

# CERTAIN CONDITIONS DETERMINING ENHANCED INFECTION WITH THE RABBIT PAPILLOMA VIRUS

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PLATE 5

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The conditions determining the effectiveness of the Shope papilloma virus (1) have a special interest because of the frequency with which extracts of vigorous growths produced with this virus fail to yield it on test. In many cases this is because antiviral antibodies have extravasated into the tissue, which neutralize the virus when the latter is free from the cells during extraction (2); but there are other instances, notably in domestic rabbits, for which the reason is not plain. Even though the virus can be usually recovered from growths in its native host, the cottontail rabbit, the titer is low; according to a recent report  $10^{-8.355}$  gm. of purified virus protein per 0.1 cc. of inoculum is the minimum with which papillomas can be produced, an amount which corresponds on calculation to about 56 million virus particles (3).

In a previous paper (4) the fact has been brought out that the way in which the virus is usually inoculated, namely by scarification and inunction, is in no small degree responsible for its low titer. By preliminary treatment of the skin with agents which stimulate the production of cells susceptible to the action of the virus, the effectiveness of the inoculum is greatly enhanced. Experiments have now been done to increase further the number of virus entities that come into association with susceptible cells, and it has been found that the prevention of drying and scabbing which ensues following scarification results in a far higher titer of the inoculum and an earlier appearance of the growths. The experiments demonstrating these facts will now be described.

## *Methods*

Papilloma virus suspensions were prepared from naturally occurring growths of cottontail rabbits, which had been preserved in equal parts of glycerin-Locke's solution at 4°C. Weighed portions were thoroughly ground with sand, suspended in 0.9 per cent saline to make 5 or 10 per cent extracts, and cleared by spinning in an angle-head centrifuge at about 4,500 R.P.M. for 20 minutes. For test they were rubbed into areas scarified with sandpaper, on the side and belly of adult domestic rabbits of agouti breed, after which each area was separately dressed (5). To increase susceptibility the skin of the areas had been rendered hyperplastic by applications of a mixture in equal parts of turpentine and acetone prior to inoculation (4). The character of the papillomas arising was recorded at frequent intervals 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.

*Infectivity of Papilloma Virus on Hyperplastic Epidermis*

Experiments were first done to test the infectivity of papilloma virus for hyperplastic epidermis under various conditions of inoculation.

*Experiment 1.*—Skin areas measuring about 4 by 6 cm. on the abdomens of four normal domestic rabbits were painted with the turpentine-acetone mixture at 2 day intervals for a total of four times. A day after the last application two of the treated skin areas of each animal were lightly scarified with sandpaper, while the third was left as such. A virus extract (W.R. 1-28) in dilution of 1:20,000, in terms of weight of glycerolated papilloma tissue extracted, was then applied to all three areas and rubbed in with the rounded end of a sterilized small glass test tube. The unscarified area and one of the scarified areas were allowed to dry and were then covered individually with sterile gauze patches (5), whereas the other scarified area was covered, immediately after virus inoculation, with a gauze layer that had been impregnated with a thin film of paraffin (parawax, boiling point 48°C.). The paraffined gauze was moored to the skin along each edge with 1 inch adhesive tape, to prevent drying and possible transfer of some of the inoculum to other areas. Under these circumstances a skim of virus-containing fluid remained in contact with the scarified surface, and after 24 hours the surface was still moist. A large gauze pad and many-tailed binder were put over all, and the bandages and paraffined gauze were left on for 5 days.

Table I shows the results of the experiment. The number of papillomas that arose on the skin areas under the various conditions of inoculation was strikingly different. On the 13th day no papillomas were yet visible on the unscarified skin areas and only one of the areas which had been scarified and dried showed any and this but a few. The scarified areas which had been covered with paraffined gauze on the other hand had many discrete papillomas. By the 27th day the unscarified areas showed a few discrete growths and semi-confluent papillomas were present on the scarified and dried areas while the scarified and protected areas had confluent masses of much higher papillomatous growth.

In the next experiment a comparison was made of the results of inoculating various dilutions of a papilloma virus extract into hyperplastic skin which in some instances was allowed to dry after inoculation and in others covered with paraffined gauze.

*Experiment 2.*—Hair was clipped from eight areas about 5 by 7 cm. on the abdomens of six normal domestic rabbits and a mixture of turpentine and acetone in equal parts was applied to the areas four times at 2 day intervals. Two days after the last treatment a virus extract (W.R. 2-95) in serial tenfold dilutions from  $10^{-5}$  to  $10^{-8}$  was applied to paired areas on each animal after the usual scarification. The areas which received one set of the serial dilutions were covered with paraffined gauze immediately after inoculation, as in the preceding experiment, whereas the other areas comparable with them in size, situation, and inocula received were allowed to dry and were then covered with gauze pads in the usual way.

The results are set forth in Table II. On the 14th day after inoculation three of the six rabbits had a few small papillomas on the hyperplastic areas inoculated with the  $10^{-5}$  dilution of virus and then allowed to dry. No growths had

TABLE I  
Effectiveness of Papilloma Virus on Hyperplastic Epidermis under Various  
Conditions of Inoculation

Turpentine and acetone treated skin*		Pathogenicity tests											
Virus extract W.R. 1-28 (1:20,000) rubbed into	Later treatment	13th day				18th day				27th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Unscarified skin	Skin areas allowed to dry; then covered with dry dressing	0	0	0	0	+	±	0	+	+	+	±	+
Scarified skin	As above	±	0	0	0	±±	++	±±	++	+++	+++±	+++	+++
	Skin areas covered with paraffined gauze†	++	+	±	++	+++	+++±	+++	+++	++++	++++	++++	++++

\* Turpentine and acetone in equal parts applied to skin four times at 2 day intervals before virus inoculation.

† Gauze layers impregnated with a thin film of paraffin were sealed at each border with wide adhesive tape immediately after virus application (see text).

++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = 5 to 15 papillomas, ± = 2, 3, or 4 papillomas, ± = 1 papilloma, 0 = negative.

TABLE II  
Titration of a Papilloma Virus Extract on Hyperplastic Epidermis with and  
without Paraffined Gauze Dressing

Treatment before virus inoculation	Treatment following scarification of skin and virus application	Dilution of virus extract (W.R. 2-95) used for inoculation	Pathogenicity tests														
			14th day						20th day								
Test rabbits						a	b	c	d	e	f	a	b	c	d	e	f
Skin rendered hyperplastic with a mixture of turpentine and acetone	Areas allowed to dry	10 <sup>-5</sup>	+	0	±	0	0	+	++	++	±±	+	±±	+++			
		10 <sup>-6</sup>	0	0	0	0	0	0	+	±±	±	±	±±				
		10 <sup>-7</sup>	0	0	0	0	0	0	±	+	0	0	±	+			
		10 <sup>-8</sup>	0	0	0	0	0	0	0	0	0	0	0	0			
	Areas covered with paraffined gauze	10 <sup>-5</sup>	+++	+	++	±	++	++	+++±	+++	+++±	+++±	+++±	+++±			
		10 <sup>-6</sup>	++	±	±	0	+	±±	+++	+++	+++	±±	++	+++			
		10 <sup>-7</sup>	±	0	0	0	0	±	+++	+	±±	+	+	++			
		10 <sup>-8</sup>	0	0	0	0	0	0	±±	±	±	±	±	±			

appeared in response to the higher dilutions. The areas which had been covered with paraffined gauze had many papillomas as result of the  $10^{-5}$  dilution, and also some where dilutions of  $10^{-6}$  and  $10^{-7}$  had been put. By the 20th day the areas which received the  $10^{-7}$  inoculum with drying afterwards

Rabbit No.	Treatment following scarification and virus inoculation	Pathogenicity tests			
		Dilution of virus extract (WR.295) used for inoculation			
		$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$
8-08	A Skin allowed to dry, then bandaged				
	B Skin covered with paraffined gauze, then bandaged				
8-10	A As above				
	B As above				
8-12	A As above				
	B As above				

CHART 1. Infection with a papilloma virus extract as tested on hyperplastic rabbit skin.

showed a few discrete papillomas, but many more were present on the areas covered with paraffined gauze and here all dilutions through  $10^{-8}$  had elicited growths. The papillomas of each rabbit were carefully traced in outline on sheets of cellophane with a wax pencil on the 20th day. Three instances which are representative of the group are shown in Chart 1.

The experiments make plain that the infectivity of a papilloma virus extract on hyperplastic epidermis can be greatly enhanced by protecting the virus-inoculated area with paraffined gauze. The mechanism of this effect will be considered further on.

#### *Infectivity of Papilloma Virus on Normal Skin*

Tests were now done to learn whether infection with the papilloma virus could be enhanced in scarified normal skin by the paraffined gauze method.

*Experiment 3.*—A saline extract of the glycerolated papillomas of W.R. 2-95 was rubbed in serial tenfold dilutions from  $10^{-3}$  to  $10^{-8}$  into twelve scarified areas on the abdomens of four normal domestic rabbits. Each dilution of virus was inoculated into two comparable skin areas. One was allowed to dry and was then covered with a sterile gauze pad, while the other was immediately covered with paraffined gauze, as described in the preceding experiments. A large gauze layer was then applied over all and held in place with a many-tailed binder. All of the bandages were removed 5 days after virus inoculation, at which time the areas were completely healed.

The growths of each rabbit were traced on sheets of cellophane. Chart 2 shows the number and size of the discrete papillomas and of the confluent masses of them on the 24th day after inoculation. It is apparent that the virus extract was much more effective in producing papillomas on the skin areas covered with paraffined gauze. A comparison of the number of papillomas and the highest dilution of the virus extract producing such growths indicates roughly a tenfold increase in effectiveness of the virus in the paraffin-covered skin areas. When compared with the findings of the preceding experiment in which an extract of the same papillomas (W.R. 2-95) was tested on hyperplastic epidermis (Chart 1), it is clear that paraffin-covered hyperplastic skin areas provide exceedingly favorable conditions for papilloma virus infection.

#### *Histological Findings*

In extension of the findings the histological changes in paraffin-covered skin areas were compared with those occurring in the hyperplastic epidermis under the ordinary conditions of inoculation.

*Experiment 4.*—Six skin areas measuring about 4 by 6 cm. in diameter on the abdomens of two normal domestic rabbits were rendered hyperplastic by four applications of a mixture of turpentine and acetone at 2 day intervals. A 10 per cent virus extract (W.R. 2-95) was then rubbed into each, following scarification with sandpaper. Three of the areas were allowed to dry and then covered with sterile gauze pads. The other three areas were covered with paraffined gauze immediately following virus inoculation, as described in Experiment 1. Representative pieces of skin were removed from the areas with sterile instruments 1, 2, 4, 6, and 8 days following virus inoculation. After each biopsy the dressings were replaced. The specimens were fixed in acid Zenker and stained with eosin-methylene blue.

As previously reported (4) scarification largely removes the surface epidermis, leaving the hair follicle shafts. The skin areas covered with paraffined gauze



















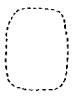
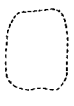






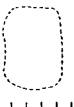





Rabbit No.	Treatment following scarification and virus inoculation	Pathogenicity tests			
		Dilution of virus extract (WR.295) used for inoculation			
		$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$
8-63	A Skin allowed to dry, then bandaged				
	B Skin covered with paraffined gauze, then bandaged				
8-64	A As above				
	B As above				
8-62	A As above				
	B As above				
8-65	A As above				
	B As above				

CHART 2. Infection with a papilloma virus extract as tested on normal rabbit skin.

showed 24 hours afterwards a regenerating epithelial layer derived from the hair follicles extending along the exposed surface of the connective tissue (Fig. 1). This surface was remarkably clean, with little or no scabbing or

necrosis of the connective tissue. The areas which had been allowed to dry after inoculation showed, on the other hand, much scabbing and necrosis (Fig. 2). Furthermore epithelial regeneration was not so far advanced as in the paraffin-protected areas. Within 48 hours after scarification the epidermis of the paraffin-covered areas had completely regenerated and the new hyperplastic epithelium was rapidly differentiating (Fig. 3). The epidermis of the skin areas which had dried was not completely regenerated at this time (Fig. 4), and scabbing and necrosis were prominent and had obviously retarded the regeneration in many places. 4 days after scarification the paraffin-covered areas showed a greatly thickened stratified squamous epithelium and several areas of beginning papillomatosis. The skin areas which were not protected had now largely healed, but the epidermis was irregular and comparatively thin. No sign of papillomas could be seen. After 6 days the paraffin-covered skin showed an almost solid, thick sheet of characteristic papillomatosis (Fig. 5). The unprotected areas on the other hand had a thin epidermis and in a few areas beginning papillomas (Fig. 6). Confluent and semiconfluent papillomatous masses were visible in the gross on the paraffin-covered areas on the 8th day, whereas only a few tiny discrete papillomas could be seen on the unprotected areas which had received the same inoculum.

*Effect of Immune Serum on Papilloma Virus Inoculated into Normal and Hyperplastic Skin*

It has been shown that young, actively regenerating cells are essential to papilloma virus infection (4). Furthermore epithelial regeneration occurs far more rapidly in scarified hyperplastic skin than in scarified normal skin, and papillomas appear earlier and in greater number. Together these findings warrant the supposition that the papilloma virus becomes associated with susceptible cells at an earlier time in hyperplastic skin than in normal skin. To test whether this is the case specific immune serum capable of neutralizing papilloma virus (6) was applied to scarified normal and hyperplastic skin areas at various times after virus inoculation. It is known that this antibody has no effect on the virus associated with epidermal cells (5) and it was thought that the test would yield evidence as to how soon such association occurred.

Six areas approximately 3 by 5 cm. in diameter on one lateral half of the abdomen of each of four normal domestic rabbits were rendered hyperplastic by four applications of a mixture of turpentine and acetone at 2 day intervals. Six comparable areas on the other half of the abdomen of each rabbit were not treated. Each of the areas was then lightly scarified with sandpaper and 0.2 cc. of a 0.02 per cent papilloma virus extract (W.R. D) was inoculated into each area by inunction, as usual, after which 0.2 cc. of a 1:4 dilution of an immune serum (D.R. 14-72) was immediately rubbed into one of the inoculated normal and hyperplastic skin areas. The immune serum had been obtained from a rabbit twice injected intraperitoneally with 10 cc. of a 10 per cent papilloma virus extract (W.R. 1-28). All of the inoculated areas

were then covered individually with paraffined gauze squares but they were temporarily removed from one area of each sort after 1, 5, 24, and 48 hours so that 0.2 cc. of the immune serum could be rubbed into it. As control a normal and a hyperplastic skin area on each rabbit were treated with a normal rabbit serum 1 hour after virus inoculation. Such serum is known to have no neutralizing ability (6). The skin areas were still moist at 24 hours, but after 48 hours they were dry yet without sign of scabbing. The normal and hyperplastic areas showed no difference in this respect. The bandages were removed from all of the areas on the 6th day.

Table III shows the results. On the 15th day after inoculation only one of the four rabbits showed any papillomas on the normal skin areas and these only where it had been treated with normal serum or with immune serum 24 and 48 hours after virus inoculation. The growths were few. The hyperplastic

TABLE III  
*Effect of Immune Serum Applied to Normal and Hyperplastic Skin at Various Times after the Inoculation of Papilloma Virus*

Time from virus inoculation* to application of immune serum†	Pathogenicity tests															
	Normal skin				15th day Hyperplastic skin				Normal skin				30th day Hyperplastic skin			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Test rabbits																
Control 1 hr. (normal serum)	0	0	+	0	+	++	++	+	++	++	+++	++	+++±	+++	+++±	+++
Immediately	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 hr.	0	0	0	0	0	0	±	0	0	0	0	0	0	±	±	±
5 hrs.	0	0	0	0	±	+	±±	±	±	0	0	0	+	++	++	+
24 "	0	0	±	0	+	±	++	±±	++	0	+	0	+++±	++	+++±	+++
48 "	0	0	±±	0	+	+++	+++	±±	++	++	+++	±±	+++	+++±	++++	+++

\* Virus extract W.R. D, 1:5000. ,

† Immune serum D.R. 14-72, 1:3.

areas, on the other hand, had in every case many papillomas on the areas treated with normal serum and with immune serum 5, 24, and 48 hours after virus inoculation. None were present on the areas treated with immune serum immediately after inoculation and this was true also of all but one of those receiving it 1 hour after inoculation, this showing a few. On the 30th day there was no difference in the number of papillomas on the normal skin areas treated with normal serum and those treated with immune serum 48 hours after virus inoculation. Two of the four areas receiving immune serum after 24 hours now showed some papillomas and one of the areas treated at 5 hours had a single discrete growth but there were none on the areas treated immediately and 1 hour after virus inoculation. The hyperplastic areas at this time showed large semiconfluent papillomatous masses on the control areas and on the areas treated with immune serum after 24 and 48 hours. There were



fewer growths, and these discrete, on the areas treated after 5 hours, yet still a considerable number, and three of the four areas treated with immune serum 1 hour after virus inoculation also showed them. No growths were present on the areas treated with immune serum immediately after virus inoculation.

It is plain from these findings that the papilloma virus gains the protection of susceptible cells much earlier when inoculated into hyperplastic skin than into normal skin. The association of the two in hyperplastic epidermis appears to have begun within an hour after inoculation, whereas in the case of normal skin it did not occur until about 24 hours had elapsed.

#### DISCUSSION

The various procedures previously found to be effective in enhancing infection with the papilloma virus all turn upon the provision to it of many susceptible cells. The present work has had this aim too, in so far as it has made for an earlier regeneration of the hyperplastic epidermis of the area exposed to the virus, and it has also had the object of preventing the large losses of inoculum incidental to previous techniques. The new method heightens the effective titer of the virus so greatly that whenever it is used care must be taken to protect test areas from droplet infection as the virus is rubbed into neighboring ones. Until this precaution was taken discordant titration results were obtained.

As already stated it has been reported that about 56,000,000 papilloma virus entities in each 0.1 cc. of inoculum are necessary to produce papillomas in normal scarified skin (3). By rendering the skin hyperplastic in ways already reported upon (4) the effective titer of the virus was increased about 100 times and by the present improvement of method a further 10- to 100-fold increase in titer has been achieved. In the most favorable instances this would reduce the minimum effective number of virus particles to about 5,600. Actually in some experiments with virus suspensions obtained by differential centrifugation the titers attained were so high as to indicate on calculation that no more than 2,000 entities per 0.1 cc. were required to produce growths. This is still a considerable number of entities but the problem here touched upon, of the need for so many, is not peculiar to the papilloma virus. It is well known, for example, that only a small proportion of the tobacco mosaic virus particles rubbed onto a leaf actually infect the cells (7).

The histological studies of the skin following scarification and virus inoculation have revealed the reasons for the success of the method of infection now described. As previously shown (4) the virus fluid is ordinarily placed upon a surface wholly denuded of epidermis following scarification except where the hair follicles have been cut across, and only after several days, when re-

generation takes place, does the virus have opportunity to become associated with cells of the sort susceptible to its action. By preliminary treatments of the skin with agents which render the epithelium hyperplastic and excite it so that it is capable of covering a denuded surface rapidly, this interval can be much cut down and many more cells than ordinary provided for the virus to act upon. Yet a considerable time still elapses before the cells proper to the virus are provided in quantity after scarification, and during this period drying of the raw surface occurs and scabbing. A great deal of the inoculum is lost as a result of these latter occurrences, since the scab which forms consists not only of exudate but of the very layer of connective tissue upon which the virus was spread. The application of a paraffined gauze dressing immediately after inoculation of the scarified area prevents the drying and necrosis that ordinarily ensue and permits a more rapid regeneration of the epidermis with subsequent early papillomatosis,—doubtless because of unimpeded association of the virus and susceptible cells.

#### SUMMARY

The infection of normal or hyperplastic rabbit skin with the papilloma virus can be greatly enhanced by protecting the scarified and inoculated area with a layer of paraffined gauze until healing occurs. In this way the necrosis which follows upon scarification and also the scabbing are almost entirely prevented and in consequence epithelial regeneration is usually complete within 24 hours. Not only are many susceptible cells provided to the virus far earlier than would otherwise be the case,—and collateral tests have shown that it becomes associated with them within a few hours instead of after many,—but the inoculum is itself conserved, instead of becoming largely lost amidst necrotic tissue and scab, as under ordinary circumstances. The effective titer of the virus is increased by the procedure from 10 to 100 times over that attained when hyperplastic skin is allowed to dry after inoculation. Since the results under the latter circumstances are 10 to 100 times better than those when normal skin is treated in the same way it follows that a 100- to 10,000-fold increase in the effectiveness of the virus has now been obtained.

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## EXPLANATION OF PLATE 5

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

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

The specimens figured were taken from a single rabbit at various times after the scarification and virus inoculation of skin prepared with turpentine and acetone.

FIG. 1. Slice taken after 24 hours from an area which had been inoculated and covered with paraffined gauze. The scarification has largely removed the surface epithelium. There is little or no necrosis and epithelial regeneration from the hair follicles is already well under way.  $\times 70$ .

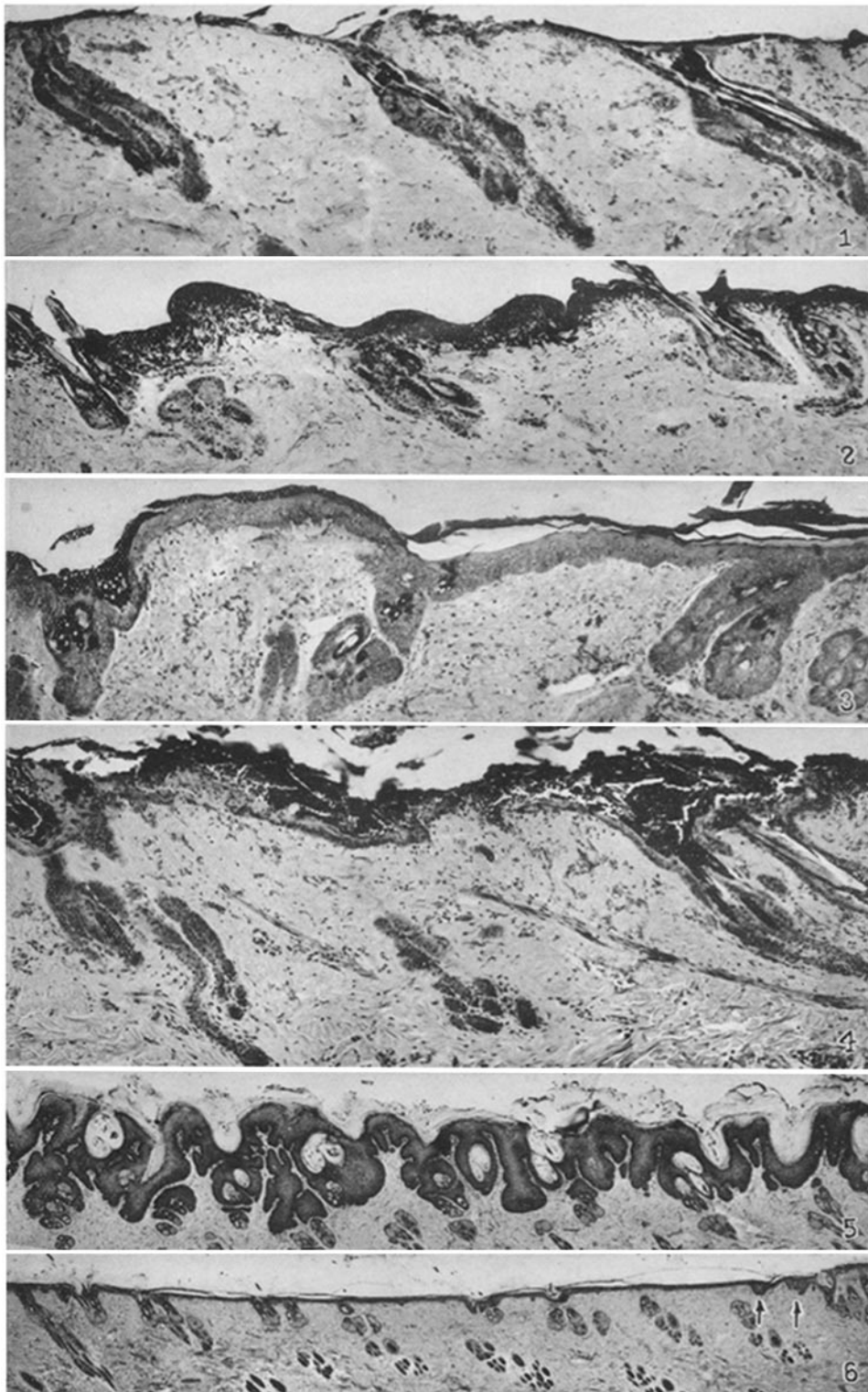
FIG. 2. For comparison with Fig. 1. A similar area which had been let dry and then covered with dry gauze: 24 hours after virus inoculation. There is much necrosis and scabbing of the superficial connective tissue and upper portion of the hair follicle shafts. No epithelial regeneration can be seen.  $\times 70$ .

FIG. 3. Same conditions as in Fig. 1, but specimen procured 48 hours after virus inoculation. The surface is now completely covered with hyperplastic, actively proliferating epidermis. Already a layer of keratinized cells is present on its surface.  $\times 70$ .

FIG. 4. Specimen procured 48 hours after virus inoculation from an area which had been allowed to dry. There is marked necrosis and the epithelium is extending in a thin layer along the surface of the connective tissue. Compare with Fig. 3.  $\times 70$ .

FIG. 5. Same conditions as in Fig. 1: specimen taken 6 days after virus inoculation. A solid sheet of characteristic papillomatosis is now present.  $\times 18$ .

FIG. 6. Same conditions as in Fig. 2: specimen taken 6 days after virus inoculation. The epidermis has completely regenerated but it is almost normally shallow. The scab has come away. At a few spots there are beginning papillomas (arrows). Compare with Fig. 5.  $\times 18$ .



(Friedewald: Enhanced infection with rabbit papilloma virus)