THE ROENTGEN RADIATION OF PAPILLOMA VIRUS (SHOPE)

II. The Effect of X-Rays upon Papilloma Virus in Vitro*

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A benign, infectious, cutaneous tumor of rabbits, the Shope papilloma, is caused by a virus that upon inoculation in either domestic or cottontail rabbits induces papillomas with great regularity (1). These tumors are not infrequently followed by cancers (2–6). On attempted recovery of the virus from these two varieties of rabbits, however, a marked difference in host reactivity becomes apparent; for the virus, which is readily recoverable from cottontail rabbits, is not recoverable, or but rarely recoverable, from domestic rabbits (1, 7–9). This finding does not prove, of course, that the virus is absent from the papillomas of domestic rabbits. On the contrary, some immunological evidence for its presence in such papillomas and in the cancers derived from them has been presented (10, 11).

If a virus were not present in the cells of papillomas on domestic rabbits, such papillomas might be expected to react to Roentgen radiation as non-infectious tumors do. This, indeed, has been shown to be the case (12). On the other hand, if a virus were contained in the tumor cells, the outcome following irradiation might be expected to be quite different. Obviously, therefore, it is important to learn the effect of Roentgen radiation on the virus itself. Data bearing on these points have been briefly recorded by Lacassagne (13) and ourselves (14, 15). The work from our laboratory that dealt with the effects of Roentgen radiation on papillomas *in vivo* has been amplified by further experimentation and presented in full (12). It was concluded that the curative effect of x-rays on domestic-rabbit papillomas (Shope) results from the direct action of the Roentgen radiation on the rabbit's cells rather than on the virus.

The studies described in the present paper deal with the effect of Roentgen radiation *in vitro* on cell-free suspensions of papilloma virus derived from cottontail rabbits. The experiments were designed to determine the dosage necessary to render papilloma virus non-infectious for both cottontail and domes-

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tic rabbits. They were planned, also, to detect any manifest alterations, if such occurred, in the characteristics of papillomas that were initiated by irradiated virus.

Materials and Methods

Virus.—The papilloma virus (Shope) was obtained from papillomas that had been incurred under natural conditions in Kansas by cottontail rabbits (genus, *Sylvilagus*). Immediately after the removal of papillomatous tissue from the rabbits, it was thoroughly triturated with Locke's solution and alundum[1] to yield a 10 per cent solution. This suspension was centrifuged horizontally at 1500 R.P.M. for 30 minutes and the supernatant fluid withdrawn by means of a pipette. Care was exercised not to disturb the sediment and to leave behind the debris floating on the supernatant fluid. The collected fluid was then subjected to three successive centrifugations at 14,000 R.P.M. (relative centrifugal force at tip of tube approximately 25,000 × gravity) in the 51°-angle head of the Multispeed Attachment for the International centrifuge, the sedimented material being discarded after each run. The final supernatant fluid, either without further treatment or after passage through a Berkefeld V filter, was used as the suspension of virus to be irradiated. Since filtration often results in a 10-fold decrease in the titer, the suspension was not filtered in the present work, except in the first two experiments.

Method of Radiation.—The suspension of virus to be irradiated was transferred to a sterile Petri dish, 4 cm. in diameter and 8 mm. in depth. To prevent contamination and evaporation, the dish was covered by a sterile piece of emulsion-free dental x-ray film. This film was used because it has essentially no filtering effect upon the x-rays. The dish was placed on a small, lead-covered table that rotated slowly under a shielded, water-cooled radiographic tube especially adapted to this purpose. During irradiation, at intervals computed to yield the dosages desired, the suspension of virus was mixed thoroughly and 0.5 ml. samples were removed for testing. The irradiation of any given sample was continuous. The use of the loosely covered Petri dish made it unnecessary to shield the suspension from the radiant energy of the filament, for the temperature of the suspension was never found to exceed the temperature of the laboratory by more than 3°C. throughout the entire period of irradiation.

The Roentgen rays were generated by a current of 40 milliamperes at 90 kilovolts. The radiation, which was unfiltered, was administered at a distance of 6 cm. from the center of the target to the center of the Petri dish, a point 4 mm. from the bottom of the dish. Although entirely accurate measurements of dosage at this short distance and high output proved to be difficult, a Victoreen r-meter, calibrated for 90 kilovolts, was used to determine the dosage. The ionization chamber (1 cm. in diameter) of the calibrated Victoreen instrument was placed horizontally in a Petri dish. This Petri dish was identical with the one used for the suspension of virus, except that one

[1] Alundum, an electrically fused crystalline alumina prepared by the Norton Company, Worcester, Massachusetts, was used because of its excellent "cutting" qualities.

side was chipped out to permit the chamber to rest in it. To reproduce the conditions of the actual test still more closely, a cover of emulsion-free dental x-ray film was placed over the container. Thus, the conditions for the measurement of dosage were the same as for the actual tests, except for a possible scattering of the x-rays by the suspension of virus. To measure the output of radiation, the radiographic tube was centered above the ionization chamber at distances of exactly 96 cm., 24 cm., 12 cm., and 6 cm., respectively. Short exposures were controlled by a radiographic interval-timer. The output of radiation, including back-scattering, was measured for each experiment. The readings showed little or no deviation from 10,400 r units per minute at a distance of 6 cm. Although the inverse-square law may not hold at very close distances, the actual readings that were obtained at a distance of 6 cm. corresponded to the theoretical readings calculated by means of the inverse-square law.

In an effort to render successive doses of x-rays as comparable as possible, all geometric factors were kept approximately constant. The only variables were the level of the suspension of virus in the Petri dish and the time of exposure, which was measured in seconds by both a timer and a stop-watch. The error in the measurement of dosage was approximately ± 5 per cent. The difference in dosage that was received at the top of the suspension and at the bottom, a difference of 8 mm., was calculated by the inverse-square law to be 7.5 per cent. Since the physical limitations of size make such changes in dosage unavoidable, however, the doses as given are comparable for the factors as specified.

Animals.—130 adult rabbits were used in the present study. 16 cottontail rabbits (*Sylvilagus floridanus alacer* Bangs[2]) from Kansas provided the virus for irradiation; 39 cottontail rabbits (*Sylvilagus floridanus mearnsi* Allen[2]) from New York and 75 domestic rabbits (genus, *Oryctolagus*) served as host animals for testing the infectivity of the irradiated samples of virus. The domestic rabbits were employed to determine the titer of the suspensions before irradiation and to measure any loss in titer that might follow irradiation. The cottontail rabbits made it possible to determine the "viability" of the virus in each irradiated sample, since papilloma virus is readily recoverable from this host.

Technique for Measuring the "Viability" and Titer of the Irradiated Virus.—After exposure to each of the specified doses of x-rays, a 0.5 ml. sample of the suspension of virus was withdrawn and diluted with Locke's solution to yield a series of decimal dilutions through 10^6. Each dilution was used for duplicate, triplicate, or quadruplicate inoculations made by rubbing 0.1 ml. on a previously prepared, lightly scarified cutaneous site (approximately 2 cm. in diameter) on the ventral surface of the body. The size of the rabbit determined the number of sites. Domestic rabbits readily accommodated 16 rectangular areas, 4 cm. square, cottontails from 8 to 12 such areas. The rabbits were observed at intervals of from 2 to 7 days for a period of 60 days, a procedure that permitted a satisfactory assessment for "viability" and titer of each sample of irradiated virus.

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[2] It is a pleasure to thank Mr. George G. Goodwin of The American Museum of Natural History, New York City, for making the specific allocation of the cottontail rabbits used.

EXPERIMENTS

That the *in vitro* irradiation of papilloma virus has had an effect on the virus becomes apparent only when the reaction of a susceptible host to infection by the virus deviates from the normal. When deviations are observed, one is justified in inferring that the virus has been changed. Such changes may involve the "viability" of the virus (as determined by its capacity to initiate papillomas), an increase in the period of time between inoculation and the appearance of tumors (the incubation period), a decrease in the titer, a decrease in the size that the tumors attain, and the recoverability of the virus from the papillomas that are induced in cottontail rabbits. To initiate our studies, therefore, it was necessary to learn the minimal Roentgen dosage that would induce some recognizable deviation. Two preliminary experiments made it clear that a dosage of at least 500,000 r was necessary.

Experiment 1.—A suspension of the virus was irradiated until a dosage of 200,000 r had been given. During the period of irradiation, samples of the suspension of virus were withdrawn at intervals computed to yield massive doses of x-rays ranging from 10,000 to 200,000 r, as indicated in Table I. The virus in each sample was tested for "viability" by the inoculation of 2 sites on each of 4 animals, 1 cottontail and 3 domestic rabbits, giving a total of 8 injections per sample.

The results of the first experiment are summarized in Table I. It can be seen that the interval of time between inoculation and the appearance of papillomas and the titer of the virus were unaffected by doses of x-rays ranging from 10,000 to 200,000 r. Virus was readily recovered from the papillomas induced on the cottontail rabbits. It was also found that irradiation with these dosages had no apparent effect on the size attained by the tumors. Thus, it was apparent that 200,000 r did not represent the minimal effective dosage capable of inducing recognizable deviations from the normal. A second experiment, employing dosages as large as 3 million r, was therefore undertaken.

Experiment 2.—A suspension of the virus was irradiated until a dosage of 3 million r had been reached. During the period of irradiation, samples of the suspension of virus were withdrawn at intervals computed to yield massive doses of x-rays ranging from 100,000 to 3 million r, as indicated in Table II. The virus in each sample was tested for "viability" and titer by the inoculation of each of 3 animals, 1 cottontail rabbit and 2 domestic rabbits. Each domestic rabbit was inoculated in duplicate with successive 10-fold dilutions of an irradiated sample, 10^1 through 10^6; the cottontail rabbit was given the 10^1 dilution at 4 sites.

The results of the second experiment are summarized in Table II. It may be seen that 3 million r reduced the titer from 10- to 1000-fold, but this amount of irradiation did not have much effect, if any, on the incubation period. It was observed, however, that the resulting tumors were much reduced in size. Virus was regularly recovered from the cottontail rabbits.

Having discovered the minimal range of dosage that affected the virus, as

measured by deviations from normal in the effects produced by the virus on susceptible hosts, it became desirable to determine the dosage of x-rays required

TABLE I

The Results of Single Massive Doses of X-Rays Ranging from 10,000 to 200,000 r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test "viability" and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
10	1	3	14–17	10^3	0
20	1	3	14–17	10^3	0
40	1	3	14–17	10^3	0
60	1	3	14–17	10^3	0
80	1	3	14–17	10^3	0
100	1	3	14–17	10^3	0
120	1	3	14–17	10^3	0
140	1	3	14–17	10^3	0
160	1	3	14–17	10^3	0
180	1	3	14–17	10^3	0
200	1	3	14–17	10^3	0
Control	4	3	14–17	10^3*	

* Before filtration, the titer was 10^3 when tested on 4 cottontail rabbits.

TABLE II

The Results of Single Massive Doses of X-Rays Ranging from 100,000 to 3 Million r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test "viability" and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
100	0	2	13–14	10^3–10^5	0–10-fold
500	1	2	10–22	10^3–10^5	0–10-fold
1000	1	2	10–23	10^2	10–100-fold
2000	1	2	17–19	10^3	0–10-fold
3000	1	2	17–23	10^1–10^2	10–1000-fold

to inactivate the virus completely. Accordingly, a third experiment was undertaken, employing doses up to 7 million r.

Experiment 3.—A suspension of the virus was irradiated until a dosage of 7,302,000 r had been given. Samples from the irradiated suspension were withdrawn at intervals computed to yield massive doses of x-rays ranging from 500,000 to 7,302,000 r, as

indicated in Table III. The virus in each sample was tested as in Experiment 2 by the inoculation of 4 animals, 2 cottontail rabbits and 2 domestic rabbits.

The results of the third experiment are set forth in Table III. Dosages of less than 2 million r had no observable effect; 2 million r probably had elicited some effect, as shown by some prolongation of the incubation period and possibly by a 100-fold loss in titer; and 3 million r, or more, resulted in from a 10- to 100,000-fold loss in titer and in a change from an incubation period of 12-32 days to 15-34 days. The effect of 4 million r was more pronounced. Finally, 5 million r inactivated most of the virus, for only a single cottontail of the 4

TABLE III

The Results of Single Massive Doses of X-Rays Ranging from 500,000 to 7 Million r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test "viability" and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
500	1	2	12–23	10^4–10^5	0
1000	2	2	15–23	10^4–10^5	0
2000	2	2	15–23	10^3	0–100-fold
3000	2	2	15–26	10^1–10^2	10–10,000-fold
4000	2	2	26–34	10^1	100–10,000-fold
5000	2 (2)*	2 (1)	26	10^1	100–10,000-fold
6500	2 (2)	2 (2)	—	—	1000–100,000-fold
7302	2 (2)	2 (2)	—	—	1000–100,000-fold
Control		2	15	10^3–10^5	

* Numbers in parentheses signify the number of rabbits that gave no reaction following the inoculation of irradiated virus.

cottontail rabbits that received virus irradiated with this dosage reacted to the undiluted material (at only 2 of 4 sites). Doses of approximately 6 and 7 million r were uniformly effective in inactivating the virus. Virus was regularly recovered from the papillomas induced on cottontail rabbits.

In evaluating the effect of irradiation in the third experiment, it should be noted that one of the domestic rabbits employed to titrate the suspension of virus for irradiation responded to the virus in a normal manner, while the other was relatively refractory to infection, as indicated by the low titer and by the fact that the papillomas regressed completely soon after their appearance. Such a regression rarely occurs in the domestic rabbit within 6 weeks.[3] An

[3] The decreased reactivity of one of these 2 domestic rabbits to infection by the virus may possibly be attributed to an increased tissue resistance, for the neutralizing

initial titer of 10^5, therefore, would probably represent the infectivity of the material more closely than 10^3.

A fourth experiment was undertaken to determine the reproducibility of the results obtained in Experiment 3, using a different preparation of virus.

Experiment 4.—A suspension of the virus was irradiated continuously until a dosage of 14,325,000 had been reached. During the period of irradiation, 4 samples, representing dosages of 1,500,000 r, 5,025,000 r, 13,875,000 r, and 14,325,000 r were withdrawn and tested, as indicated in Table IV. The virus in each sample was tested as in Experiment 2 by the inoculation of 4 animals, 2 cottontail rabbits and 2 domestic rabbits. Three of the 4 cottontail rabbits used to test the samples of virus irradiated

TABLE IV

The Results of Single Massive Doses of X-Rays Ranging from 1.5 to 14 Million r on a Suspension of Papilloma Virus

Dosage in thousands of r units	No. of rabbits used to test "viability" and titer of virus		Incubation period	Titer	Probable loss in titer
	Cottontail	Domestic			
			days		
1,500	0	2	14	10^2–10^6	0–10,000-fold
5,025	1	2 (1)*	30–41	10^1–10^2	10,000–1-million-fold
13,875	2 (1)	2 (2)	30	10^1	100,000–1-million-fold
14,325	2 (2)	2 (2)	—	0	
Control	0	3	10–13	10^5–10^6	

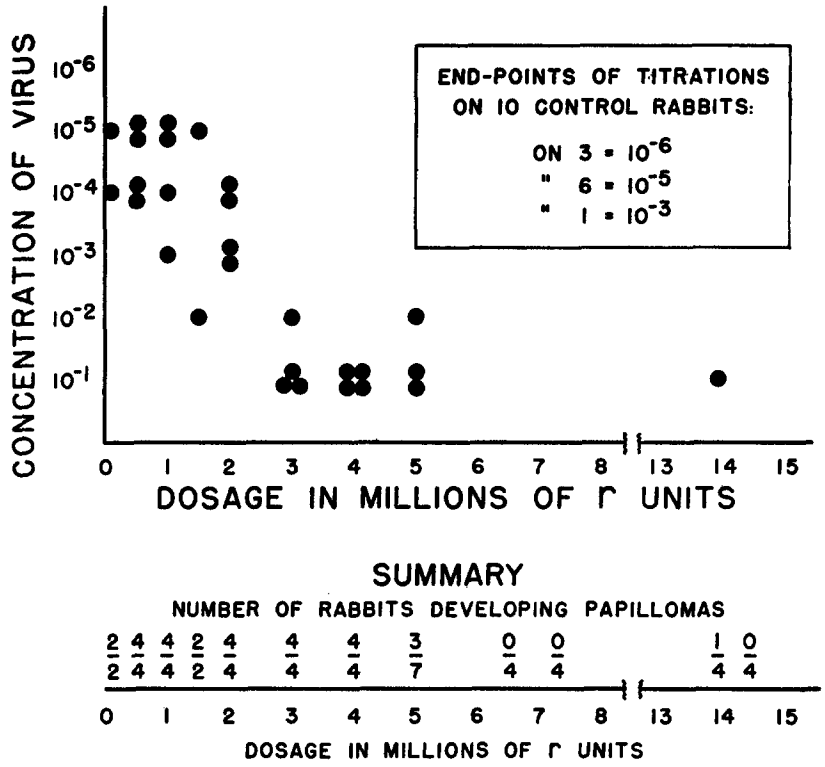
* Numbers in parentheses signify the number of rabbits that gave no reaction following the inoculation of irradiated virus.

with 1,500,000 r and 5,025,000 r died from extraneous causes before papillomas developed.

The results of the fourth experiment are summarized in Table IV. It may be seen that the results obtained in Experiment 3 were confirmed and extended. A dose of a million and a half r was without obvious effect. A dose of 5 million r, on the other hand, resulted in a significant loss of infectivity (from 10,000- to 1,000,000-fold) and in a prolongation of the incubation period (from 10-13 days to 30-41 days). Moreover, papillomas were induced on only a single rabbit of the 4 that received material irradiated with the enormous dosage of almost 14 million r. On this single animal, furthermore, papillomas appeared at but 2 of the 4 sites inoculated, and then only after a prolonged incubation period of 30

capacity of sera withdrawn from the 2 animals before inoculation was identical, as was the capacity of similar samples withdrawn 60 days after injection. Nevertheless, the papillomas on one rabbit had regressed completely within 40 days, whereas those on the other rabbit grew progressively, as usual.

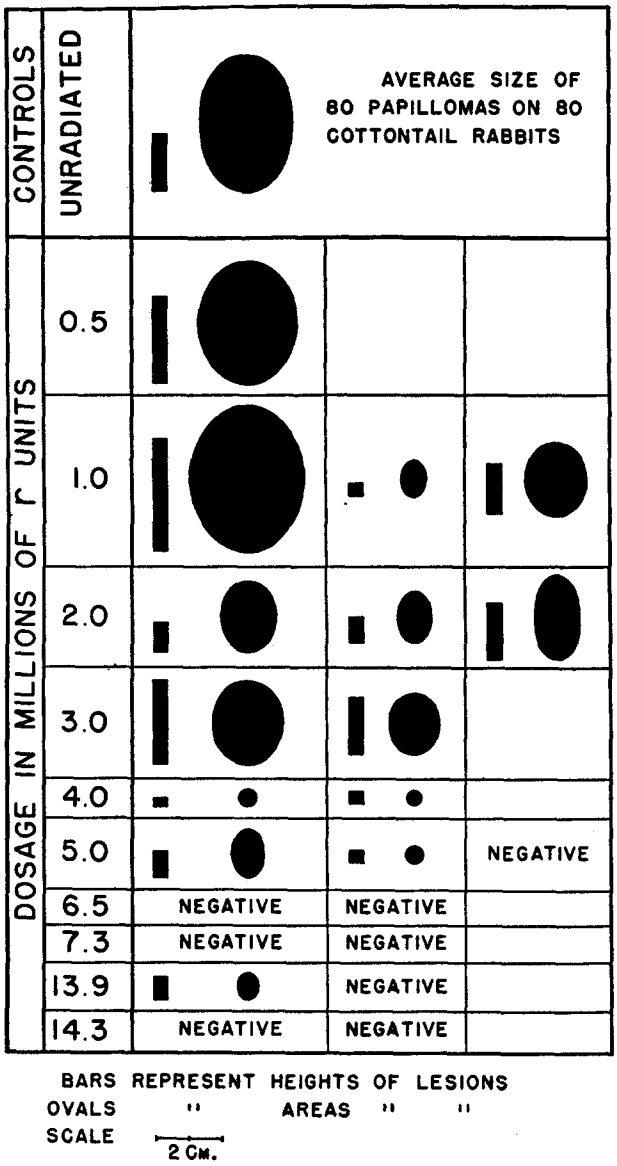
days. (Because this positive finding concerns a single animal only, one must not overlook the possibility that it may have been exceptional.) With the next larger dose, over 14 million r, no reaction in any of the injected rabbits was



TEXT-FIG. 1. The effect of Roentgen radiation on the infectivity of papilloma virus in cell-free suspensions. Each dot shows the highest titer attained on an individual positive rabbit. The concentration of the virus is plotted against the dosage of x-rays, a concentration of 10^{-1} equalling a 10 per cent suspension of tumor tissue. In the summary at the bottom of the figure, the lots of animals inoculated with each sample of irradiated virus are recorded as the denominators of the fractions, those developing papillomas as the numerators.

noted. Virus was regularly recovered from papillomas induced in cottontail rabbits.

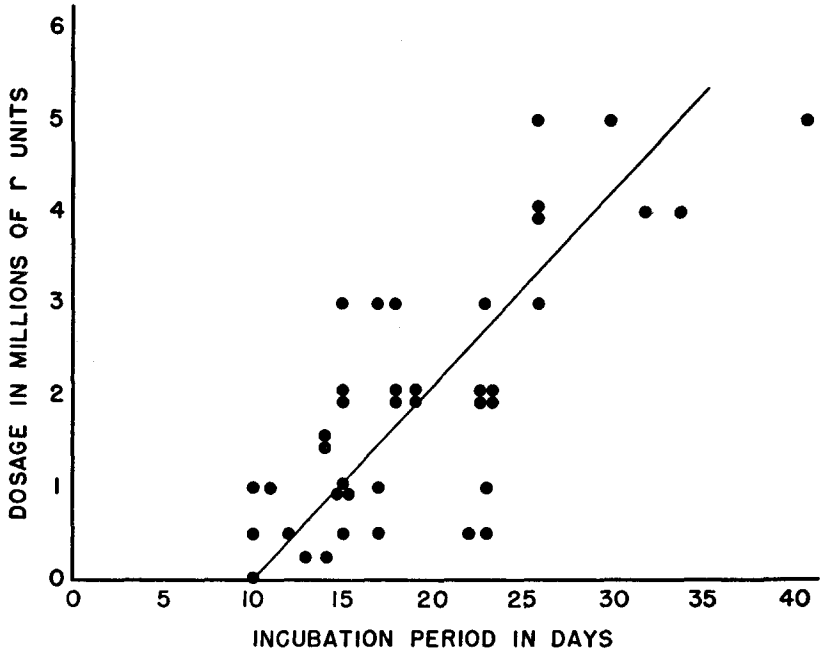
When the four experiments were considered collectively, it was evident that large doses of Roentgen radiation result in a lowered titer, a prolongation of the incubation period, and a decrease in the size attained by the papillomas. These effects are readily apparent when the results that follow irradiation with graded doses of from 100,000 to 14 million r are depicted graphically. In Text-fig. 1



TEXT-FIG. 2. The sizes attained by papillomas on cottontail rabbits inoculated with irradiated papilloma virus. Each rectangular area of the figure represents an individual rabbit (except in the case of the controls). The effect of the x-rays in decreasing the size of the tumors is apparent.

the loss in the infective capacity of the virus that occurred when the dosage of radiation was increased is graphically shown. (The results of Experiment 1,

the preliminary experiment, are not included.) It may be noted that a demonstrable effect on the virus was not elicited until the dosage had reached 1 million r. With increasing dosage, the reduction in the infective titer was progressively greater. In Text-fig. 2 the progressive decrease in the size of the tumors that resulted from the inoculation of the irradiated virus (in 10 per cent suspension) is shown. The effect on the size of the papillomas following successively greater dosages of x-rays is obvious. Other variations from norma



TEXT-FIG. 3. The effect of x-rays in prolonging the period of time between the inoculation of rabbits with irradiated papilloma virus and the appearance of the tumors. The dosage of x-rays is plotted against the incubation period.

accompanied the progressive decrease in the size of the tumors. Thus, the incubation period was prolonged from 10 to 41 days, as shown in Text-fig. 3. (In this text-figure, as in the others, the graphs necessarily represent approximations, for the variation that characterizes the host-virus relationship renders a more precise representation impossible.) Although it is difficult to measure it is our impression that the "viability" of the papilloma cells may have been affected when the virus used for inoculation had received large doses of x-rays, for the resultant tumors frequently were dry and regressed early. Such tumors, nevertheless, when present on cottontail rabbits, regularly yielded virus.

DISCUSSION

In the present experiments the results have clearly shown that cell-free suspensions of papilloma virus (Shope) require for their complete inactivation amounts of Roentgen irradiation much greater than those needed to inactivate other infectious agents. These amounts, millions of r units, are several thousand times greater than those required to bring about the permanent regression of tumors induced by the virus in domestic rabbits (12), and they are many times greater than those needed for the inactivation of certain other viruses, bacteria, and a yeast (14). The reason for this greater resistance to x-rays is not known.

Friedewald and Anderson (16) have recently reported studies that appear to be similar to ours. They found that papilloma virus, when contained in Berkefeld filtrates, is inactivated by from 2 to 4 million r, whereas preparations of virus partially purified by repeated differential centrifugations require only from 400,000 to 800,000 r for inactivation. They attribute this difference to the greater concentration of extraneous material and virus in Berkefeld filtrates. Quite possibly the same explanation might account, at least in part, for the larger doses that were required in our investigations. It seems not unlikely, however, that the differences in the results obtained by Friedewald and Anderson and by us were caused in part by differences in the methods of irradiation and in the animal hosts used for testing the irradiated samples of virus. We used continuous irradiation and employed only a single x-ray tube. Each irradiated sample of virus was tested for "viability" and titer using domestic and cottontail rabbits. It should be remembered that the cottontail rabbits used for testing the irradiated samples were of the Eastern subspecies (*Syvilagus floridanus mearnsi* Allen), a host that is uniformly highly susceptible to the virus. As a rule, it reacts more profusely and to a higher titer with a given sample of virus than do either domestic rabbits or Western cottontail rabbits (*Sylvilagus floridanus alacer* Bangs). Papillomas induced on the Eastern cottontail rabbit, furthermore, readily yield virus for further study. It should be noted, finally, that it was on this host that papillomas were produced with the samples of virus irradiated with the greater dosages.

It is apparent from the present work that the Roentgen irradiation of materials containing papilloma virus provides a method whereby bacterial invaders can be eliminated from papillomatous material. The bacteria will be destroyed by 2 million r without observably injuring the papilloma virus. The same procedure may be useful in limiting extraneous viruses, for several are known to be inactivated by this amount of irradiation (14, 15). Using these large doses of x-rays, the treatment of the cancers that often follow the papillomas are providing us currently with a further approach to the study of the rôle of papilloma virus in the papilloma-to-carcinoma sequence.

CONCLUSIONS

Cell-free suspensions of papilloma virus (Shope) required for their inactivation *in vitro* amounts of Roentgen irradiation that are much greater than those needed to inactivate other infectious agents previously described. These amounts, millions of r units, are several thousand times greater than those required to eradicate permanently papillomas induced by the virus in domestic rabbits. Large doses of Roentgen radiation reduce the titer of papilloma virus, lengthen the period of time between inoculation and the appearance of papillomas, and decrease the size attained by the papillomas.

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