make 5 per cent extracts, and these were centrifuged at about 3,000 R.P.M. for 5 minutes and the supernatant fluids again cleared at about 4,000 R.P.M. for 10 minutes. The final supernatant fluids had a clear amber color. Nitrogen determinations showed that the D.R. 8-70 extract contained 0.248 mg. per cc., whereas the D.R. 8-71 extract contained 0.381 mg. per cc. Duplicate 3 cc. portions of each final fluid were exposed to 10,000 r and 50,000 r of x-ray irradiation in celluloid tubes, and control portions were treated identically except that they were not irradiated. The irradiated fluids showed no visible change. All the materials were now tested in dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} by inoculating 0.1 cc. of each dilution in duplicate intradermally into four normal domestic rabbits.

Table V shows the results of the experiment. The findings with the two extracts were essentially similar and they can be considered together. On the 4th day after inoculation the highest dilution of the unirradiated extracts that elicited growths was 10^{-3}, whereas those that were exposed to 10,000 r of x-ray irradiation were not infectious beyond a dilution of 10^{-2}. The extracts receiving 50,000 r had not caused growths at this time. On the 7th day, after which no more lesions appeared, the unirradiated extracts had elicited growths in dilution of 10^{-5}, whereas the extracts that received 10,000 r did not cause growths in dilutions beyond 10^{-4} while those exposed to 50,000 r were not infectious in dilutions beyond 10^{-3}. It should be noted that the numbers of growths elicited by the duplicate materials were in close agreement throughout the experiment.

Manifestly 10,000 r of x-ray irradiation is sufficient to inactivate about 90 per cent of the fibroma virus in saline extracts, and 50,000 r causes a still greater decrease in effective virus. These findings have been confirmed in two additional experiments with different virus extracts, the amount of virus inactivation produced by 10,000 r and 50,000 r being about the same as in the experiment described.

Previous work (4) has shown that the amount of x-rays required to inactivate the papilloma virus is greatly increased by the presence of extraneous protein in the virus preparation. The fibroma virus had been irradiated in crude extracts in the experiment just described, yet the virus was inactivated by relatively small amounts of x-rays. Hence it became desirable to learn whether or not further addition of protein to the extracts would alter its sensitivity.

Experiment 7.—A 10 per cent saline extract of pooled 7 day old intradermal fibromas of D.R. 7-62 was prepared as in the preceding experiment. To a portion of the extract an equal volume of 0.9 per cent saline was added and to two other portions an equal volume of saline containing crystalline egg albumin solution in two different concentrations. Determinations showed that the fibroma extract mixed with saline contained 0.13 mg. nitrogen per cc., whereas the two portions containing egg albumin

TABLE V

Effect of Irradiation on Fibroma Virus in Saline Extracts of Intradermal Growths

10 per cent saline extract of fibromas from rabbit No.	X-ray irradiation	Infectivity tests* No. of growths resulting from 8 intradermal inoculations of 0.1 cc. of extract							
		4th day				7th day			
		Dilution of extract				Dilution of extract			
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
8-71	Control	5	1	0	0	8	8	7	1
	"	6	2	0	0	8	8	8	2
	10,000 r	2	0	0	0	8	8	2	0
	"	2	0	0	0	8	8	4	0
	50,000 r	0	0	0	0	6	1	0	0
	"	0	0	0	0	7	2	0	0
8-70	Control	7	4	0	0	8	8	8	3
	"	7	3	0	0	8	8	7	5
	10,000 r	3	0	0	0	8	7	2	0
	"	3	0	0	0	8	8	3	0
	50,000 r	0	0	0	0	7	3	0	0
	"	0	0	0	0	8	3	0	0

* 0.1 cc. of each dilution was inoculated intradermally in duplicate into four normal domestic rabbits.

had 0.87 and 3.81 mg. Normal rabbit serum was added to another 10 per cent fibroma extract (D.R. 7-89) with the result that the mixture contained 13.15 mg. of nitrogen per cc. The control virus-saline mixture contained only 0.24 mg. of nitrogen per cc. Now 2 cc. portions of each fluid were exposed to 10,000 r and 50,000 r of x-ray irradiation and comparable amounts were left unirradiated as controls. All were tested for virus in dilutions from 10^{-2} to 10^{-5} by inoculation, as described in the preceding experiment.

The results are shown in Table VI. The amount of virus inactivation in the saline extracts produced by 10,000 r and 50,000 r was similar to that

observed in the preceding experiment (Table V). The addition of crystalline egg albumin or concentrated normal rabbit serum to the extract had no detectable effect on the inactivation. Manifestly the inactivation of fibroma virus in extracts, within the error of the method used, is constant over a wide range of protein concentration above 0.13 mg. of nitrogen per cc.

TABLE VI

Irradiation of Fibroma Extracts Containing Various Amounts of Extraneous Protein

Virus extract No.	Medium	Nitrogen	X-ray irradiation	Infectivity tests* No. of growths resulting from 8 intradermal inoculations of 0.1 cc. of extract Dilution of extract 10^{-3}	10^{-3}	10^{-4}	10^{-5}
		mg. per cc.					
7-62	Saline	0.138	Control	8	8	5	0
			10,000 r	8	6	0	0
			50,000 "	2	0	0	0
	Egg albumin solution	0.873	Control	8	8	7	0
			10,000 r	8	6	0	0
			50,000 "	1	0	0	0
		3.813	Control	8	8	4	0
			10,000 r	8	6	0	0
			50,000 "	1	0	0	0
7-89	Saline	0.248	Control	8	7	3	0
			10,000 r	8	5	0	0
			50,000 "	2	0	0	0
	Normal rabbit serum (concentrated)	13.151	Control	8	7	2	0
			10,000 r	7	2	0	0
			50,000 "	1	0	0	0

* Growth recorded on 7th day after inoculation.

Recoverability of Virus from Fibromas after Irradiation

The fact that 10,000 r regularly inactivates about 90 per cent of the virus in fibroma extracts, even in the presence of large concentrations of extraneous protein, made it of added interest to study the effect of x-rays on the virus in fibroma growths *in vivo*. In the next experiment subcutaneous fibromas were exposed to 10,000 r of irradiation and the amount of virus obtained upon extraction was compared with that recovered from comparable unirradiated growths from the same rabbit.

Experiment 8.—A 10 per cent saline extract of frozen testicular fibroma (D.R. 6-86) was inoculated intradermally (0.1 cc.) into twelve sites on the abdomen of a normal domestic rabbit. On the 6th day after inoculation four comparable growths were removed under ether anesthesia. Three were placed in a celluloid dish on gauze moist with physiological saline and exposed to 10,000 r of irradiation while the remaining one was kept under similar conditions but not irradiated. Three other fibromas were exposed to 10,000 r *in situ* while a fourth served as control. Immediately after the irradiation 5 per cent saline extracts of all the growths were prepared as usual, and centrifuged at about 4,000 R.P.M. for 10 minutes. The fluids obtained from the irradiated growths in this and subsequent experiments were noted to be distinctly less

TABLE VII

Recoverability of Virus from Fibromas Irradiated in Vivo and in Vitro

Intradermal fibroma growths irradiated	X-ray irradiation	Infectivity tests* No. of growths resulting from 8 intradermal inoculations of 0.1 cc. of extract			
		Dilution of extract			
		10^{-2}	10^{-3}	10^{-4}	10^{-5}
In vivo	Control	8	8	7	1
	10,000 r	8	5	0	0
	"	6	1	0	0
	"	6	0	0	0
In vitro	Control	8	8	8	2
	10,000 r	1	0	0	0
	"	4	0	0	0
	"	8	6	0	0

10 per cent saline extracts of the growths were prepared immediately after irradiation.
* Results on 6th day after inoculation.

opalescent than those from the controls. Serial tenfold dilutions of each extract, from 10^{-2} to 10^{-5}, were made with 0.9 per cent saline, and 0.1 cc. of each dilution was inoculated intradermally in duplicate into four normal domestic rabbits.

The results are recorded in Table VII. All of the fibromas, both those irradiated *in vivo* and *in vitro*, yielded from one-tenth to one-thousandth as much virus as the controls. Three additional experiments gave similar results.[1]

[1] 10,000 r of irradiation has not detectably altered the course or character of intracutaneous fibromas irradiated *in situ* on the 5th to 7th day after virus inoculation. It should be noted, however, that regression of such growths begins spontaneously about 10 days after inoculation and is usually completed within 2 to 3 weeks. But even when the growths were irradiated 2 days after the inoculation, no differences from the controls were obtained.

Effect of 400 r and 2,000 r on Recoverability of Virus from Fibroma Growths

Smaller amounts of radiation caused a variable effect on the recoverability of virus from fibromas as shown by the next experiments.

Experiment 9.—A normal domestic rabbit was inoculated intradermally with 0.1 cc. of a 10 per cent saline extract of frozen testicular fibroma into eight sites on each side of the abdomen. Five days after inoculation three of the growths were exposed to 2,000 r of irradiation while three others received 400 r. The rabbit was killed about 20 hours later. All the irradiated and two unirradiated growths were removed. They were comparable in size and character with no perceptible differences due to

TABLE VIII

Recoverability of Virus from Fibromas Following 400 r and 2,000 r of Irradiation

Treatment	Infectivity tests* No. of growths resulting from 8 intradermal inoculations of 0.1 cc. of extract Dilution of extract			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	8	8	2	0
"	8	8	5	0
2,000 r	8	8	8	7
"	8	8	8	6
"	8	8	8	7
400 r	8	8	8	1
"	8	8	6	0
"	8	8	7	2

10 per cent saline extracts of growths prepared about 20 hours after irradiation.
* Growths recorded on 5th day after inoculation.

the irradiation. 5 per cent saline extracts were immediately prepared, spun at 4,000 R.P.M. for 10 minutes, and the supernatant fluids were diluted with saline to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. 0.1 cc. of each dilution was inoculated intradermally into two spots on each of four domestic rabbits.

The findings on the 5th day after inoculation are given in Table VIII. Both control growths yielded virus in dilution of 10^{-4}. The growths that received 2,000 r of x-ray irradiation, however, were highly infectious in dilution of 10^{-5} and the growths exposed to 400 r yielded slightly more virus than the controls. It would appear that the fibromas which received 2,000 r prior to extraction yielded about 10 times more virus than the unirradiated growths from the same rabbit.

In another similar experiment an increased amount of virus was also obtained

from fibromas after 2,000 r of irradiation. In three more experiments, however, 2,000 r had no such effect. The results of one of these tests are shown in Table IX. The inconstancy of the findings, as concerns increased virus yield, is like that observed with rabbit papillomas.

TABLE IX

Recoverability of Virus from Fibromas Following 2,000 r of Irradiation

Treatment	Infectivity tests* No. of growths resulting from 8 intradermal inoculations of 0.1 cc. of extract			
	Dilution of extract			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	8	8	5	1
"	8	8	4	0
"	8	8	5	2
"	8	8	7	2
2,000 r	8	8	5	2
"	8	8	7	1
"	8	8	5	1
"	8	8	7	2

10 per cent saline extracts of growths prepared about 20 hours after irradiation.

* Growths recorded on 5th day after inoculation.

DISCUSSION

The regression of irradiated papillomas is obviously due to a cessation of cellular division accompanied by pathological changes in them with continuing differentiation of the cells. The result is that within a few weeks only a dry keratinized mass remains, which flakes away leaving a smooth scar covered with epidermis. The histological changes in virus papillomas subsequent to irradiation are like those seen in proliferating non-neoplastic tissue which has been irradiated and in the tumors of unknown cause (10). In the gross the regression of the papillomas resembles natural regression (11) but takes place more swiftly. In both instances the tumors dry to the base, dwindle in size, and flake away. But natural regression is the expression of a generalized resistance which affects all of the papillomas on the individual animal (12) whereas that due to x-rays is confined to the exposed growth, unirradiated papillomas on the same animal continuing to proliferate and enlarge. During natural regression the rate of cell proliferation becomes less than that of keratinization, just as in irradiated growths, but the papilloma cells show no abnormalities beyond those evident in "healthy" growths. Those which have been irradiated show on the other hand striking pathological changes (multiple nuclei, gigantism, etc.).

The papilloma virus is not detectably decreased *in vivo* by amounts of x-rays which destroy the papilloma cells. This has been repeatedly demonstrated not only with domestic rabbit growths but also with the papillomas of cottontail rabbits which contain an abundance of virus. Indeed the virus present in the papillomas of both species usually persists in undiminished amount during regression induced by 5,000 r of irradiation. Some workers have concluded that because the tumors of unknown cause are rendered non-viable by amounts of x-rays which most viruses resist the latter can have no share in the causation of such tumors. The present findings show this assumption to be unwarranted, for the papilloma is destroyed by small amounts of x-rays, yet it is a virus-actuated growth, and continues to harbor active virus until the last.

It was noted in some instances that the yield of virus from papillomas extracted at various periods following irradiation was considerably greater than from comparable non-irradiated papillomas. The nature of this phenomenon has still to be determined but the findings suggest several posibilities. The x-rays might have so altered the cells as to favor rapid multiplication of the virus. Against this possibility is the fact that an increased yield of virus was found in papillomas removed within an hour after irradiation. Furthermore the papilloma virus is extremely resistant, retaining its activity well in keratinized tissues (13), yet no increase in the amount of it was found when the growths had been removed several days after irradiation. These findings suggest that the x-rays caused a temporary change in the virus-cell relationship such that more virus came away on extraction, a possibility that seems the more likely in view of the findings of Ladewig *et al.* (14) who showed that a single extraction by grinding in sand and suspending in saline does not remove all of the virus from the tissue and that several successive extractions still yield virus though in diminishing amount. A similar temporary increase in the yield of virus was also observed in the irradiated fibromas.

The fibroma virus in crude extracts or *in vivo* was inactivated by far less irradiation than the papilloma virus under comparable conditions. The infectivity of fibroma virus extracts, or the amount of virus recovered from fibromas irradiated *in vivo*, was reduced 70 to 90 per cent by 10,000 r, whereas at least 100,000 r was required to inactivate 50 per cent of the virus in papilloma extracts containing roughly the same amount of protein (4). Moreover further addition of extraneous protein,—egg albumin or normal serum,—to crude fibroma extracts up to a final concentration of 13.1 mg. per cc. had no detectable influence on the amount of inactivation. The rate of inactivation in solutions containing this much protein is not only far greater than for papilloma virus but is at least four- or fivefold greater than that for any enzyme, virus, or bacteriophage so far studied. It has been reported, on the basis of tests within a small range of sizes, that the amount of x-rays required to inacti-

vate certain viruses and bacteriophages is inversely proportional to the particle size although the data are not entirely consistent (15). However, this seems to be the most reasonable explanation of the greater sensitivity of the fibroma virus, for the volume of the fibroma virus particle has been shown to be 25 to 50 times greater than the volume of the papilloma virus particle (16), and furthermore, irradiation of the extracts was done in the presence of much extraneous protein, which greatly reduces the indirect water reaction of the x-rays. Other explanations of the sensitivity of the fibroma virus to x-rays cannot be ruled out at the present time, however.

Throughout these experiments the papilloma virus was repeatedly inoculated in high dilutions, plated out as it were on expanses of scarified skin, to learn whether any variant of the virus had been produced as a result of the x-ray irradiation. Neither irradiation *in vivo* or *in vitro* altered it in any perceptible qualitative respect. Nor was any variant of the fibroma virus observed following irradiation.

SUMMARY

The virus-induced papillomas of cottontail as well as domestic rabbits regress completely within a few weeks when exposed to 5,000 r of x-ray irradiation. The x-rays do not immediately kill the papilloma cells, but lead to death by inhibiting cellular division and producing pathological changes in the cells which then continue to differentiate. The virus associated with the growths, however, not only persists in undiminished amount during regression, but often an increased yield of it can be obtained on extraction.

The fibroma virus in crude extracts or *in vivo* is inactivated by far less irradiation than the papilloma virus. 10,000 r destroys 90 per cent or more of the infectivity of the fibroma virus, whereas at least 100,000 r is required to inactivate 50 per cent of the papilloma virus in extracts containing about the same amount of protein.

No variant of the papilloma virus or fibroma virus has been encountered as a result of the irradiation.

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EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeek.

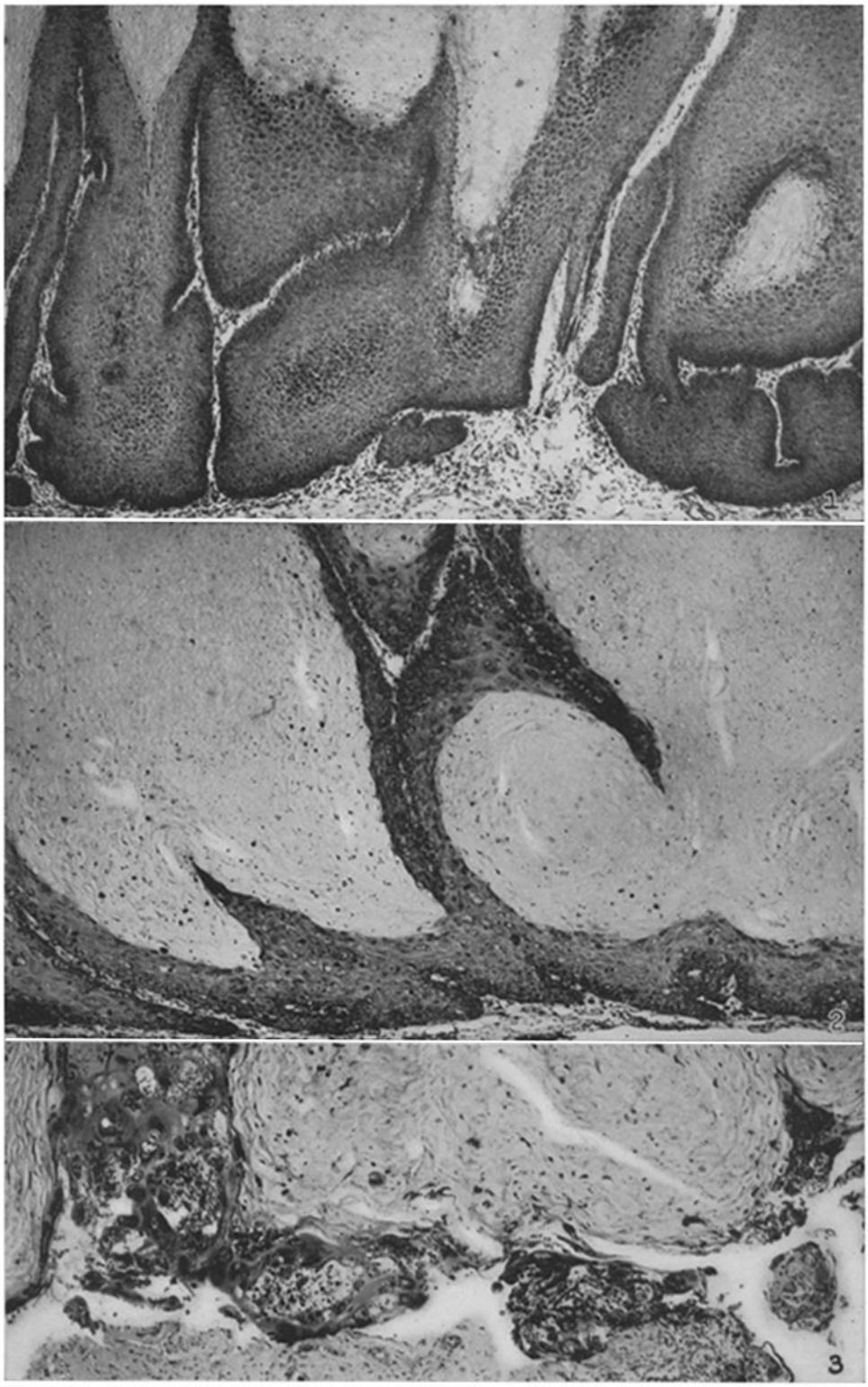
PLATE 8

FIGS. 1 to 3. To illustrate the changes induced by x-rays in virus-induced cottontail rabbit papillomas. All growths were taken from the same rabbit. × 65.

FIG. 1. Base of an unirradiated papilloma. The layer of living cells contains numerous mitoses not visible at the magnification given.

FIG. 2. Base of papilloma 5 days after exposure to 5,000 r. The living layer of cells is much thinner and the individual cells are greatly swollen. Giant and multinuclei can be seen.

FIG. 3. Base of papilloma 10 days after exposure to 5,000 r. Only a few living elements persist, all the rest having keratinized. The living cells are enormous, with giant or multiple nuclei in some instances. The mass is loose along the base and is about to flake off.

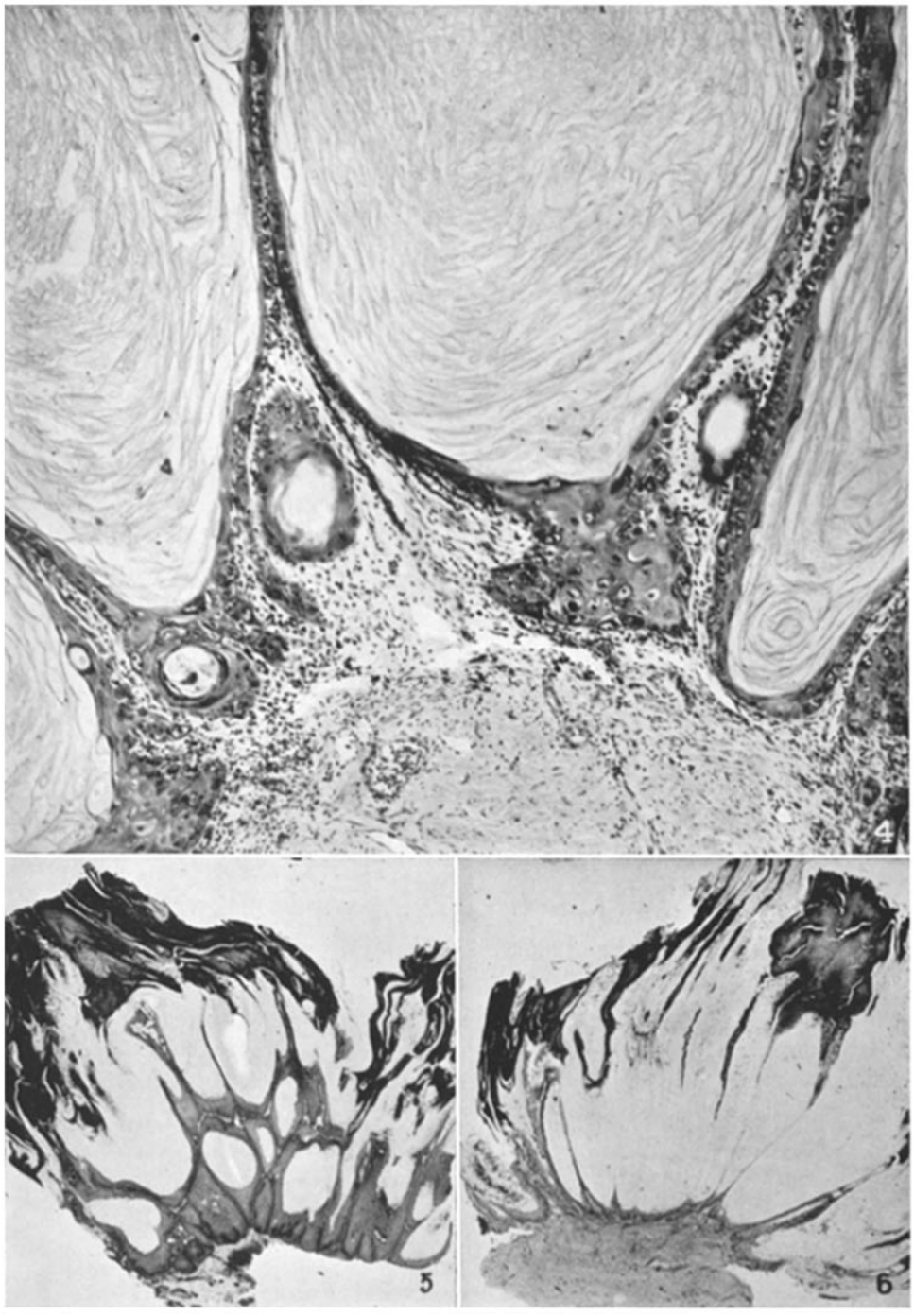


(Friedewald and Anderson: Effects of Roentgen rays on cell-virus associations)

PLATE 9

FIG. 4. Cottontail rabbit papilloma 10 days after irradiation (10,000 r),—to show the cellular reaction in the connective tissue stroma beneath the papilloma, and the thin layer of highly pathological living cells. × 85.

FIGS. 5 and 6. Unirradiated and irradiated papillomas (5,000 r) excised 9 days after irradiation,—to show the thinned layer of living cells and the correspondingly increased keratinized material which result from x-ray irradiation. In the gross the irradiated papilloma could not be distinguished from the control. × 85.



(Friedewald and Anderson: Effects of Roentgen rays on cell-virus associations)