

## THE EFFECT OF CHEMICAL CARCINOGENS ON VIRUS-INDUCED RABBIT PAPILLOMAS\*

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PLATES 9 TO 14

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The fact is now widely realized that the known chemical carcinogens are not the actuating causes of the growths they engender, and many surmises have been put forward as to how they induce neoplastic change. The evidence that viruses play a rôle in the causation of tumors has led to the supposition that there are agents of this sort in the animal body which cause no harm unless pathological alterations in their cell milieu occur or are induced, as by the action of chemical carcinogens for example, when they undergo variation, becoming tumor-producing viruses. In line with this conception is the fact that the papillomas evoked by the Shope papilloma virus on rabbit skin resemble in all essential neoplastic respects those called forth by tarring (1), while furthermore carcinomas of identical kind arise from them both by processes which involve the same train of morphological events. Continued tarring brings on malignant changes in tar papillomas, most of the cancers coming about in such way. The object of the present investigation has been to learn whether tar and methylcholanthrene will bring on cancers in papillomas of virus cause.

### *Materials and Methods*

Cancer sometimes arises from tar papillomas after tarring has been stopped, especially if the growths are crowded and inflamed. A similar "spontaneous" development of cancer often takes place in virus-induced papillomas after they have proliferated for some months; and various intercurrent disturbances, crowding and inflammation amongst them, may precipitate it then (2). The malignant change is rare in cottontail papillomas but exceedingly frequent in those produced by inoculation of the virus into domestic rabbits,—which as foreign hosts provide an unnatural milieu to the virus. The time before cancer arises varies with the vigor of the growths, and this depends upon peculiarities of the individual animal. Hence in testing the effects of the carcinogens on the papillomas of domestic rabbits it has been necessary to provide duplicate untreated growths on the same individuals as controls.

Virus papillomas become thickly covered with keratin in the first weeks of growth and it must be removed if the carcinogens are to reach the living cells. Growths on the abdomen or low on the sides often become almost denuded after a while because of maceration, and hence we inoculated there. Before maceration began the keratinized

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material was cut away with scissors at intervals, and at first there was some hemorrhage; but later the keratinized layer tended to separate and could be pulled off *en masse*, exposing a tripe-like living expanse on which the carcinogen could be directly painted. Usually all of the growths of the rabbit were stripped at the same time. In some animals they eventually became exuberant, moist, fleshy discs wholly bare of keratin.

Horizontal retort tar from the Ostergasfabrik of Amsterdam was used. It was the last of a barrel and much thicker than that previously utilized from the same container.<sup>1</sup> The methylcholanthrene was procured from the Eastman Kodak Company.

As already mentioned crowding and inflammation may hasten the appearance of cancer in virus papillomas, and so too will various intercurrent disturbances which stimulate proliferation (2). Tarring markedly stimulates the papillomas of unknown cause which it induces and hence there was reason to suppose it might have such an effect on virus papillomas. As a control on this possible factor in the results some of the growths of Experiment 1 were swabbed with a turpentine-acetone mixture which causes great proliferation of ordinary epidermal cells (3).

### *The Major Experiments*

*Experiment 1.*—The papilloma virus was inoculated into three well separated rectangular areas about 3 cm. across, on one side of the shaved abdomen of each of eight strong agouti rabbits weighing about 3 kilos, and into a fourth, similar area over the ensiform region in the midline. The skin was scarified with sandpaper everywhere in the areas and a 10 per cent extract of the glycerolated papillomas of W.R. 2-95, cleared by centrifugation, was rubbed into them, with drying afterwards in a blast of warm air.

Healing was complete after 7 days in four rabbits (group A) and the test substances were applied then. These were tar, turpentine and acetone mixed in equal parts, and a 0.3 per cent solution of methylcholanthrene in a 1 in 50 mixture of mineral oil with ether,—Crabtree's solvent which renders the carcinogenic hydrocarbons notably effective on mouse skin (4). Each was applied to one of the inoculated areas, its situation varying from animal to animal, the area on the chest serving as control. The tar was smeared on, the fluids painted on; and after brief drying a gauze square several layers thick was moored over each area with adhesive, followed by a gauze pad reaching from shoulder to groin, and a many-tailed binder. The applications were repeated thrice a week, with removal of the accumulated tar on the third occasion.

To learn the response of the uninoculated skin of the animals to the test solutions, three areas like those inoculated with virus and situated directly opposite them across the midline were scarified on the 7th day as if for inoculation, and after another 7 days, when healing had been completed, they were submitted to the same treatments as the squares that had received the virus.

Healing was not finished until the 10th day in the other four rabbits (group B). All was then done as with group A except that the tar contained 0.3 per cent of powdered methylcholanthrene, which had been worked into it, and the methylcholanthrene utilized as such had been dissolved in 49 parts of ether with 1 of liquid lard instead of mineral oil. As in group A three comparable squares of skin across the midline were scarified on the day the carcinogens were first applied, and when after a further 10 days these squares had healed they too received the test solutions.

Papillomas appeared on some of the animals of group A within 12 days, and a little later on those of group B (Table I). Their time of appearance and number were recorded with +

<sup>1</sup> The tar was the generous gift of Dr. Karl Landsteiner.

TABLE I  
Exp. 1.—Effects of Tar, Methylcholanthrene, and a Turpentine-Acetone Mixture on the Incidence and Early Growth of Virus Papillomas

Group A						Group B					
Rab- bit No.	Treatment	Day begun	Papillomas			Rab- bit	Treatment	Day begun	Papillomas		
			Incidence		Early character				Incidence		Early character
			12	14	18				12	14	18
1-56	Turp.-acetone Tar Mch. Control		+	++± ++± ++± ++±	Relatively low Low and ill-defined Higher than control	1-54	Turp.-acetone Tar-mch. Mch. Control			++± ++± ++± ++±	All similar
1-52	Turp.-acetone Tar Mch. Control		++ ++± ++±	++± ++± ++± ++±	Low Much higher than control	1-58	Turp.-acetone Tar-mch. Mch. Control	10	+	++ ++ ++ ++	Low Low and defined
1-59	Turp.-acetone Tar Mch. Control	7	++ ++± ++±	++ ++± ++± ++±	Much higher than control	1-55	Turp.-acetone Tar-mch. Mch. Control		++ ++ ++	++ ++ ++ ++	All similar
1-53	Turp.-acetone Tar-mch. Mch. Control		++ ++± ++± ++±	++ ++± ++± ++±	Low Higher than control	1-57	Turp.-acetone Tar-mch. Mch. Control		++± ++± ++±	++ ++ ++ ++	Low Very low

++++ = confluent papillomas, +++ = semiconfluent papillomas, ++ = many discrete growths, + = 5 to 15 papillomas, ± = 2, 3, or 4 papillomas, ± = apilloma, 0 = negative. Mch. = methylcholanthrene.

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signs as usual, but later as they became confluent their outlines were traced on cellophane sheets and transferred to cards, with notation of their height and other features. This was done as often as they underwent any significant change, every week in some instances.

Table I shows that the treatments much affected the incidence and early development of the papillomas. In most instances they appeared late where tar as such or with methylcholanthrene had been applied, as also where the turpentine-acetone mixture had been painted on, and instead of arising as more or less completely confluent outcroppings like those on the methylcholanthrene and control areas they were usually scattered growths.

Further differences soon became evident. The first few applications of tar and turpentine-acetone had resulted in acute inflammation and swelling of the skin, followed by scurfing which in some animals soon became pronounced on the tarred areas, the papillomas in such instances remaining patchy and lower for several weeks than those elsewhere, and covering the inoculated areas slowly by lateral extension. Not infrequently it was difficult to tell precisely where the growths ended and hyperkeratotic rugosity began. Some of the papillomas receiving turpentine and acetone also did poorly for a while. The control growths and those painted with methylcholanthrene on the other hand soon covered the entire areas of inoculation, when they had not done so on first arising, and they frequently reached a height of 5-6 mm. at a time when the tarred or turpentine growths were still only 1-2 mm. high.

The early changes in the areas of treated skin on the other side of the animals were like those of the inoculated areas receiving the same applications. The tarred skin became inflamed and hyperkeratotic, that submitted to turpentine did so to a less degree, whereas methylcholanthrene caused only a slight scurf, this gradually increasing later.

The dressings were left off a month after the inoculations. This was a mistake since soon all of the growths and skin squares came to have tar on them, the amount depending on how far they were from where it had been directly applied. Wherever it got changes rapidly ensued, obscuring the effects of the other treatments, and hence these were stopped on the 71st day, though tarring was kept up. Much earlier, between the 24th and 36th days, 0.3 per cent of powdered methylcholanthrene had been added to the tar applied to the rabbits of group A,—this with the aim of hastening carcinogenesis; but after the 71st day ordinary tar was employed again.

By the end of 6 weeks almost all of the inoculated squares were completely occupied by confluent papillomatous masses which in certain individuals slowly broadened afterwards by lateral extension until they had become as much as 5 cm. across. Some of the rabbits gnawed them repeatedly, usually all at one time, and occasionally cut them nearly to the skin level. The growths had at first the ordinary aspect, that is to say were dark gray expanses with a rough, dry, jagged surface, and they keratinized almost to the skin in certain individuals, while in others they were more or less fleshy. Under most of them as time passed scattered rounded knobs could be felt, such as usually form when proliferation is vigorous,—cysts lined with papillomatous epithelium and full of keratin. These pearls were most numerous beneath the tarred growths and somewhat less so under those getting methylcholanthrene. But another and more important phenomenon was observed toward the end of the 2nd month, namely, a deepening of the base of the tarred masses as if by general extension downward. This deepening became pronounced in five animals, and it took place not only under the tarred papillomas but wherever tar reached the other masses in quantity secondarily. In two animals the growths never extended down, but became raised raw discs. In the eighth rabbit the treatments had no evident effect on the growths, which remained dry and superficial and had retrogressed far when the animal was killed on the 152nd day. All of the rabbits rapidly weakened and lost weight owing to the toxicity of the tar (5); and two from group B died on the 82nd and 92nd days, respectively, while two of group A were killed on the 95th and 99th days when almost moribund. Their growths had for several weeks been fungoid and foul.

At the autopsies each papillomatous mass was sliced vertically at 3 mm. intervals. A singular state of affairs was revealed. The superficial layer of the tarred masses was very shallow and consisted of soft pale tissue with some patches of persisting gray: it showed little or none of the vertical striation characteristic of ordinary virus papillomatosis in agouti rabbits (Figs. 1, 2). At many spots deep extension had occurred into a profuse reactive tissue, with the formation of neoplastic islands and large and small cysts, containing keratin or a creamy grumous material. The deep tissue was almost free from melanosis, as is usually true, for that matter, of ordinary papillomatous tissue when penetrating downwards to form pearls (6, 7), and in some regions it was firm, close-textured, finely dotted with yellow opacities and what appeared to be serpiginous necroses. The corium,—which, on the rabbit belly, is a dense, sharply defined layer 1–2 mm. thick,—was lost amidst the growth, and this had extended into the underlying areolar layer at numerous situations. The gross findings seemed indicative of a wildly irregular, invasive papillomatous proliferation with secondary carcinomatosis at not a few situations. The deep musculature had not been invaded, but at several points the growths had extended out into the skin or under it,—to the distance of 1.3 cm. at one situation.

Wherever tar had reached the other papillomatous masses in quantity the same state of affairs was found, and this irrespective of whether the growth was a control (Figs. 3, 4) or had been exposed to methylcholanthrene or turpentine-acetone. Where no tar had yet got there was vigorous, superficial, palisade papillomatosis only and the usual keratinized pearls. The methylcholanthrened masses were somewhat higher than the others.

The two surviving rabbits of group A stood the tar well. In them the papillomas grew less rapidly and those of one animal were shallow and retrogressing, as already mentioned, when it was killed on the 140th day. In the case of the other (D.R. 1–52) all were fungoid, ruddy discs by the 117th day, the treated masses more fleshy than the controls, and irregular extensions downward could be felt beneath those which had received tar and methylcholanthrene. To learn what was happening a narrow slice through each mass was now taken, with ether anesthesia. All showed ordinary papillomatosis with occasional deep pearls, except the slice from the tarred mass, more than half of which consisted of deep-lying tissue which appeared carcinomatous. No further tarring was done and soon all of the growths except the one which had been tarred began to get smaller, and this did so too save in the region occupied by the presumptive cancer which ulcerated and enlarged until the animal was killed on the 140th day.

One of the remaining two animals of group B died on the 111th day. Its growths had been little affected by the treatments and were stationary, low, and superficial. Slicing and the microscope showed nothing but papillomatosis. All of the growths of the other animal (1–57) had become raised raw discs by the 92nd day, with some keratinized pearls amidst and under them. On the 108th day discrete, bulging, pink, fungoid protrusions, apparently expressive of change, were noted on the jagged dark surface of the mass treated with methylcholanthrene, and a slice to include some of them was removed, with the help of ether anesthesia, as also a slice across each of the other growths. In the region of the protrusions the tissue was found to have the gross character already described as suggestive of malignancy. The rabbit was killed on the 124th day because of purulent infection of the biopsy wounds.

In every rabbit except one tiny tumors arose toward the end of the test on the uninoculated patches of skin, which were tarred at the same time as the virus-induced growths, and similar neoplasms appeared also at several spots to which the tar had spread. Some of the little tumors were removed during life, others at autopsy, and they proved to be of the kinds that tar commonly calls forth,—benign papillomas of distinctive morphology and carcinomatoids (Fig. 5) (1). The largest was 4 mm. across.

One or more slices, usually two or three, of each papillomatous mass were sectioned and

stained with eosin and methylene blue. The microscope showed that in most regions where cancer had seemed to be present there was only wildly proliferating papilloma tissue (Figs. 3, 4). Frequently it had penetrated the cutaneous sheet of voluntary muscle and, extending beneath it, had formed irregular islands of differentiating epithelial cells like those squamous cell carcinomas often form. Anyone unfamiliar with the ability of aggressive virus papillomas to simulate cancer might have concluded that it was present, and so indeed it may have been in some instances (*e.g.* in the methylcholanthrene mass of rabbit 1-57); for the malignant growths which derive from aggressive virus papillomas may differ scarcely at all from the latter histologically (7). But these dubious instances can be left to one side since cancer seemed present unquestionably in several of the animals. The ulcerating growth which had replaced much of the tarred papillomatous mass of 1-52 and continued to enlarge while the rest of the mass was retrogressing had everywhere the microscopic aspect of a typical, moderately anaplastic squamous cell carcinoma; a similar, invasive tumor was responsible for an ulcer which had formed in the methylcholanthrene mass of 1-55; while in 1-59, killed when moribund on the 95th day, the methylcholanthrene mass contained in four separate regions completely anaplastic epithelial growths (Fig. 6) such as ordinary virus papilloma tissue never forms even when most active (7); and at numerous other spots changes of the same sort had just begun. In 1-54, which died on the 82nd day, and 1-53, killed on the 99th day, similar anaplastic growth had taken place, both from the surface layer of the methylcholanthrene masses and from deep-lying cysts lined for the most part with papillomatous epithelium. The control papillomas and those painted with the turpentine-acetone mixture, though invasive in the regions which tar had reached, were still in all instances mere virus papillomas.

In this experiment the chemical carcinogens caused growths amidst the papillomatous tissue which did not occur in the controls or result from application of a turpentine-acetone mixture; and some had the morphology of cancers (Fig. 6) and behaved like them. Yet the test was unsatisfactory in several ways. No metastases formed before the rabbits died or were killed. The secondary spread of the tar after bandaging was stopped obscured the effects of the other agents, and even its influence could not be certainly appraised since methylcholanthrene had been added to it at one time or another. There were further complications also. Some of the little growths arising on the tarred skin had the microscopic appearance of carcinomas (Fig. 5), though our previous experience goes to show that they were all carcinomatoids, tar papillomas stimulated to mimic cancer (1). Such growths arise on tarred skin as soon as papillomas, and if the tarring is kept up they may form ulcerated mounds or craters as much as 2 cm. across, though eventually they disappear, or round up into cysts lined with squamous epithelium, or become benign papillomas. In the present instance they were tiny, whereas the growths developing amidst the virus papillomas became big and destructive in certain instances. Nevertheless it seemed possible that some might have been carcinomatoids due to the action of the chemical carcinogens upon ordinary epidermal elements included amidst the horde of virus-infected cells and stimulated by local conditions.<sup>2</sup> When the

<sup>2</sup> This possibility could be ruled out for one of the presumptive cancers since its cells showed the stigmata (6-8) indicative of origin from the virus papilloma cells.

papilloma virus is inoculated broadcast into scarified areas inclusion of some uninfected epidermis in the resulting growths generally occurs.

And there was another uncertainty. It may be recalled that various interferences which encourage the proliferation of virus papillomas after these are some months old may be followed by the rapid appearance of carcinomas. In Experiment 1 not only did the tar prodigiously stimulate the papillomas (Figs. 1-4) but they were traumatized by repeated stripping away of the overlying keratin. It was conceivable that the presumptive cancers had thus been brought on.

In the next experiment steps were taken to exclude these complexities. According to Woglom (9) and Hieger (10) the carcinogenicity of tar is lessened but little when it is diluted tenfold. To reduce its non-specific, stimulating effect and its spread it was now diluted seven times.

*Experiment 2.*—Three rectangular areas 5 by 7 cm. across, stretching from the abdomen toward the back with strips of fur between, were shaved on each side of eight agouti rabbits of about 3 kilos. A 3 cm. square in the midst of each area was scarified and inoculated with a 10 per cent saline extract, cleared by centrifuging, of the pooled and glycerolated, naturally occurring papillomas of seven cottontail rabbits. The scarification was done thoroughly with a view to obtaining epidermal infection everywhere on the areas. Healing was complete within 7 days and treatment was then begun and repeated three times a week. With a soft brush the healed areas on one side of each animal were swabbed respectively with (a) a 0.3 per cent solution of methylcholanthrene in Crabtree's solvent, (b) tar diluted with 7 parts of mixture "a," and (c) tar diluted to the same extent with Crabtree's solvent as such. Of the inoculated expanses on the opposite side of the animal one was painted with the solvent only and the remaining two were left untreated, all three serving as controls. The test fluids were applied not only to the inoculated areas but to a zone of skin about a centimeter wide immediately about them, and drying was done afterwards with an electric fan. The situation of the areas receiving the same treatment was varied from animal to animal.

The solution of tar dried rapidly to a thin, tenacious skim, but nevertheless separate bandaging was done. A layer of several thicknesses of gauze was cut to fit into each of the shaved rectangles and was painted with the same fluid as the treated area and dried just before it was put on. Each of these individual dressings was fixed in position, together with the corresponding one across the belly, by means of broad strips of adhesive running around the trunk over the edge of the gauze and the adjacent fur; and when all had been moored an enveloping pad of gauze was placed around the body and a many-tailed binder. Thus each area was walled off from the others. At every treatment the adhesive along one side of each rectangle was cut through, the gauze patch everted, and the test fluid was swabbed on both skin and gauze and allowed to dry. The procedure proved increasingly tedious because as time went on the skin round about the growths had to be cleansed and dried with pledgets of cotton to prevent maceration.

Papillomas were visible by the 14th day at most situations. The first tracings were made then except of the areas painted with the solutions containing tar, where inflammatory swelling and hyperkeratosis obscured the findings. In some animals this keratosis rapidly took the form of a stiff, brown "pile" 2-4 mm. high, like rough dry velvet. In the individuals in which the pile was thickest the virus papillomas did not become clearly visible amidst it until the 21st day and then as small patches scarcely raised above it when at all. By this time the

other areas were nearly or quite covered with higher, confluent growth. The papillomas receiving tar did best in the two animals in which the surrounding skin was least changed by this agent.

The methylcholanthrene solution had at first no perceptible effect on the papillomas or else a slight stimulating one, but by the 4th or 5th week they had mostly become higher than the control masses, in some cases much higher, 6-10 mm. as compared with 3-4 mm. for the controls. The treated skin about them only gradually became hyperkeratotic but in several cases a rough pile eventually formed on it, almost or quite equaling that due to tar. The ether-oil solvent, applied as such, sometimes caused a very slight scurfing at first, and in three animals the early development of the papillomas seemed to be slightly hastened; but no differences from the untreated controls could be made out later.

At the end of the 5th week the methylcholanthrened growths were still considerably higher in most instances than the controls but resembled these otherwise, being orderly, superficial, and vigorous. The growths exposed to the tar solutions, with and without methylcholanthrene, had not entirely covered the areas of inoculation even yet in four animals although in three of the four they had attained the height of the controls, which however occupied the areas of inoculation wholly. In the other four rabbits the tarred papillomas were as high as those painted with methylcholanthrene, that is to say, were definitely stimulated. All of the masses underwent a slow lateral extension, and in several cases the controls did best in this respect for a long while (Charts 2, 6, 7).

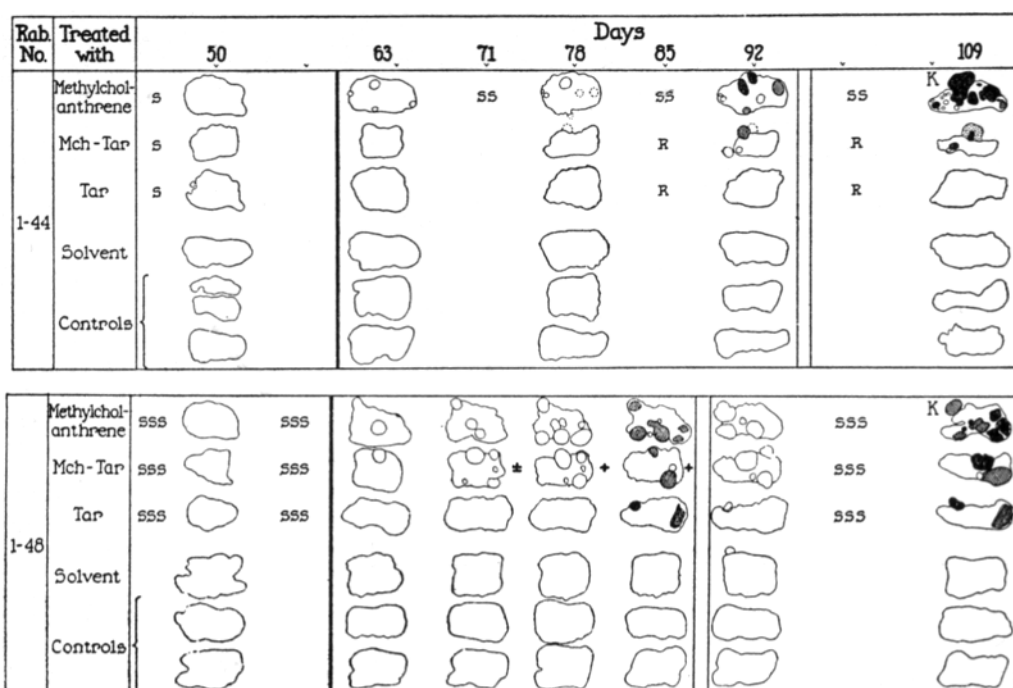
Stripping away of the keratinized papillomatous tissue was started as soon as it had accumulated sufficiently for this to be practicable, that is to say between the 38th and 50th days, and the process was repeated every week or two. As in Experiment 1, the dead layer separated easily after two or three strippings. Maceration of the surface of the masses,—most marked toward the belly,—had begun by the 50th to 58th day and the dressings were left off then and the fur between the growths shaved away. No transfer of tar from mass to mass took place afterwards, but some of the animals gnawed all of them from time to time and maceration became so pronounced in some instances that it was necessary to wash the whole under side of the animal with green soap and water to remove pultaceous matter prior to application of the test substances. Thorough rinsing and drying was done before these were put on.

One animal died on the 67th day of tar toxemia: its growths were orderly, retrogressing, and superficial. The course of events in the other rabbits, from the 50th-63rd day until death, is set forth in Charts 1-7.

The first changes suggestive of malignancy were observed on the 63rd day in two rabbits.<sup>3</sup> Until then all of the growths had seemed ordinary paps., drier and shallower than those of Experiment 1, with occasional pearls beneath them. Now, however, the mch.'d mass of 1-44, which had not been inspected for a week, was found to have a rosette 8 mm. across of fleshy, pink tissue protruding from amidst its jagged, dark gray surface, and three discrete pink mounds at its margin (Chart 1). 1-48 (Chart 2) had a pink, flat-topped disc 1 cm. across amidst the mch.'d mass, projecting 2 mm. above its level, while in the mass receiving mch.-tar there was a disc of approximately the same size and character, with a raw surface because it had been selectively gnawed. Cancers arising in virus paps. are often first brought to attention by such gnawing (2). In both rabbits the masses painted with the carcinogens were somewhat higher than the others and had more pearls beneath them, though they were still superficial.

By the 71st day a raised, raw disc, 16 mm. across, with a central crater, was present in the

<sup>3</sup> The abbreviations mch. and mch.-tar will be used to designate the methylcholanthrene and methylcholanthrene-tar mixtures, as also pap. for papilloma, malig. pap. for malignant papilloma, and squam. carc. for squamous cell carcinoma.



CHARTS 1 AND 2

CHARTS 1-7 (Experiment 2). *The Development of New Tumors amidst Virus Papilloma Tissue.*—The expanses of papilloma tissue are shown in outline and those of each animal are grouped according to treatment received, not in the spatial relationships to one another that actually obtained. The end of each expanse that was nearest the abdomen is toward the left. For the sake of simplicity only a few of the many records are reproduced. The horizontal spacing between them is roughly expressive of time elapsed, and the letters S or R indicate that stimulation or repression of the papillomatous proliferation was observed, S, SS, SSS meaning slight, moderate, and marked stimulation, respectively. The insertion of such letters prior to the first outline of an expanse means that stimulation or repression was observed during the early weeks of growth, and repetition further on means that it was present throughout the period indicated. Where the symbols are missing from the gaps between the outlines, it is to be assumed that the treated growths behaved as did the controls.

The tumors which appeared amidst the expanses are drawn in outline until biopsy or autopsy had disclosed their character, and the outlines are broken where their margins were indistinct. Only those tumors have been depicted which were 5 mm. across or more. A heavy vertical line has been placed just to the right of the tracings made at the time when new tumors were first observed amidst the papillomatous tissue, and two thin vertical lines with a space between have been inserted immediately after the tracings obtained at the time biopsies were done and the treatment with carcinogens stopped. The tumors which the biopsy (or autopsy) specimens showed to be squamous cell carcinomas are blacked in, those which proved to be malignant papillomas are hatched, and those which had the morphology of merely aggressive papillomas are set down in outline as previously. A stippling in the chart for rabbit 1-44 tells where cancer had extended out into the subcutaneous tissue without involving the skin. The keratinized papillomatous pearls which were not infrequently present just outside the edges of the expanses have been omitted from the charts.

The occurrence of tumors due to application of the chemical carcinogens to the skin round about the papilloma masses is indicated by + signs, the number of these signs denoting the number of growths present when the outline tracings were made which just precede them. K = killed.

mass receiving mch.-tar of 1-39 (Chart 3). It had already extended out from this mass toward the axilla, infiltrating and replacing the skin, and a firm, prong-like downgrowth could be felt under it in the subcutaneous tissue. The mch.'d mass showed a growth of similar surface aspect and approximate size, scarcely raised and with a central crater. Neither had been perceived on the 67th day and the regional glands had not been palpated then, but now a firm nodule, 12 mm. across, was found in the neighboring axilla. Except for the new tumors the masses all appeared to be ordinary paps. still; but those getting the carcinogens had become higher than the controls, though only since the last stripping, and they were superficial except for some pearls and the prong already mentioned. The rabbit was in fair condition when killed on the 78th day. The malig. growths, for such they proved to be, had enlarged notably within the week, and the invasive tumor in the mch.-tar mass had invaded the musculature. It had the histological aspect of an active squam. carc. (Fig. 7), and so too had the crateriform tumor in the mch.'d mass. The slicing of this last at the usual 3 mm. intervals disclosed several spots amidst the ordinary gray palisade pap. where the tissue was close-













Rab. No.	Treated with	Days		
		50	71	78
1-39	Methylchol- anthrene		s	 K
	Mch-Tar		s	 + + +
	Tar		s	 + + +
	Solvent			
	Controls			
				

CHART 3

textured, devoid of the pap. striation, and dotted with yellow necroses, as if cancer were present; and sections of the numerous blocks showed, in addition to the tumor noted during life, four separate growths, 6-15 mm. in diameter, which had the morphology of active squam. carc. All were mushrooming out beneath the pap. mass in which they had arisen and two had penetrated to the subcutaneous tissue. The axillary nodule, football-shaped and 20 by 8 by 8 mm. at autopsy, consisted of three lymph glands, two of them small and negative for tumor, while the third had been almost entirely replaced by grayish firm tissue with a cyst in its midst which had a ragged wall and contained pultaceous matter. Microscopically the wall consisted of typical squam. carc., partly anaplastic, partly keratinizing, amidst profuse reactive tissue (Figs. 8, 9). It was impossible to tell from which cancer this metastasis had derived because all had nearly the same morphology. The other pap. masses of the animal were everywhere merely such microscopically.

It will be recalled that several fleshy pink excrescences had appeared by the 63rd day amidst the papillomatous tissue of rabbits 1-44 and 1-48, which had been treated with the carcinogens. Their aspect was the more striking because the masses amidst which they arose had the usual jagged dry surface and slaty hue. By the 78th day new tumors were numerous amidst the growths of 1-48 which were receiving mch. and mch.-tar, but none appeared in the tarred mass until the 85th day when two were noted (Chart 2). The rabbit was now etherized, narrow slices 12-20 mm. in length were removed from the pink components of each mass,

together with the adjacent ordinary pap. tissue, and slices were taken as well through the highest portion of the mass receiving ether-oil, and through one of the untreated controls. Bleeding was stopped by packing the clefts with sterile absorbent cotton for an hour or two and the strippings and applications were permanently discontinued. Healing was swift, the clefts in the pink tissue closing with notable rapidity. Under the microscope all five of the new tumors in the mch.'d mass were found to have the morphology of malig. paps., as also the two in the mass painted with mch.-tar and one of those in that which was tarred, the other appearing to be a squam. carc. There was ordinary papillomatosis only in the slices from the other masses. The animal was killed on the 110th day. Specimens taken then showed that six of the tumors which had seemed to be malig. paps. were now squam. carc. to all appearance. The others had not altered in histology though some had grown swiftly, replacing the ordinary virus pap. tissue.

Rabbit 1-44 was submitted to biopsies on the 92nd day. It had fewer new tumors and none in the tarred mass. Of the four in the mch.'d mass which were biopsied, two had the character of squam. carc. while the other two seemed to be malig. paps. The growth in the mass receiving mch.-tar had the aspect of a cystic squam. carc. New tumors continued to appear until the animal was killed on the 109th day (Chart 1). Plate 12 shows the condition at autopsy of the growths. One of the color prints has been made in reverse, for the readier comparison of the masses situated directly opposite each other across the belly.

The jagged sooty expanses of the upper color print are the control paps. Keratin had built up over them since the last stripping and they were 3-8 mm. high at autopsy, virus growths of ordinary aspect except for keratinized pearls visible here and there, like white pebbles in a dark conglomerate. Such pearls are ordinarily covered with dry matter but the strippings had bared them. An unusually large one, walled as usual with pap. tissue, had formed in the skin next the left edge of the control growth at the left and there was inflammation over it as a local reddening shows. The masses proved superficial on slicing and they had the characteristic palisade striation.

The growths treated with the carcinogens presented a different picture. Those which had been painted with tar and mch.-tar still consisted mostly of sooty tissue with pink patches amidst it, some of them fleshy and bulging. The sooty layer of the tarred mass was now a mere skim, less than 1 mm. thick toward the belly but rising to 3 mm. toward the back. The pink patches amidst it were mostly at the level of the surrounding skin, and the microscope showed them to be covered with a thin papillomatous layer devoid of melanosis. It was residual to the eruption of large pearls,—several of which can still be seen,—the non-pigmented papillomatous tissue on the under side of the pearls persisting afterwards as a surface covering.<sup>4</sup> The only mounds present were due to pearls and nowhere was there any sign of cancer.

The growth that had been swabbed with mch.-tar was the smallest and like the tarred mass in its sooty portions, though slightly higher and with an especially large pearl in the skin next its edge on the right. Near this pearl a fungoid, fleshy mound protruded from amidst the sooty tissue to 5 mm. above its level, while from the left border of the mass a wedge-shaped pink growth, fleshy and firm, had extended out into the skin and 8 mm. down under it. A slice procured from this last growth on the 92nd day had shown it to have the histology of a cystic squam. carc. (2) and it had preserved this character as the autopsy specimens proved. The protruding mound had the aspect of an anaplastic squam. carc. The other pink areas visible in the mass were erupting pearls.

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<sup>4</sup>A similar sequence of events has been observed not infrequently in cottontail rabbits after extrusion of the huge pearls which sometimes form spontaneously under the paps. (7).

More than half of the mass treated with mch. now consisted of ruddy, fungoid, close-textured growths, bulging from amidst the dark pap. layer, which showed exposed white pearls here and there. This layer was only about 5 mm. thick but owing to proliferation under it of the pink growths was raised 2 cm. in some situations, as can be seen where the mass is viewed end on in the figure depicting the controls. The largest pink growth had resulted from the fusion of two, widely separate at first (Chart 1). Amongst the fourteen tumors visible in the gross at autopsy, seven had the microscopic appearance of more or less anaplastic squam. carcs. (Figs. 10, 11, 12), the others having the morphology of malig. paps. or aggressive paps. of ordinary virus type. At numerous situations cancer seemed to be just beginning. There were no metastases. The zones of skin treated with the chemical carcinogens had remained wholly free from growths though there had been inflammation and a marked hyperkeratosis, now gone. Throughout the period of treatment the tar and mch.-tar had exerted a depressant effect on the virus pap. tissue. The mch. solution on the contrary had stimulated it.

The remaining four rabbits developed malig. tumors later on. A large, gristly, pink rosette had appeared in the mch.'d mass of 1-45 by the 85th day (Chart 4), and already was replacing the adjacent skin. It soon ulcerated, and biopsy on the 92nd day disclosed what seemed to be carcinomatosis of squamous type, ranging from the cystic to the anaplastic. When the animal was killed, on the 113th day, two other pink growths had arisen, in the masses receiving tar and mch.-tar respectively. They too had the morphology of squam. carcs.

The paps. of 1-42 (Chart 5) grew less vigorously than those of most of the group, though mch. and mch.-tar had a pronounced stimulating effect on them, as evidenced by the height they attained between strippings. On the 80th day a pink mound was noted in the mch.-tar mass, and during the next 5 days it became a raised rosette 12 mm. across, covered with foul exudate and with a crateriform center. Thereafter it enlarged more slowly and all of the masses,—which were shallow,—began to dwindle, the controls in special. On the 102nd day about half of the mass treated with mch. was noted to be firmer and fleshier than the rest and to have extended down irregularly. Slices were taken on the 106th day through the new tumors and across all of the other masses, and the animal was found dead next day. The microscope showed the rosette in the mch.-tar mass to have the morphology of a squam. carc., while the changed portion of the mch. mass seemed made up of malig. pap. tissue, sharply different from the ordinary retrogressing pap. next it. Elsewhere there was only this latter. No metastases were found.

The growths of 1-47 (Chart 6) became the most vigorous in any animal, and by the 80th day they were fleshy, discoid "raw hamburgers," flecked with gray instead of almost black as in the early weeks. The tarred mass, still somewhat smaller than the others and less active, had now at one end a bulging protuberance consisting of a tissue denser and pinker than that of the rest of the mass and devoid of the gray flecks there present. The protuberance had become much larger by the 101st day when an area of similar tissue with a firm prong extending downwards from it was noted in the midst of the mass getting mch.-tar. By the 106th day two other pink growths had arisen in the latter mass and the tumor already present in the tarred one had extended a centimeter down. The mass receiving mch. had become nearly twice as high as the others, 13 mm. as compared with 7-8 mm., yet everywhere it still had the appearance of ordinary pap., and biopsy showed its height to be largely due to underlying reactive tissue. Slices were now taken through each of the pink growths and through the highest portions of all of the control masses (two or three slices from each mass, the routine procedure). The new tumors as thus exposed appeared in the gross to consist of malig. tissue, but the microscope showed that only the growth which had arisen first in the mch.-tar mass had the aspect of a squam. carc. All of the others, including the one which had extended deep below the tarred mass, seemed to be composed merely of exceptionally aggressive pap. tissue, such as might have been formed by the proliferation of unusually active "families" of pap.

Rab. No.	Treated with	Days				
		50	63	85	92	113
1-45	Mch		SS	SS		SS K
	Mch-Tar R	R		R		SS
	Tar R	R		R		SS
	Solvent S	S	SS			
	Controls					

5 cm.

CHART 4

Rab. No.	Treated with	Days				
		63	80	102	106	120
1-42	Methylcholanthrene SSS		SSS	SSS		
	Mch-Tar			SSS		
	Tar			SSS		
	Solvent SSS		SSS			
	Controls					

1-47	Mch	SS		SS		SS K
	Mch-Tar R					S
	Tar R					S
	Solvent SS					
	Controls					

5 cm.

CHARTS 5 AND 6

Rab. No.	Treated with	Days				
		50	63	80	113	122
1-49	Mch S					
	Mch-Tar R	R				
	Tar R	R				
	Solvent					
	Controls					

5 cm.

CHART 7

cells (6, 7). Healing was swift, the pap. tissue continued to proliferate, and the new tumors enlarged and protruded; yet on the 113th day the mass which had received mch. still showed no significant changes. But two pink tumors appeared on its surface before the rabbit was killed on the 120th day and one of these was already large and had begun to extend out under the skin. During the 2 weeks since the treatments had been discontinued all of the masses had come to be similar save where the new growths were present; and all had the same height, and scattered pearls had formed under and along the edges of all. The specimens procured at autopsy showed the tumor which had appeared first in the mch.-tar mass, and become the largest later, to be a keratinizing squam. carc. microscopically, just as at biopsy. All of the other new pink growths had the morphology of greatly stimulated ordinary paps., even where they had extended deep.

The last of the group to develop new tumors was 1-49 (Chart 7). It had less active paps. than any of the others, but by the 80th day they had formed macerating, discoid expanses, fleshy, superficial, and all apparently alike. The control expanses were the largest, owing to lateral extension, despite the fact that the mch. solution had stimulated the virus-infected cells in the beginning, as evidenced by the rapidity with which they built up. The solutions containing tar had been depressant at this time. Shortly after the 80th day all of the masses began to get smaller and by the 122nd day, when the animal was killed, they were very shallow. A pink mound had lately appeared in the one receiving mch., but on section it was found to be made up merely of highly aggressive papillomatous tissue of virus type. Elsewhere in the mass however the slicing disclosed a small growth with the histology of an anaplastic squam. carc.

The neoplastic responses of the uninoculated skin areas treated with the chemical carcinogens will be considered further on. Tumors appeared in some instances, all tiny and of the benign sorts usually called forth first by the chemical agents. Most of them were paps. and frill horns, with an occasional, indolent carcinomatoid.

Dilution of the tar had the desired results in this experiment. It did not undergo transfer, caused little stimulation, when any, of the virus papillomas, and seldom elicited carcinomatoids from the skin.

One rabbit died with retrogressing papillomas on the 67th day, but in the other seven they did well, and invasive and destructive growths with the morphology of carcinomas (Figs. 7-12) arose in every instance amidst some of them. The new tumors appeared only in those receiving the chemical carcinogens, and were multiple in six of the animals (Charts 1-7). They arose within a very short time. Several had appeared by the 63rd day after virus inoculation, less than 50 days after papillomas had first become visible and on the 56th day after treatment with the carcinogens was begun. Some were more than a centimeter across when first noted. By the 71st day metastasis had taken place in one instance while in others the tumors had ulcerated and were rapidly replacing the papillomatous tissue, with extension beneath it and into and under the adjacent skin.

#### *Were the New Tumors Cancers?*

Active virus-induced papillomas not infrequently send down tongues into

the connective tissue, which round out and form keratinizing cysts lined with papillomatous epithelium. Such tongues may penetrate into the blood and lymph vessels and, in cottontail rabbits, extend along these latter to the nearest gland with pearl formation there (7, 11, 12). Both in this species and domestic rabbits the invasive cells may replace the voluntary muscle fibers of the skin layer individually on occasion, just as active squamous cell carcinomas often do, though with a rounding up eventually into orderly pearls. Stimulation of the papillomatous tissue by the injection of Scharlach R in olive oil may cause it to proliferate wildly and take on a carcinomatous aspect temporarily (2, 7); but ordinary epidermis will respond in this way (13), though to a less extent. In view of these facts the first question as concerns the present experiments is whether the growths which appeared to be carcinomas were truly such. The findings leave no doubt of this. Many of them became completely anaplastic (Figs. 6, 10, 11), as never happens with stimulated papillomas (7). They did not revert to the papillomatous state when the applications were stopped, as happens after mere stimulation, but continued malignant. One (Fig. 7) metastasized and the metastasis had the character of a squamous cell carcinoma (Figs. 8, 9). Besides the growths which appeared to be of this sort there were others of all the various kinds which arise when virus papillomas become malignant spontaneously (2, 7). Some of the tumors appearing under the latter circumstances differ so little from virus papillomas histologically that it may be impossible to tell,—except when they metastasize or are widely destructive,—whether they are more than mere stimulated papillomas; while others retain the structure of the latter though exhibiting the cytological features indicative of cancerous change (7). All this held true of the growths of the present experiment.

#### *Derivation of the Cancers*

The carcinogenic agents had opportunity to act not only on papilloma cells but on uninfected epidermal elements included amongst these, as already pointed out. It follows that the cancers might conceivably have taken origin from cells uninfected with the virus.

Microscopic study of the new tumors has told much in this relation. Many retained the papillomatous structure and exhibited the cytological stigmata (6-8) indicative of the persisting influence of the virus; and growths expressive of all stages in the morphological transition between ordinary papillomatosis and anaplastic carcinomatosis were encountered, just as when virus papillomas undergo spontaneous malignant change. To this evidence should be added that obtained by applying the carcinogens to the skin round about the papillomas. Here the opportunities to produce a carcinomatous change in epidermal cells uninfected with virus were far greater than where only individual elements

of the sort, or small islands, had been included amidst the papillomatous tissue. Yet no cancers were induced, and carcinomatoids were so exceptional as to make it unlikely that the tumors arising amidst this tissue were of such sort. Indeed the carcinogens produced no growths or very few on the skin of certain animals, while causing many to appear amidst the virus papillomas (Charts 1, 2; Plate 12).

#### *The Results of Brief Exposure to the Carcinogens*

Many unavailing efforts have been made in our laboratory to induce variation of the papilloma virus with result in unusual growths. The effect of the chemical carcinogens in the present work has led us to employ them in further attempts to procure variants. Methylcholanthrene was applied to virus papillomas, and after about a month some were removed and dilute saline extracts of them were inoculated into expanses of skin rendered highly susceptible to infection by previous swabbings with a turpentine-acetone mixture (3). This plating out yielded ordinary papillomas only, and the later course of the treated growths left *in situ* showed that it had been done too soon, these failing for months to become cancerous. The findings will be detailed here since they show that methylcholanthrene has little effect on virus papillomas unless applied directly to the living cells. The negative results of injecting methylcholanthrene under the growths (14) can be readily understood in the light of this finding.

*Experiment 3.*—Saline extracts of the glycerolated paps. of W.R. 1-96 and W.R. 1-52 were tattooed into 12 spots, 2 mm. across, on the right and left sides respectively of twelve large agouti rabbits, and the situation of the spots was marked with a dab of carmine near each one. 7 days later the treatments were begun, although no paps. had appeared. A 0.3 per cent solution of mch. in Crabtree's fluid was dropped from a pipette on 9 of the 12 spots on each side of the animals, whence it spread to the surrounding skin. The treatments were repeated thrice a week for 134 days.

The strains of virus employed were "recoverable," producing growths in domestic rabbits from which active virus could be got again by extraction,—as is far from always the case in these alien hosts. Recoverable strains take long to act and the paps. they produce grow but slowly (15). None appeared where the mch. had been applied until the 17th to 20th days after the inoculation, and only after several further days at the control sites,—which had been varied from rabbit to rabbit. In some of the latter the control growths were still small mounds at the 30th day after the inoculation, recently erupted and only slightly melanotic at most, whereas those receiving mch. were large, discoid or conical, dry-topped, and sooty gray (Fig. 13), owing to stimulation of the included melanoblasts (16). The shaved skin to which the carcinogen had spread was also gray though less so, and it had become scurfy and hairy, unlike that elsewhere. In every case the treated growths soon became covered with a layer of dense keratin several millimeters thick through which the mch. could scarcely be supposed to penetrate; and that it did not was evident from the fact that they no longer grew

faster than the controls, which by the 66th day had become as large as they or larger and wholly similar in the gross.

The test for variant virus entities was made between the 27th and 35th days. Some of the most rapidly growing of the treated papillomas were dissected away from the connective tissue with a sharp knife, and the living layer was washed free from blood, ground with sand, and extracted with saline. Each extract was inoculated into a patch of scarified skin on three rabbits, previously prepared by several paintings with the usual turpentine and acetone mixture. There resulted only discrete, ordinary paps. Microscopic examination of slices of the growths subjected to test showed them to have the ordinary morphology.

Between the 51st and 134th days eight of the methylcholanthrened rabbits were discarded because general retrogression of the paps. had begun despite the applications. These were kept up until the 134th day in the case of the others. Some of the paps. taken for test had recurred long before then, by growth from tiny fragments left behind; and stimulated by renewed direct exposure to the mch. these recurrences in most instances soon reached the size of the other treated growths. Outline tracings of all were made from time to time.

The earliest cancer was noted on the 118th day in rabbit 1-85, as a sanguineous, fleshy disc in the midst of one of three mch.'d paps. recurrent from the excisions of the 34th day. By the 134th day, when mch. was discontinued, a firm prong had extended down from the new tumor into the subcutaneous tissue, and the surface growth had greatly enlarged. It was an ulcerated mass 6.5 by 12 cm. across when the animal died on the 160th day, and it had involved and destroyed several of the neighboring treated paps. Sections showed moderately anaplastic squam. carc. Metastases were absent. The other treated growths were 2.5—4.5 cm. across and none had become malignant, nor had any of the control paps.

In rabbit 1-80 the control growths were definitely the larger and more fleshy when the mch. was stopped, averaging 18 by 30 mm. in diameter as compared with 13 by 20 mm. Recurrence had taken place at 4 of the 6 sites from which treated growths had been excised. At death on the 182nd day one of the six control paps. and one of the sixteen exposed to mch. had been mostly replaced by discoid, invasive, ulcerated new tumors. There were no gross signs of malignancy in the other growths, yet nearly all, both treated and control, showed small areas of squamous cell carcinomatosis microscopically. The ulcerated tumors were of this sort, and a metastasis was present in one of the axillary glands.

None of the growths of rabbit 1-88 was excised and all were of about the same size when the treatments were stopped. They remained without sign of malignancy until the animal was killed on the 295th day. By then the control growths were much the more fleshy and somewhat the larger, from 4-6 cm. in greatest diameter as compared with 3-5 cm. Under the microscope sagittal sections disclosed squam. carc. in five of the eighteen growths that had been treated with methylcholanthrene whereas all of the six controls were still ordinary paps.

The findings in rabbit 1-84 are summed up in Chart 8. In this animal four of the control growths did better eventually than the treated ones but the fifth remained tiny and the sixth never appeared. By the time mch. was stopped ulcerating cancer had appeared in one of the nine treated growths on the animal's left side and by the 232nd day it had almost wholly replaced another. Two more of the treated paps. showed it on the 346th day, as did the sole control growth that had grown. The animal was killed then. The cancers longest present had become huge destructive growths (Fig. 13) with extensions far out in the subcutaneous tissue.

Three of the treated paps. on the right side of rabbit 1-84 had been excised on the 34th day without recurrence. Of the six that had been left, two had been partially replaced by ulcerating cancers by the 262nd day, a third by the 295th, while a fourth and fifth were found to

Rab. 1-84

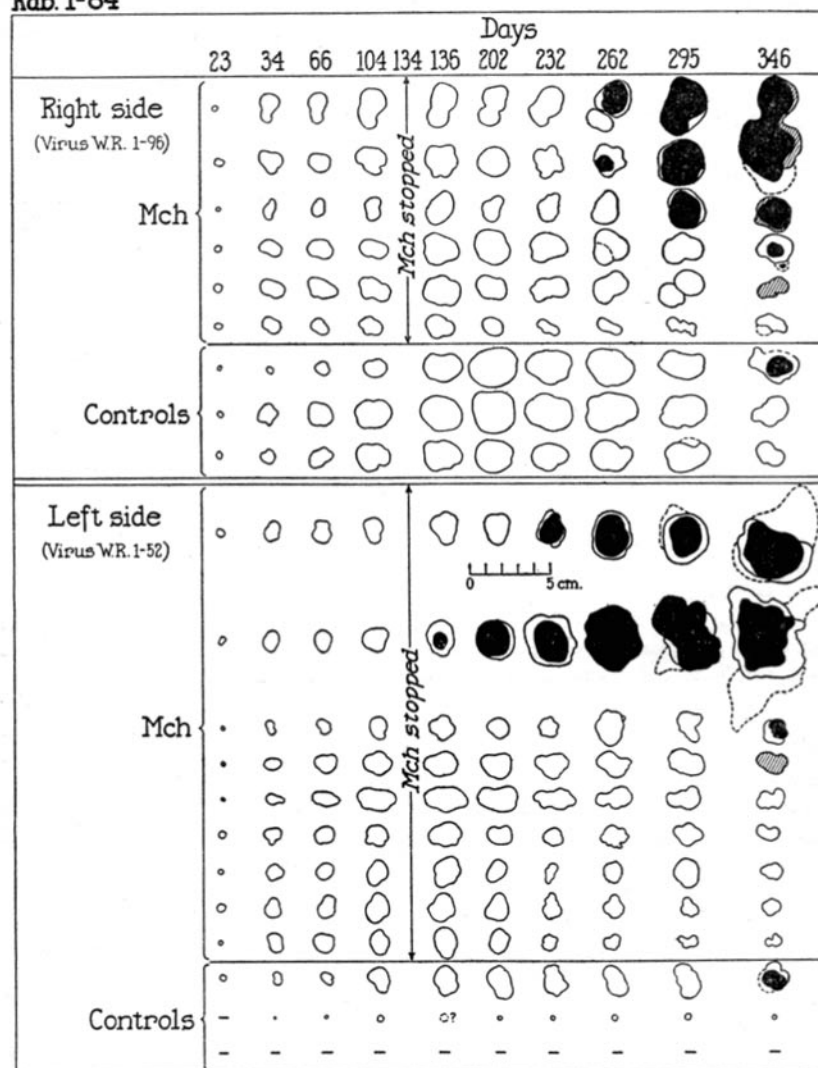


CHART 8. *Effects on Virus Papillomas of Relatively Brief Exposure to Methylcholanthrene (Rabbit 1-84, Experiment 3).*—Of the twelve papillomas on each side of the animal nine were painted with methylcholanthrene, and on the 34th day three of those on the right were excised for test. They did not recur. The control growths on this side did rather better than those treated with the carcinogen, whereas one of the controls on the left,—where a different inoculum had been used,—fared badly and at another site none appeared.

The growths are grouped in the chart with reference to treatment and cancer incidence, not in their spatial relations. Frank, ulcerating cancers are recorded in black, non-ulcerating malignant papillomas (as disclosed with the microscope) by hatching, and papillomas which showed no gross signs of cancer are drawn in outline. Subcutaneous extension is indicated by a broken line. The two largest cancers on the right side had fused before the animal died and from one of them such extension had taken place. The largest malignant tumors on the left side, two huge destructive growths, had penetrated far into the subcutaneous tissue (*vide* Fig. 14).

contain much malig. tissue at death, as was true also of one of the three controls. There were metastases in the axillary lymph nodes on both sides. The findings were all confirmed with the microscope.

This experiment had not been planned for the quantitative appraisal of the effects of methylcholanthrene and the controls were too few for this purpose, yet the results support those of the previous experiments. Cancer developed in one animal by the 118th day, in a papilloma recurring after excision and hence with cells directly exposed to methylcholanthrene for an exceptionally long period. In a second rabbit five of eighteen treated papillomas were carcinomatous by the 295th day but none of the six controls, although latterly they had grown the faster. In a third individual cancer developed in some of the treated growths months before it arose in any control (Chart 8). In a fourth animal the chemical carcinogen had no evident effect to hasten malignancy.

One of the two largest carcinomas arising in the rabbit of Chart 8 (left side, top growth), and the next to the earliest to appear, closely resembled a stimulated virus papilloma microscopically (Fig. 14), its cells differentiating in the same peculiar way (Fig. 15); and it was identified as malignant only by its destructive course and by certain cytological abnormalities associated with the cancerous state (2, 7). It ulcerated soon and extended into the subcutaneous tissue, eventually becoming huge. None of the other treated papillomas behaved so. The instance points a difficulty already brought up in connection with Experiment 2, of determining which growths are truly cancerous, and it raises the larger question of the etiology of the carcinomas arising from virus papillomas.

#### *Exposure of the Virus in Vitro to Methylcholanthrene*

It seems unlikely from what is known of cell permeability that the effect of methylcholanthrene to bring on cancer in virus papillomas can be due to a direct action of the carcinogen on the virus. Nevertheless two tests of this possibility were carried out.

In the first test an extract in salt solution of glycerinated cottontail papillomas, containing highly pathogenic virus, was passed through a Berkefeld filter and mixed with pooled serum from several normal domestic rabbits, and finely ground methylcholanthrene crystals were added in some instances. All of the sera exhibited a brilliant purple fluorescence under ultraviolet light after the crystalline material had settled out, most of it remaining undissolved. One batch of pooled sera came from rabbits which had been given a mixture of heavy cream and ground peanuts by stomach tube 24 hours previously, with result in lipemia, since it was thought that in this way the exposure to methylcholanthrene might be rendered more considerable, owing to solution of it in the finely divided fat. Duplicate mixtures of the sera and the virus-containing extract, with and without the carcinogen, were sealed in test

tubes and placed at 4° and 37°C., respectively, with occasional agitation, for 26 days when they were inoculated into the scarified skin of four rabbits, each animal having 12 shaved patches, previously prepared by three paintings with 0.3 per cent methylcholanthrene in benzene (3), for test of the 12 specimens. All the mixtures had remained free from bacterial infection. Those kept in the cold gave rise to many growths, whereas the incubated specimens caused but a few and these slowly. All of the growths were ordinary papillomas and the methylcholanthrene present in the serum had had no share in reducing the pathogenicity of the mixtures, as shown by a comparison with the yield from incubated controls. The incubated fluids proved slightly alkaline, pH 8-8.4, and the papilloma virus is known to deteriorate under such circumstances (17).

In the second test a different filtered virus extract and pool of sera were utilized and hydrochloric acid was added to the serum-virus mixture to render the pH optimal for the virus. Two portions of the mixture were thus brought to pH 6.0 and 5.5 respectively, as determined with the potentiometer,<sup>5</sup> and to some of the material thus altered the phosphate-phthalate buffer of Best and Samuel (18) was added as a stabilizer. Duplicate specimens of the mixtures,—of which there were 10, with and without methylcholanthrene crystals,—were placed in the cold and at 37°C. Potentiometer readings on the 93rd day showed that the pH of the specimens had remained nearly unchanged. Each was then inoculated into three rabbits having patches of skin prepared by four applications of turpentine-acetone mixture. Two groups of three animals were required. The materials kept in the cold at pH 6.0 gave rise slowly to fairly numerous scattered papillomas, whereas the corresponding specimens incubated 93 days produced few growths or none. The yield of papillomas was larger with the pH at 5.5 but there were similar differences. None of the growths had peculiar features and the results with the incubated controls made plain that the methylcholanthrene had had no part in reducing the pathogenicity.

Variation of viruses is most likely to come about when these are functioning, and this was not the case in the tests just described. In extensive experiments previously unreported we have submitted rapidly enlarging virus papillomas on the skin of cottontail rabbits to the Roentgen rays, according to the method of Friedewald and Anderson (19). The raying ranged from the few thousand r which will produce cell injury, to the far larger amount required to effect partial inactivation of the virus (19). Immediately or within the next few days the growths were removed, extracted, and various dilutions of the extracts were plated out on broad scarified expanses of the prepared skin of groups of domestic rabbits. Apart from a reduction in pathogenicity when the raying had been copious, as compared with that of extracts of unrayed control growths from the same animals, no alterations in the virus could be discerned.

<sup>5</sup> All of the potentiometer readings were made by Dr. Theodore Shedlovsky to whom our warm thanks are due.

## DISCUSSION

There would seem to be no doubt that the chemical carcinogens brought on cancerous changes in the virus papillomas, and that the cancers took origin from the virus-infected cells. Numerous questions arise in connection with these findings.

*Did the Carcinogens Act Merely by Stimulating Proliferation of the Growths?*—Papillomas produced by the same inoculum do poorly in some animals but well in others and those which grow most vigorously are most likely to become malignant spontaneously (2). Various procedures which stimulate proliferation hasten the appearance of cancer, providing they are brought to bear when the papillomas have already arrived at the precancerous state (2, 7). Hence the possibility must be considered that tar and methylcholanthrene may have acted in some such way.

For both theoretical and practical reasons we have tried during several years to obtain cancers quickly by stimulating virus papillomas from the time of first appearance. They were repeatedly incised at the same spot, or pieces of them were removed again and again from the same region and implanted in the muscles, or Scharlach R in olive oil was injected at frequent intervals into or under them. All was to no purpose. However active the induced proliferation,—and that resulting from Scharlach R was prodigious (12, 20),—more than 4 months always went by before cancer appeared. Against this background the findings of Experiment 2 stand out sharply. The growths receiving the carcinogens were only moderately vigorous, that is to say would not ordinarily have undergone spontaneous malignant change for many months, and the controls manifested none of the alterations indicative of arrival at the precancerous state (2). The methylcholanthrene solution in most instances caused slight to pronounced stimulation at one time or another, as the charts show, but this had no evident connection with the occurrence of cancer. In the animal of Chart 3 which had a metastasis by the 71st day and multiple carcinomas in the methylcholanthrened growth by the 78th day, the papilloma tissue had been only slightly and transiently stimulated by the applications. In the rabbits of Charts 4 and 5 the stimulation of some growths was marked and prolonged, yet few malignant tumors arose and these relatively late. The tar had a repressive effect on the papillomas in several instances<sup>6</sup>

<sup>6</sup> The tar used in Experiment 2 was the dregs of the barrel and it was applied in solution. No repressive effect like that obtaining in this test had been noted in previous work in which tar of the same lot was applied to virus papillomas situated on rabbit ears (5, 21), but on the contrary wild proliferation (like that of Experiment 1) with extension to the other side of the ears. The animals were killed early: cancer had not appeared.

whereas in other cases it was slightly to markedly stimulating; and as the charts show, cancer arose independently of these effects. In rabbit 1-44 (Chart 1) the mixture of tar and methylcholanthrene was distinctly repressive yet cancers occurred.

On surveying the results of Experiment 2, one perceives that methylcholanthrene was far more effective than tar in bringing on malignancy, a fact which accords with what is known of their carcinogenic powers. When they were applied together the papillomas underwent precisely the same repression or stimulation as if tar had been put on alone, this substance wholly determining the changes in such respect. Yet the outcome of the two treatments in terms of malignancy was very different, cancers arising much earlier and oftener when the agents were combined (Charts 1-7). The difference can only have been due to the methylcholanthrene component of the mixture. The fact that methylcholanthrene was less effective in carcinogenesis when mixed with tar than when alone can be accounted for by inclusion of much of it in the dried, tarry skim.

In sum the findings rule out the possibility that the cancerous changes were brought on by ordinary stimulation of the papilloma cells.

*Did the Chemical Agents Initiate Neoplastic Changes of a Different Sort from Those Resulting from Virus Infection?*—When the papilloma virus is injected into the circulation of domestic rabbits carrying tar papillomas and carcinoma-toids,—growths of the sorts which arose on the skin in the course of the present work,—it frequently localizes in them, with result that some are converted into virus papillomas, others retain their morphology but proliferate with unprecedented rapidity, others still become mongrel growths in which the traits of both tumors are blended, while not a few turn into frank squamous cell carcinomas, often highly malignant (5, 21). These changes are so numerous and immediate that one can scarcely suppose the virus, a notably stable agent, to have undergone variation. The most reasonable explanation is that the tar tumor cells, after infection with the virus, are under the compulsion of two distinct neoplastic influences which find differing expression under ordinary circumstances but which when working together give rise to growths that neither can produce separately, growths varying in character according as the influence of the virus dominates or that of the unknown cause of the tar tumors.<sup>7</sup> The same state of affairs might conceivably obtain if the virus influenced the

<sup>7</sup> Rabbits with tar papillomas which have undergone morphological alteration as result of infection with the virus develop also papillomas of the sort ordinarily caused by this agent, and in some instances these latter have been observed to undergo a general retrogression after a while as result of a suddenly developing resistance on the part of the host. Many of the altered tar papillomas regained their pristine character concurrently (21), as if the virus were no longer influencing them.

epidermal cells first and the chemical carcinogen did so later, as under the conditions of the present work. On this assumption the cells of the papilloma, though rendered tumor cells by the virus, would have retained the ability to respond to the chemical carcinogens like ordinary epidermal elements and consequently in some instances would have undergone neoplastic changes distinct from the papillomatous alteration already existing as result of virus infection, yet additional thereto.

The possibility outlined had been foreseen and our tests were planned to disclose individual differences in the liability of the epidermis to undergo neoplastic change in response to the chemical carcinogens. This liability is known to vary widely from animal to animal, and observation has shown that the neoplastic response of the epidermis at one situation furnishes an index to what may be expected at other, related areas on the same individual (5, 22). Hence expanses of skin directly opposite those occupied by the virus papillomas or immediately about them were given the same treatments they received. Secondary spread of the tar obscured the results in Experiment 1, but in Experiment 2 the findings were explicit. The carcinogens failed to call forth growths anywhere on the treated epidermis of two rabbits (Charts 1, 5); a few appeared on that of a third for a brief while, where the mixture of tar and methylcholanthrene was put but not elsewhere (Chart 2); while in a fourth many tumors developed on all of the treated expanses (Chart 7). In two of the other three animals both the solutions containing tar brought out growths whereas methylcholanthrene did not (Charts 3, 6), as would follow from the fact that it has much less power than tar to promote the formation of growths by the cells it renders neoplastic (23). The remaining animal (Chart 4) provided an exception to the rule. Its skin proved exceedingly susceptible to methylcholanthrene, which caused a profuse hyperkeratosis and amidst it many small growths, whereas the mixture with tar produced few and none resulted from tar applied alone. The ether-oil solvent as such never gave rise to tumors.

There was no correlation between these skin responses to the carcinogens and the incidence of malignancy in the treated virus papillomas. The agents failed to elicit any growths on the epidermis of one of the animals which had most cancers (Chart 1) and gave rise to only a very few on that of the other (Chart 2). They induced many growths on the skin of the individual which developed cancer last of all and at one spot only (Chart 7), as also on that of another in which malignancy developed very late (Chart 4) and at few situations.

It would appear from all this that the cancers deriving from the virus papilloma cells as result of exposure to the chemical carcinogens cannot have been due to neoplastic changes of the sorts these agents induce in uninfected epidermis,—changes distinct from that which the virus brings about,—unless indeed it be assumed that the latter so disordered the cells that their response

to the chemical agents was no longer like that of ordinary epidermal elements but wholly irregular, the very cells which would ordinarily have proven refractory to the influence of the chemical carcinogens now being in some cases the most responsive and *vice versa*.<sup>8</sup>

*Did the Virus and the Chemical Carcinogens Act upon the Same Cell Mechanism in Producing the Cancers?*—The papilloma virus, like other neoplastic viruses, is the actuating cause of the growths it induces whereas the chemical carcinogens merely provoke neoplastic changes, these being motivated in some way as yet undetermined (25). The difference, while basic, does not exclude the possibility that the rabbit virus, in addition to functioning as the cause for the papillomatous state of the cells infected with it, may also act upon them as a provocative carcinogen. The cancers which arise spontaneously from such elements can readily be accounted for on this assumption, and in extension of it one might suppose that those brought on by tar or methylcholanthrene are consequent upon the summated influence of two provocative carcinogens, the virus and the hydrocarbon, acting upon a single cell mechanism. Additive or summative effects have been frequently reported for the provocative carcinogens, those of widely differing type sometimes supplementing each other (26).

If the virus and the chemical agents played upon the same mechanism one would expect that the rabbits with skins most responsive to the latter, as manifested by neoplasms called forth, would also have most cancers where their influence was added to that of the virus in the papillomas: but this was not the case, as has already been brought out. Here though a difference must be recognized in the character of events at the two situations, the skin growths being the outcome of changes from the normal state to the neoplastic, whereas the carcinomas arising from the papillomas are consequent on alterations taking place secondarily in cells already in a neoplastic state as result of the influence of the virus. Obviously the initiation of this state is not the same thing as a shift in its character, and hence one must ask whether both are due to the same influence on the part of the carcinogen. The question is not peculiar to the phenomena now under consideration. It comes up whenever a provocative carcinogen—tar, methylcholanthrene, ultraviolet light, or another—acts on the epidermis; for here these agents usually elicit benign papillomas some while before cancers arise, and from the benign growths many of the malignant tumors

<sup>8</sup> In a preliminary report on the present work (24) it was pointed out that the tumors arising from the treated virus papillomas were less diverse in type than those resulting from the infection of tar tumor cells with papilloma virus (5, 21). But the virus was already in the saddle when the chemical agents were applied and the cells infected with it might then have been incapable of undergoing the morphological changes expressive of the neoplastic states induced by these agents.

derive secondarily, just as they do from virus papillomas. Yet the question is raised infrequently, because of the general experience that those agents which are capable of eliciting benign papillomas will also bring on the secondary malignant change and furthermore produce cancers which are primary, at least to all appearance.

*Did the Chemical Carcinogens Act upon the Virus?*—There remains the possibility that the tar and methylcholanthrene may have influenced the papilloma virus itself, either directly or by altering its cell environment with eventual result in cancer-producing variants of it. The virus is the most powerful of known carcinogens for rabbit skin, and it was obviously the main factor in the production of the cancers of the present experiments, the chemical agents inducing none where it had not infected the epidermis. Furthermore many facts point to virus variation as the reason for the carcinomas which arise spontaneously from the papillomas (8). One of these facts has found illustration in the present work, namely, the occurrence of malignant growths which retain the cytological peculiarities associated with the virus' action (1, 8) (Figs. 14, 15).

The recent discovery that viruses of some sorts lie latent for long periods, causing disease only on special occasion, coupled with the realization that some tumors have viruses as their cause, has led to a supposition already mentioned that agents of this sort may reside in animal tissues, perhaps throughout the lifetime of the organism, doing no harm unless the cells with which they are associated undergo special pathological changes, when they undergo variation as result of the new, abnormal milieu and render the cells neoplastic. According to this supposition, tar and methylcholanthrene are carcinogens because they alter the environment of viruses; and in certain tissues, notably the epidermis, they produce conditions which ordinarily result first in a slight virus variation, such that benign growths are produced, with sometimes another change later on, superimposed on the first or else occurring "at one jump" and in either case finding expression in cancer. Evidence has recently accumulated to indicate that the milk factor responsible for the liability to mammary cancer of certain strains of mice is a virus (27); and many if not all of the cancers due to the factor are now known to be the outcome of successive changes in the gland cells, benign adenomas forming first in the breast tissue of the animals concerned, with cancers deriving from them subsequently (28). The application of methylcholanthrene to the skin of mice of a strain known to carry the milk factor (Little's dba mice) much hastens the occurrence and markedly increases the frequency of the mammary cancers (29). The likeness to the findings when virus papillomas are exposed to the action of chemical carcinogens is close and may prove significant. For these growths are also benign tumors capable of becoming cancerous spontaneously after a time, and the change is much furthered by the influence of chemical carcinogens, as our experiments show.

## SUMMARY

The application of methylcholanthrene and tar to virus-induced papillomas of the domestic rabbit caused them to become carcinomatous with great rapidity, and the malignant changes were frequently multiple. In bringing on the cancers the chemical agents acted in their specific capacity as carcinogens, not as ordinary stimulants of cell proliferation. The cancers derived from the virus-infected cells and were of the same types as arise from these elements spontaneously after a much longer time.

The evidence would seem to indicate that the chemical carcinogens acted by way of the virus.

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## EXPLANATION OF PLATES

All of the sections were stained with eosin and methylene blue.  
The photographs were made by Mr. Joseph B. Haulenbeek.

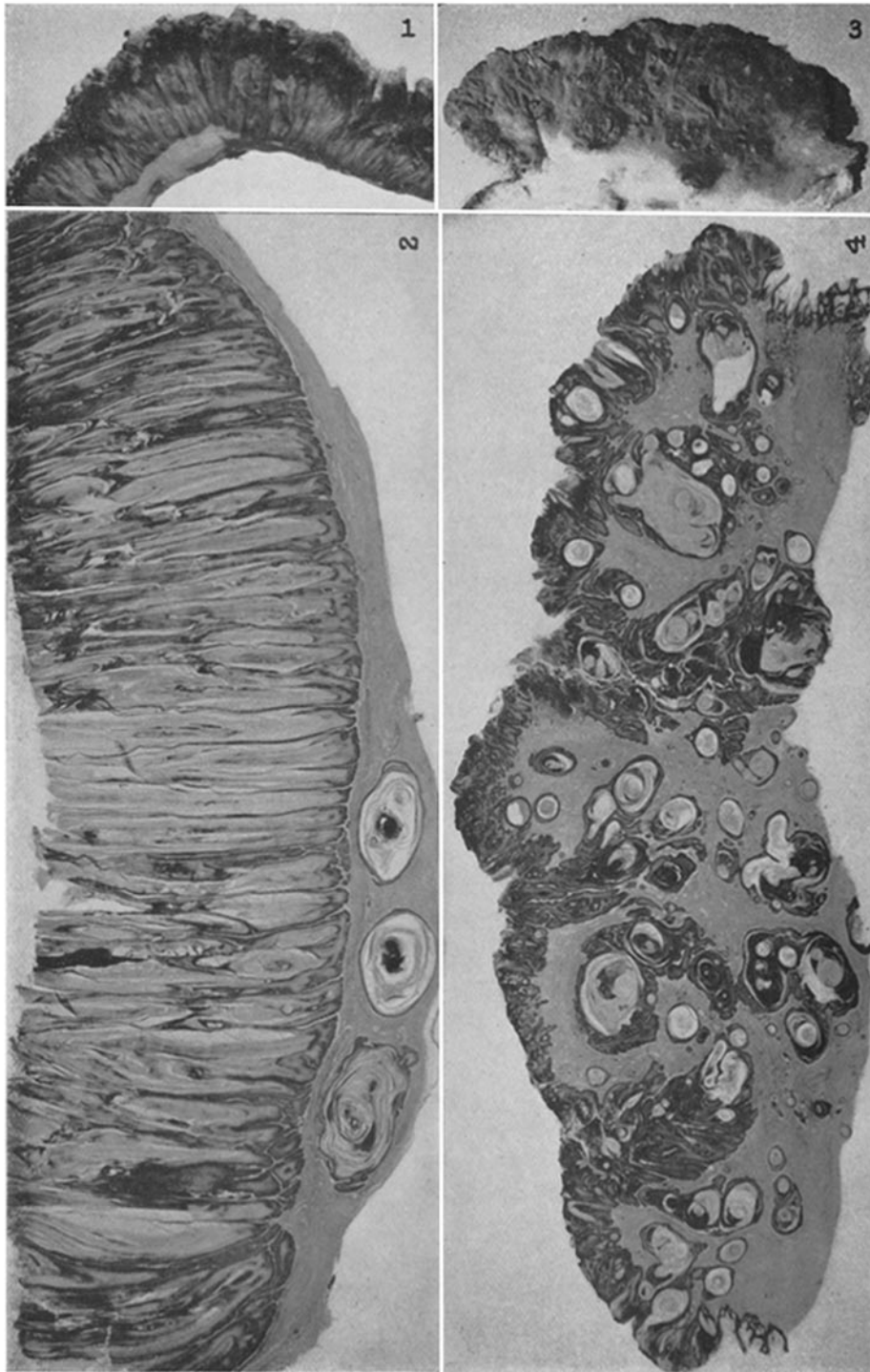
## PLATE 9

FIG. 1. Ordinary virus papillomatosis (95th day),—for comparison with Fig. 3. The growth came from a rabbit utilized for a virus titration. The palisade striation and sharply demarcated base are characteristic. The superficial layer is darker than the rest because compacted by drying.  $\times 1\frac{1}{2}$ .

FIG. 2. Ordinary virus papillomatosis produced with virus of the strain used in Experiment 1 (99th day, titration rabbit). Much keratinized material has been cut away for ease in sectioning. A few cysts lined with papillomatous epithelium and containing keratinized pearls lie beneath the growth.  $\times 4.3$ .

FIG. 3. The effect of transferred tar to cause disorganization and downgrowth in a control mass of papillomatous tissue (rabbit 1-59, Experiment 1, 95th day). The mass projected more than 1.5 cm. above the surrounding skin,—which is to be seen at either end of the slice,—but it was devoid of keratin and its surface layer was only 1-2 mm. thick, all the rest consisting of invasive downgrowth amidst profuse reactive tissue. Nowhere is there the palisade striation typical of virus papillomas (see Fig. 1). In the gross cancer seemed to be present at many situations, but the microscope showed only aggressive virus papillomatosis (see Fig. 4). The specimen has become tar-stained during its sojourn in Kaiserling's solution.  $\times 1$ .

FIG. 4. Adjacent slice through the same growth. The shallow surface layer is disorderly, the numerous downgrowths convoluted or cystic, and the narrow tongues extending from them appear in cross-section as nests of cells simulating squamous cell carcinomatosis. Yet the growth had everywhere the cytological character of virus papillomatosis, as higher magnifications showed. The tar has caused the skin next it to be hyperplastic.  $\times 4.3$ .



(Rous and Friedewald: Chemical carcinogens and virus papillomas)

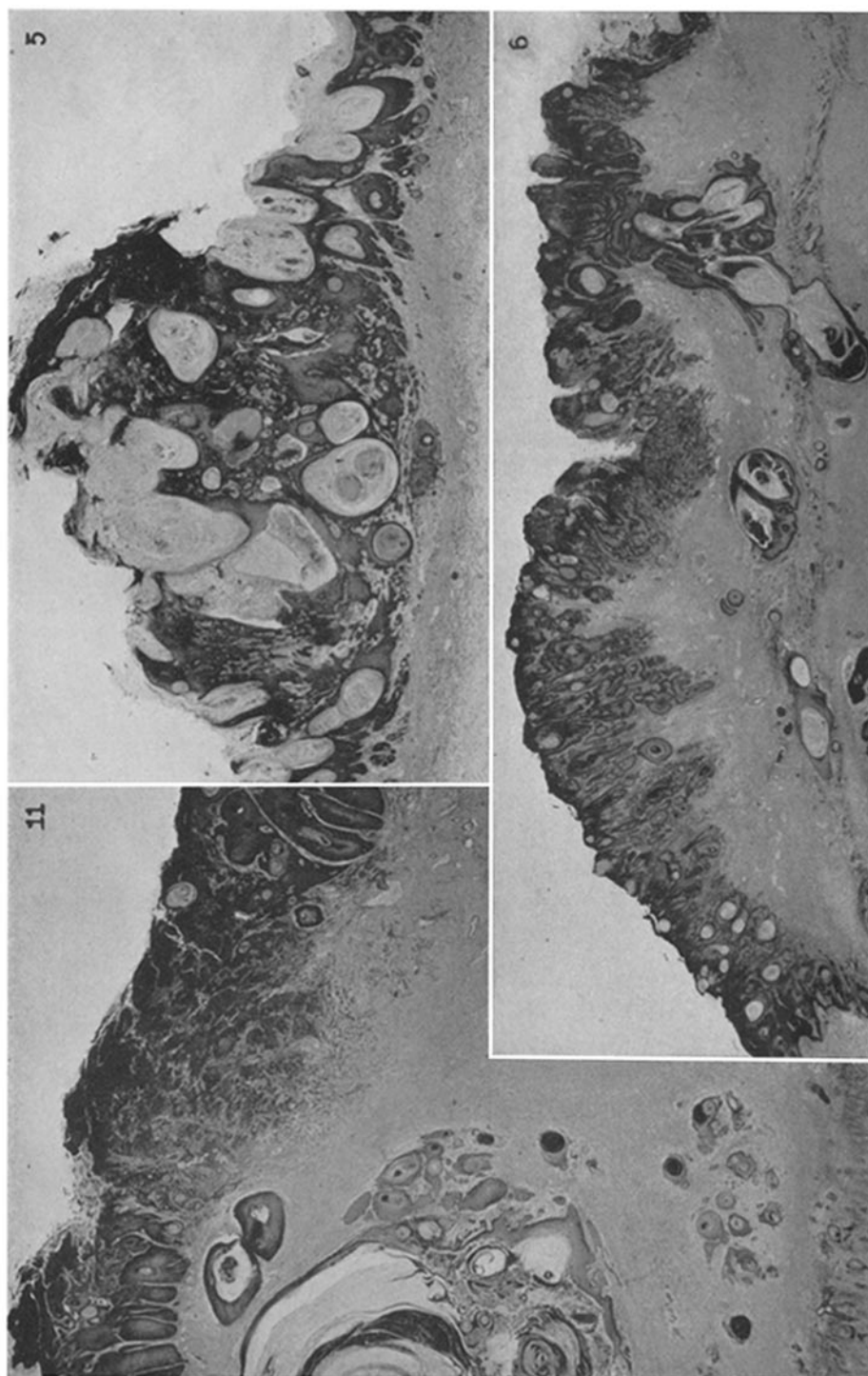
PLATE 10

FIG. 5. Carcinomatoid which arose on skin treated with tar containing methylcholanthrene (rabbit 1-58, Experiment 1, 82nd day of treatment). The epidermis round about the growth is unusually hyperplastic.  $\times 17$ .

The tar greatly stimulated the virus papillomas present on the same animal but nowhere did these undergo malignant change.

FIG. 6. Half of a slice through the methylcholanthrened mass of rabbit 1-59, Experiment 1. The block was taken through a depressed, ulcerated area 6 mm. across which had lately appeared. Here there is what looks like anaplastic squamous cell carcinomatosis and elsewhere along the slice cancer seems to be just arising at numerous situations. At one spot where ordinary papillomatosis is still present it has grown down irregularly, penetrating the cutaneous musculature; and amidst it there are some papillomatous cysts, either residual from extension of a surface layer now replaced or consequent on lateral extension from near by. There were four good-sized discrete growths with the aspect of anaplastic squamous cell cancers in the mass here shown in part.  $\times 5.8$ .

FIG. 11. An anaplastic carcinoma in the methylcholanthrened mass of rabbit 1-44: autopsy specimen. Deep in the reactive tissue are islands of keratinizing squamous cell carcinomatosis, lateral extensions from a growth not included in the specimen. The voluntary muscle along the lower edge of the picture has been invaded by this growth. The surface layer of papillomatous tissue to either side of the anaplastic cancer is orderly, and just beneath it on the left are a few papilloma cysts, one of them invaded by the keratinizing cancer.  $\times 10.5$ .



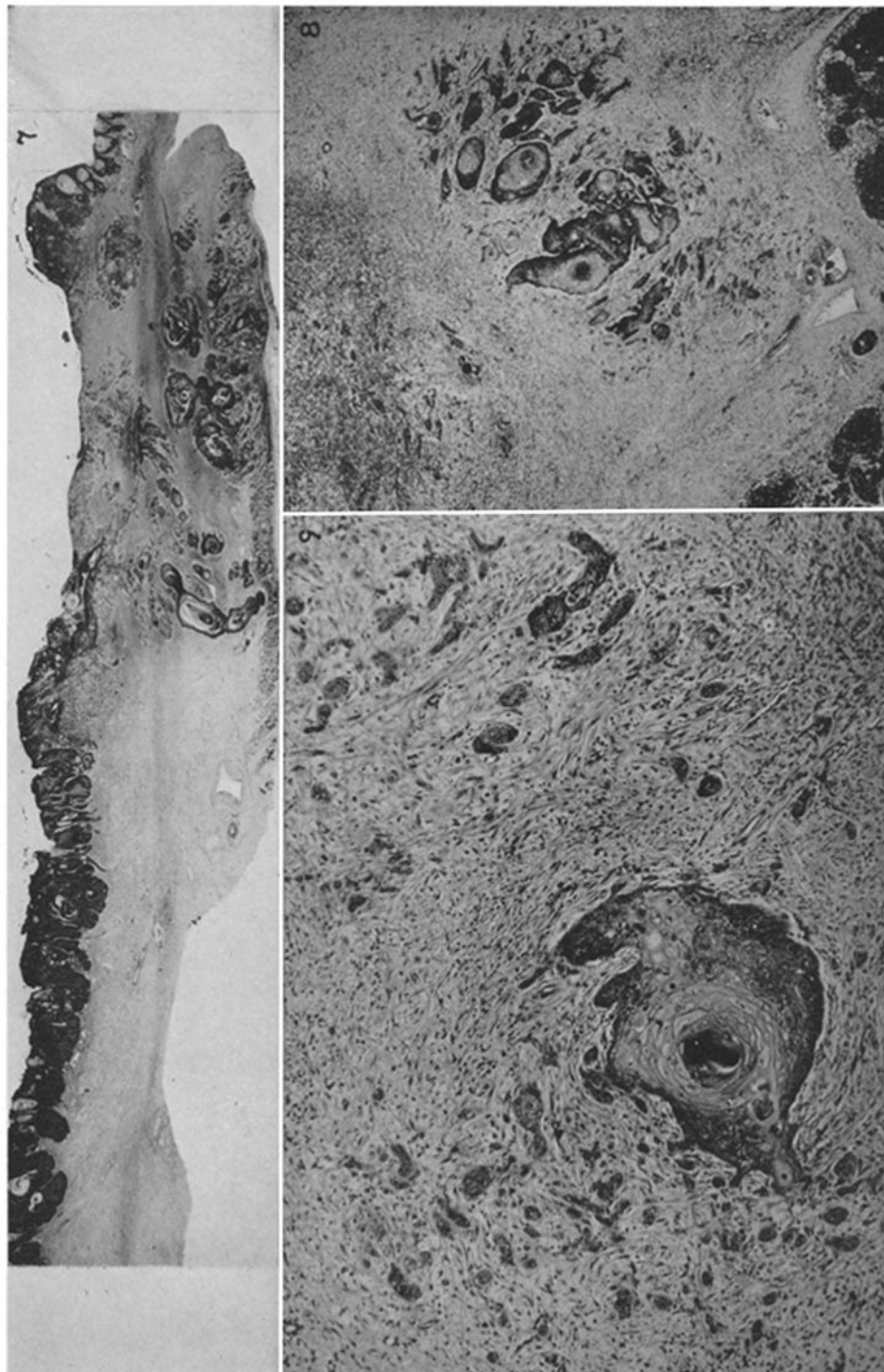
(Rous and Friedewald: Chemical carcinogens and virus papillomas)

PLATE 11

FIG. 7. Ulcerating cancer in a papillomatous expanse treated with a mixture of tar and methylcholanthrene (rabbit 1-39, Experiment 2, 78th day). The growth was already big when first noted on the 71st day after the inoculation, and had extended toward the axilla (Chart 3). The figure shows it to have the morphology of a squamous cell carcinoma, anaplastic in some regions and replacing the sheet of voluntary muscle along the lower edge of the block. Here and there in the profuse reactive tissue are keratinizing cysts lined with ordinary papillomatous epithelium. The surface layer of this latter is shallow and irregular.  $\times 4.3$ .

FIG. 8. Metastasis in an axillary lymph node of the rabbit with the cancer of Fig. 7. It may have derived from this growth or from one of the malignant tumors which had arisen in the methylcholanthrened mass, for they had a similar morphology. The metastasis is partly keratinizing, partly anaplastic. The gland tissue had been replaced entirely but portions of two neighboring glands can be seen.  $\times 18$ .

FIG. 9. Part of the same metastasis, to show the character of the malignant cells.  $\times 70$ .



(Rous and Friedewald: Chemical carcinogens and virus papillomas)

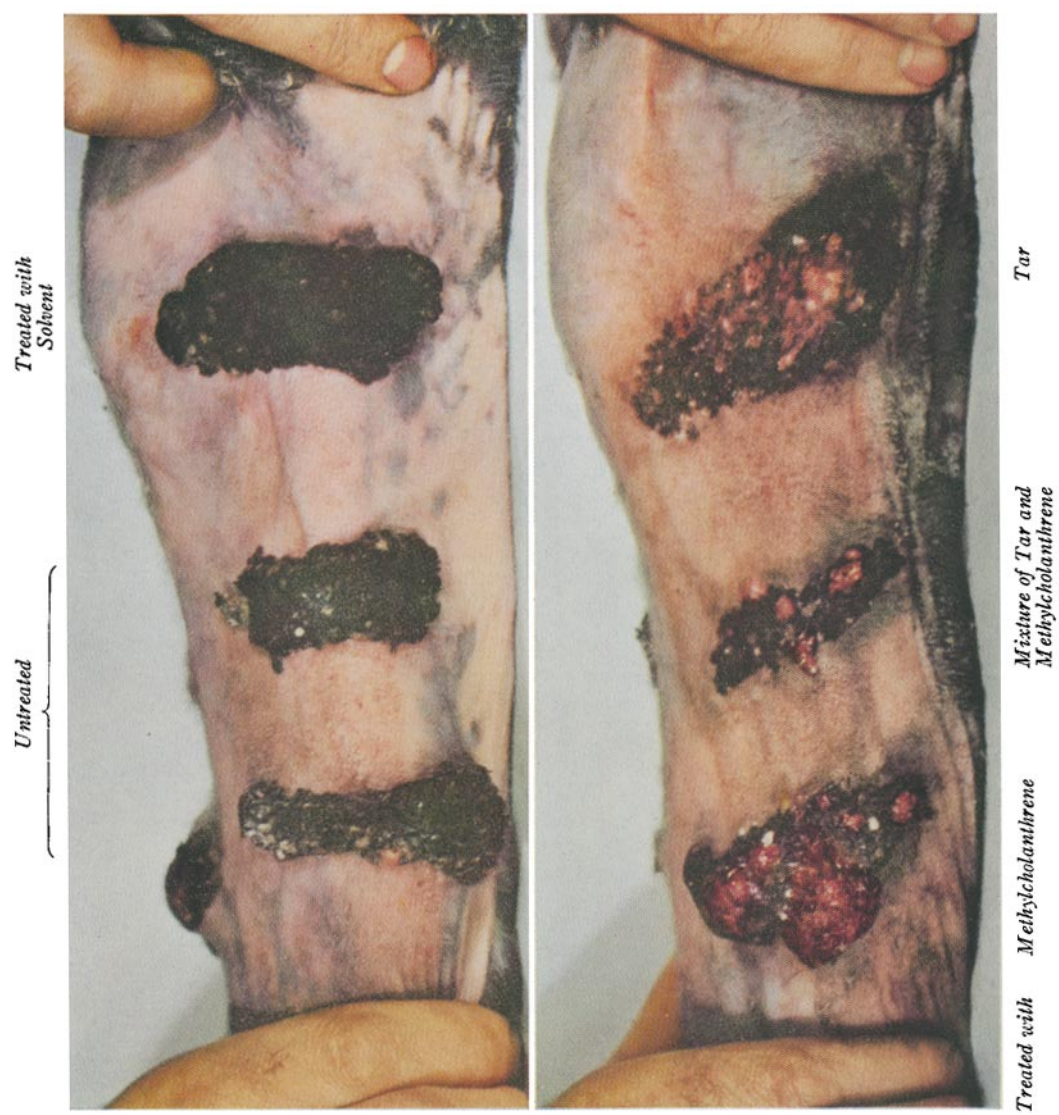
*Effect of Chemical Carcinogens on Virus-Induced Papillomas*

The virus was inoculated into six scarified areas on the sides of rabbit 1-44, Experiment 2, and 7 days later, when healing had taken place, three of the areas, as also the skin immediately about them, were painted respectively with (a) 0.3 per cent methylcholanthrene in Crabtree's solvent, (b) tar dissolved in 7 parts of solution "a," and (c) tar in 7 parts of Crabtree's solvent. To a fourth area the solvent was applied while the remaining two served as controls. The applications were repeated thrice a week until the 90th day, and the photographs were taken on the 109th day, when the animal was killed. One of the prints has been made through the back of the negative for the readier comparison of growths at corresponding situations. Hence the animal appears to have two left sides.

The control masses of craggy papillomatous tissue are so heavily melanotic as to appear black. The white spots amidst them are due to erupted pearls of keratin, and the pink bulge at the edge of the untreated growth on the left is a papillomatous cyst containing such a pearl, with inflammation about it. The ruddy fungoid protrusions from the growths which received methylcholanthrene as such or mixed with tar are nearly all cancers, as the microscope showed (see Figs. 10-12). There was none in the tarred growth, the pink areas visible amidst the black papillomatous tissue being residual to the extrusion of keratinized pearls or due to some as yet not erupted (see text).

By the 63rd day after virus inoculation several new tumors had appeared amidst the papillomatous tissue receiving methylcholanthrene (see Chart 1), and on the 78th day one was present in that treated with the mixture of methylcholanthrene and tar. They grew rapidly: when the color photographs were taken fourteen sizable cancers were present in the mass that had received methylcholanthrene, three in that treated with the methylcholanthrene-tar mixture, and one in the tarred mass. Also there were many papillomatous cysts containing pearls, notably in the mass that had received tar. These introduce a confusing element into the pictures. No metastases were found.

The skin immediately about the growths, which had also been painted with the carcinogens, is wholly bare of tumors but somewhat inflamed.



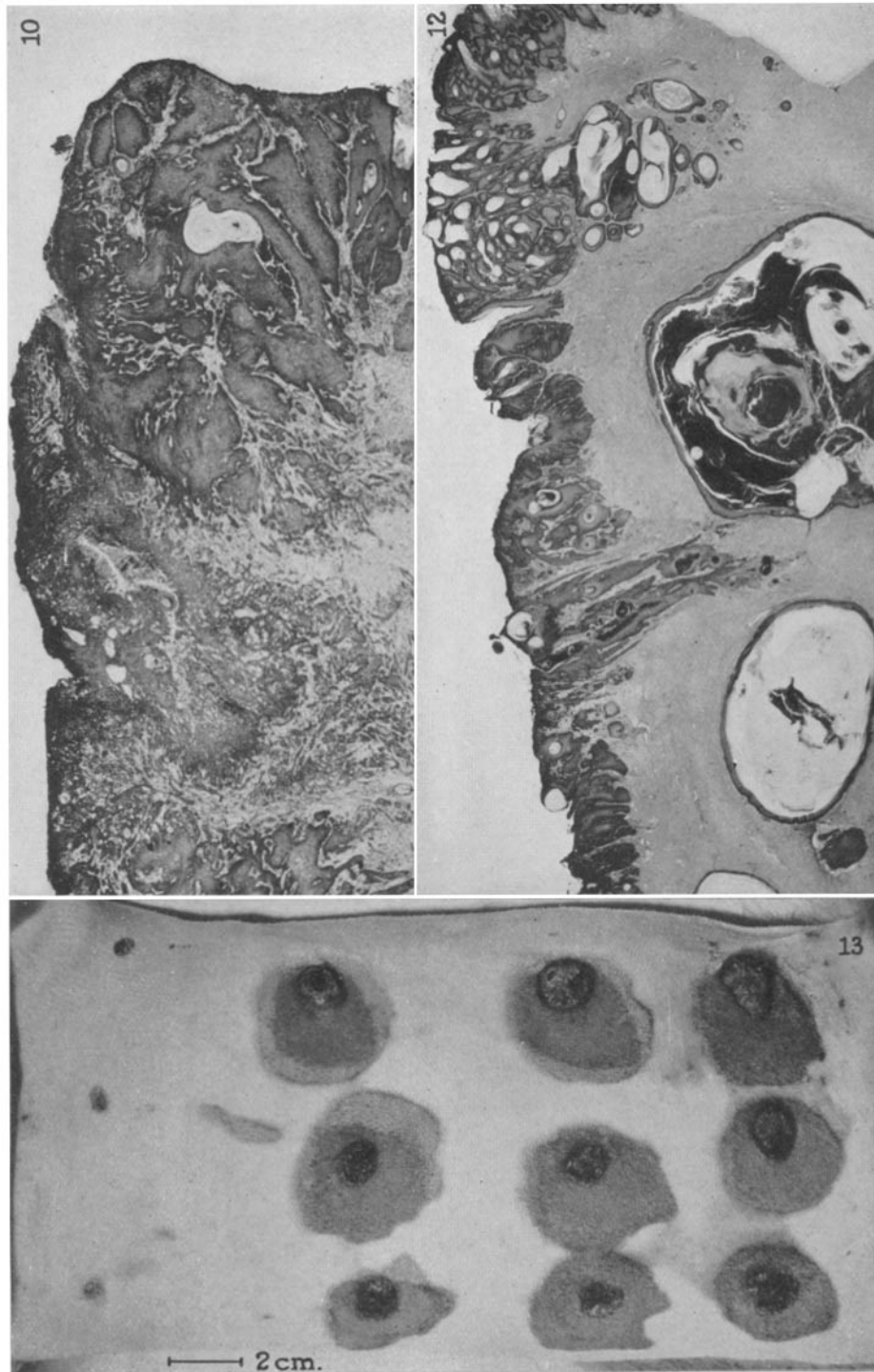
(Rous and Friedewald: Chemical carcinogens and virus papillomas)

### PLATE 13

FIG. 10. Biopsy specimen procured on the 92nd day after virus inoculation, from a fungoid growth which had appeared amidst the methylcholanthrene papillomatous tissue of rabbit 1-44 (see Plate 12). It shows a wholly anaplastic squamous cell carcinoma, separated from the very hyperplastic skin (on right) by a little papilloma tissue. The tumor was first noted on the 63rd day and it coalesced with another to become the largest of the ruddy growths shown in Plate 12.  $\times 24$ .

FIG. 12. Another slice from the same methylcholanthrene mass, showing the diversity of the malignant changes. At the left there is a thin layer of virus papilloma tissue, as also at the middle of the picture. The region between is occupied by a keratinizing squamous cell carcinoma, while toward the right a cystic squamous cell carcinoma has built up. Beyond that again is virus papilloma tissue, and deep in the profuse reactive tissue are several large cysts lined with virus papilloma cells and filled with concentric keratinized lamellae.  $\times 5.8$ .

FIG. 13. Stimulating effect of methylcholanthrene in Crabtree's solvent on virus papillomas and the skin about them (rabbit 1-77, Experiment 3, 34th day after virus inoculation and 27th of treatment with methylcholanthrene). The whole right side of the animal is shown. The papillomas receiving the methylcholanthrene solution are thick, dry-topped, keratin-covered, dark gray discs, and the shaved skin round about them, to which the solution spread, is medium gray, somewhat scurfy, and already becoming hairy again. The three control growths, those in a vertical row on the left of the picture, are small, unkeratinized, and devoid of pigment, the dark spots on them being due to hemorrhage from abrasions. No hair has come in on the untreated skin and it shows only traces of melanosis.  $\times \frac{1}{2}$ .

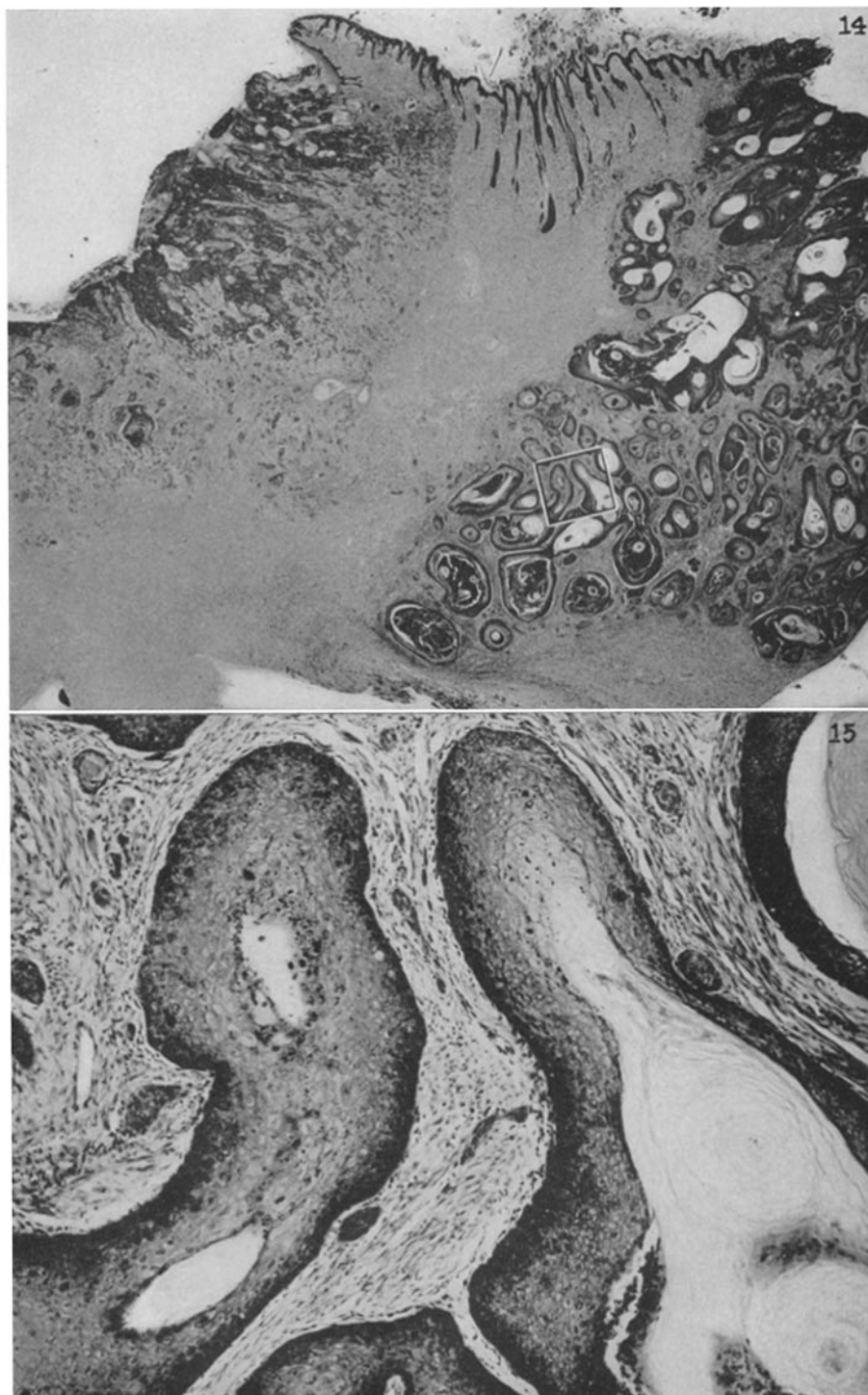


(Rous and Friedewald: Chemical carcinogens and virus papillomas)

PLATE 14

FIG. 14. To show two malignant growths of widely differing types, which originated in adjacent papillomas exposed to the action of methylcholanthrene (rabbit 1-84, Experiment 3, Chart 8, largest growths on left side of the animal). The huge ulcerated tumors extended far out into the subcutaneous tissue and almost met, though at the surface they were still separated by a zone of hyperplastic skin as the photograph shows. One of them is a wholly anaplastic carcinoma, whereas the other, despite its malignant behavior, has epithelium nearly resembling that of an ordinary virus papilloma.  $\times 6.5$ .

FIG. 15. Part of the malignant growth of Fig. 14 (region enclosed in white rectangle) which had retained the features of a virus papilloma. The cells are differentiating and keratinizing as in such a papilloma but are less well ordered, and both they and their nuclei show distinguishing abnormalities not visible in the photograph (wide variations in size and in nuclear size and character, occasional multiple nuclei).  $\times 80$ .



(Rous and Friedewald: Chemical carcinogens and virus papillomas)