CELL STATE AS AFFECTING SUSCEPTIBILITY TO A VIRUS

Enhanced Effectiveness of the Rabbit Papilloma Virus on Hyperplastic Epidermis

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Plates 2 and 3

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The effects of some viruses are known to be altered when they act upon cells which have been previously rendered abnormal. The virus of herpes simplex induces lesions resembling those of herpes zoster in rabbit skin altered by tar (1), and ultraviolet irradiation of rabbit skin modifies the local susceptibility to inoculation with vaccine virus (2). The Shope papilloma virus (3) elicits carcinomas forthwith, as well as various papillomas of unusual sort, when it is brought into association with the epidermis of domestic rabbits, which has been tarred for some weeks (4). The experiments reported in the present paper were undertaken to learn whether preliminary alterations of rabbit epidermis would provide a more favorable soil for the demonstration of the papilloma virus. Often no virus can be got from the papillomas of domestic rabbits directly produced therewith though its presence in the growths is readily demonstrable serologically (5, 6) as also in the cancers deriving from them (7).

Material and Methods

Papilloma virus was obtained from the naturally occurring growths of cottontail rabbits which had been preserved in 50 per cent glycerin-Locke's solution at about 4°C. Weighed portions of the tissue were passed through several changes of saline, ground with sand, and suspended in 10 to 20 volumes of 0.9 per cent saline. The crude extracts thus obtained were spun in an angle-head centrifuge at about 3500 R.P.M. for 5 minutes and the supernatant fluids were again spun at about 4500 R.P.M. for 20 minutes. They were now clear amber and were usually highly infectious as such and after filtration through Berkefeld V candles. For test they were inoculated intradermally or rubbed into the skins of adult domestic rabbits of the gray-brown (agouti) breed or cottontail rabbits (genus *Sylvilagus*) after scarification with sandpaper, according to a method already described (5, 6). The character of the papillomas arising was recorded at frequent intervals from the 8th day to about the 42nd day after inoculation according to a standard scale: ++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = 5 to 15 papillomas, ± = 2, 3, or 4 papillomas, ± = 1 papilloma, 0 = negative.

To conserve space, the readings of only a few days are given in the tables. They are representative of the findings as a whole.

Influence of Carcinogenic Agents to Alter Skin Susceptibility

In a first experiment the results of rubbing a suspension of papilloma virus into scarified skin were compared with those consequent on infiltrating tarred rabbit ears with the suspension by way of a marginal vein.

Experiment 1.—The ears of fourteen normal cottontail rabbits were tarred over the inner and outer surfaces on three occasions at intervals of 3 or 4 days. This amount of tarring caused the ears to become acutely inflamed, thickened, and moist, but elicited no tumors. A strong rubber band was placed about the base of the ear, to stop the circulation, and 5 cc. of a virus filtrate (W.R. 1-28) diluted from 1:500 to 1:480,000,—in terms of the papilloma material originally extracted,—was then slowly injected into a marginal vein of the ear. The rubber bands were removed 5 minutes after injection. The ears of each rabbit received different dilutions of the virus. 0.2 cc. of the same dilutions was then rubbed into scarified areas of normal skin on the abdomens of the same rabbits.

The results can be briefly summarized. Six of the rabbits proved resistant to infection, only a few growths arising from either the tarred or the normal epidermis. Eight rabbits were susceptible and in every one many more papillomas developed from the tarred epidermis than from the normal skin inoculated with the same dilution of virus. The ratio of the number of papillomas on the tarred skin to that on the normal skin was as high as 135 or more to 1. Furthermore papillomas were elicited in the tarred ears by a considerably higher dilution of virus. In one instance a dilution of 1:80,000 elicited papillomas on the tarred epidermis, yet 1:500 failed to do so on the untreated skin. In five animals papillomas appeared on the tarred ears and none on the scarified skin in response to the same dilution of virus. All of the growths were benign papillomas.

The findings indicate that rabbit skin to which tar has been applied a few times is more susceptible to infection with the papilloma virus than normal scarified skin. The objections can be raised, however, that the total amount of virus introduced into the tarred ears was greater than that rubbed into the skin of the abdomen and that neither the skin situations nor the methods of inoculation were comparable. To control these variables an experiment was done next in which virus was rubbed into areas of skin, some of which had been tar-treated while others had been left untreated prior to scarification. As enlarging the test, other areas which had been treated previously with methylcholanthrene or benzpyrene were inoculated with virus.

Experiment 2.—The hair was clipped from twelve rectangular areas of skin measuring about 3 × 5 cm. on the abdomens of four domestic rabbits. Small hairy strips were left between the areas which were in three anteroposterior rows of four areas each. One area of each row was painted with a carcinogenic tar, another with 0.3

per cent methylcholanthrene in benzene, and a third patch with 0.3 per cent benzpyrene in benzene.[1] Small camel's hair brushes were used to apply the agents. The remaining area of each row served as control. The situations of the control and treated areas were varied from animal to animal. After each treatment they were covered individually with sterile gauze pads, moored with adhesive to prevent spread of the reagents, after which a gauze pad and many-tailed binder were put over all. The skin treatments were repeated twice per week for 2 weeks. They elicited no tumors.

Two days after the last treatments the patches were greatly changed. Those that had been tarred showed on stripping an acutely inflamed and thickened epidermis covered with a moist yellowish-brown scurf. The methylcholanthrene-treated skin was also inflamed and greatly thickened, but less so, and was covered with a dry brown scurf. The benzene-treated skin had a similar appearance. To learn the nature of the changes a narrow slice of a normal area and of each of the treated patches was taken under ether anesthesia from each rabbit, fixed in acid Zenker solution, and stained with eosin and methylene blue.

Immediately after the biopsies the areas were scarified with sandpaper and a 0.5 per cent virus extract (W.R. 1-28) was rubbed into one row of the normal and treated skin areas, and another similarly prepared extract (W.R. 11-69) into another row. The treated areas proved more difficult to scarify than the normal skin, the tar-treated areas especially, because of the moist adherent scurf. Immediately after inoculation the patches were dried in a blast of warm air and bandaged as before.

The results of the experiment are shown in Table I. The findings with the two virus extracts can be considered together. On the 10th day after virus inoculation, discrete papillomas had appeared on most of the methylcholanthrene-treated skin areas and on a few of the benzpyrene- and tar-treated areas. No growths were visible on the normal skin at this time. By the 15th day the papillomas on the treated skin areas had greatly increased in number and had enlarged rapidly, many of them now being semiconfluent papillomatous masses. The normal skin areas, on the other hand, showed relatively few papillomas and they were small and discrete. On the 20th day most of the treated skin areas showed large, vigorous, confluent or semiconfluent papillomatous masses up to 1.0 cm. high, while the growths on the normal skin were still discrete, in some instances just appearing, and only occasionally were as much as 0.3 cm. high. The papillomas on the treated patches were in many instances dark gray while those on the healed normal skin of the same animal were pink or light gray. All were ordinary virus-induced papillomas.

The experiment shows (Table I) that a few preliminary applications to rabbit skin of tar, benzpyrene, or methylcholanthrene greatly enhance the susceptibility of the skin to papilloma virus infection. Growths appeared earlier in the treated skin, were more numerous, tended to be more pigmented,

[1] The tar came from the Ostergasfabrik of Amsterdam, and was the generous gift of Dr. Karl Landsteiner. It has been employed in much work in this laboratory. The methylcholanthrene was obtained from the Eastman Kodak Company and the benzpyrene from Hoffmann La Roche, Inc.

and grew more rapidly, forming large papillomatous masses at a time when the growths in the normal skin were just appearing or were still small and discrete. Similar findings were obtained in another experiment when virus was inoculated into normal and methylcholanthrene-treated skin by means of a tattoo machine (Figs. 6 and 7).

TABLE I

Susceptibility to the Papilloma Virus of Normal Skin and Skin Treated with Carcinogenic Agents

Papilloma virus extract	Agent applied to skin before virus inoculation*	Pathogenicity tests											
		10th day				15th day				20th day			
Test rabbits.....		a	b	c	d	a	b	c	d	a	b	c	d
No.													
	Normal skin	0	0	0	0	+	±	±	+±	++	++	+±	++±
W.R. 1-28	Tar	0	0	0	+	+±	+++	+	+++	+++±	++++	+++	++++
(0.5 per	Benzpyrene	+	0	0	±	++±	+++	++	+++±	+++	+++±	++++	++++
cent)	Methylcholan-threne	+±	±	±	+±	+++	+++	+++	+++±	++++	++++	++++	++++
	Normal skin	0	0	0	0	+	±	±	+±	++	+±	+	++
W.R. 11-69	Tar	0	0	0	0	±	++±	±	+++	++	++++	++	+++±
(0.5 per	Benzpyrene	0	0	0	±	++	++±	+	++±	+++±	++++	+++	+++±
cent)	Methylcholan-threne	+	±	0	±	+++	+++	++	+++	++++	++++	+++	+++±

* Benzpyrene and methylcholanthrene (0.3 per cent in benzene) and tar applied twice per week for 2 weeks. Virus inoculated 2 days after last treatments.

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

Effect of Non-Carcinogenic Agents to Alter Skin Susceptibility

The agents used in the preceding experiment were potent carcinogens, but were employed for so brief a period that they elicited no tumors. A test was next done to find whether non-carcinogenic agents, inflammatory for rabbit skin, would have the same effect.

Experiment 3.—Twelve skin areas on the abdomens of four domestic rabbits were clipped as in Experiment 2. Three on each animal were painted with benzene as such and three others with 0.3 per cent methylcholanthrene in benzene three times per week for 2 weeks. Three more areas were exposed to 1500 r of x-ray irradiation 48 hours before virus inoculation. The rabbits were protected by flexible lead foil during irradiation, leaving only the areas exposed, and the rays came from a single tube run at 5 milliamperes and at a peak voltage of 135 kilovolts, without filtration.

The distance from tube to skin was 50 cm. The three remaining skin areas served as controls. A representative piece of the normal and treated skin areas was taken for microscopic study 2 days after the last treatments. The methylcholanthrene-treated skin showed the same gross changes described in Experiment 2. A 5 per cent virus filtrate (W.R. 1-30) was then rubbed into one area of each sort after scarification and into another after dilution of the filtrate to 0.1 per cent. The inoculations were made immediately after the biopsies.

Table II shows the results of the experiment. On the *12th day* after virus inoculation the benzene- and methylcholanthrene-treated patches inoculated with 0.1 per cent filtrate showed discrete papillomas in three of the four test rabbits. No growths could be seen on the normal or x-rayed skin areas at this time. All of the areas inoculated with 5 per cent filtrate showed papillomas, the growths on the methylcholanthrene-treated areas being large, semiconfluent masses in contrast to the few small discrete growths just appearing on the normal and x-rayed patches. Many more discrete papillomas were present on the benzene-treated areas than on the normal skin, but they were fewer in number and smaller than on the skin areas which had received methylcholanthrene. By the *35th day* the papillomas on the benzene- and methylcholanthrene-treated areas inoculated with the dilute filtrate had developed into confluent and semiconfluent papillomatous masses, whereas the growths on the normal and x-rayed skin areas produced with the same inoculum were still small and discrete though increasing in number. The skin areas inoculated with 5 per cent filtrate all showed confluent papillomas, but those on the normal and x-rayed patches were low mounds, up to 0.6 cm. high, whereas on the benzene-treated areas they were jagged peaks up to 1.0 cm. high and on the skin treated with methylcholanthrene they were up to 1.6 cm. high. The last mentioned growths were deeply pigmented, while the others were pink or merely streaked with gray. Microscopic sections of the growths showed them all to be ordinary papillomas.

To extend the findings another experiment was done. This time the skin areas were treated with ultraviolet light, methylcholanthrene, and a mixture of turpentine and acetone.

Experiment 4.—A mixture of turpentine and acetone in equal parts,—which had proved non-carcinogenic (8),—was applied to three clipped areas on the abdomens of four domestic rabbits five times at 2 day intervals and 0.3 per cent methylcholanthrene in benzene in like manner to another three. Three other areas were exposed to a quartz mercury vapor lamp[2] for 40 minutes at a distance of 25 cm. 48 hours before virus inoculation. This caused a marked erythema. Three untreated skin areas served as controls. The turpentine and acetone-treated skin became acutely inflamed, thickened and hyperkeratotic, as greatly changed in the gross as the methylcholanthrene-treated skin. A virus filtrate (W.R. 1-70) in concentrations of 0.1 per cent and 5 per cent was rubbed into two rows of the areas after scarification.

The results are shown in Table III. It will be seen that the papillomas on the methylcholanthrene- and turpentine and acetone-treated areas arose earlier and in

[2] Alpine sun lamp, Hanovia Chemical and Manufacturing Company.

TABLE II

Susceptibility of Normal Skin and Skin Treated with X-Rays, Benzene, and Methylcholanthrene

Dilution of virus filtrate W.R. 1-30	Skin treatment before virus inoculation*	Pathogenicity tests										
		12th day				18th day				35th day		
Test rabbits		a	b	c	d	a	b	c	d	a	b	c
per cent												
0.1	Normal skin	0	0	0	0	±	±	+	±	++	+±	++
	X-rays	0	0	0	0	±	+	0	0	+±	++±	+±
	Benzene	+	+	0	+	+±	++	++	++	+++±	+++±	+++
	Methylcholanthrene	±	+	0	±	+±	++	++±	+±	+++±	+++±	++++
5	Normal skin	±	+	±	+	++	+++	++±	++±	++++	++++	++++
	X-rays	±	+	±	±	++	+++	+++	+±	+++±	++++	++++
	Benzene	++	++±	++	++	+++±	++++	+++±	+++±	++++	++++	++++
	Methylcholanthrene	+++	+++±	+++	+++±	++++	++++	++++	++++	++++	++++	++++

* Benzene applied to skin areas three times per week for 2 weeks before virus inoculation.

0.3 per cent methylcholanthrene in benzene applied three times per week for 2 weeks before virus inoculation.

X-ray irradiation, 1500 r.

Virus inoculated 2 days after last treatments.

TABLE III

Susceptibility of Normal Skin and Skin Treated with Ultraviolet Light, Turpentine and Acetone, and Methylcholanthrene

Dilution of virus filtrate W.R. 1-70	Skin treatment before virus inoculation*	Pathogenicity tests											
		11th day				18th day				35th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
per cent													
0.1	Normal skin	0	0	0	0	±	+±	+	+±	++	+++±	++±	+++±
	Ultraviolet light	0	0	0	0	+±	+±	+	+	+++	+++±	++±	+++
	Turpentine-acetone	±	+	0	±	+++	++++	++	+++	++++	++++	++++	++++
	Methylcholanthrene	0	±	+	0	+++	+++	+++	++±	++++	++++	++++	++++
5	Normal skin	0	++	+	+	+++	++++	+++	++++	++++	++++	++++	++++
	Ultraviolet light	+	+++	++	++	++++	++++	+++	++++	++++	++++	++++	++++
	Turpentine-acetone	+±	+++	++±	+++	++++	++++	++++	++++	++++	++++	++++	++++
	Methylcholanthrene	+±	+++	++++	+++	++++	++++	++++	++++	++++	++++	++++	++++

* Ultraviolet light, 40 minutes irradiation from a carbon lamp 24 hours before virus inoculation.

Turpentine and acetone in equal parts applied to skin five times at 2 day intervals.

0.3 per cent methylcholanthrene in benzene applied to skin five times at 2 day intervals.

greater numbers than on those which had been normal. There was no significant difference between the growths on the treated areas and they all soon became large, confluent papillomatous masses. The papillomas arising on the areas which had been exposed to ultraviolet light were like those on the normal skin in number and size.

The results of Experiments 2, 3, and 4 (Tables I, II, and III) show that a variety of agents, some carcinogenic, others not, will render the skin abnormally susceptible to virus infection. A mixture of turpentine and acetone was as effective in this respect as was tar or methylcholanthrene. These findings have been confirmed in many subsequent tests in which the procedures have been utilized for various purposes. Mere acute inflammation produced by ultraviolet light did not render the skin more susceptible, nor did the Roentgen rays.

Optimal Preparation of the Skin for Virus Infection

Steps were now taken to determine the number of applications of methylcholanthrene or of turpentine and acetone which renders the skin most susceptible to the papilloma virus.

Experiment 5.—0.3 per cent methylcholanthrene in benzene was painted onto a skin area of each of four domestic rabbits at 2 day intervals for a total of six times, and another comparable skin area was simultaneously treated with a mixture of turpentine and acetone. Two other skin areas of the same animals were treated with the agents three times at 2 day intervals, while another two areas received but a single application. The schedule was so arranged that the last application was made 24 hours prior to virus inoculation. Two skin areas on each rabbit were left untreated. As usual the situation of the treated areas was varied. A single application of methylcholanthrene caused only a reddening of the skin after 24 hours. After three applications, however, the skin was acutely inflamed, thickened, and covered with a branny scurf. Six treatments caused even greater changes with marked thickening and much scurf, some of which could be flaked away. Turpentine and acetone caused similar changes, but the skin was slightly less thickened, although more inflamed. Biopsy specimens were taken from all of the areas. Immediately afterward the areas were scarified and a 0.5 per cent virus extract (W.R. 1-28) was rubbed into them.

The results are summarized in Table IV. It will be seen that a single application of methylcholanthrene or turpentine and acetone did not alter the susceptibility of the skin to papilloma virus infection; the incubation period and the number and size of growths were about the same on the normal and treated skin areas. Those treated three times, however, showed numerous papillomas before any could be seen on the normal areas and they had become large, confluent masses, up to 1.6 cm. high, at a time when growths on the normal skin were still discrete or semiconfluent and no more than 0.4 cm. high at most. Six applications of the agents rendered the skin only slightly more susceptible to virus infection than did three applications, in spite of the fact that it appeared much more changed, and histologically was really so, as will be shown further on.

Six applications of methylcholanthrene or turpentine and acetone at 2 day intervals are about the maximum that the skin of the rabbit's abdomen will bear without becoming macerated.[3] It will withstand many applications of methylcholanthrene, however, if applied at 3 to 4 day intervals. Further experiments have shown that many treatments with these agents fail to render the epidermis more susceptible than do three to six applications at 2 day intervals.

TABLE IV

Susceptibility of Skin Prepared for Various Lengths of Time

Agent applied to skin before virus inoculation*	No. of applications	Pathogenicity tests‡											
		12th day				16th day				24th day			
Test rabbits.....		a	b	c	d	a	b	c	d	a	b	c	d
Normal skin		0	0	0	0	+	+±	±	++	+++	+++	++	+++
Turpentine-acetone	One	0	0	0	0	++	+	+	+	+++±	++±	++	+++
	Three	+±	+±	+±	++	+++	+++	+++	+++	++++	++++	++++	++++
	Six	+	+++	+++	+	+++	+++±	+++±	+++	++++	++++	++++	++++
Methylcholanthrene	One	0	0	0	0	+	±	±	+±	+++	++	++	+++
	Three	+±	+±	+	++	++	+++	++±	+++	++++	++++	++++	++++
	Six	+±	++	+++±	++	+++	+++	+++±	+++	++++	++++	++++	++++

* Turpentine and acetone in equal parts.

Methylcholanthrene, 0.3 per cent in benzene.

‡ Virus extract, W.R. 1-28, 0.5 per cent, rubbed into scarified skin 24 hours after preparation.

Duration of the Abnormal Susceptibility

Does the increased susceptibility of methylcholanthrene-treated skin to papilloma virus infection persist when applications of methylcholanthrene are discontinued? To answer this question skin areas were treated with methylcholanthrene in the next experiment and then inoculated with papilloma virus at various intervals from 1 day to 1 month after completion of the treatments.

Experiment 6.—Five areas on each of five rabbits were painted with 0.3 per cent methylcholanthrene in benzene at intervals of 2 days for a total of four times. The treatment of each skin area was begun on a different date so that from 24 hours to 4

[3] The agents mentioned induce a profuse growth of hair. It has been found that removal of this with clippers prior to each application provides a better prepared epidermis which can be readily scarified.

weeks had elapsed on the day when they were all inoculated with 0.1 per cent virus filtrate (W.R. E). Just prior to inoculation biopsies were made as usual.

Table V shows the results of the Experiment. As noted in the preceding tests, papillomas appeared earlier and in greater number on the methylcholanthrene-treated skin inoculated 24 hours after completion of the treatments than on scarified normal skin. The areas inoculated with virus 1 week afterwards showed slightly fewer papillomas in four of the five test rabbits but in one instance there was no difference. The skin inoculated 3 weeks after discontinuation of the methylcholanthrene applications showed more growths than the normal skin in two instances only, and the patches inoculated 4 weeks after the applications had been stopped yielded the same results as the normal

TABLE V

Duration of the Increased Susceptibility of Methylcholanthrene-Treated Skin

Time from skin treatment* to virus inoculation‡	Pathogenicity tests														
	14th day					16th day					21st day				
Test rabbits.	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
1 day	+	++	+++	+++	+±	++	++	+++	+++	++±	+++	++++	++++	++++	+++±
1 wk.	±	+±	+±	+++	+±	++	+±	++	+++	++	+++	+++	++++	++++	+++
2 wks.	±	+	0	++	+	++	+	±	++±	+±	++	+±	+±	+++	++±
3 wks.	0	0	0	+±	±	±	±	±	++	+±	+	+±	+	++	++±
4 wks.	0	0	0	0	0	+	±	±	+±	±	+	+	+±	++	+±
Normal skin	0	0	0	0	0	+	±	±	+	±	++	+	+±	++	+

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals.
‡ Virus filtrate, W.R. E, 1:1000.

skin. It is thus apparent that the increased reactivity of methylcholanthrene-treated skin to papilloma virus infection is transitory and is lost within 2 to 4 weeks after the treatments are stopped.

Comparative Titrations of the Virus on Normal and Altered Skin

The increased number, shortened incubation period, and the rapidity with which the papillomas enlarged when virus was inoculated into properly prepared skin pointed to a markedly increased effectiveness of the virus. Experiments were next undertaken to learn whether it would be infectious in dilutions which yield no growths under ordinary circumstances. The virus is rarely infectious on the scarified normal skin of domestic rabbits in dilutions beyond 1:100,000 (in terms of weight of papilloma tissue extracted) (6).

Experiment 7.—Six squares were clipped on one side of the belly of four rabbits, and painted with a mixture of turpentine and acetone five times at 2 day intervals. They

showed the usual alterations. Six corresponding squares on the other side were not treated. All were scarified and a papilloma virus extract (W.R. 1-28) in dilutions of from 1:10,000 to 1:10,000,000 was rubbed into corresponding normal and treated squares. The inoculations were made 24 hours after the last of the turpentine-acetone applications.

It will be seen (Table VI) that by the 16th day after inoculation all of the treated areas which had received the 1:50,000 dilution of virus showed papillomas and the 1:100,000 had also caused them in one animal. No growths whatever had appeared at this time on the control squares. Later the control areas showed papillomas in every case as result of the 1:50,000 dilution, and the 1:100,000 dilution also caused them in the more susceptible animals. But

TABLE VI

Titration of a Virus Preparation in Normal and in Turpentine and Ace one-Treated Skin

Dilution of virus extract W.R. 1-28	Pathogenicity tests															
	16th day								42nd day							
	Normal skin				Turpentine and acetone-treated skin*				Normal skin				Turpentine and acetone-treated skin*			
Test rabbits...	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
1:10,000	0	0	0	0	+	+	++±	+±	+++±	+++±	+++	++±	++++	++++	++++	++++
1:50,000	0	0	0	0	±	±	±	±	+	+++	+	±	+++	+++	+++	++±
1:100,000	0	0	0	0	0	±	0	0	±	++±	±	0	+++	+++	++	+
1:500,000	0	0	0	0	0	0	0	0	0	0	0	0	+	++	++	±
1:1,000,000	0	0	0	0	0	0	0	0	0	0	0	0	±	+	±	0
1:10,000,000	0	0	0	0	0	0	0	0	0	0	0	0	0	±	0	0

* Turpentine and acetone in equal parts applied three times per week for a total of five times before virus inoculation.

the virus had proved greatly more effective on the treated areas. At 1:100,000 it regularly produced papillomas, as did the 1:500,000 dilution, while in three of the four animals the 1:1,000,000 dilution proved effective, and in one individual the virus extract yielded papillomas when diluted 10,000,000 times.

The experiment shows that not only was the number of papillomas increased and the incubation period shortened by the use of treated skin but the titer of the virus was much stepped up—from 10- to 100-fold. Another, similar experiment, using methylcholanthrene-treated skin and another virus extract, yielded practically identical results. In a subsequent paper many instances will be given, incidentally to other work, of the enhanced susceptibility of treated skin.

Effect of Skin Alterations before and after Virus Inoculation

It seemed possible that methylcholanthrene or turpentine and acetone applications to the epidermis after virus inoculation might have the same

effect as preliminary treatment. An experiment was done to test the possibility.

Experiment 8.—Nine areas in three parallel rows were clipped on the abdomens of four rabbits. Those of one row were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times, with result in the usual changes. The other six patches were not treated. Two days after the last treatment all were scarified and inoculated with a virus extract (W.R. 2-95), in dilutions of 1:50,000,

TABLE VII

Infectivity of Virus in Skin Treated with Methylcholanthrene before and after Inoculation

Skin treatment	Dilution of virus extract W.R. 2-95 used for inoculation	Pathogenicity tests											
		18th day				28th day				35th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Methylcholanthrene applied to skin four times *before* inoculation*	1:50,000	++±	++	++±	++	+++	++++	+++	+++±	+++±	++++	+++	+++±
	1:500,000	±	+	±	+	+±	++	+	++	++	++	+	++
	1:5,000,000	0	±	0	0	+	+	±	±	+	+±	±	±
Methylcholanthrene applied to skin four times *after* virus inoculation‡	1:50,000	±	0	0	±	±	+	0	±	±	+	0	±
	1:500,000	0	0	0	0	0	0	0	0	0	0	0	0
	1:5,000,000	0	0	0	0	0	0	0	0	0	0	0	0
Normal skin	1:50,000	±	0	0	0	++	+	±	0	++	+	±	0
	1:500,000	0	0	0	0	±	0	0	0	±	0	0	0
	1:5,000,000	0	0	0	0	0	0	0	0	0	0	0	0

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals before virus inoculation.

‡ 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals beginning 5 days after virus inoculation.

1:500,000, and 1:5,000,000. One area of each row received the same dilution of virus. Five days later, when the skin areas had almost healed, one of the rows of patches which had not been treated prior to inoculation was painted with 0.3 per cent methylcholanthrene in benzene, and the applications were kept up at 2 day intervals for a total of four times, again inducing the familiar changes. To the other rows of patches nothing further was done.

The results of the experiment are set down in Table VII. On the 18th day after inoculation the virus diluted 1:500,000 had caused papillomas on all of the patches treated with methylcholanthrene prior to inoculation and so too had a dilution of 1:5,000,000 in one rabbit. The control patches and those treated with methylcholanthrene after inoculation showed at this time no

papillomas where virus had been inoculated in dilutions above 1:50,000, and they were only just appearing where this latter had been put. By the 35th day (after which no growths appeared) the 1:5,000,000 dilution of the virus had produced papillomas on all of the skin areas treated before inoculation, but had failed to cause growths on untreated areas or on those that were treated with methylcholanthrene after virus inoculation. 1:50,000 caused large, confluent or semiconfluent papillomatous masses on all of the areas prepared before inoculation, but it produced only a few discrete growths on the other areas.

Manifestly, methylcholanthrene treatment of the skin after virus inoculation did not enhance the effects of the virus. A similar experiment, using a mixture of turpentine and acetone instead of methylcholanthrene, yielded similar results except that fewer growths arose on the skin treated with the turpentine and acetone after scarification than on the untreated skin.

Results of Altering the Skin of Cottontail Rabbits

The work was now extended to cottontail rabbits, the natural hosts of the papilloma virus, to learn whether their skin can be rendered more susceptible to the virus.[4]

Several experiments of the sort already described were frustrated by skin injury, maceration and bacterial infection occurring when two to four applications of methylcholanthrene or the mixture of turpentine and acetone were applied at 2 day intervals, —procedures well tolerated in the domestic rabbit. However, when the intervals were lengthened to 4 days the skin underwent the same gross changes as in these latter animals. But unfortunately it also became notably susceptible to bacterial infection and scarification frequently resulted in broad abscesses. To avoid these, resort was had to intradermal inoculations of the virus. These gave irregular results, yet by and large the several experiments gave clear indications that methylcholanthrene, benzene, and a mixture of turpentine and acetone, all render the epidermis of the cottontail rabbit unusually susceptible to the papilloma virus.

Nature of the Skin Changes

The agents that enhanced the susceptibility of the skin for the papilloma virus all caused marked alterations of the same general sort. In the gross they consisted of thickening, scurfing, increased pigmentation, and more or less

[4] The papilloma virus ordinarily titers slightly higher in susceptible cottontails than in the domestic species. Table VIII shows the results of rubbing graded dilutions of a virus extract (W.R. 1-28) into scarified skin areas of six normal domestic rabbits and six cottontail rabbits. One cottontail proved immune to the virus and hence was not included in the table. It will be seen that the virus extract produced growths in all of the cottontail rabbits in dilutions up to 1:800,000, whereas this last caused none in any of the domestic rabbits, and in only two of the six did a dilution of 1:400,000 cause any.

acute inflammation. The extent of the changes, however, varied with the agent used.

The microscopic findings can be briefly summarized:—

The *normal skin* of the abdomen of the domestic rabbit has a thick connective tissue stroma and a mere skim of superficial epidermis (Fig. 1), consisting of a Malpighian layer one to three cells thick which keratinizes abruptly without differentiation. The hair follicles do not go deep and the sebaceous glands are small and inconspicuous. Three to four applications of *tar* to the skin were found to cause striking changes (Fig. 2). The Malphighian layer became greatly thickened and the cells appeared larger than normal and showed many mitoses. They differentiated gradually, forming a granular layer and a thick stratum of keratinization. The hair follicles became greatly distended with keratin in many instances. Numerous sebaceous glands appeared and the dermis showed an acute inflammatory reaction with edema, small hemorrhages, and cellular infiltration. All these changes have been often described before. A single application of *methylcholanthrene* caused a mild inflammatory reaction in the dermis and the basal cells of the epidermis appeared to be slightly larger than normal but were not increased in number. After three or four applications the epidermis was markedly hyperplastic and the changes were similar to those seen in the tarred skin, but lesser in degree (Fig. 8). After six applications there was even greater thickening and irregularity of the epidermis and portions of the skin were necrotic. When the treatments were discontinued the skin gradually reverted to the normal. Within 1 week after the skin had received four applications of methylcholanthrene, regression was evident. The epidermis was still thicker than normal and exhibited a graded differentiation but the cells showed far fewer mitoses than 24 hours after the treatments and were smaller and less abnormal. Within 2 weeks involution was advanced, and after 3 weeks the epidermis had returned to normal save for a slight hyperkeratosis. The hair follicles appeared normal now except at the bases, where there was still some thickening of the epithelial layer and the cells showed numerous mitoses and were slightly larger than normal. A few polymorphonuclear leucocytes could still be seen in the connective tissue. After 4 weeks the skin seemed wholly normal. *Benzpyrene* caused changes similar to those induced by methylcholanthrene except that the sebaceous glands were more hyperplastic and occasionally cystic.

The non-carcinogenic agents which were effective in increasing the susceptibility of the skin for the virus brought about much the same histological changes. *Turpentine and acetone* caused less hyperplasia of the epidermis than methylcholanthrene (Fig. 3) but a more acute inflammatory reaction in the subcutaneous tissue. The *benzene-treated skin* was less hyperplastic than that altered by methylcholanthrene, but the general changes were similar. Occasionally there were small patches of epidermal necrosis. The sebaceous glands were markedly hyperplastic and showed differentiation.

Ultraviolet light caused an acute inflammatory reaction 48 hours after irradiation, but the epidermis was still as thin as usual at this time and sometimes showed small areas of necrosis. *X-ray irradiation* elicited no significant changes in the skin under the conditions employed (Experiment 3). These agents failed to alter the susceptibility of the skin for the virus, as Experiments 3 and 4 showed.

The microscopic changes elicited in cottontail rabbit skin by the carcinogenic and non-carcinogenic agents were at least as marked as those seen in the domestic rabbit and usually more so. Two to three applications of methylcholanthrene, benzene, or turpentine and acetone at intervals of 4 days changed the epidermis of the wild rabbit from a thin, delicate epithelium one to two cells thick, to a greatly thickened and irregular, differentiating sheet, with distended, hyperplastic hair follicles and numerous sebaceous glands. Further applications usually rendered the epidermis necrotic.

The findings plainly showed that the various agents which enhanced the susceptibility of rabbit skin all caused the epidermis to become hyperplastic and to proliferate actively. The agents which proved ineffective,—ultraviolet light and x-ray,—did not do so, although the former produced an acute inflammatory reaction in the skin.

The Skin Changes Induced by Scarification

Several authors have described the early stages in virus-induced papillomatosis (9) but no one has inquired into the changes which follow immediately upon scarification of the skin and virus inunction. It seemed probable that a knowledge of these changes would aid toward an understanding of the reasons for the increased susceptibility of altered skin, and consequently a study of them was undertaken. Papilloma virus was rubbed into some scarified areas and a solution of minute graphite particles (hydrokollag (10)) into others, to learn the fate of particulate matter as such.

Experiment 9.—Nine skin areas on the abdomens of three domestic rabbits were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times. 24 hours after the last treatment a representative piece of the changed skin was removed from each animal. All of the areas were then scarified with sandpaper to the usual extent, that is to say, until there was oozing of serum, sometimes blood-tinged, and a 5 per cent papilloma virus extract (W.R. D) was rubbed into three of the patches, a dilute suspension of hydrokollag in saline into three others, while the remaining three areas were left as such. In addition nine untreated areas on each of three other rabbits were similarly scarified and the virus and the hydrokollag suspension were rubbed into them. Slices of skin were taken from all of the various areas 5 hours after scarification, and additional pieces were procured after 1, 2, 4, 6, 8, 11, and 14 days. They were fixed in acid Zenker's and stained with eosin and methylene blue. Duplicate sections from the areas receiving hydrokollag were stained with Lichtgrün as well.

It was found that scarification of the *normal skin* to the extent ordinarily employed for virus inoculation removed practically all of the epithelial covering and some of the superficial connective tissue as well, cutting across the hair follicle shafts near the surface. Only occasional small islands of surface epidermis were left. After 24 hours the connective tissue was edematous and showed a few polymorphonuclear leucocytes and a thin scab had formed on the surface, but as yet no epithelial repair had taken place. The scab had become much thicker after 24 hours, owing not only to the ac-

cumulation of dried exudate but mostly to necrosis of the superficial connective tissue layer with incorporation of the dead material in the scab. After 48 hours epithelial regeneration was for the first time plainly evident. It took origin almost entirely from the cells of the hair follicle shafts, the epithelial cells extending laterally between scab and living connective tissue and sometimes into crevices in the latter. The first extension seemed to be mostly migration though occasional mitoses could be seen. The newly formed cells showed many mitoses later. After 2 to 4 days they had multiplied and spread laterally to such extent as to form umbrella-like expanses with the hair follicles as the staffs of the umbrellas. At this time the epidermis had not nearly covered all of the denuded surface. The connective tissue was still edematous and showed some round cells and polymorphonuclear leucocytes but the scab had begun to separate here and there. After 4 to 8 days the surface was entirely covered with a layer of hyperplastic epithelium (Fig. 5) three to six cells thick which had differentiated into granular and keratinized layers. The picture was markedly different from the normal (Fig. 1). The hair follicle shafts had thickened only slightly except next the orifices where the epithelium was hyperplastic. There was no evident stimulation of the sebaceous glands. At this time the surface scab had only just come away and remnants of it could be seen. Later the hyperplastic epidermis slowly took on a normal appearance and by the 14th day it had nearly done so, though here and there it was still slightly thickened and hyperkeratotic.

The skin areas inoculated with papilloma virus showed similar general changes during the first days, and with the inoculum employed,—which gave rise to growths relatively late,—changes referable to the virus were not discerned until 6 to 8 days had elapsed. Then the characteristic alterations of early papillomatosis (9) could be made out here and there. By the 11th to the 14th day there were discrete characteristic growths. The microscope showed most of them to have arisen from the basal layers of the new hyperplastic epidermis which now covered the surface between the hair follicles.

The areas into which hydrokollag had been rubbed showed the black particles in close contact with the abraded surface immediately after the inunction and in direct contact with the epithelium of the hair follicles that had been cut across. But as the scab thickened, owing to the necrosis of the superficial tissue with incorporation in it of the dead layer, almost all of the hydrokollag became incorporated too, only traces remaining in crevices here and there where it might come in contact later with the regenerating epithelial cells. Careful search was made for phagocytosis of it by these elements but none could be found, and when the newly formed epidermis keratinized, by the 4th to the 8th day after scarification, it was all cast off.

These findings show that the scarification of normal skin as ordinarily done removes nearly all of the surface epidermal cells and entails a loss of most of the virus (if one can judge from what happens to hydrokollag) in the scab which forms, the result being that the chance for it to reach susceptible cells is greatly reduced. Almost the only spots at which virus is directly brought into contact with epithelium are where the hair follicles have been cut across and even here the epithelium commonly becomes implicated in the later

necrosis and is lost. Papillomas derived from the hair follicle epithelium are relatively infrequent. Instead they usually appear on the surface between the hair follicles and one must suppose they principally arise from young, regenerating cells which have extended laterally from the follicles to cover the denuded surface.

Scarification of the *methylcholanthrene-treated skin* (Fig. 8) was done to the same extent as with normal skin, that is to say, until the surface of the patch seemed everywhere abraded and serum oozed out. Though the surface epithelium was markedly hyperplastic, the microscope showed that practically all of it had been removed as in the case of the normal skin, exposing the fibrous corium and the hair follicle shafts (Fig. 9). These latter were hyperplastic. The connective tissue became edematous as usual, polymorphonuclear leucocytes wandered into it, and scabbing took place with increase in the thickness of the scab by superficial necrosis (Fig. 10). But epithelial regeneration was far more rapid than under ordinary circumstances. Within 24 hours after scarification it was already far advanced (Fig. 10) and within 48 hours the entire abraded surface was covered (Fig. 11). Migration began by lateral extension from the hair follicles, and was attended by such active multiplication that very soon large, thick, umbrella-like structures with the follicles as the shafts of the umbrellas had formed beneath the surface scab (Fig. 12). They consisted of great numbers of epidermal cells, many of them in mitotic division, and as these spread along the surface beneath the scab they frequently invaded irregularly the crevices in the fibrous corium. The sheet of hyperplastic, actively regenerating epidermis that rapidly formed was much thicker than that produced by the regeneration of ordinary epidermis (Fig. 5) and consisted of a shallow keratinized layer and a Malpighian layer ten to fifteen cells deep, showing numerous mitoses. Irregular papillae extended down into the connective tissue. The hair follicles were distended with keratinized epithelium and the epithelial lining was thickened. There appeared to be a notable increase in the number of sebaceous glands and they were rendered prominent not only by hyperplasia but by retention of secretion which caused many of them to be actually cystic. It seems probable that the hyperplastic surface epithelium had become so crowded through active proliferation as to prevent escape of their contents. Four to 6 days after scarification the epidermis was more orderly, differentiating into wide granular and keratinized layers, and as keratinization progressed the scab came away. Involution took place rapidly and by the 14th day the epithelium had returned practically to normal save for irregularities of basal contour and local thickenings where the layer of living cells was still three to four cells deep. The hyperkeratosis was now in general less than that where the normal skin had been scarified 14 days previously. While involution was going on the sebaceous glands at first became still more cystic, some of them rounding out into small spheres but later the distention of them disappeared and they again became inconspicuous.

The methylcholanthrene-treated areas which were inoculated with virus after scarification underwent similar changes. The effects of the virus were evident in them earlier than when scarified normal skin had received the same inoculum, the characteristic cytological changes indicative of the beginning of papillomatosis being perceptible in one rabbit 4 days after inoculation and on the 6th day in others. The fate of the

hydrokollag was the same as when rubbed into scarified normal skin. Practically all of it was caught in the scab and came away when this did.

From these findings it is plain that the changes which render skin especially susceptible to the virus provide to the latter young, actively proliferating epidermal cells in unusually great number and at a much earlier stage in events than when normal skin has been scarified. Where the hair follicles have been cut across by the sandpaper many more cells are exposed to direct infection with the virus, and where it persists under the scab the regenerating epithelium soon reaches it.

Intradermal Inoculation of Virus into Prepared Rabbit Skin

Despite the defects of inoculation into scarified areas as just disclosed, it has proved the most certain method to the present for titrating the papilloma virus. Intradermal injection of active virus into normal skin does not always result in growths, and the incubation period is regularly longer than in scarified skin (4).[5] It has seemed worth while nevertheless to determine the effects of the intradermal inoculation of virus in skin altered by methylcholanthrene. Accordingly papilloma virus was so inoculated and was also rubbed into scarified normal and methylcholanthrene-treated skin areas at comparable situations in the same animals. Incidentally, to enlarge the general findings, a mixture of methylcholanthrene and Scharlach R was applied to some areas, and the dye was injected intradermally into other areas that had also been treated with methylcholanthrene prior to virus inoculation. Scharlach R is known to cause a profuse epidermal proliferation and sometimes temporary downgrowths simulating early carcinomatosis (11).

Experiment 10.—Four rectangular areas were clipped on each side of the abdomens of four rabbits. One on each side was painted with 0.3 per cent methylcholanthrene in benzene, another with a saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene, while a third was painted with 0.3 per cent methylcholanthrene in benzene and 0.1 to 0.2 cc. of a saturated solution of Scharlach R in olive oil was injected intradermally at several places. The treatments were repeated four times at 2 day intervals. The fourth area on each side served as control. Two days after the last treatments pieces were taken from each area of two of the rabbits for microscopic study. The mixture of methylcholanthrene and Scharlach R caused much greater skin alterations than did methylcholanthrene alone. The thickened skin was rendered far more rugose and became so redundant as to be thrown into folds. Microscopically, the changes resembled in many ways those seen in the tarred epidermis, already described, but the thickening of the surface layer of epithelium

[5] This is also true of tattoo inoculations.

TABLE VIII

Comparison of the Infectivity of the Virus in Domestic and in Cottontail Rabbits

Rabbit species	Dilution of virus extract W.R. 1-28	Pathogenicity tests																	
		16th day						28th day						42nd day					
Test rabbits		a	b	c	d	e	f	a	b	c	d	e	f	a	b	c	d	e	f
Domestic rabbits	1:10,000	0	0	±	±	+	++	+±	+±	+±	++	+±	+++	+±	+±	+±	++±	+±	+++±
	1:100,000	0	0	0	±	0	±	0	+	±	++	+	+	±	+	+	++±	±	++
	1:400,000	0	0	0	0	0	0	0	0	0	±	0	+	0	0	0	±	0	+
	1:800,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Test rabbits		g	h	i	j	k		g	h	i	j	k		g	h	i	j	k	
Cottontail rabbits	1:10,000	±	±	+	++	±		++	++	++±	+++	+++		+++	++	+++	+++	++++	
	1:100,000	±	±	0	+	0		+	+	+	+±	+±		+	+	+	++	+±	
	1:400,000	0	0	0	±	0		±	0	+	+	±		±	±	+	+	±	
	1:800,000	0	0	0	0	0		0	0	±	±	±		±	±	±	±	±	

TABLE IX

Comparison of Intradermal Virus Inoculation and Virus Inunction after Scarification of Normal and Prepared Rabbit Skin

Method of inoculation of virus extract W.R. 1-28, 1:500,000	Skin treatment before virus inoculation	Pathogenicity tests											
		20th day				28th day				35th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Intradermal	Normal (untreated)	0	0	0	0	0	0	0	0	0	0	0	0
	Methylcholanthrene*	±	+	±	±	+	+	+	±	+	+±	+	±
	Methylcholanthrene and Scharlach R‡	+	+	+	+	+±	+	+±	+	+±	++	+	+
	Methylcholanthrene and intradermal injections of Scharlach R§	+	±	±	+	+±	+	±	+±	+±	+±	+±	+±
Scarification	Normal (untreated)	0	0	0	0	0	0	±	±	0	0	±	±
	Methylcholanthrene*	+±	±	±	0	+±	+	++	±	+±	+	++	+
	Methylcholanthrene and Scharlack R‡	±	±	±	±	±	+	+±	+±	±	+	++	+
	Methylcholanthrene and intradermal injections of Scharlach R§	±	+±	±	±	+	+±	+±	+	+	+±	+±	+±

* 0.3 per cent methylcholanthrene in benzene.

‡ Saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene.

§ 0.3 per cent methylcholanthrene in benzene plus intradermal injections of a saturated solution of Scharlach R in olive oil.

was not so considerable (Figs. 2 and 4). The intradermal inoculation of Scharlach R in olive oil into the the methylcholanthrene-treated skin caused small nodules at the points of injection, consisting of much new formed connective tissue about the oil globules and what appeared to be malignant downgrowth in one instance,—spurious carcinomatosis as the event proved.

A virus extract (W.R. 1-28) diluted 1:500,000 in saline was inoculated into the patches 24 hours after the last treatment. It was rubbed into the normal and treated skin areas on one side of the abdomen after the usual scarification and injected intradermally (0.1 to 0.2 cc. in eight places) into those of the other side.

Table IX shows the results of the experiment. No papillomas resulted from the intradermal inoculation of the very dilute virus into normal skin, but where it had been rubbed into scarified areas a few growths slowly appeared in two of the four rabbits. The areas treated with methylcholanthrene or methylcholanthrene and Scharlach R, on the other hand, all yielded discrete papillomas in more or less considerable number and in general they were as numerous and grew as fast on the intradermally injected areas as on those that were scarified.

In this experiment the addition of Scharlach R, though producing more marked skin changes, failed to increase the susceptibility of the methylcholanthrene-treated skin to virus rubbed into it after scarification. It seems possible that methylcholanthrene brings about maximal susceptibility to the virus. When the virus was introduced intradermally into hyperplastic epidermis, it produced as many growths as when rubbed into the hyperplastic skin following scarification.

DISCUSSION

The experiments make plain that those methods of papilloma virus inoculation which result in growths all provide young, actively regenerating cells in abundance. Cells in this condition would appear to be essential for infection. As already stated, normal skin can be infiltrated with large amounts of highly pathogenic virus and yet no papillomas be caused. Furthermore when virus-containing fluid is injected in quantity into normal skin, producing a broad wheal, papillomatosis arises only where the needle has caused injury although the epidermis over a considerable area must have been brought into contact with the inoculum. The experiments of Kidd and Rous (7) have already been mentioned, in which normal rabbit ears were infiltrated with virus fluid by way of a marginal vein, without result in growths save at points of injury. Tarring the ears within the next 7 days, however, to such extent as to induce hyperplasia, brought out a horde of growths. One may recall in this general connection that bacteriophage multiplies only in the presence of young, actively growing bacteria (12) and that the first evidence of infection with vaccine

virus, herpes virus, and virus III is found in the young cells filling in the defects consequent on scarification (13).

The experiments here reported to learn the effects of the scarification preliminary to virus inoculation and the stages in its repair have disclosed that most of the surface epithelium is scraped away and that practically all of what is left, and the superficial connective tissue as well, is destroyed in the scab which forms within the next 2 days. As already pointed out, the virus rubbed into the raw surface must also be lost in the scabbing and but little can remain here or there underneath the dead material to infect the young, regenerating epidermal cells which extend from the hair follicles. It is possible that some direct infection of the preexisting hair follicle cells may occur at the time when the shafts of the follicles are cut across by the sandpaper and the virus is rubbed in, but this seems unlikely because the scabbing involves them too. Indeed, microscopically one seldom sees papillomas beginning at the hair follicles, these ordinarily arising from the reconstituted surface sheet of epithelium which lies between them.

The various agents which render the skin more susceptible to infection with the virus all effect changes which should bring young, actively regenerating cells into association with the virus in much greater number than ordinary and at a much earlier time. They also increase the local vascularity, and to this circumstance as well as to a much more abundant initial cell infection the shortened incubation period and subsequent rapid enlargement of the papillomas can be laid. Olitsky and Schlesinger (14) have recently shown that local edema produced by the subcutaneous injection of hypertonic solutions prior to inoculation of the skin with herpes virus greatly increases the effectiveness of the latter; and Sprunt (15) has brought evidence to show that susceptibility to infection with vaccine virus is influenced by the number of cells exposed to the latter. But the increased susceptibility of the altered skin for papilloma virus may be due to more than the provision of a richer vascularization and of an increased number of susceptible cells for infection. The individual young cells of an epidermis regenerating after alteration by the preparatives of the present work may be especially susceptible to the virus.

Under the ordinary circumstances of scarification and virus inunction infection takes place at scattered points with secondary coalescence, the result being separate more or less broad-based papillomas or confluent papillomatous expanses. The scattered character of the initial infection explains why confluent masses usually have craggy peaks with clefts between that frequently extend down to the skin level, and why on cross-section local differences can be perceived, which are expressions of the proliferation of differing infected cell families (16). Only occasionally does infection by scarification result in growths so compacted as to suggest that a broadcast, primary infection has

taken place. Naturally occurring papillomas in cottontails are not infrequently compacted, however, having the form of solid discs or "onions," presumably because they are the outcome of primary punctate infection with expansile enlargement. Growths of similar sort can be produced experimentally in both cottontails and domestic rabbits by intradermal or tattoo inoculation.

Bryan and Beard (17) have lately laid stress on the length of the incubation period (time elapsing after inoculation before papillomas are visible in the gross) as a reliable indicator of the amount of active virus present in the inoculum. There can, of course, be no doubt that the greater the number of virus entities distributed upon a scarified area the greater the chance will be for cells to become infected, up to a certain maximum, other things being equal and,—since most papillomas are consequent on multiple cell infection,—the sooner should the papillomas appear. But if the skin is abnormal other factors enter into the matter, as the present work shows, notably the regenerative activities of the epidermis and the local vascular state. The rate of appearance of papillomas is conditioned not only by virus quantity and pathogenicity but by the state of the tissue acted upon.

The experiments here reported provide a practicable method to render papilloma virus many times more effective on experimental inoculation. Papilloma extracts that do not elicit growths in normal skin in dilutions beyond 1:100,000 produce growths in methylcholanthrene- or turpentine and acetone-treated skin in dilutions of 1:1,000,000 or 1:10,000,000, while furthermore the incubation period of the papillomas in the altered skin is considerably shorter than in normal skin. It has been calculated that about 94,000,000 papilloma protein molecules are present in the dilution giving the 50 per cent point (18). The findings of the present experiments suggest that this figure mostly expresses the difficulty of bringing virus into association with susceptible cells. By the means described in the present work the number of entities in the inoculum requisite to infection can be reduced considerably, although under the circumstances of virus inunction into scarified skin most of the virus is still lost by the way. To gauge the effectiveness of individual papilloma virus entities one would have to be certain that these reached the appropriate cells and that the latter were in a state to be infected. Needless to say, these considerations apply to other viruses as well.

Experiments carried out with Dr. John G. Kidd have shown that skin preparation by the methods here described is useful in demonstrating papilloma virus in materials which fail to give rise to growths when inoculated in the ordinary way. Extracts of domestic rabbit papillomas which are non-pathogenic when so tested, in which, that is to say, the virus appears to be "masked," produce growths in most instances when inoculated into methylcholanthrene-

or turpentine and acetone-treated skin. These findings will be reported in detail in a forthcoming paper.

SUMMARY

Rabbit skin can be rendered abnormally susceptible to papilloma virus infection by preliminary treatments with a variety of agents. The most effective agents thus far found are 0.3 per cent methylcholanthrene in benzene and a mixture in equal parts of turpentine and acetone, applied four or five times at 2 day intervals. When virus is inoculated into skin altered by these agents, either intradermally or by inunction after scarification, papillomas appear earlier and in greater number than on normal skin, and much higher dilutions give rise to growths. The method provides a means of detecting amounts of virus which cause no papillomas upon inoculation into normal skin.

Papilloma virus material which is rubbed into scarified normal or hyperplastic skin is largely lost in the scabbing which ensues, and nearly all of it fails to reach susceptible cells. The preparatory agents which increase the effectiveness of the virus bring about marked epidermal hyperplasia, and the hyperplastic tissue regenerates with greater rapidity when scarified. The agents evidently act in large part by providing young epidermal cells in quantity to the virus, as also by inducing a richer vascularization than ordinary in support of the papillomatous proliferation. It is possible that they also act by providing especially susceptible cells. The implications of the findings are discussed.

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EXPLANATION OF PLATES

All of the specimens were stained with eosin and methylene blue.

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

PLATE 2

FIG. 1. Normal skin, from the abdomen of a normal domestic rabbit. It has been overstained with hematoxylin to make the thin epidermal layer more evident. × 18.

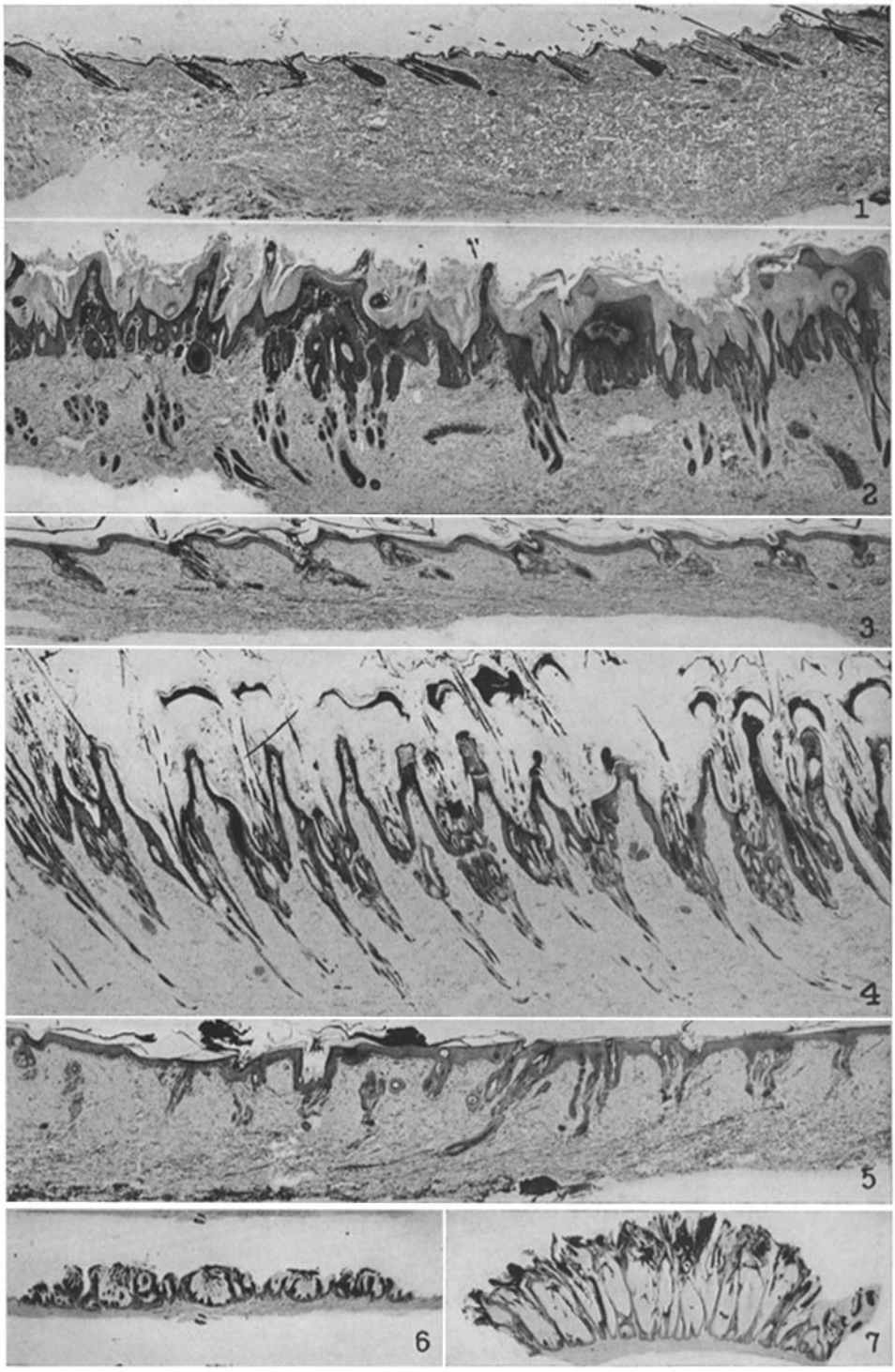
FIG. 2. Portion of an area of abdominal skin which had been tarred twice a week for 2 weeks. Biopsy 2 days after last application. × 18.

FIG. 3. Skin treated with turpentine and acetone. The mixture had been applied five times at 2 day intervals. Biopsy 24 hours after last treatment. × 18.

FIG. 4. To show the skin alterations induced by four applications at 2 day intervals of a saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene. × 18.

FIG. 5. Illustrating the marked hyperplasia of normal skin after scarification. Specimen taken 8 days after scarification. × 18.

FIGS. 6 and 7. Cross-section of the papillomatous masses formed after broadcast tattoo inoculation of normal skin (Fig. 6) and of methylcholanthrene-treated skin (Fig. 7) of the same rabbit. The growths were removed and sectioned 42 days after virus inoculation. Only a portion of the mass on the methylcholanthrene-treated skin is shown. × 2½.



(Friedewald: Cell state as affecting susceptibility to virus)

PLATE 3

FIGS. 8 to 12. To illustrate the changes induced by scarification of methylcholanthrene-treated skin. All the specimens were taken from the same rabbit.

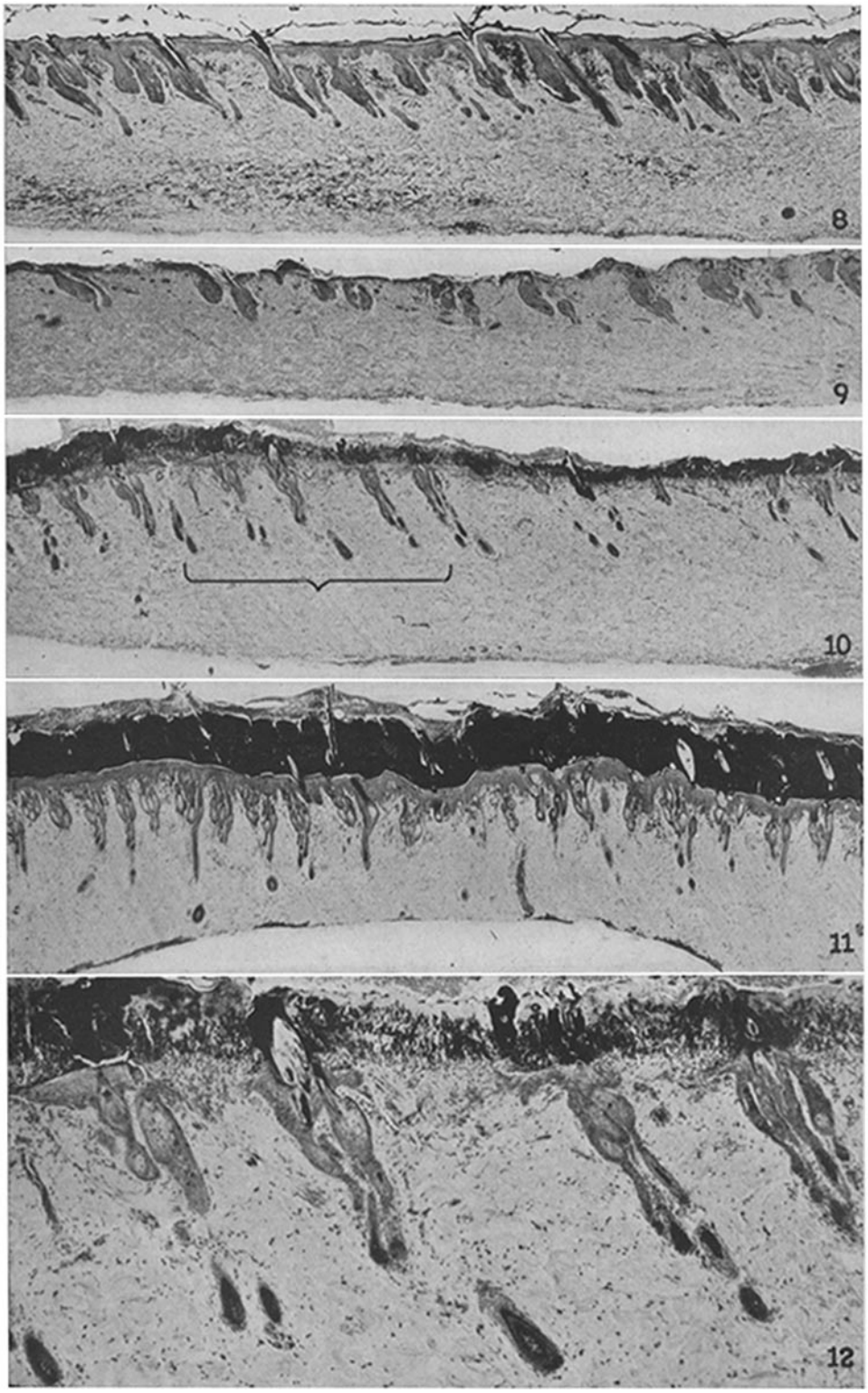
FIG. 8. Methylcholanthrene-treated skin before scarification. 0.3 per cent methylcholanthrene in benzene had been applied to it four times at 2 day intervals. Biopsy 24 hours after last treatment. × 18.

FIG. 9. 5 hours after scarification. The surface epithelium has been almost completely removed together with some connective tissue and the upper portion of the hair follicle shafts. A scab is forming on the surface. × 18.

FIG. 10. 24 hours after scarification. The scab is unusually thick as result of superficial necrosis involving both the connective tissue and hair follicle shafts. From the latter epithelial regeneration is already taking place, the new cells extending between scab and connective tissue. The bracketed region is shown at greater magnification in Fig. 12. × 18.

FIG. 11. 48 hours after scarification. Epithelial regeneration is now complete and the denuded surface is covered with markedly hyperplastic epidermis. The sebaceous glands are also hyperplastic and many are distended with secretion. Hence their prominence as compared with those of Fig. 8. × 18.

FIG. 12. The bracketed region of Fig. 10 at higher magnification. The epithelium is spreading laterally from the shafts of the hair follicles to form umbrella-like structures. Some of the new epidermal cells can be seen extending irregularly into crevices in the fibrous corium. × 60.



(Friedewald: Cell state as affecting susceptibility to virus)