A VIRUS-INDUCED MAMMALIAN GROWTH WITH THE CHARACTERS OF A TUMOR (THE SHOPE RABBIT PAPILLOMA)

III. Further Characters of the Growth: General Discussion

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In Papers I and II the behavior and appearance of the papilloma under various circumstances have been described. They are those characterizing the tumors. Yet the cause of the growth is a filterable virus. Are there no distinguishing signs of its presence? This question will now be taken up.

Concerning Inclusion Bodies

The morphology of the growing or retrogressing papilloma does not suggest the presence of a virus. But in one important respect it has been insufficiently dealt with,—the possible presence within the affected epithelial cells of inclusion bodies such as occur in certain virus diseases has not been considered.

Hurst (1) could find no inclusions but we have renewed the search and have compared the cytological findings with those in some epidermoid carcinomas of man and tar cancers of mice. The sections of human tumors we owe to the kindness of several pathologists. A large variety of mouse tumors were available, deriving from previous work in this laboratory. The immediate point to be determined was not whether "bodies" can be discovered in the cytoplasm or nucleus of the papilloma cells but whether any exist that are not present in the recognized neoplasms as well. The sections of the papillomas and tar tumors were stained with methylene blue and eosin, or with Giemsa; those of the human epitheliomas in several ways.[1]

[1] The interpretation of the presence of inclusion bodies in tumors has of late become complicated with the recognition that these may not only harbor viruses

The literature on strange bodies in tumor cells is a teeming one, most of it dating back thirty years or more (2) to the time when some investigators thought that they could perceive an intracellular cancer parasite. It need not be reviewed. DeMonbreun and Goodpasture have recently summarized the findings on inclusion bodies in the warts and papillomas caused by viruses (3). It is remarkable for the diversity of the forms described, amphoteric, acidophilic or basophilic, cytoplasmic or intranuclear, sometimes occurring in certain regions only, or supposedly at special stages in the development of the growths. There has been no consistency in the findings.

In addition to the large eosinophilic inclusions, described by Hurst, which are the remains of dead cells, a variety of cytoplasmic bodies can be seen in some of the papillomas studied by us; but they are wholly lacking in others. They occur especially where the cells are differentiating into the granular layer, and within this layer, which is unusually rich in the granules that give it its name.

Sometimes the bodies take the form of pyknotic, crescentic masses such as Kyrle has described in warts, psoriasis, and other skin conditions (4). These lie just outside one or both ends of the nucleus, as if extruded therefrom. Again they appear as evenly staining bodies, dark with methylene blue, filling the nucleus where keratinization is in progress, "like an egg within its shell" (Lipschütz (5)). Others are moderately refractile, rounded or oval, alkaline or amphoteric, and are first noted in the cytoplasm of cells immediately beneath the granular layer, reaching their greatest size in the latter (Fig. 40) and fading and disappearing at the same time as the nucleus, when the cells become keratinized and die. All these various bodies are encountered in surface growths, being seldom seen in interior ones or in those that are retrogressing and orderly. They are most pronounced where the cells are unhealthy as result of infection or maceration, being found especially in the deep crypts between papillae, where the differentiating epithelium has undergone a localized swelling with hydropic enlargement of the nucleus. None of them resembles the distinctive virus inclusions, and all appear to have originated from ordinary cell constituents,—to be the outcome of excessive granulation and granular fusion, of nuclear pyknosis, or of localized cytoplasmic keratohyalinization. Similar bodies have been present in some of the epidermoid cancers of man and the mouse that we have examined, notably where there was a heavily granulated layer of differentiating neoplastic cells at the skin surface or just beneath it. Often they were present in the thickened, neighboring epidermis as well.

DeMonbreun and Goodpasture have described groups of large, clear epithelial cells in the tissue of a transmissible dog papilloma caused by a virus, which failed to differentiate with the rest of the layer in which they were situated (6). These they

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(Levaditi and Nicolau (8), Rivers and Pearce (9)), but that the latter may cause within the tumor cells the inclusions characteristic of them (10).

regard as characteristic of the disease, reporting their presence in human warts also, where Lipschütz found cells having much the same appearance (7). No such elements are present in the Shope papilloma, but hydropic changes are frequent in unhealthy growths, as also in epidermoid tumors.

In sum we have failed to discover in the papilloma inclusion bodies such as indicate the presence of a virus, though various peculiar intracellular elements are to be found like those in the cells of the epidermoid tumors of man and the mouse. Certain characters to be noted in the papilloma when growing on the skin, namely great proliferative energy yet slight capacity for aggression, a thick layered and folded epithelium with an accentuated granular stage of differentiation, together suggest a virus cause, for the reason that they are present together in some of the warts, condylomas, and papillomas known to be due to such cause. Individually these characters are not distinctive, however, and their association is far less evident and frequently missing when the papilloma is growing inside the body.

The Reaction about Beginning Papillomas

The study of precancerous conditions and of beginning cancers has shown that pronounced changes in the connective tissue usually precede and nearly always accompany the first appearance of the growth (11); and the suggestion has frequently been made that to them the latter is really due. It seemed conceivable that the first changes produced by the papilloma virus in and about the epithelium might differ in significant ways from those occurring in and about epidermoid tumors.

Virus was rubbed into broad scarified areas on the sides of a group of domestic rabbits, and each day thereafter, until the papillomatous roughening showed itself, a narrow strip of skin was removed from the etherized animal. Charts were kept of their location as a check upon whether they came from the midst of skin in which the growth subsequently developed. The sections were stained with eosin and methylene blue.

The scarification was found to have caused definite breaks through the epithelial layer, as a rule almost midway between the openings of the hair follicles. Within the next few days the layer reunited beneath a light scab of dried serum containing some red cells. This soon flaked off exposing a skin surface that in the gross appeared smooth and normally pale. But within 7 to 12 days after the inoculation a patchy reddening took place, and the strips removed at this time showed the

virus changes in the epithelium to be well under way. Its earliest effects were a thickening of the deeper cell layers by mitosis, localized almost midway between hair follicles, that is to say to the places where scarification had caused breaks. The capillaries in the connective tissue immediately beneath the thickening epithelium were greatly dilated, as not elsewhere below it, and to this the surface reddening was attributable. The subepidermal tissue, slightly more cellular than normal, as result of the initial injury, sometimes showed no other change; but usually a few lymphocytes and makrophages, with rarely a polymorphonuclear leukocyte, collected under the proliferating epithelium. This graded insensibly into the normal layer next it. Its first extension was downwards, as Hurst noted, and soon the growth had advanced beyond the region of cellular reaction when one was present. The keratinizing cells on its surface failed to desquamate as ordinarily, and piled up, causing the first slight roughening of the skin surface. Fig. 41 is from an instance in which the lymphocytic reaction about beginning growths was unusually marked.

It is plain from these data that the early stages in the development of the papilloma are usually though not always attended by changes in the surrounding tissue. But they are trivial as compared with those about most beginning epidermoid carcinomas, though in both instances there is local vascular dilatation with lymphocytic accumulation. Nothing distinctive has been found to suggest the presence of a virus.

The frequent lack of all cellular reaction about the papillomas during the early period of development bears out our conclusion derived from a study of implantation growths, that the inflammatory changes present beneath well established skin papillomas are due to trauma, necrosis, and bacterial action.

The Conditions Determining Effectiveness of the Virus

The virus regularly produces growths in domestic rabbits, and in nearly all wild ones. Animals carrying the papilloma can be successfully reinoculated with virus during some weeks at least. Can one infer that the growth enlarges in part by contact infection of the epithelial cells immediately about it? Are there histological signs of such a process? What conditions are requisite to effectiveness of the virus? This last question is largely comprehensive of the others and hence will be taken up forthwith.

The virus does not induce a papilloma when it is placed as virus fluid on the intact skin; and if rubbed into a large area that has been but lightly scarified it causes scattered, discrete growths. Either it fails to act on the outer cells of the epidermis or it acts too slowly, the cells differentiating and dying before the characteristic proliferation begins. It must have access to the deeper layers such as trauma provides; and the growth appears precisely where the necessary injury has been inflicted. Intradermal inoculation of virus fluid results in a solitary papule localized to the old needle wound, unless indeed the fluid has been forcibly introduced, in which case a crowded group of small papules may appear where the epithelium was torn apart at the site of injection. We have infiltrated large areas of normal skin with active virus fluid, traumatizing them with sterilized tattooing needles at weekly intervals thereafter; but growths appeared nowhere except at the immediate points of injection and within a few millimeters of these, where the skin had been tattooed on the day of infiltration.

The following experiment shows that other injury besides trauma will furnish the needed conditions for effectiveness of the virus:—

A saturated solution of Scharlach R in olive oil (0.1 to 0.2 cc.) was injected intradermally into the shaved ears of three domestic rabbits at several well separated points, and 5 days later drops of virus fluid were placed on the reactive thickenings, and tattooed into spots about 0.4 mm. broad, the thickenings themselves being more than 1 cm. across. Numerous similar inoculations were also done into the normal skin. The papilloma appeared far sooner in the regions of Scharlach R reaction; and there were other differences. Where the skin had been affected by the dye the growth appeared not only at the point of inoculation but often almost everywhere at once in the thickened epidermis (Fig. 45), and sometimes as deep nodules covered by epithelium that did not itself show any papillomatous change (Fig. 45, *S*, *P*). Occasionally the nodules were several millimeters away (*P*) from the spot inoculated. At the control sites in normal skin the usual small, sharply localized epidermal lesions developed. The gross findings were confirmed by the sections from biopsies.

Under the conditions of this experiment some of the virus fluid through which the tattooing was done may have entered fissures in the epidermis altered by Scharlach R, thus accounting for the diffuse papillomatous change. But this will not explain the development of nodules of papillomatous tissue some distance away and beneath an epidermis that showed dye changes only. It is possible that the nodules were local metastases; but more likely the virus introduced by the tattooing was carried through the tissue to epithelial processes that had extended down in the well known fashion as result of Scharlach R stimulation.

Shope noted that the intravenous inoculation of virus, while not giving rise ordinarily to growths, may induce them where the epidermis has been traumatized. It will do so as well where the skin has been damaged by Scharlach R, as the following test makes plain.

Scharlach R in olive oil (0.2 cc.) was injected at a number of points arranged in a circle about 7 cm. broad on each of the shaved sides of a domestic rabbit; and the injections were repeated after 18 days. 8 days later, when the local discoid thickenings were about 1 cm. across but stationary in size, or beginning to dwindle in some instances, 5 cc. of 5 per cent virus fluid was injected into an ear vein. Papillomas developed within a few weeks in seven of the fourteen thickenings on the right side and three of fifteen on the left. None appeared anywhere else. The growths were first noted as an increased rugosity and heightening throughout certain of the reactive areas (Fig. 46), occurring at a time when the others were disappearing because the effects of the dye were wearing off.

The following experiment was done to find whether regenerating epithelium is especially susceptible to the virus.

Virus fluid was rubbed into large scarified areas on the abdomens of four domestic rabbits, and after 26 days, when the sharply defined "pancakes" of confluent papillomatous tissue were already 2 to 5 mm. high, two circular discs about 4 cm. broad were excised at their periphery, about half of each disc being normal skin and half papillomatous. The normal skin was cut through first, under ether anesthesia, and great care was taken not to sow fragments of papilloma on the raw surface. Under a dressing of sterile paraffin with a low melting point (Ambrine) clean healing took place, a smooth sheet of epithelium extending from both the normal and the papillomatous sides. But within a few days that from the latter source became rugose and developed abundant papillae, the result being that soon the old contour of the growth was approximately restored, as successive tracings showed. There was never any creeping of the papillomatous condition around the margin of the healing disc, such as might have resulted from a spread of the virus infection by continuity to the regenerating cells, though at a few places where the papillomatous tissue was exuberant the new epithelium growing out from it upon the denuded surface advanced more rapidly than did the normal epithelium and covered a larger area eventually. With the development of papillae a sharp demarcation became evident, the new papillomatous tissue rising cliff-like next the regenerated, normal epidermis to which it was joined.

No signs were encountered in this experiment of enlargement of the growths by contact infection of the regenerating epidermis. But their absence might be accounted for by the change the virus undergoes in the tissue of domestic rabbits which renders it incapable in

most instances of causing the growth anew. This explanation will not cover the results of the tests now to be described.

A circular disc of skin about 4 cm. across was cut from each side of the abdomen of three etherized, domestic rabbits, and the raw surface was dressed with paraffin which was replaced at intervals. After 12 days, when the raw surfaces were just covered with new, thin epithelium, the whole surface and the adjacent normal skin were flooded with virus fluid, and through it two strips about 2 mm. wide were tattooed at right angles to each other, from normal skin to normal skin through the middle of the healing area, the result being a cross of skin punctures, each arm of the cross from 2½ to 3 cm. long. The fluid was now blotted off and a dressing put on as before. When the discs were excised India ink had been tattooed into the skin at four equidistant points about 0.5 cm. outside them; and the dark spots now lay just beyond the ends of each cross, serving to show its situation. After 2 weeks the papilloma began to appear, and it soon assumed the exact shape of the cross, being restricted at first to the tattooed strips. It increased somewhat more rapidly in height in the newly covered areas, as would follow from the more abundant vascularization, but did not appear sooner or broaden more rapidly there.

In this test the papilloma appeared only where virus had been directly introduced into the old and new epidermis. No spread occurred in the new-formed epithelium to indicate that it was especially susceptible.

In the large number of wild and domestic rabbits thus far observed by us, some of them carrying the papilloma at many situations, no secondary growths have appeared under circumstances indicative of spontaneous infection with virus from an existing focus. Enlargement never occurred by saltation, by an appearance of separate small papillomata near the growing edge.

The surface growths of wild and domestic rabbits have been incised on many occasions, and on others implantation nodules have been removed from the muscles. The experiments sometimes involved large skin incisions. During the implantations (Paper I) no pains were taken to prevent the suspension of tissue fragments from coming in contact with the traumatized epidermis. That it must often have done so was indicated by the frequent development of implantation nodules in the healed wound. Yet only once did a growth appear on the epidermis, and then as a solitary small papilloma after laparotomy, occurring under circumstances which made implantation probable (Paper I).

Numerous attempts were made to induce malignancy in both wild and domestic rabbits by repeatedly infiltrating the base of the papilloma and the surrounding tissue with Scharlach R (Paper II). The dye injections were begun when the

growth was not more than 1 or 2 mm. across, and the injecting needle was thrust into the neighboring skin at several points, thus adding trauma to the effects of the dye. The latter sometimes caused a more or less widespread, shagreen-like thickening of the skin for a centimeter or more about the papilloma, with epithelial downgrowths of the well known sort. Yet though tattooing the virus itself directly into skin thus changed resulted in "takes" that were often extensive,—in animals previously free from the papilloma,—no secondary growths ever developed about existing ones. True, the papilloma grew rapidly as result of the dye stimulation, but in the main by proliferation beneath the surface (Paper II).

As a whole the experiments demonstrated the following points. The virus acts only upon cells that have been rendered susceptible in some way; intradermal injections of Scharlach R, as well as trauma, will bring about the susceptible state; the virus can localize out of the blood stream upon susceptible cells; and when introduced directly into skin containing such cells it may undergo some local distribution. Such are the findings with normal animals and virus fluid obtained by the grinding and extraction of papillomatous tissue. Significantly different are those when the source of the virus is a papilloma *in situ*. No evidence has been obtained of secondary infection with the virus from an existing growth, even when the adjacent epidermis has been rendered susceptible. In regenerating epithelium no extension of the papillomatous change takes place suggestive of cell to cell infection.

Shope proved that animals carrying the papilloma become resistant to secondary inoculation with the virus and have neutralizing antibodies in circulation (12). These were demonstrable within 14 days after inoculation and 6 days after the growth appeared. They may prevent the transfer of infection from an existing papilloma to the adjoining cells. The further possibility exists that under natural conditions the virus is confined to the affected cells.

Histological Relations to the Surrounding Epithelium

The best opportunity for the virus associated with a papilloma to affect the neighboring epidermis may come in the early days, before antibodies have been induced. We have examined sections of many early papillomas with this possibility in mind. Their proliferating epithelium grades into that of the normal epidermis rather abruptly, as Hurst observed of older growths, yet still so gradually that

it is impossible as a rule to say where one leaves off and the other begins (Fig. 41). So too where the growth is surrounded by an epidermis that is thickened and proliferating as result of the influence of Scharlach R (Fig. 23). No foci of papillomatous change can be perceived in the tissue thus altered. Occasionally when established papillomas are pigmented, or their cells are especially crowded, a fairly sharp histological distinction does exist between the virus-affected epithelium and the normal layer continuous with it (Fig. 47). In other, more frequent, instances the connection with the surrounding epidermis is accidentally torn through or macerated, and the enlarging growth is isolated by a cleft (Fig. 48). In yet other cases the papilloma undermines the epithelium next it, with result in a necrosis that destroys the continuity of the two, and henceforth it proliferates independently.

All these various relations are found at the edges of the epitheliomata of mice and men. The histological evidence for a conversion of normal to papillomatous epithelium at the edge of the papilloma is neither less nor greater than in the case of these neoplasms. Such conversion was greatly debated amongst the older students of cancer. Ribbert, the main advocate of the view which now prevails, that tumors once well started grow only by their own intrinsic proliferation,—*aus sich heraus*,—himself produced evidence that epidermoid cancers may begin at several neighboring centers (13), a fact now generally conceded, as result more especially of the study of tar tumors. He pictured epitheliomata that were directly connected with the epidermis, grading into it by a gradual transition; others in which, though the epithelial layers of the two were continuous, pronounced histological differences distinguished them; and yet others in which a secondary break had occurred to separate them.[2] In no feature does the papilloma more closely resemble the classical epitheliomata than in these

[2] That a connection between two widely differing sorts of epithelium may come about secondarily has been shown by experiments in which cell groups of the Flexner-Jobling adenocarcinoma of the rat united with regenerating epidermis (Rous, Peyton, *J. Exp. Med.*, 1913, **17**, 494). In the course of the present work we have observed instances in which the papillomatous epithelium of implantation growths became directly connected with the epithelium of the kidney pelvis (Fig. 49), with that of the mammary duct, and with the peritoneal lining.

relations to the neighboring epithelium. To all appearance once the growth has been started it enlarges by its own proliferation. Our experimental observations, recorded a few pages back, provide reasons for the supposition that in general it really does so.

Attempts at Transplantation to Other Individuals

The presence in the papillomatous tissue of a causative virus almost uniformly infective for rabbits lends special significance to the fate of this tissue on transplantation. Bits of it placed in the inner organs of the host grow with such regularity that one might suppose transplantation to other individuals would be readily accomplished.

Experiment 1.—In order to free the papillomatous tissue, so far as possible, from contaminating bacteria, a preliminary subcutaneous implantation was made in the axilla of a wild rabbit (W.R. 10) of bits shaved from a papilloma on the skin of the abdomen. There resulted a large growth. It was removed, under ether, 65 days after the original inoculation with virus and 24 after implantation; the living epithelial rind was cut up; and part was suspended in Tyrode solution and injected into the extensor muscles of the upper forelegs of the donor, three other wild rabbits, and three domestic ones. With the remainder 5 per cent virus fluid was made in the usual way and centrifuged; and some was withdrawn from the center of the column of fluid and heated at 53°C. for 15 minutes to kill any tissue cells present.[3] It was then rubbed into a large, freshly scarified area on the lower side of W.R. 10 and into areas of similar size on the abdomen of the other animals. Sections proved that the tissue utilized had been actively proliferating.

All of the rabbits except W.R. 10 developed papillomas as result of the skin inoculations with virus, this individual showing itself completely resistant. The growths thus caused retrogressed in one of the wild rabbits; and in one of the domestic species they were discrete, though numerous and rapidly growing. The other four animals promptly developed confluent "pancakes" of papillomatous tissue. Only in W.R. 10, however, did growths result from the tissue implantations in the forelegs. They appeared within 10 days in this individual, and when it was sacrificed, 79 days after implantation, they measured 3 cm. and 2.5 cm. in diameter respectively, and had the characteristic make up. The rabbits were kept under observation for months. In some of them nodules 2 to 4 mm. in diameter soon appeared at the sites of transplantation but after several weeks could no longer be felt.

The rabbit providing the tissue for transplantation proved insusceptible to infection with the virus extracted from this tissue, yet

[3] Shope has shown that the virus in 5 per cent suspension is unaffected by heating for 30 minutes at 65°C.

favorable to implantation with the cells. The other rabbits showed themselves susceptible to the virus though the tissue providing it failed to grow in them.

Experiment 2.—The general procedure was the same as in Experiment 1. The material for transplantation came from Domestic Rabbit 1-34 which had large implantation growths in all four legs. One was removed piecemeal, by operation under ether, and minced and suspended in Tyrode. Part of the suspension was filtered through gauze and injected into an ear vein and artery of the host. The unfiltered remainder was injected into the extensors of the upper forelegs of three wild rabbits and three normal domestic ones, into another domestic rabbit recently recovered from vaccinia, and into one in which a previously induced skin papilloma had retrogressed. No muscle injections were made in D.R. 1-34. To rule out the possibility that the needle might carry some epidermal cells before it into the deeper tissue, infecting them with virus simultaneously and thus causing growths which were not transplants at all, a small slit was made in the skin at each implantation and its lips were held apart so that the entering needle did not come in contact with the epidermis. A 5 per cent extract of some of the papilloma tissue was prepared in the usual way, heated at 53°C. for 15 minutes after centrifugation, and rubbed into the scarified abdomen of all of the rabbits.

The donor, D.R. 1-34, died of intercurrent causes 14 days after the intravascular inoculation,—which had produced no visible growths. It carried a large, foul abdominal "pancake," and implantation nodules up to 3 cm. in diameter in all of the legs except that furnishing the material for transplantation, which showed small recurrences in the subcutaneous tissue of the healed incision. The virus inoculations failed to cause the papilloma in any animal, and the transplantations resulted merely in transient, small nodules in some of them. One such nodule, taken from a domestic rabbit that died of intercurrent illness 7 days after the transplantation, contained proliferating and well vascularized epithelium of the characteristic sort (Fig. 42), surrounded by new connective tissue in which were a few lymphocytes. At several points epithelial processes were extending out.

In one of the wild rabbits receiving implants, which died of intercurrent causes 37 days later, sections showed the transplanted tissue to be surrounded by an accumulation of small round cells so dense as to have the superficial appearance of a lymph node (Fig. 43). The tissue was now necrotic, but the shape of its largest fragment showed that it had undergone some early proliferation. The lymphocytic reaction was far more pronounced than that about dead autoimplants in domestic rabbits (Fig. 44).

The papillomatous tissue utilized in this experiment had been actively invading the muscle, as sections showed. That it was capable of surviving on transplantation to another individual was proven by the findings (Fig. 42) in the domestic rabbit that died early; yet it

failed to cause growths in any of the surviving animals except the host, in which nodules developed as result of accidental dissemination when the transplantation material was procured. The wholly negative results of the virus inoculations accord with Shope's experience that neither wild nor domestic rabbits can be infected ordinarily with an extract made from the papilloma of a domestic animal.

Experiment 3.—A wild rabbit (W.R. 18) with a papillomatous "pancake" and rapidly enlarging implantation nodules in the foreleg muscles, right kidney, liver, spleen, stomach, and groin, was killed and material taken from the surface growth. This was large and high with a dry, tough, fibrous, outer portion which was sliced off separately, and extracted in the usual way, yielding what may be termed Virus Fluid I. The base of the growth was similarly treated to procure Virus Fluid II, the intermediate part in which dead and living epithelium were mixed being discarded. Portions of the living rind of the leg nodule that had enlarged most rapidly were extracted for Virus Fluid III, and other portions of it were added for transplantation purposes to parts of a nodule found on the splenic omentum, and one from the parietal peritoneum. The pooled material was minced, suspended, and injected into the upper forelegs of three normal wild rabbits, three in which a previous skin inoculation with virus had failed, and three normal domestic animals. The skin was slit just beforehand, as in Experiment 2. The virus extracts (5 per cent in each case, unheated) were rubbed into scarified areas on the sides and abdomen respectively of three other normal domestic rabbits and three wild ones, the site being varied for each extract.

The animal furnishing the experimental materials had large, actively growing nodules at several implantation sites in the viscera, and one of these (from the splenic mesentery) yielded a virus of unusual activity after some weeks glycerinization, as animal tests proved.

The transplantations resulted in nodules as much as 4 mm. across in some instances, but all had disappeared within 3 weeks, though the microscope showed that vigorously growing material had been implanted. No attempt had been made to prevent the suspension fluid from coming in contact with the slit skin when the needle was withdrawn, and in one of the domestic rabbits a papilloma developed in the linear scar. Virus Fluid I gave rise to skin papillomas in two of the wild rabbits and one domestic; Fluid II caused them in these same wild rabbits and the two other domestic ones; while Fluid III produced the papilloma only in one of these last. Although skin areas about 5 cm. across had been scarified and inoculated broadcast, discrete, slowly growing, cone-shaped growths appeared which were obviously the result of scattered, punctate infection. In several of the rabbits they retrogressed.

In this experiment, like the others, the transplantations failed to

yield growths. Five of the six rabbits of the group inoculated only with virus developed papillomas in consequence.

Prior to the efforts at transplantation it had been supposed that the papillomatous tissue might grow on transfer to rabbits that were resistant to the action of the causative virus as such; for animals with flourishing growths frequently prove refractory when reinoculated with the virus,—a fact exemplified in W. R. 10 of Experiment 1. To test the point in Experiment 3 grafts were placed in several proven immunes to the virus, but with negative results. It had been further assumed that the virus would "take" when grafts could scarcely be expected to do so, for example in domestic animals receiving papillomatous tissue from cottontails; and for this reason transplantations to some rabbits of alien species, together with virus inoculations, were included in Experiments 1 and 2. The results justified the assumption, the virus producing growths in individuals unfavorable to transplantation (Experiment 1). It seemed conceivable that the inoculations with virus might affect the outcome of implantations made simultaneously. For this reason a separate group of animals was employed for the inoculations of Experiment 3.

The total number of rabbits implanted with papillomatous tissue deriving from other individuals of the same species was only fourteen, nine of these previously normal, three recently refractory to the virus as such, one immune to vaccinia, and one in which a previous papilloma had retrogressed and disappeared. In the one normal animal that died early the implanted material had survived and proliferated slightly. No extensive test this; yet since it has shown that the papillomatous tissue can live for a little while at least in the new host, there is a possibility that transplantation into rabbits of a single strain, or into a large number picked at random, may yield transplantable growths.

The evidence is convincing that the fate of the papilloma on transplantation is not determined by the presence of the virus associated with the tissue and inducing its proliferation. This virus as such will "take" vigorously, causing an exuberant papilloma, on the skin of individuals unfavorable to implants of the tissue with which it is associated (Experiment 1). This tissue, though failing to grow on transplantation to other individuals, will proliferate rapidly when grafted in the host, though the latter is now insusceptible to the direct action of the contained virus (Experiment 1). It is plain that two

sorts of resistance, occurring independently of each other, may be manifested by individuals to which pieces of the growth are transferred, these being directed against the implanted cells and the agent causing their proliferation, respectively. Such a state of affairs has already been demonstrated for the chicken tumors (14).

The fate of engrafted tumor cells is known to be determined by the laws of compatibility which influence the fate of transplanted tissues generally. This is evidently true of engrafted papilloma cells as well. Though the proliferative activity of the growth is great, it is possessed of but slight capacity for aggression (Paper II),—a fact which probably has some subsidiary influence on the outcome of transplantation. But many spontaneous mouse tumors that are highly malignant for the host and can be readily grafted within its body fail to grow on transfer to other individuals picked at random.

The cellular changes about implants that survive or die respectively would seem from the scanty evidence at hand to be identical with those about tumor grafts,—which is scarcely surprising since the reactions about the latter are elicited by them as tissue not as tumor.

Influence of Other Disease Processes, and of the Host's Age and Condition

Certain additional observations and experiments deserve record, though unconnected with the immediate purposes of this report.

Not only does the virus fail to affect embryonic epithelium (Paper I) but the skin of new-born rabbits appears to be relatively refractory to it.

Two litters from gray-brown, domestic does were inoculated a few days after birth by rubbing virus fluid into the scarified skin of the abdomen, and in some instances injecting the skin of the ears. The does were similarly treated and some other domestic rabbits as well. All the adults promptly developed vigorous growths. In the five young rabbits on the other hand the papilloma appeared much later and at relatively few points.

The rabbit pox (15), a virus disease having many similarities to vaccinia, was epidemic in our animal room during part of the period of experimentation, and many of the animals bearing the papilloma, or in which it was just appearing, showed pocking with the "snuffles" and conjunctivitis that accompany the malady in its pronounced form.

Some became seriously ill; but in none was any effect manifest on the papilloma. To test the influence of vaccinia a group of domestic rabbits were injected intradermally at several points on the sides with the New York Board of Health strain, as propagated in tissue cultures by Dr. Rivers, at a time when the papilloma was just appearing in them and in an appropriate control group, as result of virus inunction into the skin of the abdomen. Large vaccinial lesions developed, but the disease had no evident effect upon the development of the papilloma. In studying the growth we have of late used many domestic rabbits recently recovered from experimental vaccinia. The findings were regularly the same as in the normal animals employed with them.

Many spontaneous and transplanted tumors of mice and rats are adversely affected by ill health or loss of weight of the host, and this is the case with Chicken Tumor I as well. The Shope rabbit fibroma, a growth caused by a virus, is in our experience remarkably susceptible to such influences. The papilloma on the other hand is not notably affected either by malnutrition of the host or by intercurrent illness. But this is the case, for that matter, with not a few of the recognized mammalian tumors.

Shope could elicit no cross-immunity between the papilloma and his rabbit fibroma caused by a virus. A test with the Brown-Pearce rabbit tumor seemed desirable,—the more so since the cells of the latter growth are of epithelial nature.

Thirteen adult, domestic, gray-brown rabbits of the same size were procured, three of them set aside as controls, and ten inoculated intradermally with 0.2 cc. of Tyrode suspension of Brown-Pearce tumor at four sites on the back of the neck, as well as with 0.3 cc. intramuscularly in each hind leg. The object was to cause tumors which would undergo retrogression because in unfavorable situations. Within the succeeding 46 days this was the course of events in four animals, the others either developing progressive tumors or failing to show any. Most of the intramuscular growths that retrogressed were large at one time. Now the four animals were injected again intramuscularly with Brown-Pearce suspension, this time into the muscle of the forelegs, as were the controls, and on the same day virus fluid prepared as usual from glycerinated papilloma tissue was rubbed into the scarified abdominal skin of them all. Intradermal titrations of the papilloma virus were not employed because of the uncertainty of the method and the long period elapsing prior to the appearance of growths,—a period during which

induced resistance to the Brown-Pearce tumor might have fallen off. The local variations in the conditions of virus inunction upon the broad areas that were scarified may have served as a rough substitute for titration, the papilloma appearing late and at scattered points in some parts of such areas, whereas at others a confluent papillomatous change took place early.

The rabbits in which the Brown-Pearce tumor had retrogressed did not develop tumors from the material of the second injection whereas the controls did so, the growths in two of them enlarging rapidly. The papilloma appeared in every animal, and there were no differences in its incubation period (about 12 days), or its character or course, to indicate that acquired resistance to the Brown-Pearce tumor influenced it. But more extensive and delicate tests are called for.

GENERAL DISCUSSION

Some of the characters of the papilloma have been compared with those of the neoplasms as they were described. It remains to survey the findings in their general relation to the tumor problem.

The Shope papilloma, as occurring in nature, manifestly falls into the group of the infectious warts, condylomas, and papillomas, pathological processes of such dubious character that they have no fixed habitation in textbooks, some authorities classing them with the tumors and others with the hypertrophies or the inflammations. Two recent reviews deal with those known to be due to viruses (16). They have been encountered in several animal species. In 1920 Magalhaes stated in a preliminary report (17) that he had produced generalized papillomatosis in steers by the intravenous injections of filtrates and suspensions of tissue from a spontaneous instance of the disease. No detailed account of the work has appeared. Ullmann (18) demonstrated the presence of a virus in a laryngeal papilloma of a boy, by two successive transfers to the skin of adult volunteers, the second of which gave rise to a papillomatous growth which developed so swiftly that excision seemed the safest course.

The rabbit papilloma differs in several important respects from those previously studied experimentally. No development of secondary growths takes place by infection from an existing papilloma, a frequent happening in human beings with warts or condylomas, and in dogs carrying the pointed condyloma of Borst (19) or the infectious papilloma described by DeMonbreun and Goodpasture. The rabbit growth does not show any morphological peculiarities that enable one

to distinguish it from the tumors. Unlike the generality of infectious warts, papillomas, and condylomas it does not tend to retrogress and disappear, but usually, in experimental instances at least, continues its proliferation indefinitely.[4] Furthermore the virus is capable of causing vigorous, progressive growths in animals of an alien genus (domestic rabbits).

Tumor Attributes of the Growth

Whatever one calls or however one classes the papilloma it possesses, as our experiments have shown, the traits of behavior and appearance which characterize the tumors. It is an autonomous new growth, purposeless, parasitic, and, on occasion, progressive. Its extension on the skin ceases after a time because local conditions become unfavorable to it; but it continues to proliferate there and can be stimulated to renewed aggression. When implanted within the host it often looks and acts like a highly malignant neoplasm, directly invading and destroying the normal tissues, and causing death. On excision it recurs unless removed entirely; and during operation its cells may undergo implantation in the tissue of the wound or upon the peritoneal surface, with result in secondary nodules. Its own proliferation invariably involves it in widespread necrosis, and it may retrogress at some situations while progressing at others. Scharlach R stimulates it and bacterial infection sometimes causes it to become pronouncedly malignant. Its morphology as well as its behavior are influenced by the peculiarities of the individual host. It frequently penetrates into the blood and lymph vessels; but though the injection of fragments of it into the circulation not infrequently results in lung nodules, owing to cell survival and proliferation, conclusive instances of metastasis formation as distinguished from accidental implantation have not thus far been encountered.

[4] The occasional retrogression in cottontails of the experimentally induced growth, and the occurrence of individuals resistant to the virus and possessing neutralizing antiviral bodies in the blood, suggest that under natural conditions retrogression may sometimes occur, or else that the papilloma may be torn off the skin, or gnawed away completely, after the hosts have developed neutralizing antibodies for the virus as such.

The papilloma produces no distinctive cytological alterations in the blood or in the other organs. It elicits a stroma and usually some new formation of connective tissue attributable to bacterial contaminants, but no characteristic histological reaction occurs about it and sometimes none of any sort when it is growing rapidly. The inflammatory changes found at its base, when long established on the skin, are referable to trauma, necrosis, and incidental infection.

The morphology of the growth when proliferating within the body under favorable circumstances is that of an epidermoid carcinoma. Its cells no more give indication of the presence of an extraneous cause than do those of such tumors. When situated on the skin it closely resembles them in its histological relations to the surrounding epidermis. Virus inoculations may cause it to develop at one point or many, according to the technique employed; but the established growths appear to enlarge solely by a multiplication of their own cells, and the experimental evidence provides reasons for supposing this to be the case. In some animals the experimentally induced papilloma retrogresses, and when so doing it goes through the identical histological changes recorded for the epidermoid carcinomas of man and of rabbits, with a similar cellular reaction in the surrounding tissue. On transfer to other hosts the fate of the papillomatous tissue appears to be wholly uninfluenced by the association with it of a causative virus to which as such the host may be susceptible, but is determined instead by the laws governing the transplantation of tissues generally, including tumor tissue. As in the case of the chicken tumors, two sorts of resistance can be detected in animals to which the growth is transferred, these being directed against the strange cells and the agent responsible for their pathological behavior, respectively.

The habit of the tar tumors to become malignant or retrogress in response to local stimulating or repressing influences has been many times invoked as proving that neoplasms in general are the consequence of intrinsic cellular modifications. The papilloma, a growth due to a virus, behaves in precisely the same way. Tar cancers of rabbits frequently cease to enlarge after a time, like the papilloma when it is situated on the skin. In both cases a dense layer of newformed connective tissue is to be found next the growth, and in both there is evidence that it has checked the latter (20).

Comparison with the Chicken Tumors

Andrewes (21) has recently assembled and discussed a graded series of disease processes due to viruses, ranging from such as are necrotizing to those causing growths of neoplastic character, namely the chicken tumors. The Shope papilloma belongs at the further end of the series; and some comparison of it with the chicken tumors would seem called for, though of necessity this must be confined to major differences and likenesses. The growths of both sorts resemble the recognized tumors in their manifestations within the individual host, even as regards characters demonstrable only by experiment; yet both are due to filterable agents which can readily be separated from the tissue. Each causative agent is specific for cells of a certain kind, affecting no others and making these behave in ways from which there is remarkably little deviation, the result being the regular production of an osteochondrosarcoma, for example, or a fissured sarcoma of intracanalicular pattern in fowls, or a papillomatous epithelial growth in rabbits. Nevertheless some of the agents will act upon the cells of an alien species (duck (22), pheasant (23) in the case of certain chicken tumors, domestic rabbits in that of the Shope papilloma). Once the cells have been acted upon, however, their character and activities are responsible for the obvious disease phenomena. Cell injury is requisite to the action of the agent causing Chicken Tumor I (24), as also to that of the virus producing the rabbit papilloma.

Theoretical difficulties in supposing tumors to be due to viruses, and certain characters of the agents causing the growths in fowls, have led some workers to assume that these agents are substances elaborated by the cells themselves; but experiments in this direction have but enlarged the similarities to the viruses, as Andrewes has pointed out. The agent causing the rabbit papilloma has the typical characters of a virus, and the disease to which it gives rise is endemic in the wild rabbits of certain localities, multiple growths being not infrequent. The incidence of the chicken tumors on the other hand does not suggest an infectious source. But the conditions favor transfer of the papilloma virus. It persists in active form in the dried tissue covering the growth (a fact demonstrated incidentally in Experiment 3 of our transplantation series), and some of this must often be

rubbed off into the dust of the rabbit's burrow or form. It is also notably resistant to heat (25),—which it must frequently have to withstand on the prairies where the disease is found. Acting as it does upon freshly injured epidermis, every rabbit with a scratch provides an opportunity for it, while furthermore the great majority are susceptible. The activity of the agents present in chicken tumors, those thus far studied at least, is easily destroyed, and their distribution to the outside world is beset with difficulties. The growths lie in the interior of the body, and the causative agents, if entering or leaving cells, must in many cases have to run the gauntlet of neutralizing substances present in the blood.[5] Yet if they are to produce the disease in other fowls they must somehow reach injured cells that are beneath the body surface. Such differences as these in the conditions determining effectiveness may account for a difference in incidence that at first glance seems fundamental.

Virus Changes and the Behavior of the Growth

There is good reason to believe that once the agents causing the rabbit papilloma and chicken tumors have become associated with the cells they, like other viruses (26) and certain intracellular bacteria (27), are protected from the influence of neutralizing principles in the body fluids. Growths of noteworthy vigor are to be found in individuals completely resistant on inoculation with the causative agent as such and possessing antibodies effective against it in their blood. The behavior of both the papilloma and the chicken tumors is directly referable to that of the cells composing the growths. Yet alterations in the causative entities cannot but influence the latter profoundly. Attenuation of the agent responsible for Chicken Tumor I, by heating, prolonged desiccation, or a sojourn in glycerin, leads it to produce tumors that progress slowly or retrogress; and heating the virus of the papilloma at 67°C. for 30 minutes (28) results in retrogressing growths. The agent responsible for Chicken Tumor I cannot be re-

[5] In a recent, unpublished study of the age incidence of neutralizing substances for the agent causing Chicken Tumor I (McMaster, Hudack, and Rous), it was found that the majority of normal, adult Plymouth Rock fowls possess them, whereas they are much less frequent in pullets of the same stock, and in the pooled serum of new-hatched chicks they are not demonstrable.

covered from slowly enlarging sarcomas (29), and the papilloma virus, though setting up a vigorous and persistent proliferation in domestic rabbits, undergoes some change in these that robs it of infectivity even for wild rabbits. That it may be modified in yet another way is suggested by the tendency of implantation growths in some animals, domestic ones especially, to enlarge more slowly as time passes and eventually to retrogress. Secondary retrogression is frequent in the case of some of the chicken tumors also. In each case the cell or its immediate milieu is the medium in which multiplication takes place of the entity causing the disease. Presumably it becomes attenuated when this medium is not wholly suitable and in consequence the cell proliferation it induces becomes slower or stops.

Differences from the Tumors

The papilloma differs significantly from the tumors in the conditions of its natural occurrence. Its incidence shows it to be an infectious disease, and its virus cause need only be brought in contact with traumatized epidermis for a growth to result. Few facts have been so elaborately proven for the tumors as that their incidence is without sign of an infectious cause; while furthermore all efforts to demonstrate such a cause have consistently failed. Rabbits can be protected from the papilloma: when kept where there is no virus their skins can be repeatedly scarified without inducing it. But the most careful isolation fails to give the least protection from cancer. Mice which are tarred while living on sterilized bedding in sterile jars, drinking sterile water, and eating muscle removed under aseptic conditions from other species, develop tumors with the same frequency as do controls in dirty surroundings, exposed to every chance infection, and fed on a highly various diet (30).

Another difference from the tumors is to be found in the local conditions leading to occurrence of the growths. Both arise in tissue that has been rendered abnormal in some way. But the papilloma will develop on the basis of acute injury, a scratch sufficing to render the epithelium susceptible to the virus, whereas the generality of tumors follow only upon long-continued disturbance (31), the precise character of the cellular changes preliminary to the neoplastic process being not yet clear. The papilloma appears a few days after injury

attended by introduction of the virus; and about the beginning growth there is little or no cellular reaction. The typical epidermoid tumors, on the other hand, of rabbits, mice, and men, develop only after months or years respectively, of recurring local disturbance; and where they appear marked "precancerous changes" are ordinarily present in the tissue.

Implications for the Tumor Problem

In thinking upon the tumor problem facts deserve recognition before new biological possibilities are invoked. One of these facts is that an epithelial growth due to a filterable virus, namely the rabbit papilloma, as observed in the individual host, acts and looks like a tumor. Can one suppose that tumors in general are due to viruses or other extraneous entities?[6] The supposition is tenable only if such entities are widely distributed throughout the animal population, being constantly present in or upon the body, like the colon bacillus or the staphylococcus; and if their opportunity to cause tumors is restricted by the need for very special conditions. Ubiquitous such entities would have to be to produce cancer in human beings everywhere throughout the world, and with the same frequency in isolated, tarred mice as in unsheltered ones. If they acted under ordinary conditions multiple spontaneous growths would be frequent, and the general incidence of tumors would provide evidence of an infective cause for them. Each of the two requisites for the assumption that extraneous entities cause tumors is manifestly a corollary of the other. The more considerably an agent is conditioned in its activity, the more often must it be present if it is to cause disease at all. Though widely present, if heavily conditioned it will cause disease only here and there and now and again. A presumptive instance of how the combined influence of several factors modifying the effectiveness of a causative entity may determine the incidence of the neoplasms has already been discussed during the comparison of the chicken tumors with the papilloma. Evidence that the mammalian tumors are heavily conditioned in their occurrence is at hand, both in the history of indi-

[6] Andrewes has recently advanced the view that tumors are due to viruses and has discussed comprehensively the facts for and against this conception. (Andrewes, C. H., *Lancet*, 1934, **2**, 63, 117.)

vidual cases and in the data on "precancerous changes." Yet though this holds for most instances, where the local circumstances are exceptionally propitious numerous spontaneous tumors may arise, as where a broad expanse of skin has been damaged by the Roentgen ray, by tar, or other physical or chemical agency; and where the necessary general conditions are fulfilled, as in certain races or families, case incidence may be high. The demonstration of the cause for the generality of tumors, whatever this is, waits upon the provision by the investigator of the conditions necessary to its effectiveness.

SUMMARY

Experimental study of the rabbit papilloma of Shope, a growth caused by a virus, has shown that it possesses the immediate characters whereby tumors are recognized. Often it looks and acts like a malignant neoplasm. It differs from the tumors as a group, however, in its incidence which is that of an infectious process, and from other mammalian tumors in that its cause has been demonstrated. The possible bearing of the findings upon the problem of tumor causation is discussed. The morphology and behavior of the generality of tumors can no longer be taken to exclude the possibility that these are produced by extraneous, living entities. The incidence of some of the tumors at least, and the failure to demonstrate their cause can both be explained on the assumption that they are due to such entities, widely distributed in or upon the animal population but effective only under special circumstances. Present knowledge makes this assumption reasonable as a basis for further work.

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EXPLANATION OF PLATES

PLATE 45

FIG. 40. Cells in the granular layer of epithelium at the bottom of a crypt between two papillae,—from a papilloma on the skin of a wild rabbit (W.R. 19)

killed 60 days after inoculation. In the cytoplasm are numerous moderately refractile bodies, which appear to have developed from the granules characteristic of the differentiating cell layer. × 625.

FIG. 41. Unusually marked cellular reaction about beginning growths. The specimen was excised 12 days after virus fluid had been rubbed into the scarified skin of the abdomen of a domestic rabbit. The four little growths here shown are extending down into the connective tissue, and keratinized epithelium has begun to heap up over them. About each is an accumulation of round cells, with an occasional polymorphonuclear leukocyte. The marked capillary dilatation is barely visible. × 105.

FIG. 42. Graft from the leg muscle of a domestic rabbit dying of intercurrent causes 7 days after transplantation to it of fragments of papilloma from another domestic rabbit. The proliferating graft has rounded out and some extension from it into the surrounding tissue has occurred. A capsule is forming in which lymphocytes are present. The dark strip is epithelium overstained with methylene blue. × 65.

FIG. 43. Reaction about papilloma fragments transplanted from a domestic rabbit into the leg muscle of a wild one 37 days previously. All of the implanted tissue is now dead and fails to stain; but the size and shape of the largest fragment shows that it had undergone some proliferation. Lymphocytes have accumulated in enormous numbers and encapsulation has taken place. × 40.

FIG. 44. Edge of a retrogressed nodule situated in an old laparotomy wound (Domestic Rabbit 1-21). The dead squamous epithelium is surrounded by granulation tissue containing giant cells. × 100.

PLATE 46

FIG. 45. To show the influence of Scharlach R on the development of the papilloma. Virus was tattooed into five spots in a line across the middle of the ear (*A*), and also into three spots near the tip, 5 days after an intradermal injection of the dye into the middle one (*M*) of the three. Four similar tattooings were also done into the skin near the base, which had been widely infiltrated with Scharlach R at the same time as the tip. The photograph was taken 26 days later when the growths in the normal skin were still small. Wherever the virus was introduced into tissue affected by the dye relatively large growths have appeared, one near the base of the ear developing as a spherical nodule (*S*) under intact skin. Still nearer the base, where there had been no inoculation, two smaller nodules have also appeared, one papillomatous in character (*P*), the other an epithelial cyst (*C*) referable merely to the Scharlach R. The character of the growths was determined by biopsies. × ½.

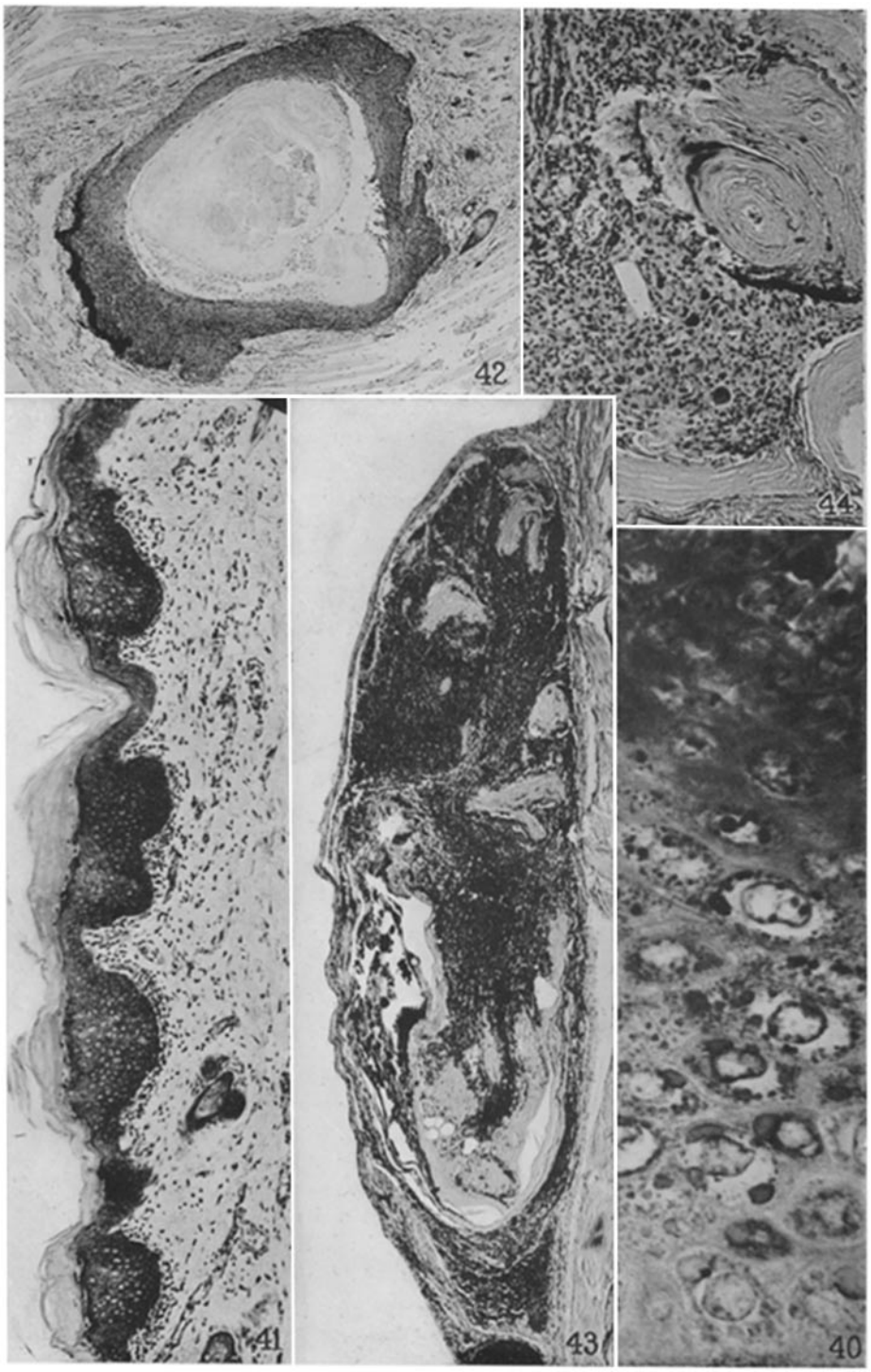
FIG. 46. The localization of circulating papilloma virus. Scharlach R was injected intradermally into fourteen spots in a circle on the side of a domestic rabbit, and 18 days later the injection was repeated. Well defined, discoid thickenings of the skin resulted, with marked scurfiness. 8 days after the second

injection, 5 cc. of virus fluid was injected into the blood. After another 10 days seven of the discs were noted to be everywhere thickened and raised, whereas the others were disappearing; and soon the papillomatous change had declared itself. The photograph was taken 14 days later. × ⅔.

FIG. 47. Unusually sharp demarcation between the normal epidermis and that affected by the virus. Note the pathological enlargement of the cells and nuclei, the crowding, and the darker staining with methylene blue. The section was taken from the edge of an enlarging, pigmented papilloma 22 days after it had appeared on the skin of a domestic rabbit subjected to punctate inoculation. × 500.

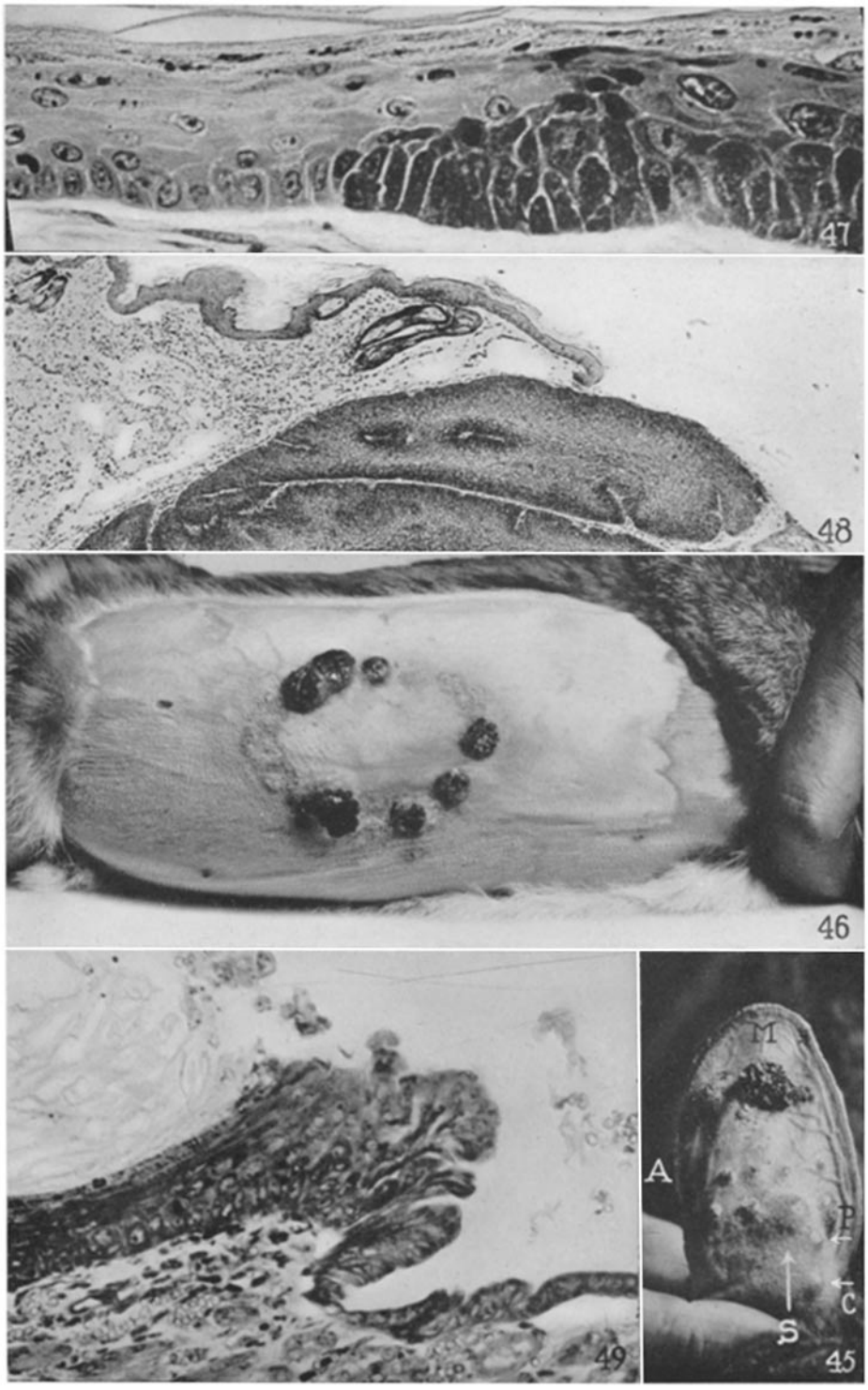
FIG. 48. Edge of a growing skin papilloma isolated from the surrounding epidermis by a tear. Specimen taken from a domestic rabbit inoculated 48 days previously. × 37.

FIG. 49. Junction of papillomatous epithelium and that of the kidney pelvis. The animal (Wild Rabbit 10) had a narrow horn of keratinized epithelium projecting into the pelvis, as result of an implantation made 79 days previously through a hollow needle (see Paper I). The section is from the edge of the base of the horn. × 312.



Photographed by Louis Schmidt

(Rous and Beard: Virus-induced mammalian growth. III)



Photographed by Louis Schmidt

(Rous and Beard: Virus-induced mammalian growth. III)