

STUDIES ON THE SHOPE RABBIT PAPILOMA VIRUS*

II. THE LOCATION OF INFECTIVE VIRUS IN PAPILOMAS OF THE COTTONTAIL RABBIT

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PLATES 48 TO 50

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A study, using the fluorescent antibody method to locate the Shope papilloma virus antigen in the papillomas of the cottontail and domestic rabbit, has shown the antigen to be present in demonstrable quantity only in the cells of the keratohyaline and keratinized layers of the papillomas, with no detectable antigen in the thick, underlying layer of proliferating epithelial cells (1). Since antigen detected with fluorescent antibody is not necessarily infective, an obvious next step has been to determine whether the location of infective Shope virus is the same as that of the fluorescence-detected antigen. For this purpose it was necessary to develop a special method of assay for the Shope virus. It depends in brief on the direct application of frozen sections of cottontail rabbit papillomas to the sensitized skin of domestic rabbits, after selective destruction of either the keratinized or proliferating portions of the papilloma sections by means of a microcautery-micromanipulator. In the present paper the method will be described and the findings reported.

Materials and Methods

Papillomas.—Naturally occurring papillomas of cottontail rabbits from Kansas were studied, as were papillomas induced in normal cottontails with fresh or glycerinated virus from fresh, naturally occurring papillomas or from glycerinated growths of the same derivation. The virus preparation was obtained by grinding the papillomas in a mortar with the gradual addition of pH 7 buffered saline to give a 20 per cent tissue suspension, which was centrifuged at 2500 r.p.m. for 5 minutes. The supernatant fluid thus obtained, in the amount of 0.1 to 0.05 ml., was rubbed into an area of the skin 2 cm. square which had been shaved and scarified lightly with sandpaper. Papillomas appeared after an incubation period of 7 to 14 days and were removed from the animal for sectioning 2 to 4 weeks after their appearance, at which time they were overlain with keratin and were 6 to 10 mm. in height. The naturally occurring growths were in the same height range when removed.

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Frozen Sections.—The specimens for sectioning were rapidly frozen in glass tubes in a dry ice-acetone bath, and cut in a cryostat at -20°C ., as described by Coons (2). The sections were cut vertically at 10 microns for the microcautery-micromanipulation technique and at 5 microns for routine hematoxylin and eosin staining. Results were the same whether the microtome knife passed first through the keratin or through the living tissues. The sections were placed on the slides in the frozen state and were then thawed by the application of finger heat to the underside of the slide. The blocks providing the frozen sections varied from 6 to 10 mm. in height and from 5 to 9 mm. in width. All the sections used in one experiment were adjacent or serial sections of the same papilloma and all were cut at 10 microns. Sections from the same papillomas have been used immediately or kept at 4°C . for 4 weeks before use, with no difference in the results.

Microcautery-Micromanipulator Apparatus and Technique.—The complete apparatus, consisting of a standard microscope, microcautery loop, de Fronbrune micromanipulator, dry cell batteries, and ammeter, is illustrated in Fig. 1. Fig. 2 is a 100 magnification of the cautery loop which was made from a 25 gauge needle-cleaning wire, and constricted at the tip to provide a very fine point 0.18 mm. in diameter. This loop was soldered to wires leading to two $1\frac{1}{2}$ volt dry cell batteries connected in series, and the loop and wires were taped to a wooden dissecting needle handle and this handle fitted to the pneumatic de Fonbrune micromanipulator. A knife switch, 100 watt rheostat, and an ammeter were interposed in the system. A current flow of 1.5 amperes was sufficient to bring the cautery loop to a dull red heat, which was optimal for these experiments. A standard microscope with a $3.5\times$ objective and $10\times$ ocular provided sufficient magnification and a good working distance.

It was difficult to cauterize sections of papillomas which had been induced by a high concentration of virus as these were usually broad based, with numerous keratinized and interspersed proliferating cell areas. The ideal specimens for this purpose resulted from inoculation of a high dilution of virus and hence from a small number of cells. These growths were constricted at the base and had relatively few keratinized and proliferating cell areas in their midst. Small, naturally occurring papillomas were also excellent for the cautery technique.

To effect a selective destruction of the keratinized or proliferating cell areas of the papilloma sections it was necessary to perceive their boundaries. This was done by tilting the mirror of the microscope to give a semidark field. An unstained frozen section of a typical cottontail papilloma is shown in Fig. 3. Note that the central branched area composed of proliferating cells is easily differentiated from the surrounding darker keratinized area.

When the microcautery loop was first tested on unstained frozen sections it was difficult to visualize the extent of the cauterized area; however, it was found that when the section was applied over a thin layer of solidified 3 per cent nutrient agar the burned area became markedly visible owing to darkening of the agar granules. The effect of the cautery loop on an unstained section is illustrated in Fig. 4. In Fig. 5 is a section stained with hematoxylin and eosin, which had been streaked with the cautery loop. It shows an absence of any staining in the cauterized area, but the cells stain well a few cell widths from the burned area (arrows). Cauterization with the loop destroys the papilloma virus antigen, as determined by the ability to stain with fluorescent antibody (Fig. 6); yet a short distance beyond the cauterized zone (which appears white in the photograph owing to non-specific autofluorescence) nuclear viral antigen has been rendered visible by attached fluorescent antibody (arrow), illustrating the focal nature of the destruction.

An attempt was made to destroy the proliferating cell areas while leaving the keratinized ones intact, and in other instances to destroy the keratinized areas while leaving the proliferating cell areas. This selective destruction was readily accomplished because the micromanipulator gave precise control of the loop in any direction and by means of it one could burn

exceedingly small and sharply localized areas. Such destruction of the keratinized portion of a section of a papilloma is illustrated in Fig. 7.

Skin Sensitization and Slide Application.—The shaved skin of the sides of domestic albino rabbits was rendered especially sensitive to infection with the papilloma virus by repeated painting with turpentine as described by Friedewald (3). Turpentine was swabbed on areas of skin four times at 2 to 3 day intervals and the sections were applied 2 days after the last painting. The most easily infected skin was invariably found on rabbits which had developed a thick crust on the turpentine-treated area. This crust was pulled off, several areas of epidermis 1.0 cm. square were abraded with sandpaper, and a vertically cut frozen section of a cottontail rabbit papilloma on a microscope slide was applied to each area and the slide was cemented to the surrounding skin with weldwood contact cement. Thus the section was brought into close apposition with the abraded area and fixed firmly in place. Several slides were placed on each rabbit and then an ace bandage was wrapped tightly around the animal to hold them against it and was left on overnight. When the slides were removed after 18 to 24 hours almost complete disintegration of the sections had occurred, but without any shifting of them.

EXPERIMENTAL RESULTS

A typical experiment was to apply 3 serial or closely adjacent frozen sections of the same papilloma to 3 scarified areas on the sensitized skin on the same rabbit: one section not cauterized at all, served as control; in another all the proliferating cell areas had been burned, and in a third all the keratinized areas had been cauterized. Twelve experiments of this type were done, each with a different rabbit. Discrete papillomas appeared first where the control had been placed and there they grew the most rapidly, arising after incubation periods of from 7 to 12 days (Fig. 8 A.). Papillomas always developed where sections had been placed in which the proliferating cell areas had been destroyed, and they grew almost as rapidly as on the control areas, having incubation periods of 8 to 21 days (Fig. 8 C). In contrast, no papillomas appeared at the sites of application of sections in which all the keratinized areas had been destroyed (Fig. 8 B), save in the first two of the twelve experiments. In these, single growths arose after incubation periods of 33 and 37 days respectively. They grew very slowly as compared with the controls.

In some sections of a single specimen, part of the proliferating cell area was so large and so situated that it could be cut away with a scalpel without contamination by keratinized material. The material thus obtained from two sections was applied to the sensitized and abraded skin of the same rabbit utilized for the other test materials. It caused no growths, whereas control sections and sections with only the keratin left intact after burning caused growths which were typical papillomas, both in the gross and microscopically. In no instance did any papillomas appear except at the site of contact of the sections.

Needless to say, the amount of proliferating cell and keratinized tissue present in the papilloma sections was not always the same. In several instances the expanse of proliferating cells was slightly larger, and in others the ratio of proliferating to keratinized was as high as 2.5:1. This was fortunate in providing

at least as many cells in the proliferating areas as were present in the cross-sections of the flat, almost dry, keratinized elements. The experimental results were the same, whatever the difference in the ratio and irrespective of whether natural or induced papillomas had furnished the test material.

In a later series of three experiments the microscope slides bearing the cauterized sections of frozen papilloma were photographed just before application, and after each slide had been cemented on the rabbit's skin this was tattooed with India ink in four places at the edge of the slide, next to and corresponding with marks on the latter. By this means the exact location of slide and section could be determined later on. The rabbits were observed every other day and papillomas could be detected when only 0.5 mm. in diameter. At this time the photographic transparency previously made of the original slide and papilloma section was applied to the area and lined up with the four tattoo marks on the rabbit's skin. Thus one could determine under what area of the section papillomas had arisen. Without exception they were found to be situated where the keratinized portion of the section had been applied.

DISCUSSION

The method employed in the present study has involved the assumption that virus would pass from frozen sections of papilloma tissue into the highly susceptible skin to which it was closely applied, and this did indeed happen, but only when there was keratinized tissue. When the keratinized tissue was eliminated, leaving the undifferentiated layers of proliferating papilloma cells, no growths were obtained. In explanation of this finding one might suppose that the proliferating cells had retained their content of virus within their substance, were it not for the fact that the sections had obviously autolyzed during the 24 hours they were pressed against the prepared skin. They were so thin to begin with (10 microns) that many cells must have been cut across. Furthermore, some sections were stored at 4° for as long as 4 weeks, during which time they dried, with no difference in the results. For these reasons it would appear that virus, if present in any considerable quantity in the proliferating cells would have produced infection.

When the proliferating cell areas of the papilloma sections had been destroyed selectively, papillomas developed after a slightly longer time where keratinized cells had been placed than at similar situations in the controls. This slight delay may well have been due to accidental destruction of part of the keratinized area during the cauterization.

In two instances a solitary papilloma arose after long delay where a section was placed in which all the keratinized areas had presumably been burned with the cautery loop. They appeared late, as compared with those arising where the keratinized areas of control sections had been placed; whence one may infer that they were caused by a much smaller amount of virus than those due to the controls (4). It seems probable that some of the keratinized cells had escaped

destruction. This could easily have happened, since the papilloma sections were unstained and a tiny, keratinized area might well have escaped detection. It becomes all the more likely because the results now in question were obtained when the technique was being developed and were not repeated in the later ten experiments. Furthermore, in the series of three experiments in which the position of the papilloma was determined in relation to the cell type of the section, it was found that papillomas did not arise where proliferating tissue had been, but only where keratinized tissue had been placed.

While the experimental findings indicate that infective Shope papilloma virus occurs mainly in the non-dividing cells of the keratinized and keratohyaline portions of wild rabbit papillomas, they do not exclude the possibility that infective virus may have been present in the proliferating cells of the papilloma in amounts too small for demonstration with this present technique.

The results are in accord with those obtained by use of the fluorescent antibody method (1), which demonstrated the presence of Shope virus antigen only in the keratinized and keratohyaline layers of the papillomas.

SUMMARY

A method has been devised to determine the location of infective Shope virus in the papillomas of cottontail rabbits. Frozen sections of the growths were burned selectively with a microcautery to destroy either the keratinized or proliferating layer and the sections were then applied directly to the sensitized epidermis of domestic rabbits. No papillomas appeared when the keratohyaline and keratinized areas had been eliminated leaving the proliferating cell layer, whereas papillomas arose when the proliferating cell areas were destroyed leaving the keratohyaline and keratinized layers. The results indicate that infective Shope papilloma virus is situated mainly, perhaps entirely, in the keratohyaline and keratinized areas of cottontail papillomas. This is in accord with the previous disclosure by the fluorescence technique that virus antigen in demonstrable quantity is present only in these situations.

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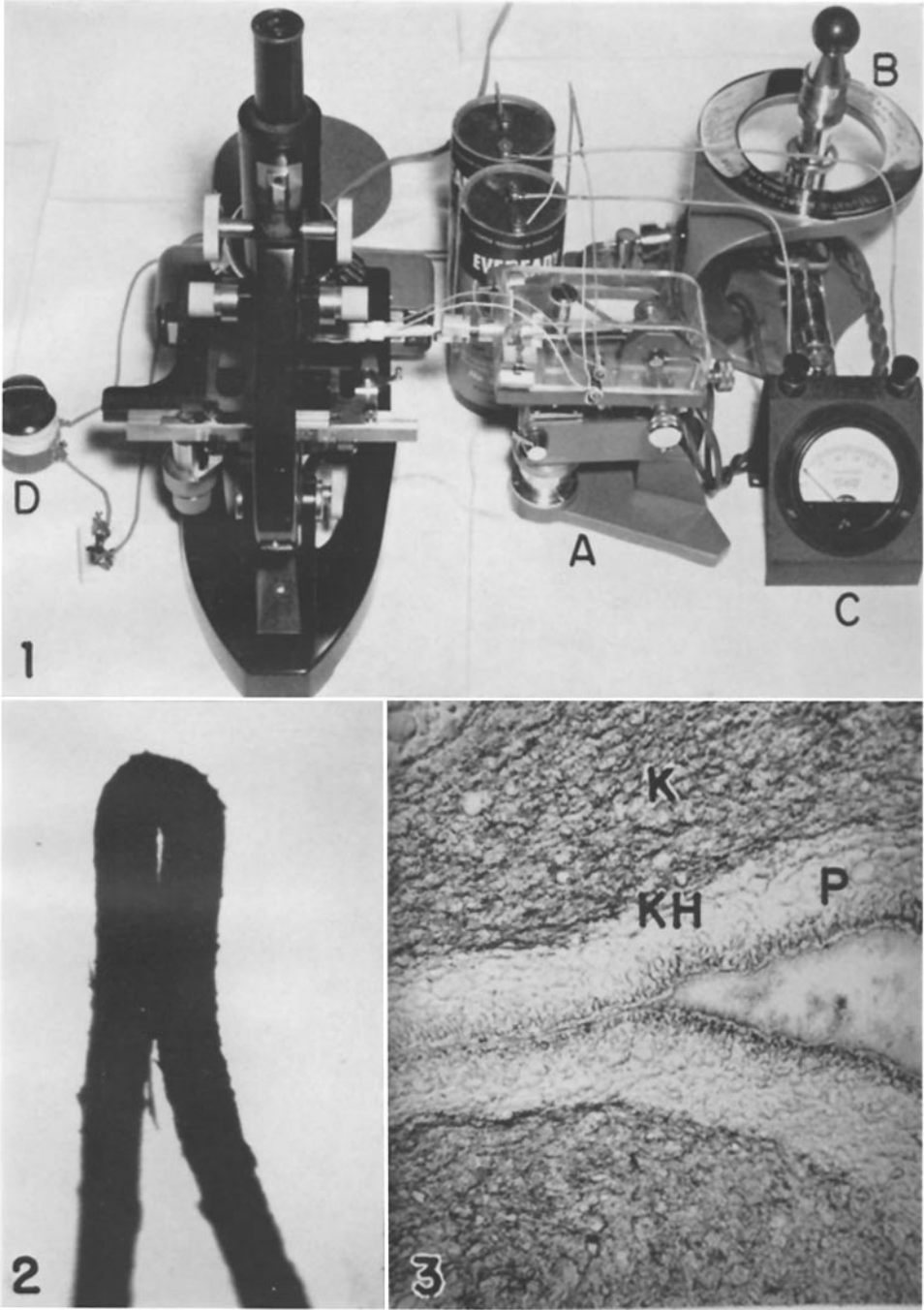
EXPLANATION OF PLATES

PLATE 48

FIG. 1. The complete microcautery-micromanipulator apparatus. The wire cautery loop is in place over the microscope stage and is attached *via* a wooden handle to the micromanipulator mechanism (*A*) which is operated by the pneumatic control (*B*). The ammeter (*C*) and rheostat (*D*) are used in adjusting the temperature of the wire loop.

FIG. 2. Microcautery loop, made from a 25 gauge, needle-clearing wire. $\times 100$.

FIG. 3. An unstained frozen section of a cottontail rabbit papilloma cut vertically. The empty looking, transverse wedge, with its base at the right consists of supporting connective tissue, and it is overlain by a relatively thick layer of epidermal papillomatous cells (*P*) which abruptly become keratohyaline (*KH*) and are overlain by a thick mass of keratin (*K*). $\times 150$.



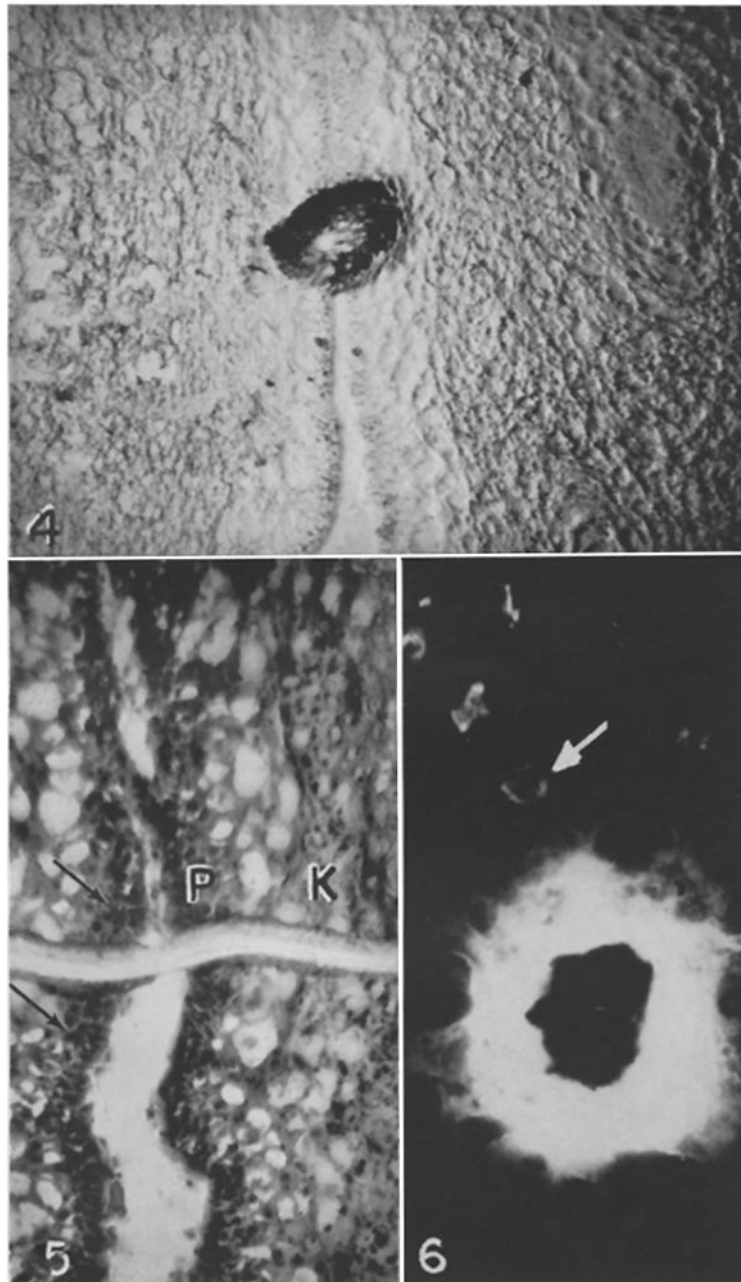
(Noyes: Studies on Shope rabbit papilloma virus)

PLATE 49

FIG. 4. An unstained frozen section of a cottontail rabbit papilloma which was placed over a nutrient agar layer and then cauterized with the microcautery loop. The central black spot is the burned area, made visible by darkening of the underlying agar granules. $\times 150$.

FIG. 5. Frozen section of a cottontail papilloma stained with hematoxylin and eosin. The cautery loop was drawn transversely through the keratinized (*K*) and papillomatous cells (*P*), leaving an unstained burned band. Note the normal staining of the papilloma cells a short distance from the edge of the burn (arrows). $\times 150$.

FIG. 6. Fluorescence photomicrograph of a frozen section of a wild rabbit papilloma stained for Shope viral antigen with a fluorescent antibody preparation. The central, ragged, black area is where the cautery loop touched the section and caused complete incineration. The surrounding white band is due to non-specific autofluorescence caused by heat. The arrow points to a cell nucleus dimly outlined by the specific fluorescence within it which is indicative of the presence of Shope viral antigen. Its survival and the high magnification of the photograph together demonstrate that the destructive effect of the loop is narrowly localized. $\times 450$.

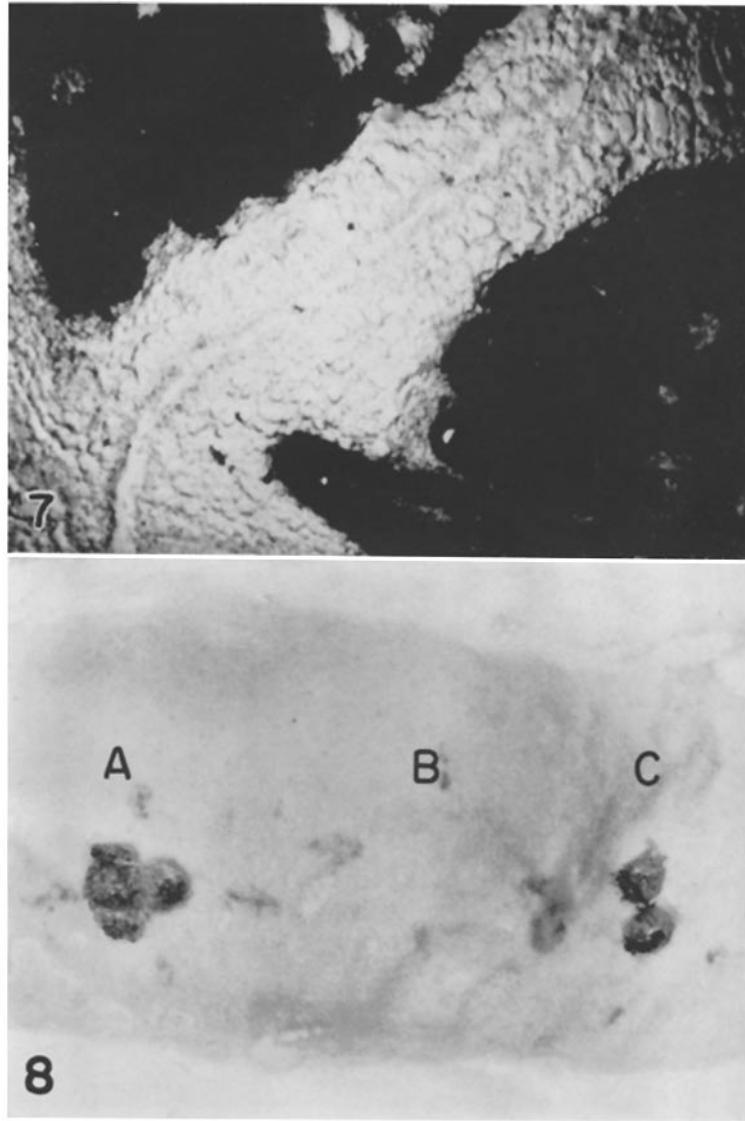


(Noyes: Studies on Shope rabbit papilloma virus)

PLATE 50

FIG. 7. A frozen section of a cottontail papilloma in which all the keratinized area (black) was destroyed with the cautery loop, leaving a slanting white zone of proliferating cells. These lie in two parallel layers with a much narrower layer of supporting connective tissue between them, as is ordinarily the case in folds of the papilloma. $\times 150$.

FIG. 8. Prepared skin of the flank of an albino domestic rabbit on which slides bearing papilloma sections from a cottontail had been placed. Photograph taken 6 weeks after the slides were applied. At site *A* an untreated control section was placed with resulting large papillomas. At site *B* an adjacent section of the same growth was placed after all keratinized areas had been destroyed by cautery, leaving much proliferating tissue. No growth has arisen. Site *C* is where an adjacent section from the same growth was placed after all the proliferating cell areas had been destroyed with the cautery loop leaving intact the keratinized areas; note the two papillomas. $\times 1$.



(Noyes: Studies on Shope rabbit papilloma virus)