JOINT ACTION OF A CHEMICAL CARCINOGEN AND A NEOPLASTIC VIRUS TO INDUCE CANCER IN RABBITS*

Results of Exposing Epidermal Cells to a Carcinogenic Hydrocarbon at Time of Infection with the Shope Papilloma Virus

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Plates 33 to 41

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When the skin tumors induced in rabbits by tar or methylcholanthrene are experimentally infected with the Shope papilloma virus, some of the growths previously benign change rapidly to carcinomas, and the carcinomas already present often undergo marked alterations in behavior and morphology (1–3). If the order of the test is reversed, that is to say if tar or methylcholanthrene or the two together are applied repeatedly to preexisting virus papillomas these become malignant far earlier than would otherwise be the case and at many more situations (4). In both cases the influence of a carcinogen of one sort is secondarily superimposed upon that of another. The present paper reports the results of concurrently exposing hyperplastic, regenerating epidermal cells to virus infection and methylcholanthrene (*MC*) or the much more powerful carcinogen, 9:10-dimethyl-1:2-benzanthracene (to be called *9:10*). When this was done the resulting virus papillomas became cancerous long before the control growths, which indeed seldom underwent malignant change during the period of observation. Collateral findings made plain that the virus and the hydrocarbons had acted in their carcinogenic capacities, not by way of any collateral effects.

Method

Numerous experiments have been made in our laboratory to learn whether growths differing from the ordinary can be obtained by bringing the papilloma virus to bear on epidermal cells rendered pathological beforehand by various means. In one such test rabbit skin, already in a hyperplastic state as result of paintings with turpentine, was scarified over several considerable areas, and a mixed suspension of papilloma virus and methylcholanthrene was rubbed into the raw surface. The resulting papillomas looked like those produced by the virus alone; yet after some months carcinomas arose from them, whereas the control growths gave no sign of malignant change. In

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a second experiment, similar save that the *MC* suspension was put on the scarified skin immediately after the virus, identical findings were obtained. Comprehensive tests were then decided upon and a technique worked out. The results with the method ultimately adopted will be described first, and then such pertinent facts as were obtained along the trial-and-error way.

A prime requisite was intimate contact of the chemical carcinogen with the regenerating epidermis under sharply controlled conditions. This was finally obtained by applying to the scarified skin, immediately after virus inoculation, gauze saturated with olive oil containing *MC* or *9:10*, keeping it closely apposed until healing was done.

Three to five large, rectangular, shaved areas on each side of several agouti rabbits were rendered hyperplastic by four to six paintings at 2-day intervals with turpentine and acetone in equal parts, and 2 to 4 days later the epidermis was removed from them by light sandpapering. Immediately afterwards in each case the area was treated in one of the three following ways: (*a*) it was inoculated broadcast with a saline suspension of virus, allowed to dry partially, and then covered with a single layer of gauze saturated with olive oil; (*b*) it was similarly inoculated, but covered with gauze saturated with olive oil containing 0.5 or 1 per cent of *MC* or *9:10;* (*c*) it was not inoculated, but covered as in (*b*).

The oiled gauze was cut to the precise size of each raw area, and over it a slightly larger rectangle of the Cilkloid[1] used to cover skin grafts was superimposed, followed by one of gauze impregnated with solid paraffin (melting point 47–49°C.), no larger than the abraded square. All were bound together to the skin by broad strips of adhesive plaster, and enveloped in a gauze binder and many-tailed bandage. The Cilkloid prevented loss of oil to the paraffined layer, and this latter was thick enough to transfer the pressure of the binder, thus holding the oiled gauze close against the skin. Healing was complete within a few days and the dressings were then removed, but sometimes binder and bandage were put on again for a brief further period to protect the new epithelium.

A single preparation of virus was used throughout, an extract in 0.9 per cent salt solution of the papillomas from cottontail rabbit W. R. 1-52. It had been run through a Berkefeld filter in 5 per cent strength and stored at −30°C. in many small lots which were thawed as required and diluted to 2.5 or 2 per cent for inoculation. The virus had only moderate pathogenicity in these dilutions, no papillomas (paps.) appearing as a rule until 9 to 11 days had elapsed, though they generally had become confluent within another week or two. When, after the lapse of months, it seemed likely that the pap. masses might soon become cancerous, outline tracings were made of them, but in several instances not soon enough. Frequent tracings were made later, the cancers drawn in, and copious notes kept.

The *MC* came from the Edcan Laboratories, and the *9:10* was the generous gift of Dr. W. E. Smith, who had received it from Dr. Engelbreth-Holm; it had the melting point of the pure compound (123°C.). A single batch of olive oil was used, autoclaved as such and with the carcinogens dissolved in it. The preparations were distributed in many small tubes and kept at 4°C. until needed.

The cotton gauze (44 by 36 meshes to the inch) had been cleansed by letting it stand in 5 per cent hydrochloric acid, with rinsing in distilled water, alcohol, and ether successively. Just before each experiment rectangles were cut, spread in Petri dishes, and the oil, heated to 100°C., was dropped on each to the amount required for saturation. The size of the inoculated areas ranged from 3 by 3 cm. in some experiments to 4 by 5 cm. in others.

Most of the rabbits were killed before the malignant growths had run their course,—this for the better analysis of the findings. At autopsy each growth was sliced at intervals of a

[1] The Cilkloid Company, Marshalltown, Iowa.

centimeter or less in the search for cancers. They could be readily discerned in most instances, even before they had invaded, through the disturbance they caused in the pattern of the vertically striated, palisade paps. A representative slice across all the latter growths was usually taken for section, and wherever there seemed to be any likelihood of malignancy further slices were fixed, and often sectioned on both sides. All the cancers were examined microscopically and numerous blocks were taken from most of those that were large. Each of the metastases,—found in the lymph glands only,—was also sectioned, and a piece of lung whenever there seemed reason. The blocks were fixed in acid Zenker's solution and stained with eosin and methylene blue.

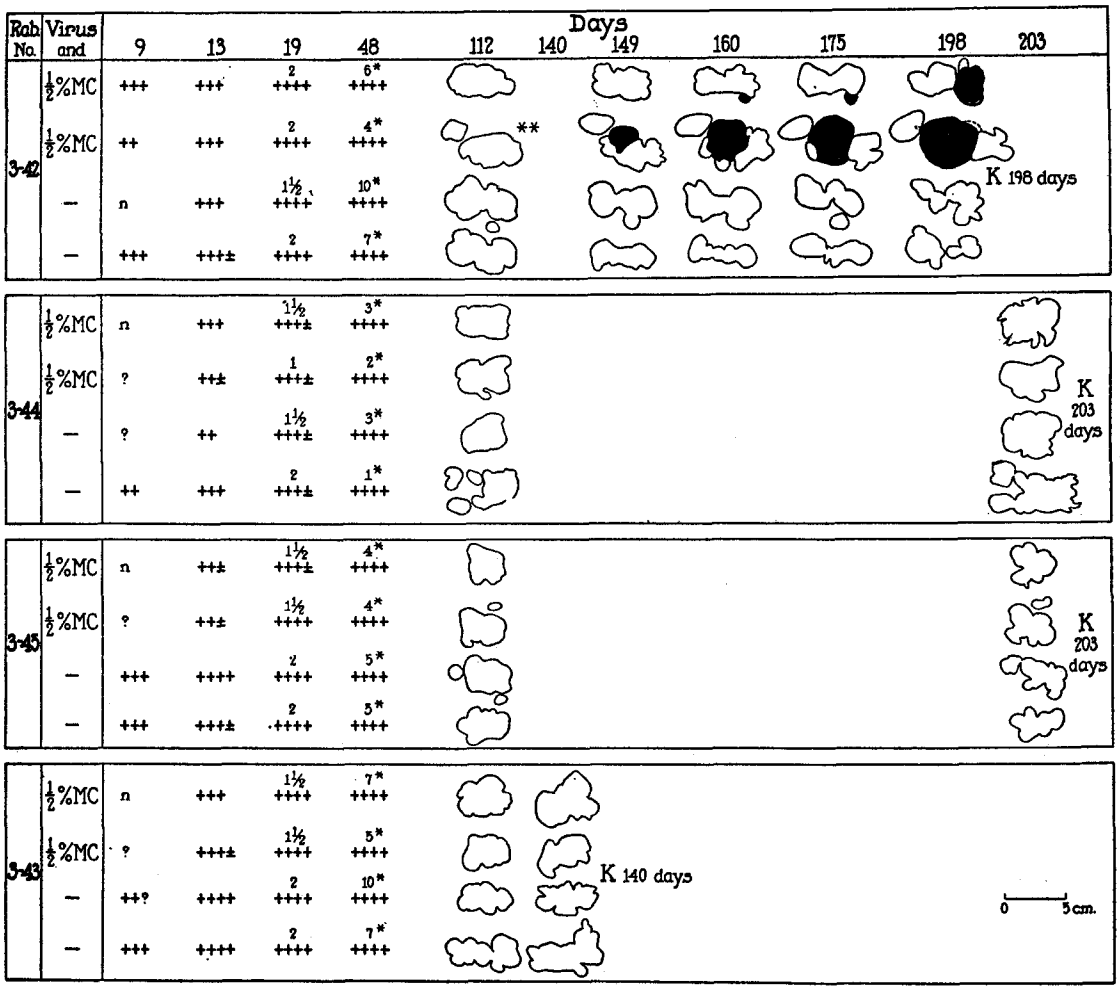
Effect of the Carcinogenic Solutions to Hasten Cancer

Two tests with *MC* were carried out as described. Charts 1 and 2 tell their outcome.

Experiment 1 (Chart 1).—Two 3 by 3 cm. areas on one side of four rabbits were inoculated with 2 per cent virus and covered with gauze saturated with oil containing 0.5 per cent of *MC*. Two other areas at corresponding situations on the other side were similarly inoculated and covered with gauze soaked in olive oil alone. When the dressings were removed on the 5th day healing had been completed, and paps. appeared soon after, those on the *MC*ed areas later in general than on the control expanses (as the plus marks on the chart make plain), and the resulting confluent masses tended to be smaller and lower for a while; but they showed no other gross difference, and no significant differences could be discerned on the 112th day when the first tracings were made. So too on the 128th day; hence no charting was done. Rabbit 3-43 was accidentally killed on the 140th day. On the 149th, when the others were next examined, an obviously malignant growth of considerable size, ulcerated and deeply invasive, had replaced part of the pap. mass on an *MC*ed area of **D. R. 3-42,**—this although all its growths had been dry practically to the base, that is to say relatively unfavorable to cancerous change, as much previous experience had shown. During the next few days a second malignant tumor appeared amidst the pap. tissue next the edge of the mass on the other *MC*ed area, and rapidly extended out into the skin. On the 182nd day a football-shaped nodule 1.2 cm. long was discovered in an axillary gland. By the time the rabbit was killed on the 198th day, the new tumors, raised, ulcerated, discoid masses, had replaced much of the pap. and pushed out laterally into the subcutaneous tissue. The microscope showed them to consist of squamous-cell carcinomas differing in character. One was due to the fusion of an ordinary cancer of this sort with a malignant growth that had retained the papillomatous structure though made up of spindle-shaped epithelial cells. The metastasis was of this latter kind. The control masses consisted merely of virus pap. tissue and had of late dwindled greatly.

The two remaining rabbits were killed on the 203rd day. Their growths were vigorous but showed no signs of cancer.

Experiment 2 (Chart 2).—Both 1 per cent and 0.5 per cent solutions of *MC* in oil were used, and one or the other was tested in duplicate on two of the four rabbits. The inoculated areas measured 3.5 by 4 cm., the virus was of 2.5 per cent strength, and the dressings were kept on 7 days, though healing had been completed earlier. Paps. appeared relatively slowly on some of the *MC*ed areas, but the masses formed were as large as the controls. None showed cancer on the 112th day, but by the 125th the mass on **D. R. 3-33,** where 1 per cent *MC* had been put, was found to have been largely replaced by an ulcerated, fleshy tumor which had already extended in the subcutaneous tissue more than 3 cm. toward the hip. Charting was begun on the 127th day. By the 150th, when the rabbit died, the mass on the other area



** Negative for cancer on the 129th day.

CHART 1

CHARTS 1 to 8. *Development of Cancers amidst Virus Papilloma Tissue.*—The occurrence of papillomas after virus inoculation, their number and course during the first weeks, are recorded in plus signs according to a standard scale: ± = 1 papilloma; ± = 2, 3, or 4 paps.; + = 5 to 15 paps.; ++ = many discrete paps.; +++ = semiconfluent paps.; ++++ = confluent paps. The height of confluent masses is given in millimeters above the plus signs and followed by a star * when the growths have been gnawed back, as often happened when they were vigorous. Those of each animal are grouped according to whether they served as controls or had arisen from epidermal cells exposed to *MC, 9:10,* or podophyllin. The masses are shown in outline later on, but for the sake of simplicity many tracings have been omitted which were merely confirmatory of those immediately preceding them, though the one made just before cancer was first noted has generally been included. In most instances the control expanses and those under test were at corresponding situations on either side of the animal. For charting purposes the traced outlines have been turned so that the end of each mass that was nearest the abdomen is toward the left. Obvious cancers, as yet unulcerated, are outlined and hatched, and ulcerated ones are in black. Broken lines indicate lateral extensions of the malignant growths in the deep corium, and stippled expanses tell where they were subepidermal (Charts 2 to 4).

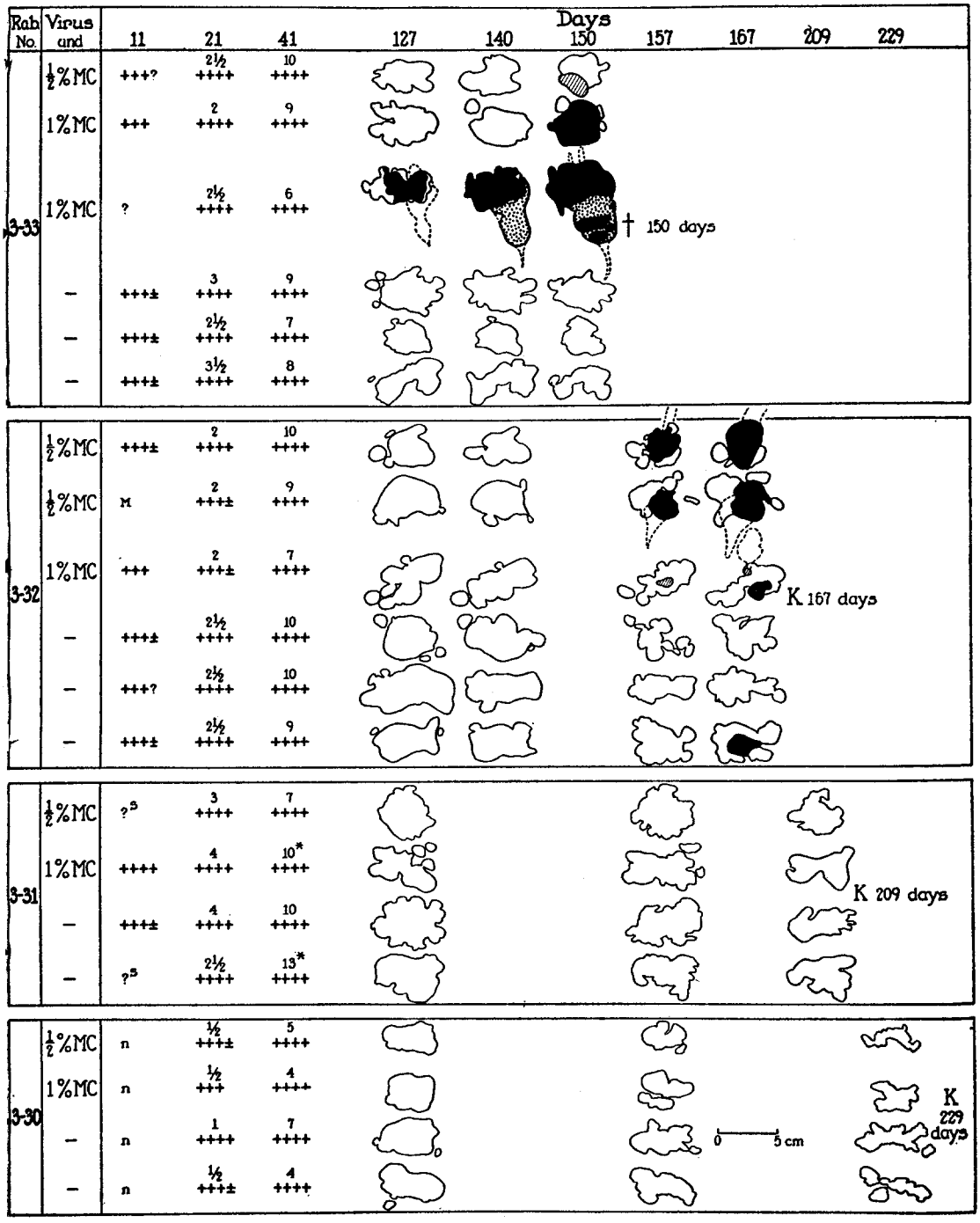

Rab. No.
Virus and
Days
11
21
41
127
140
150
157
167
209
229
3-33
† 150 days
3-32
K 167 days
3-31
K 209 days
3-30
K 229 days
0
5 cm

CHART 2

receiving 1 per cent *MC* had been almost replaced by a cancer, and a second, smaller one had arisen amidst the pap. tissue where 0.5 per cent *MC* had been put. It had extended deep, yet still was overlain by pap. tissue. By now several deep prongs had extended laterally from the first cancer to appear and it had in addition a huge intradermal extension, 2 cm. high, ulcerating at several spots (Fig. 1). The tumors were all sqaumous-cell carcinomas, each composed of more than one neoplasm, as their histological diversity made plain; yet none had metastasized. The control growths were mere indolent paps., smaller than before, black, dry to the base, and scoriaceous (Fig. 2).

In **D. R. 3-32** cancer appeared between the 140th and 157th days in the two masses where 0.5 per cent *MC* had been put; and already on the latter day there were four metastatic nodules in the axilla, and deep extension had taken place toward the groin. By the 167th day the axillary nodules had got big; cords of neoplastic tissue connected them with one another and with the nearest primary growth; and a nodule had formed in the groin, and another in an iliac gland, as autopsy showed. The pap. mass where 1 per cent *MC* had been applied had now been partly replaced by an obviously malignant tumor. Cancer had just manifested itself in a control growth when the animal was killed.

D. R. 3-31 became thin, developed purulent "snuffles," and hence was sacrificed on the 209th day. Its growths were mere papillomas, dry to the base, and had lately begun to retrogress. Retrogression of all the growths of **D. R. 3-30** was far advanced when it was killed, after 229 days.

The three experiments with *9:10* yielded similar but more striking results.

Experiment 3 (Chart 3).—Two preparations of *9:10* were used, 0.5 and 1 per cent in oil, on scarified areas measuring 4 by 4.5 cm. inoculated with 2.5 per cent virus; dressings off after 6 days.

All four rabbits developed cancers, but only where *9:10* had been applied. In **D. R. 3-26** malignancy seemed probable on the 113th day in the mass where 0.5 per cent had been put; it was indubitable by the 127th day (when charting was begun); and by the 142nd there was an axillary metastasis. The pap. mass where 1 per cent had been used underwent manifest malignant change between the 127th and 142nd days, and the resulting invasive, new growth was found to have ulcerated on the 167th, when the animal was killed (not on the 176th day, as the chart might be taken to imply). One of the cancers had by then wholly replaced the pap. tissue, the other had largely done so, and there were deep, lateral extensions from both (Fig. 3). Sections showed each to be composed of several intermingled carcinomas; five could be discerned in the ulcerated growth where 1 per cent *MC* had been put, and the axillary metastasis showed two side by side (Fig. 12).

The control masses (Fig. 4) had of late become smaller.

D. R. 3-28 developed cancers somewhat later, in both *9:10* masses. The new tumors enlarged mostly by expansile proliferation, exerting such lateral pressure on the persisting border zones of pap. tissue as to turn their peaks outwards, almost parallel with the skin. Hence the masses appear broader in the photograph taken on the 168th day (Fig. 5) than was the actual case. The rabbit was still in good condition when killed after 230 days, and the malignant growths, long since wholly destructive to the pap. tissue and now huge, had not metastasized. The control pap. masses had of late become smaller (Fig. 6).

D. R. 3-29 was killed on the 203rd day because emaciated. Its malignant tumors had appeared late but grown fast, and one showed five differing, intermingled carcinomas microscopically. Metastases were absent.

D. R. 3-27 died early of intercurrent disease. A keratin-covered carcinoma of papillomatous structure had almost completely replaced the pap. mass where 0.5 per cent *MC* had been applied.

Again the control growths had remained mere paps.

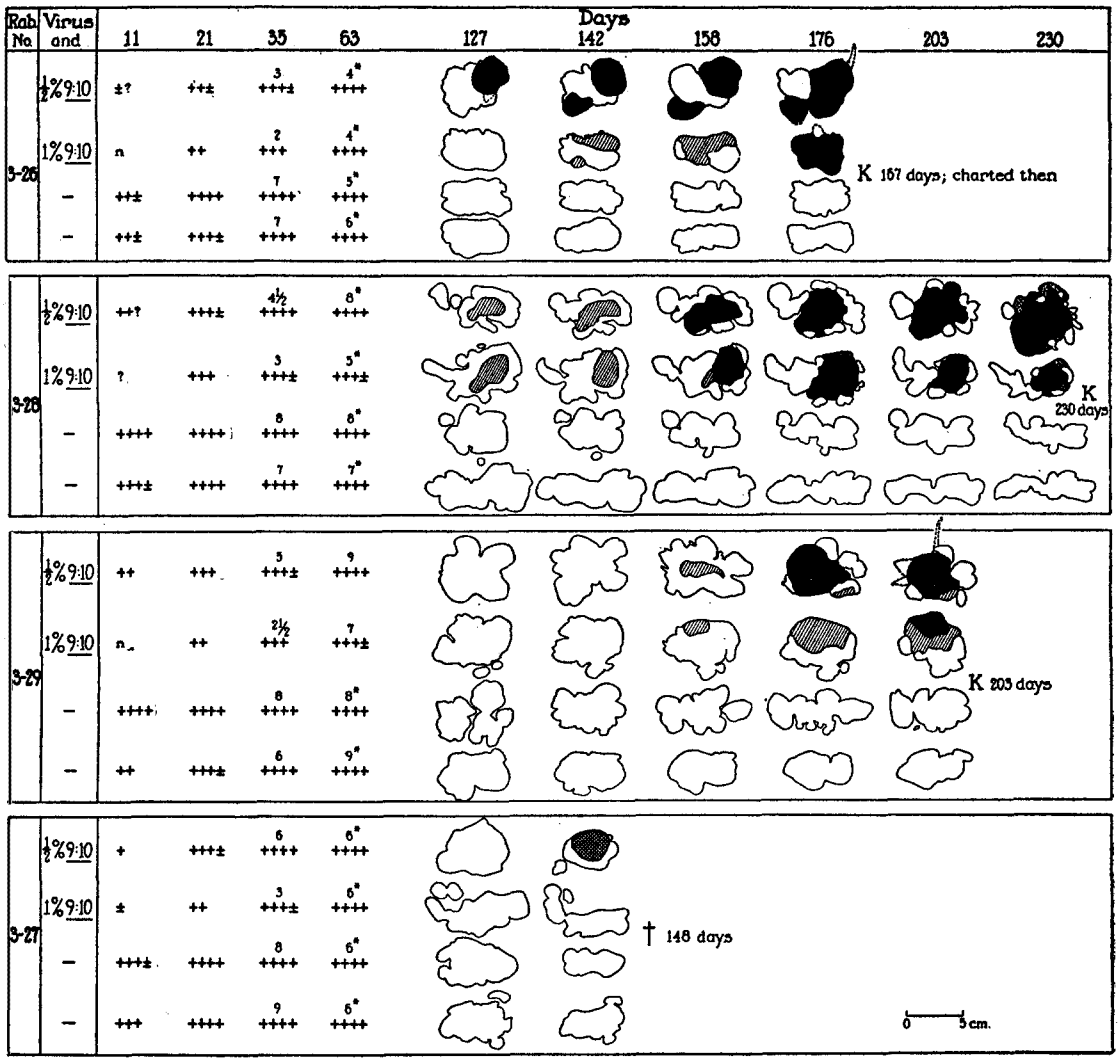

Rab No.
Virus and
11
21
35
63
Days
127
142
158
176
203
230
3-26
½%9:10
1%9:10
–
–
K 167 days; charted then
3-28
½%9:10
1%9:10
–
–
K 230 days
3-29
½%9:10
1%9:10
–
–
K 203 days
3-27
½%9:10
1%9:10
–
–
† 148 days
0 5 cm.

CHART 3

Cancer was well established by the 127th day in two rabbits of this test and developed ultimately in all four. It arose only where *9 : 10* had been applied with the virus, and was frequently multicentric in origin. The 0.5 per cent preparation of the hydrocarbon seemed perhaps the more effective in carcinogenesis, but this had not become plain before the next experiments were begun, and in them 1 per cent only was used. They were essentially repetitions of Experiment 3 in other ways, except that most of the animals were killed early in order to procure the new tumors before they had come to consist of several merged growths.

Experiment 4 (Chart 4).—The scarified areas measured 3 by 3 cm., and 2 per cent virus was employed; dressings off after 6 days. On the 101st day the growths of **D. R. 3-48** seemed still to consist of ordinary papillomas, but by the 135th, that of next examination, cancers had almost entirely replaced the two masses at the *9:10* sites, and by the 162nd deep prongs had extended toward the axilla in one case and the groin in the other. On the 179th day an axillary nodule was noted (Fig. 7). Later the malignant masses fused into a single huge one with big intradermal extensions up to 2 cm. high (Chart 4), covered with dense, purply epidermis ulcerating at several spots. The picture was very like that in D. R. 3-33 of Experiment 2 (Fig. 1). At death the axillary metastasis was 2 cm. across, and another was present in the groin. The microscope made plain that each had derived from the nearest cancer, a keratinizing, squamous-cell carcinoma with areas of fusiform cells (Fig. 17), and a cystic, malignant papilloma respectively.

The control growths had not enlarged in recent months, but were vigorous and had lost much of their initial gray pigmentation (Fig. 8). Neither showed any sign of cancer.

D. R. 3-49.—At the first charting (101st day) pap. masses scarcely covered the areas inoculated, whereas those of the other test animals had almost or quite doubled in size. Nevertheless one situated at a *9:10* site had already a discrete raw area in its midst, with a dubious bulge beneath. A month later the raw area was thickened and fleshy, had extended to the margin of the mass, and many creamy, opaque dots of necrosis could be seen in its exposed, ruddy side. Later on the cancer, for such it was, scarcely enlarged but extended deep.

All of the growths of **D. R. 3-46**, though growing fast at first, underwent marked regression between the 135th and 148th days; yet during this period cancer appeared in one of the *9:10* masses. It enlarged almost not at all until the 162nd day, but thereafter extended slowly into the deep cutaneous tissue. On the 177th day what looked like a ruddy pustule 1 mm. high and 2 mm. in diameter was noted on the skin 3 mm. away from the pap. mass containing the cancer, and at a considerable distance from this latter (Chart 4, arrow A). The other *9:10* mass had undergone less regression, and appeared to be everywhere papillomatous save for a blunt peninsula which had lately ulcerated and was suggestively thickened at the base (arrow B). The animal was killed now and slices were taken through the masses and the adjacent questionable growths. These latter proved to be moderately anaplastic, squamous-cell carcinomas, differing distinctively in morphology. A flat layer of virus pap. epithelium connected the peninsular cancer (Fig. 9) with the mass from which it had arisen. The "pustule" consisted of a keratin-filled cyst connected with the skin by a narrow opening (Fig. 10) and lined with a thick layer of keratinizing, malignant elements such as frequently derive from virus papilloma cells. Extension into the deep corium had taken place (Fig. 11). Superimposing the tracings which had been taken from time to time showed that the pap. mass had once covered the situation of the "pustule" cancer,—from which it was now separated by a stretch of merely hyperplastic epithelium, and by a second small cyst (Fig. 10) lying immediately next the mass and lined with its characteristic epithelium. The cancer amidst this

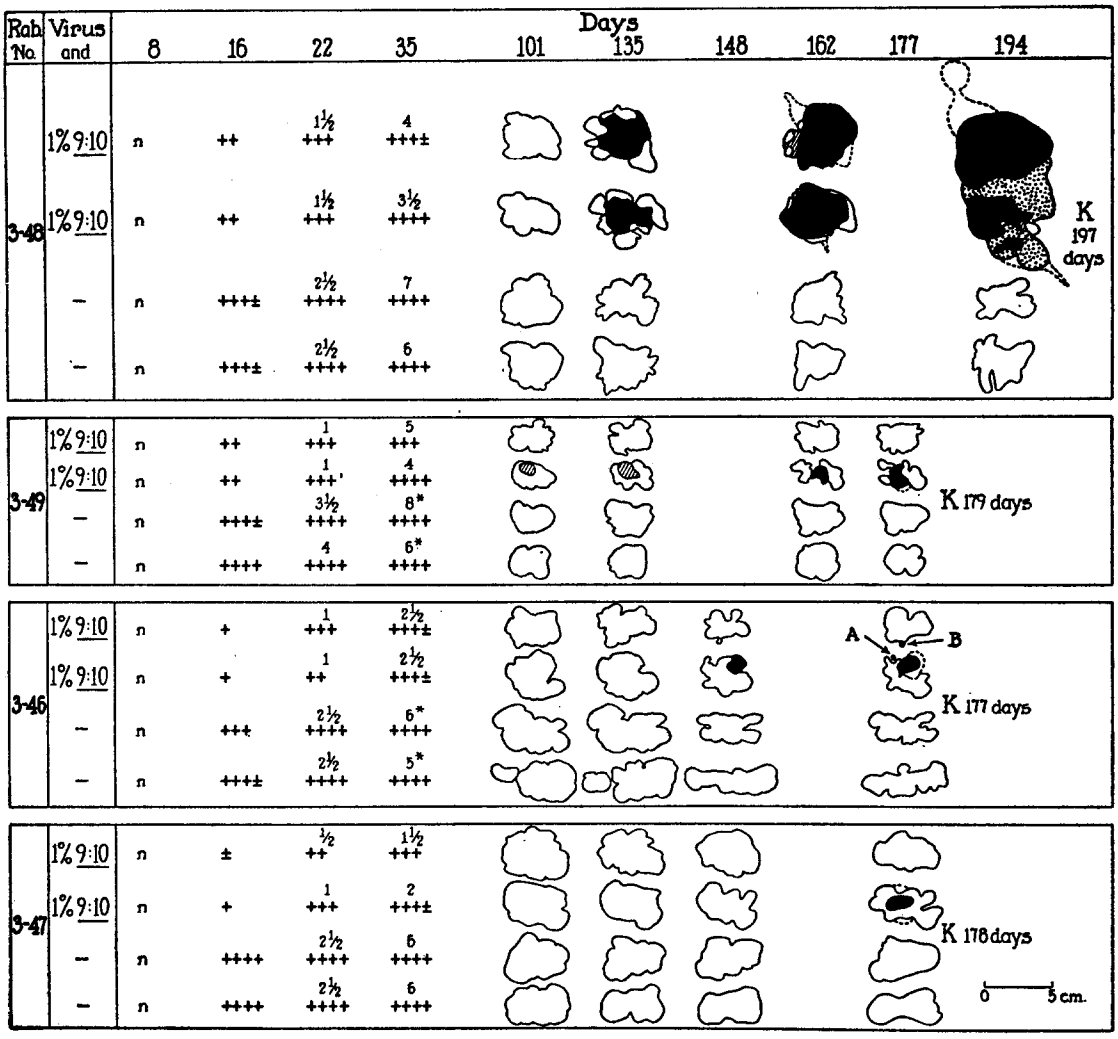

Rab. No.
Virus and
Days
8
16
22
35
101
135
148
162
177
194
3-48
1% 9:10
1% 9:10
—
—
3-49
1% 9:10
1% 9:10
—
—
3-46
1% 9:10
1% 9:10
—
—
3-47
1% 9:10
1% 9:10
—
—
K 197 days
K 179 days
K 177 days
K 178 days
A
B
0
5 cm.

CHART 4

mass, first charted on the 148th day, was a cystic, malignant papilloma, sharply different from the pustule cancer histologically.

D. R. 3-47 had notably vigorous paps. yet not until the 178th day was cancer discernible,—in one of the *9:10* masses. Already it had extended deep, and out to either side beneath the epidermis. Metastases were absent.

Again in this test cancer appeared in one or another or both of the *9:10* masses of every rabbit, yet not in any control mass. In two animals the papillomatous tissue was retrogressing when the malignant growths appeared, and in one of them (D. R. 3–46) it had lately disappeared from a situation at which cancer arose.

In the next experiment all was done in the same way, but the animals were killed still earlier.

Experiment 5 (Chart 5).—Dressings off after 5 days. The papillomatous masses all grew vigorously, and in **D. R. 3-51** cancer was present by the 120th day, in **3-52** by the 135th day, and in **3-53** by the 159th day. It appeared only in the *9:10* masses. **D. R. 3-50** had only paps. when killed; but an unexampled phenomenon had been noted in the previous week,—small verrucous growths had come into being next the masses at the *9:10* sites, in the angle where their bulging sides overhung the skin. At death the new little growths had become more numerous and larger. They were discrete, soft, pink, and delicately papillomatous, up to 4 mm. high, and with a broad or constricted base. A few were partly keratinized and rather dry. All had arisen from the skin deep in the angle, and several were somewhat flattened laterally by the pressure of the bulging mass next them,—from which however they were separated by ordinary hyperplastic epidermis. Each was sectioned in series, together with the adjacent, bulging pap. tissue (Figs. 18 and 21). The size of the little growths has been exaggerated in Chart 5 to show where they were.

In its main features this experiment corroborated the others, but the development of tiny papillomas on the merely hyperplastic skin next some of the confluent masses was wholly exceptional. More will be said of the little growths further on.

The outcome of these tests with *MC* and *9:10* was so consistent that those leading up to them need be dealt with but briefly.

Effect of Suspensions of the Hydrocarbons

The initial experiments were carried out with *MC* in suspension, as already mentioned.

Experiment A.—Separate 10 per cent extracts were made in salt solution, of the glycerinated virus paps. of five cottontail rabbits; pap. W. R. 152 was not amongst them. The fluids were cleared by repeated centrifugations; to portions of each an equal amount of acetone was added drop by drop; and light, pale flocculation followed. To other portions an equal quantity of acetone containing 0.3 per cent *MC* was added, with result in heavy, lemon-yellow flocculi containing many minute crystals of *MC*. Repeated pipetting was done to break up the flocculi and then each preparation was rubbed into a scarified square of hyperplastic skin on three rabbits, and paraffined gauze was applied directly to the raw surface. The squares were 2.5 cm. on a side, and the dressings were taken off after 7 days. One of the extracts

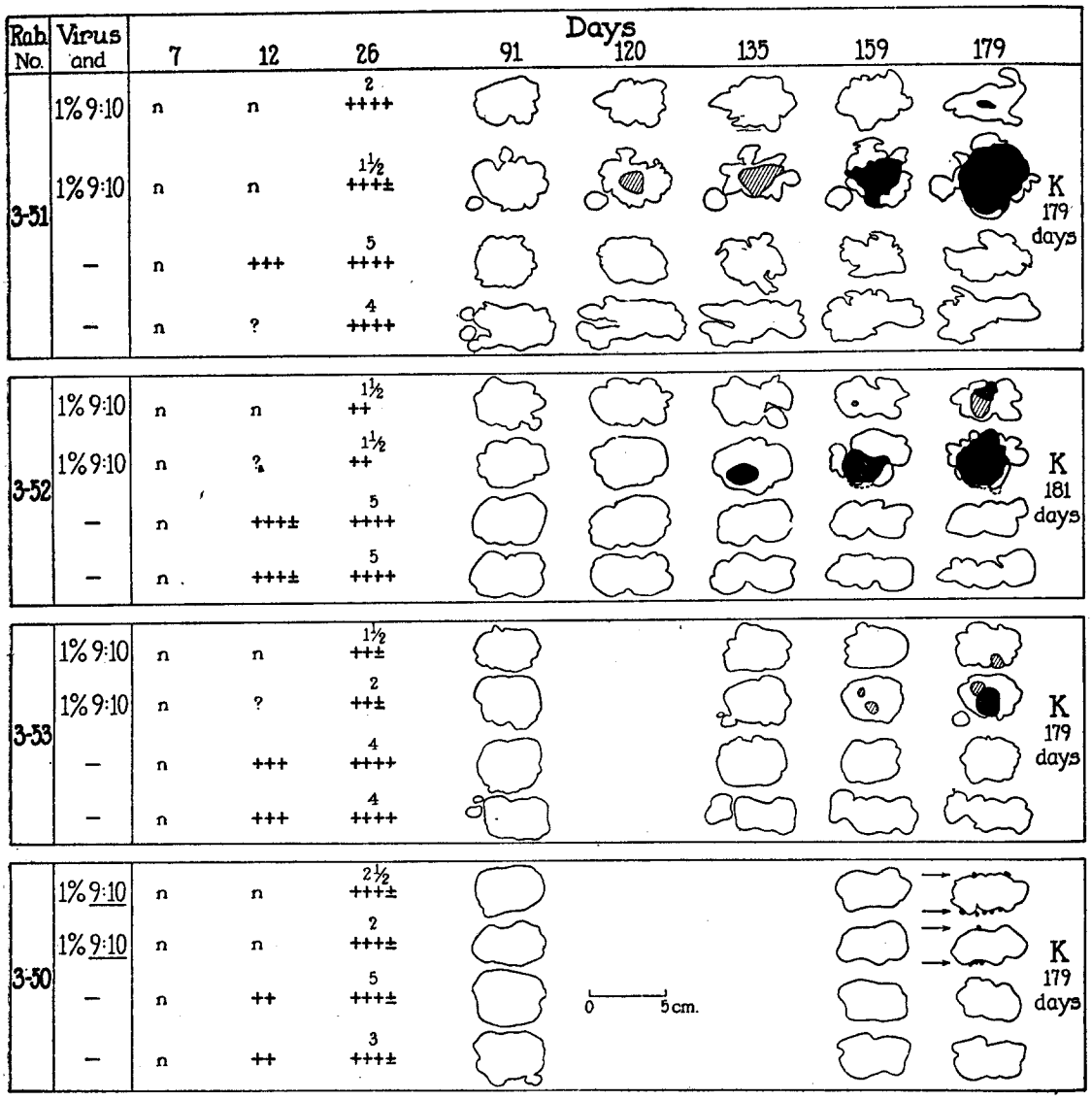

Rab. No.
Virus and
Days
7
12
26
91
120
135
159
179
3-51
1% 9:10
n
n
2 ++++
1% 9:10
n
n
1½ +++±
—
n
+++
5 ++++
—
n
?
4 ++++
K 179 days
3-52
1% 9:10
n
n
1½ ++
1% 9:10
n
?
1½ ++
—
n
+++±
5 ++++
—
n
+++±
5 ++++
K 181 days
3-53
1% 9:10
n
n
1½ ++±
1% 9:10
n
?
2 ++±
—
n
+++
4 ++++
—
n
+++
4 ++++
K 179 days
3-50
1% 9:10
n
n
2½ +++±
1% 9:10
n
n
2 +++±
—
n
++
5 +++±
—
n
++
3 +++±
0
5 cm.
K 179 days

CHART 5

proved devoid of active virus, but the others soon caused confluent pap. masses to form on the inoculated squares. The *MC* had no evident effect on the gross character of the growths, and for this reason two of the animals were killed after 18 and 35 days to learn whether it had caused histological alterations,—of which no signs were found. On the 140th day a downgrowth was noted beneath one of the actively proliferating masses on the remaining animal, produced by virus W. R. 632 together with *MC*, and by the 238th day this mass and an adjacent one, due to virus W. R. 633 with *MC*, were found to have been wholly replaced by a huge, ulcerated, obviously malignant tumor. There was a big metastasis in the axilla. The control paps., at corresponding situations on the other side, which had resulted from inocula containing the same virus materials and acetone as such, were still benign, as were all the other growths. A slice through the ulcerated mass showed everywhere a single carcinoma of peculiar morphology (Figs. 13 and 14). It was due to the virus material which had produced the most vigorous of the pap. masses (W. R. 632).

There was no sign in this experiment that the heavy flocculation caused by acetone had decreased the effectiveness of the virus; papillomas came in as soon and as abundantly as on the control areas. Nevertheless in the next test virus was added to the inocula only after flocculation had been completed.

Experiment B.—Subsidiary work had brought out the fact that the addition of rabbit serum to 0.9 per cent salt solution lessened the flocculation when an equal quantity of acetone containing 0.5 per cent *MC* was slowly stirred in; and 8 parts serum to 92 parts salt solution had proved optimal in such relation. Accordingly this was used, and when flocculation had ended 5 per cent virus W. R. 152 was added to the mixture in the same amount. The flocculi became smaller yet the microscope showed numerous tiny crystals of *MC* amidst them, although only 0.125 per cent of the carcinogen was now present. The control fluid containing acetone as such had not flocculated at all.

Inoculation was done into four rabbits with hyperplastic skin, each mixture into two 3 by 3.5 cm. squares in every case, with paraffined gauze coverage only and dressings off after 7 days. One rabbit died soon and another, killed after 239 days, had only papillomas. In a third (**D. R. 3-21**) cancer was first noted on the 141st day, in one of the confluent masses resulting from virus plus *MC*, although this mass, like its fellow, had seemed previously to differ no whit from the control masses, either in time of formation or in vigor. The malignant growth enlarged swiftly and on the 183rd day the animal succumbed. The new tumor (Figs. 15 and 16) had much the same peculiar microscopic features as that of Experiment A (Figs. 13 and 14). There were no other cancers.

The confluent masses of **D. R. 3-20** were fleshy for several months but by the 141st day had become dry and scoriaceous, and they began to dwindle soon after. On the 219th day no cancer was visible anywhere, but on the 230th an ulcerated growth more than 2 cm. across had appeared in a much diminished pap. mass situated where *MC* had long previously been applied. The animal was killed then.

The findings with D. R. 3–20 are charted because cancer had again arisen from retrogressing papillomatous tissue due to virus W. R. 152 (Chart 6). It appeared late. Only a 0.125 per cent suspension of *MC* had been applied to the inoculated area, but since it was partly in crystalline form some cells must have been exposed to it in maximal concentration.

For the next test *9:10* was used in suspension.

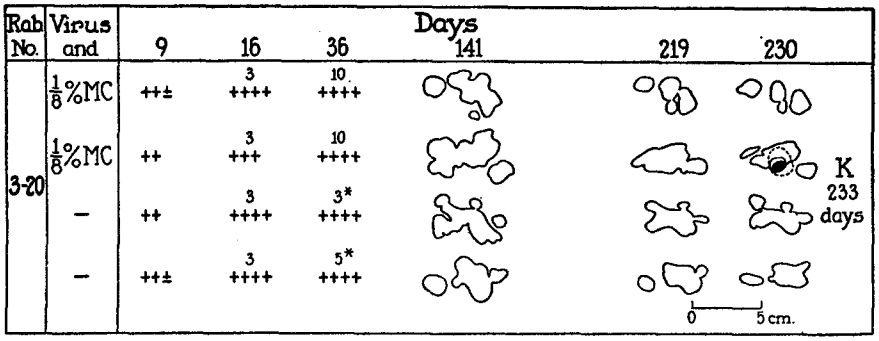


CHART 6

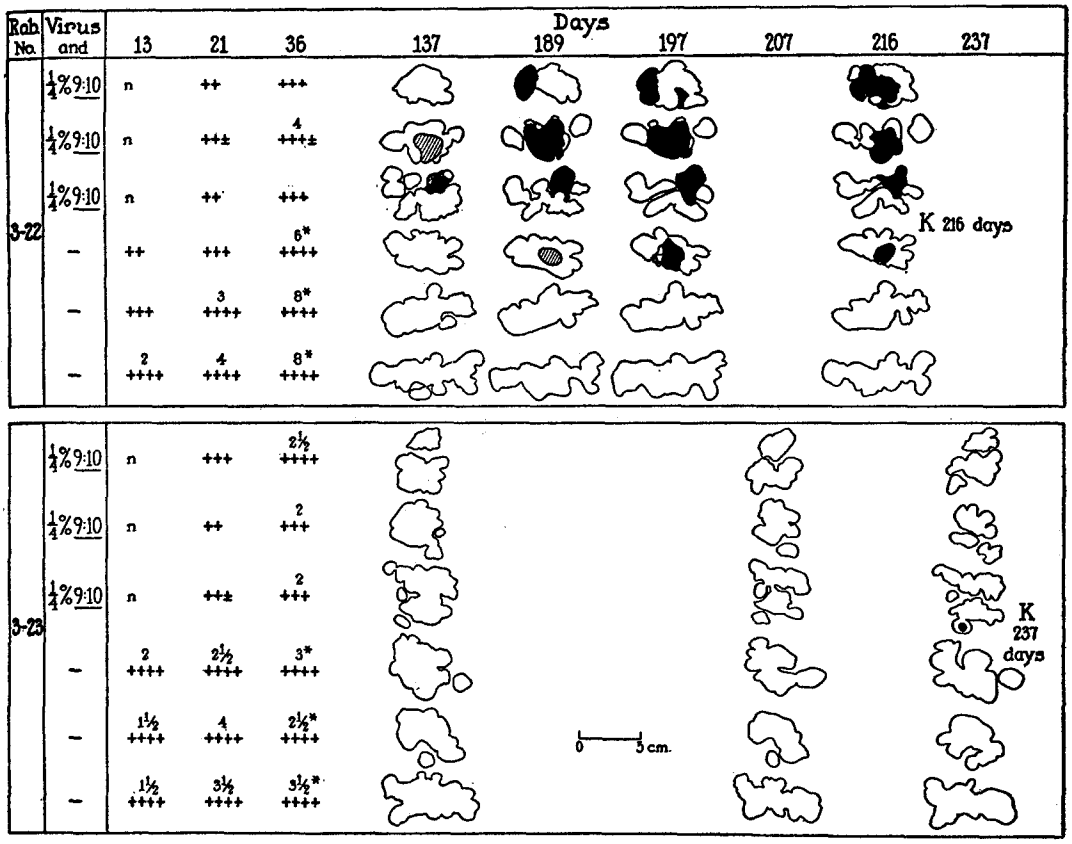


CHART 7

Experiment C (Chart 7).—The techniques, the virus material, and the number of animals were as in the experiment just described. Acetone containing 2 per cent of *9:10* was added to the serum-salt mixture, but the further addition of virus suspension diluted the hydrocarbon to 0.25 per cent. Many minute crystals of it had come out. The scarified areas, 3 by 3.5 cm., were covered with Cilkloid, followed by paraffined squares, as in Experiments 1 to 5; healing was complete when the dressings were taken off after 7 days.

By the 13th day paps. had appeared on the three control areas of **D. R. 3-22,** but none on those exposed to *9:10* (Chart 7). They had arisen by the 21st day however, and by the 36th were nearly confluent. The masses they later formed remained smaller, lower, and less fleshy than the controls, yet by the 137th day cancers had developed in two of them, and before the 189th in the third,—by which time one of the control masses looked as if it too might have become cancerous, a fact indubitable on the 197th day. All the paps. were now retrogressing. The rabbit was killed on the 216th day. Two of the control masses had remained free from cancer. No metastases were found.

In **D. R. 3-23** repression of the paps. on the *9:10* areas was still more marked. None had arisen by the 13th day although the control areas were already covered with confluent growths several millimeters high; and those that arose later were considerably less numerous and relatively indolent, though they eventually coalesced into masses. By the 207th day these latter had dwindled considerably, much more than the controls, and were separating into islands; yet autopsy 30 days later showed cancer in one, whereas it was everywhere absent from the controls,—which had remained confluent.

The other two animals died early, after 27 and 78 days respectively. A retardation and partial repression of paps. at the *9:10* sites had been noted in both, but the virus-infected cells did not differ from the ordinary microscopically.

Again the findings were consistent but the epidermal cells must have been unequally exposed to the hydrocarbon, as in Experiments A and B, because of its partly crystalline form. Furthermore there was no certainty as to how long exposure to it had lasted. These were the reasons why gauze saturated with oil containing the carcinogen was utilized in Experiments 1 to 5.

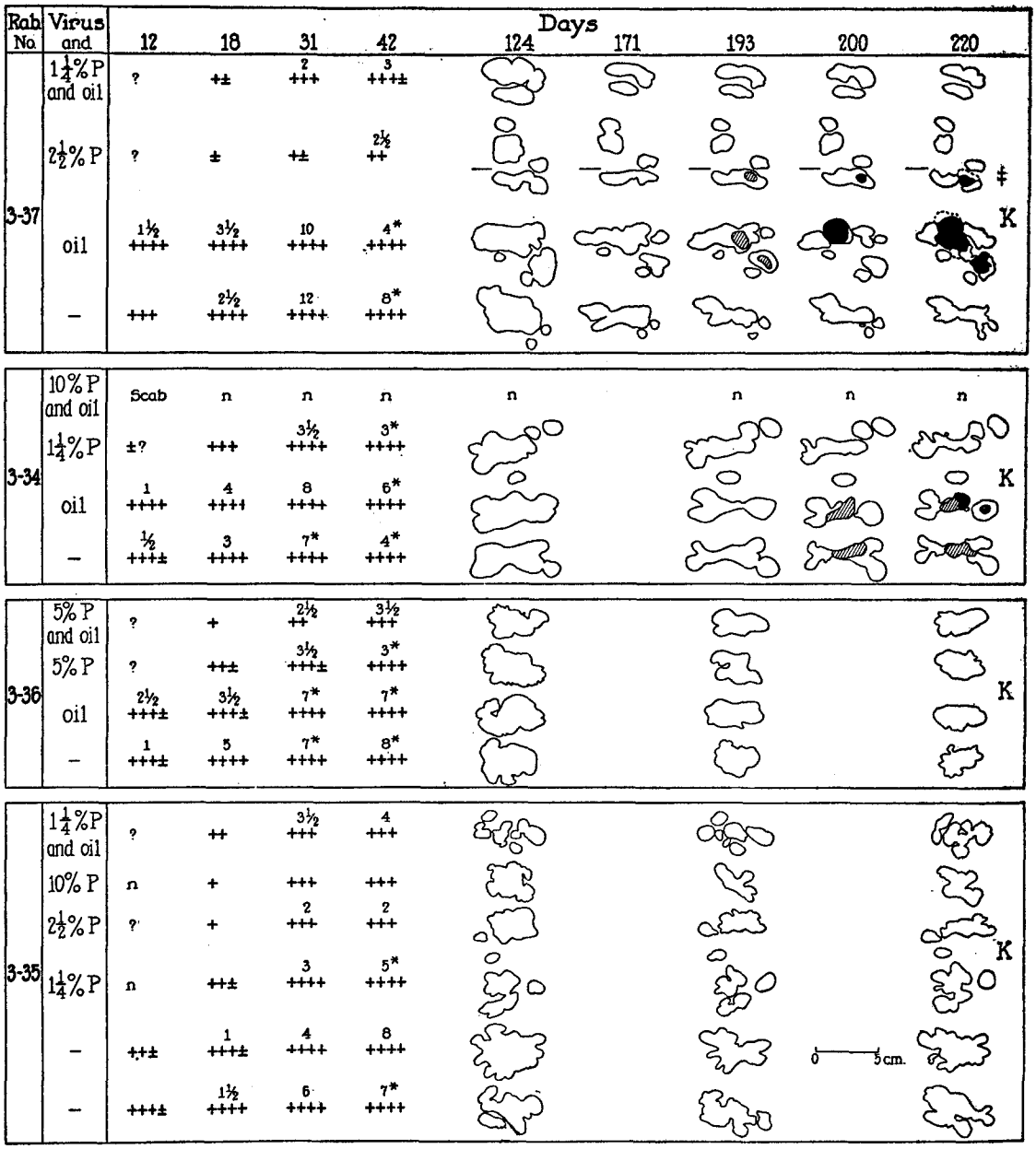
In the remaining test only one animal lived long, and it remained free from cancers.

Experiment D.—A mixture was prepared as in Experiment B, containing *9:10* in 0.25 per cent suspension and 2.5 per cent virus W. R. 152, and all was done as in this test save that the scarified areas were directly covered with Cilkloid instead of paraffined gauze. When the dressings were taken off, after 6 days, healing had been completed.

The *9:10* exerted its usual repressive effect on the paps. arising on the two inoculated areas exposed to it on each animal, yet they slowly formed masses. Two of the rabbits were killed on the 54th day to learn whether they showed any changes referable to the carcinogen,—which was not the case,—and a third died on the 81st day. The remaining animal was kept for 244 days, throughout which period its paps. did well; yet none became cancerous. A collateral finding of significance will be described further on.

The Effects of Podophyllin

Some unplanned controls to the action of the carcinogens were provided by an experiment with podophyllin (Chart 8). It had been set up to learn whether this poison, productive of mitotic abnormalities (5), would so affect epidermal cells exposed to it at time of virus infection that unusual tumors would result.



‡ The transverse lines separating some of the traced outlines from D. R. 3-37 mark the lower margin of the inoculated square exposed to 2.5 per cent podophyllin in its relation to an adjacent pap. mass due to accidental virus infection. This mass, an inadvertent control, became cancerous.

CHART 8

The podophyllin (resin podophyllum Merck N. F.) was not entirely soluble in acetone, a small residue persisting. For test it was ground in a mortar; 10, 5, 2.5, and 1.25 per cent preparations of it were made with acetone; and squares of gauze were saturated with each. The insoluble residue was kept suspended by agitation while the fluid was dripped on, and the squares were turned repeatedly as drying took place in order to distribute it evenly. Some of the impregnated squares were employed as such, and others were saturated with mineral oil just prior to use (heavy California mineral oil, Squibb).

In the experiment of Chart 8, three areas of hyperplastic skin, 3.5 by 4 cm., on each of three rabbits, were inoculated with 2.5 per cent virus W. R. 152 and covered with gauze carrying one or another of the podophyllin preparations; and three other areas, at corresponding situations on the opposite side of the body, were covered after inoculation with dry gauze or gauze soaked with mineral oil, as the control needs required. Three squares on one side of a fourth inoculated animal were dressed with podophyllin gauze, and on its opposite side there were two control squares and yet another podophyllin square. Cilkloid was put over every square, followed by paraffined gauze as usual.

When the dressings were removed after 7 days the areas on which gauze carrying 10 per cent podophyllin had been put, either as such or in oil, were found to be covered with a cheesy layer several mm. thick of dead leucocytes, with no healing as yet underneath. So too where oiled gauze carrying 5 per cent podophyllin had been put, though where it had been applied without oil the skin had healed though markedly swollen. The control areas and those exposed to podophyllin in smaller amounts had also healed and were not swollen. The cheesy layers were left in place but dressed with ordinary gauze, the over-all binder was replaced, and in rather more than another week the cheesy stuff had dried and come away from a surface covered with thin, glazed epidermis. The development of papillomas was delayed and their number cut down in rough proportion to the amount of podophyllin that had been applied, but in most cases confluent or semiconfluent masses eventually formed. The control growths were regularly the more vigorous, and in two animals they became much the larger.

On the 193rd day cancers could be seen at two spots in a control mass of **D. R. 3-37,** and also in what was, to all intents and purposes, another control growth, namely a small pap. mass due to accidental virus infection of abraded hyperplastic skin lying outside a square covered with gauze containing 2.5 per cent podophyllin. Its situation is shown in the chart. The cancers enlarged but slowly during the 27 days before the animal was killed, and the paps. themselves became smaller.

No paps. arose on **D. R. 3-34** where 10 per cent podophyllin had been put with oil, and only a few where 1.25 per cent had been applied dry, all developing late and mostly at the edge of the square. In each of the control masses there was a cancer by the 200th day, and by the 220th another had developed in one of them,—this although the papillomas were retrogressing, as in the case of D. R. 3-37.

All the growths of **D. R. 3-35** and **3-36** did fairly well, yet no cancers developed in any.

Another similar test on four rabbits, but with the 10 per cent preparation of podophyllin omitted, resulted in the same delayed appearance and reduced number of paps. where the substance had been put. The confluent masses formed later were smaller and less vigorous than the controls and cancer was still absent from all when the animals were killed on the 206th day.

In these tests the podophyllin delayed the appearance of papillomas and cut down their number just as *9:10* had done, with result in relatively small masses, sometimes only semiconfluent and in general less active than the controls; yet no malignant changes took place. Such cancers as arose were situated in the control growths and were late in appearing.

The Presence of Latent Neoplastic Potentialities in Skin Exposed to "9:10"

A single painting of mouse skin with *MC* sometimes suffices to induce cancer (6, 7), and *9:10* much exceeds *MC* in carcinogenicity, notably for the rabbit (8, 9). This being so, it had seemed possible that the exposure of regenerating epidermis to these powerful agents might induce neoplastic changes where they were applied alone under the conditions of the present experiments. Hence steps were taken in all of these latter except A and C to learn whether this was the case: on each animal two squares of hyperplastic skin were scarified but not inoculated, though dressings that contained *9:10* or *MC* were applied to them just as if they had received virus. The skin thus treated soon regained the aspect of that round about and remained bare of tumors, yet this could not be taken to mean that no neoplastic changes had taken place; for previous work with the chemical carcinogens (10) had shown that they render many cells neoplastic which remain hidden, for long periods sometimes, and that these latent elements can be made to disclose themselves not infrequently by forming tumors, if some agent or influence stimulating the hyperplastic proliferation of normal cells of the same sort is brought to bear (10–12). Chloroform is such an agent; incapable of initiating neoplastic changes in rabbit skin, it yet promotes tumor formation (11). In this knowledge it was now repeatedly applied to one of the two squares on each animal that had been exposed long previously to *MC* or *9:10* only.

The paintings were begun 3½ to 5 months after the gauze containing *MC* or *9:10* had been taken off the healed areas. Reagent chloroform was regularly used, and it was put on all but two of the rabbits of Experiments 1 to 5, and on the one animal of Experiment D which lived long. The treated areas were kept bare with the clipper, together with a broad zone round about them, and this was painted as well. The chloroform was applied lightly with a soft brush, three times a week, and whenever the inflammation it induced threatened to become severe the paintings were intermitted. The treated skin soon became bright pink and within 10 days was twice to three times as thick as before (when felt in a fold), often a little scurfed, and more or less gray; fur reappeared on it much more quickly than on the neighboring normal surface, clipped but not painted. In this state it was kept throughout the weeks or months until the animal was killed.

The chloroform, though inducing epidermal hyperplasia (Fig. 26), brought out no tumors where *MC* had long previously been applied (seven rabbits of Experiments 1 and 2); but growths appeared on the squares of three out of eleven animals, which had been exposed to *9:10* (Experiments 3 to 5). None arose on the surrounding painted zones of unexposed skin.

All of the growths were small, discrete papillomas. One developed within 16 days on the chloroformed square of 3-53 (Experiment 5, Chart 5) and on the 23rd day it was excised for section (Fig. 29). 5 weeks later cancers were found in one of the masses of virus pap. tissue derived from cells exposed to *9:10* at time of inoculation. When the animal was killed, 77 days after the first application of chloroform, two more papillomas were present on the painted square.

In 3-50 of the same experiment the chloroform had called forth a papilloma by the 42nd day of the paintings, and at death on the 179th day (78 days after the first chloroform) six more growths of the sort were present. Not only this, but some had formed, as already described, in the angle next the overhanging edge of those virus pap. masses which had originated

from the epidermis exposed to *9:10* (Figs. 18 and 21). No cancers arose either from these or from the control masses.

In D. R. 3-47 of Experiment 4 chloroform brought out a papilloma within 64 days after the paintings were begun. It was first noted at autopsy, by which time cancer had appeared in one of the virus pap. masses where *9:10* had been put at time of inoculation. The history indicates that the malignant growth had been present at least a month.

Gauze soaked in olive oil containing 1 per cent *9:10* was used as the original dressing in all these instances. To them should be added one from Experiment D, in which *9:10* had been applied in 0.25 per cent suspension, partly in crystalline form. Chloroform was first applied on the 138th day, and 44 days later a papilloma had arisen on the painted square, which was excised after 34 days more (Figs. 22 and 23). The animal was kept for 244 days in all, but no cancers appeared in any of the masses of virus papillomatosis,—which latterly had been dwindling. The other rabbits of Experiment D had been killed early.

No tumors appeared at any time on the control areas exposed to the hydrocarbons but to which nothing further was done.

All of the growths called forth by chloroform were sectioned serially.

In our experience chloroform, like turpentine, exerts but a mild and inconstant influence to promote the multiplication of epidermal tumor cells (11), and it does not stimulate the underlying connective tissue elements sufficiently for them to provide more than a meager support to the papillomas it evokes. Hence it was no surprise to find that the growths elicited in the present instance were slow growing and delicate, not rapidly enlarging and fleshy like those of similar sort which appear where tarring is done, or those which start forth where punch holes are healing in ears previously submitted to tar, benzpyrene, or *MC* (10–12). They had however, the general structure and the cytology of the papillomas induced by the hydrocarbons (7, 13),—which is to say that they differed distinctively from the papillomas caused by the Shope virus (Figs. 18 to 23, 29).

As already stated, the agents promoting the formation of tumors by latent tumor cells act in this way incidentally to their stimulation of normal elements of the same sort. The occurrence of cutaneous papillomas in the angle next the overhanging edges of the virus-induced masses of D. R. 3–50 (Experiment 5) can be understood in the light of this fact. Where the growths appeared, pronounced hyperplasia of the normal epidermis had taken place, as the result doubtless of warmth, moisture, and hyperemia. The new tumors arose only next the sides of the masses that had originated from skin exposed to *9:10* at time of virus infection and only where these were still within the limits of the exposed areas (Chart 5), not at their ends where they had extended out. None occurred next the control masses.

It will be recalled that chloroform was applied to only one of the *9:10*ed or *MC*ed squares of skin on each rabbit, not to the other. This latter appeared in no way pathological in the gross, save in some instances for a slight graying which served to define its limits. Where this was the case a slice was taken through it and the adjacent normal skin, for purposes of microscopic com-

parison, and almost regularly the epidermis long previously exposed to *9:10* was found to be 2 to 4 cells thick (Figs. 24 and 28), whereas the normal layer was only 1 to 2 cells thick, with shallower hair follicles (Figs. 25 and 27). Similar though less definite differences were to be seen in some of the specimens of *MC*ed skin. Unfortunately no controls were available of skin that had been only sandpapered and dressed with oiled gauze at time of inoculation, so one cannot tell whether the persisting slight epidermal hyperplasia may not have been merely residual to scarification and repair. Whether the exposure to *MC* had been too brief to confer neoplastic potentialities on the skin subsequently painted with chloroform, or the promoting influence of this agent was too slight to call them forth remains uncertain also. The areas repeatedly painted with chloroform were moderately to markedly hyperplastic (Figs. 26 and 29), having a many-layered epidermis and follicles deep in the corium.

Character of the Cancers

The virus papillomas formed through the proliferation of cells which had been exposed to *MC* or *9:10* at time of inoculation did not differ from the ordinary microscopically; and the cancers subsequently developing were in the main wholly like those deriving from virus papillomas under ordinary circumstances and resulting from secondary changes in virus-infected cells (14–16). They have been so often pictured that there is no need to do this again. Most of them were frank squamous-cell carcinomas, but not a few had retained many of the features of the papillomas from which they derived, although sometimes arrantly malignant. Often several were present in the same mass, and occasionally more than one metastasized to the same gland (Fig. 12). Marked expansile proliferation, taking place within some of them, acted to broaden the papillomatous masses in which they had arisen (Fig. 5).

A watch was kept for "anomalous tumors" such as develop when tar papillomas are infected experimentally with the Shope virus (1–3). These are mongrel growths exhibiting in varying proportions the mingled features of virus papillomas and those due to tar. No such tumors were encountered but the cancer of Experiment A, derived from virus-infected epidermis exposed to *MC*, had features (Figs. 13 and 14) recalling the morphological changes sometimes appearing when cancers due to tar or methylcholanthrene are acted upon secondarily by the Shope virus (4). So too with the cancer of D. R. 3–21 of Experiment B (Figs. 15 and 16). In both instances the carcinogenic hydrocarbons, *MC* in the one case, *9:10* in the other, had been applied in suspension, largely in crystalline form. Later, when they were applied in olive oil, only ordinary carcinomas were obtained. With these questionable exceptions, the findings were wholly like those when methylcholanthrene or tar, or a mixture of the two, is repeatedly applied to preexisting virus papillomas (4). On two occasions the malignant epithelial cells were fusiform (Fig. 17).

No papillomas with the morphology of those due to the chemical carcinogens

were encountered within the virus masses affected by *9:10*. Being unassertive in the absence of stimulation (as by chloroform), they either may not have formed or else they chanced to be absent from the sections studied.

DISCUSSION

Under ordinary circumstances cancers derive oftenest and earliest from the virus papillomas which proliferate most actively, those produced by the inocula of greatest pathogenicity (14–16). Had an inoculum of this sort been utilized in the present work, the control papillomas might have become cancerous so frequently and so soon as to have proved frustrating. The mild virus W. R. 152 was a fortunate choice. Such viruses give rise to growths that are especially liable to disappear, but Kidd has shown that when retrogression takes place confluent masses persist much longer than individual papillomas of the same inoculation (17); and in this knowledge virus W. R. 152 was brought to bear under such conditions that it gave rise to broad masses of papillomatous tissue. A large proportion of those serving as controls dwindled eventually, as was to have been expected, but none disappeared during the period of test. Cancers arose from them late and infrequently (in only five out of the 58 instances of the charts), making plain that the state of affairs within the papillomas was unfavorable to malignant change.

According to some workers (18, 19) virus papillomas pass through three phases, proliferative, stationary, and involuntionary, the growths either retrogressing after many months or undergoing then an "involutionary carcinomatous change." In our extensive experience with inoculated cottontails (20, 21) and domestic rabbits an initial phase of proliferation has regularly been noted, needless to say, as the papillomas came into being, and retrogression often took place early, after only 6 to 8 weeks had gone by. Well established confluent masses usually become stationary (22) or actually diminish somewhat in area, owing to cicatricial contraction of the reactive connective tissue underlying them, a change exemplified by the growths of Chart 5, and readily distinguished from true retrogression,—during which the papillomas gradually become smaller and more keratinized, break up into islands, and slowly flake away (22). The stationary state does not entail any lessening in proliferative activity, which on the contrary often increases, notably before cancerous change takes place. Cancers sometimes arise from retrogressing papillomas, as happened in the present work, but under ordinary circumstances this is highly exceptional. Where malignant change is about to take place, the keratin overlying the growth usually becomes less tenacious and desquamates, the mass getting lower as result; but concurrently the basal layer of living papillomatous tissue thickens and deepens, bulges at the sides and below, and becomes somewhat disorderly, sometimes even invasive,—changes all that betoken a stepping up of activities (14). The "spontaneous" papillomas of wild cottontails from which cancers have derived under our scrutiny were exceedingly vigorous at the time (Figs. 7, 9, and 21 of reference 20); and the greater the vigor of the papillomas of domestic rabbits the greater is the likelihood

that malignant change will occur, as has already been mentioned[2] (14–16). In sum, these facts speak wholly against the concept of a terminal "involutionary carcinomatous change" in virus papillomatosis.

The conditions prevailing in the growths derived from epidermal cells exposed to the action of *MC* or *9:10* at time of virus infection differed markedly from the ordinary, as the hastening of cancerous change attested and its frequent occurrence in retrogressing growths, an exceptional happening under ordinary circumstances. *MC* failed to hurry the appearance of papillomas and in some instances delayed it (D. R. 3–43 and 3–45, Chart 1), cut down their number, and so reduced their vigor that they did not for a while reach the height of the controls, though usually forming confluent masses eventually which occupied as large areas as these latter but sometimes remained lower and less fleshy, bulged less at the sides, and were less often gnawed down by the host animal, a common happening when growths are exuberant.[3]

The *9:10* had a marked repressive influence. Where it was put the papillomas appeared relatively late (Charts 3 to 5), were fewer, often much fewer, and formed confluent masses tardily, sometimes indeed only semiconfluent ones. These differences were more pronounced with 1 per cent *9:10* than with 0.5 per cent (Chart 3). Both had enduring effects that were greater than those of *MC*. The confluent masses frequently showed less vigor than the controls, some being dry almost to the base, whereas these latter, situated at corresponding places on the other side of the host, were fleshy. When retrogression occurred, a change which regularly implicates in greater or less degree all the growths resulting from the same inoculum on the individual rabbit (17), the papillomas influenced by *9:10* dwindled the more rapidly. This may well have been due to their less dense or imperfect confluence, since individual papillomas vanish first, as already remarked. The question arises of whether *MC* and *9:10* may not have exerted some special, lasting influence to bring on the

[2] The charts of previous work (4) in which preexisting confluent expanses of virus papilloma tissue were repeatedly tarred or painted with *MC* have pertinence in this relation. Cancers appeared soonest and were most frequently multiple in those papillomatous masses which did best.

[3] A curious finding repeatedly noted was a dark gray, or actually black, coloration of the paps. arising where *MC* had been put,—this although the control paps. at corresponding sites on the opposite side of the animal were pink or pale gray. The melanosis was not due to the slowness with which some of the growths appeared and proliferated (as providing a longer time for the melanoblasts to darken them), nor can it be laid to delayed healing of the skin with result in a relatively great migration of these cells along with the epithelium extending from the hair follicles to cover the denuded surface; for no increased pigmentation resulted from *9:10*, which had a far greater retarding influence. In experiments directed to other ends we have noted that the repeated direct application of 0.3 per cent *MC* in Crabtree's fluid to skin into which virus had been tattooed 7 days previously caused paps. to appear earlier than at control sites and to grow more rapidly, as further that the treatment frequently caused them to turn black.

observed retrogression of the papillomatous masses from which some of the cancers came; but this assumption seems unwarranted since the control growths of the same animals also retrogressed, though to a less extent (Chart 7), a difference readily understandable in view of what has just been said.

From the findings with ordinary papillomas it was to have been expected that the growths repressed in various degree by *MC* or *9:10* would become malignant less often than the controls. The outcome was all the other way. Of the 50 control papillomas on the rabbits to which the hydrocarbons were applied (Charts 1 to 7) only two became malignant during the period of observation, whereas cancer occurred in eight out of 20 *MC* masses, and in 23 out of 30 influenced by *9:10*, often appearing unusually soon and at several situations. It asserted itself before the hundredth day in D. R. 3–49 (Chart 4), before the 120th in another animal, and in three others had given rise to large tumors by the 127th day, that of first charting, and in one such instance had by then extended prongs to a considerable distance into the subcutaneous tissue. No effort was made to learn how long the control masses would remain free from cancer, but in D. R. 3–28 (Chart 3) they gave no sign of malignancy throughout more than 100 days after the *9:10* masses had become cancerous, nor did they during the 2 months' survival of D. R. 3–48 (Chart 4) and 3–51 (Chart 5). Many of the rabbits failed to live long after cancer had appeared in the *MC* or *9:10* masses, and some were killed almost at once after it was first noted, thus cutting short observation of the controls.

All of the non-specific, collateral forces recognized up to now as hastening malignancy stimulate proliferation of the papilloma cells (14). *MC* and *9:10* had the contrary effect. Again and again in the individual records the note appears that the *9:10* masses were dry to the base and scoriaceous, whereas the controls were fleshy,—with the added comment that the latter seemed much the more likely to become malignant. Yet it was from the dry, indolent, apparently unfavorable papillomas influenced by *9:10* that the carcinomas developed, even appearing in one animal at spots from which the growths had almost or quite vanished (D. R. 3–46, Chart 4 and Figs. 9 to 11). The influence of the chemical carcinogens on the virus-infected cells was so lasting and powerful as to cause them to become cancerous frequently under conditions ordinarily unfavorable to the change.

A first question to be settled, when appraising the influence of the hydrocarbons, is whether they acted in their carcinogenic capacities. The virus indubitably did this; the observed culmination in cancer was that for which it is ordinarily responsible. But the chemical carcinogens produce marked collateral changes in the tissues they act upon, causing chronic inflammation in the corium of skin exposed to them for any considerable time; whence it follows that *MC* and *9:10* might conceivably have exerted a non-specific influence on the virus papillomas by way of such disturbance, and thus have

accelerated malignant change. The experiments made plain that this was not the case:–

The hydrocarbons were applied for so brief a time as to cause little enduring change in the control expanses of skin exposed to them. Within a few weeks the areas on which they had been put were indistinguishable in the gross from the adjacent skin, save for a lingering gray pigmentation in some instances which vanished later on; and in specimens taken at autopsy the microscope disclosed no abnormalities where *9 : 10* had been applied except a slight epidermal thickening with deepening of the follicles (Figs. 24 and 28), and more dubious changes of the same sort in the case of *MC*. The corium was not perceptibly altered.

According to previous workers *9 : 10* greatly exceeds *MC* in carcinogenic power, notably in rabbits (9). The outcome of the present experiments accords with this finding. Cancers appeared in all four animals exposed to 0.5 per cent *9 : 10* in olive oil and in ten out of twelve of those that had received a 1 per cent solution (Charts 3 to 5). They derived much less often from the papillomas due to cells exposed to *MC*, three of eight rabbits developing cancers where 0.5 per cent *MC* had been applied, and two out of four of those submitted to a 1 per cent solution (Charts 1 and 2). Four of the animals with papillomatous masses that remained benign were retained unusually long, 203 to 229 days.

The oncogenic power of *9 : 10* was so great as to confer neoplastic potentialities on some epidermal cells of control areas that had been merely scarified and exposed to the substance while healing took place,—a fact proved by the appearance of papillomas like those due to the hydrocarbons (11, 13), in response to the stimulating influence of chloroform on cell proliferation (Figs. 22 and 29). Papillomas of this kind even arose where their formation had not been purposely promoted (Figs. 18, 20, and 21); they came into existence where local conditions had brought on hyperplasia of skin long previously exposed to *9 : 10* (D. R. 3–50, Experiment 5, Chart 5). In contrast to these findings, chloroform called forth no growths where *MC* had been applied. Again the results fall in with what is known of the relative powers of *9 : 10* and *MC* to bring about neoplastic change.

All in all, the conclusion seems justified that *MC* and *9 : 10* acted in their peculiar capacity as carcinogens when determining precocious malignant changes in the virus papillomas.

But on what and when did the hydrocarbons act? *MC* produces no alteration in the Shope virus when the two are kept in contact at body temperature throughout weeks (4), and hence a direct influence of it upon the inocula utilized in the present experiments seems out of the question. There is no doubt on the other hand that the epidermal cells which became infected with virus during exposure to *9 : 10* were exposed long enough for the latter to have conferred neoplastic potentialities upon some of them; for chloroform dis-

closed the presence of cells with latent neoplastic potentialities in some control areas of skin to which *9:10* had been applied for the same time. Under the experimental conditions the carcinogens had opportunity to act upon the regenerating epidermis just prior to virus infection, during it, and for one to several days afterwards. A few of the virus entities rubbed into the sandpapered surface may have reached susceptible cells at once, those of the cut ends of the hair follicles, but the vast majority were strewn on a raw stroma and must have come into contact with epithelial elements only secondarily, when these spread laterally from the follicles in coverage. Such spread occurs swiftly however, if the skin has already been rendered hyperplastic, as was the case in our experiments; under such circumstances, according to Friedewald (23), a scarified area may be overlain in 48 hours with a thick sheet of new epidermis, and in another 48 this sheet may show papillomatous changes where the Shope virus was rubbed in. True, *9:10* much slowed repair, but nevertheless healing had been well completed when the dressings were taken off after 5 to 7 days. It would appear then that one cannot localize the epidermal effects of the hydrocarbons to any special stage in the events occurring during exposure to them.

With two dubious exceptions (Figs. 13 to 16) the cancers wholly resembled those arising from preexisting virus papillomas painted week after week (4) with tar or *MC*,—which is to say they were just such as derive from papillomas becoming malignant spontaneously. Findings of quite another sort are obtained when tar tumors are experimentally infected with the Shope virus by way of the blood stream (1–3). Some of these growths keep their original morphological features, but at once begin enlarging with unprecedented rapidity, while others become "anomalous tumors," exhibiting in greater or less degree the cytological features characteristic of virus papillomatosis, while retaining others that are characteristic of papillomas due to the hydrocarbons. Even the carcinomas induced by tar or methylcholanthrene may become "anomalous" after experimental virus infection, their cells exhibiting signs of its influence.[4] In the instance first mentioned the virus produces no morphological effect, though exerting a great one on cell proliferation, and in the second and third its effect upon morphology is but partial; its influence has not entirely superseded that of the actuating cause of the tar tumors, whatever this may be. In a fourth set of instances however, only now mentioned, it takes over wholly, converting tar papillomas or frill horns (Figs. 30 and 31), or even carcinomas, into virus papillomas (1–3). This happens so frequently as to indicate that its influence upon morphology is much more powerful than that of the actuating cause of the growths due to tarring. One can comprehend in

[4] After intravenous injection of the virus, tumors of all the sorts mentioned were observed to start forth not infrequently from skin where no growths had been visible. The later demonstration that tarring often brings into being tumor cells which lie latent in the epidermis (10) makes the supposition reasonable that the virus took effect on such elements.

the light of this fact why tar, *MC*, and *9:10* fail to alter the morphology of cells in which the Shope virus has already become ensconced.

Many instances are known in which physical and chemical agents have acted together or successively to bring on cancer more often and sooner than either could alone, and there has been no obstacle to supposing that their influences were combined or summated; for when brought to bear singly on the tissues of test they have caused preliminary disturbances of nearly the same sort, so far as could be perceived, and the benign and malignant tumors eventually induced have had essentially similar morphological traits. But the case of the Shope virus and the hydrocarbons presents difficulties because of basic differences in their relation to the cells they influence (24), differences which need not be gone into here. The papillomas they both induce have a close resemblance in all major respects (11, 13), yet they exhibit cytological features whereby they can be readily distinguished. When tar papillomas become infected with the virus secondarily, the morphological effects of the two agents are not merged but are either mutually exclusive,—as in the growths which remain tar tumors morphologically or are converted into virus papillomas,—or else they are mingled, not blended, as in the cells of the "anomalous tumors." True, if one assume that the formative influences of the virus and the hydrocarbons are collateral to their carcinogenic action,—mere differing side influences unrelated to their abilities to induce neoplastic change (which may well be identical in nature),—then all would seem clear; and the conclusion would be justified on *a priori* grounds that here is just another, if unique, instance of the combined or summated effects of widely differing carcinogens. The frequency with which tar papillomas,—from which the tar cancers generally take off,—are summarily converted into carcinomas on infection with the virus might be cited in support of this conception.

Certain findings of the present experiments stand in the way of such an explanation. The rabbits which had cells most susceptible to the oncogenic influence of *9:10* when applied alone, as proven by the tumors chloroform called forth from skin exposed to it, were not those in which cancers derived soonest and oftenest from the cells influenced by virus as well. The animal of Experiment 5 which developed most papillomas on skin exposed only to *9:10* (D. R. 3–50, Chart 5) remained wholly free from cancers where the latter had acted together with virus, the sole individual of its group of four which failed to develop them. In one other rabbit of the same group (D. R. 3–53) chloroform also evoked papillomas, and it was the last to develop cancer. D. R. 3–47 (Chart 4), also the last of its group to get cancer, was the only one to yield a papilloma in response to chloroform. In Experiment D chloroform elicited a tumor where *9:10* had been applied in crystalline form; yet during 244 days of observation no cancer arose from the mass of virus papilloma tissue formed through the proliferation of cells exposed to its influence.

These instances are few and the promoting effect of chloroform on latent

tumor cells is often erratic (4), but in previous experiments a similar lack of parallelism was noted between the tumor-producing effect of tar and *MC* on normal skin and the occurrence of cancers amidst the virus papillomas of the same animals, which were treated with these substances.

It may be objected that oncogenic action is estimated on the one hand in terms of papillomas, and on the other in carcinomas; but it has been the general experience that those rabbits which are most liable to papillomas in response to the action of the carcinogenic hydrocarbons are also those most prone to have cancers,—which indeed generally take off from the papillomas.

It is possible that epidermal cells infected with the Shope virus become so altered in responsiveness to the chemical carcinogens that the effects of the latter on normal elements tell nothing concerning them; but a nearer reason why the discordance in findings now under consideration does not bulk large is to be found in the circumstance that thus far only one major factor in the carcinogenesis, namely the hydrocarbon, has been considered. Nothing has been said of the relationship existing between virus and cell as making for malignancy (25), although in some animals it determines cancer soon, whereas in others not until late or perhaps never. Cancer arose so seldom in the control masses of virus papilloma tissue of Charts 1 to 7 as to yield no information on the part played by such individual differences in the experiments now under discussion.

However all this may be, no doubt exists that the exposure of rabbit epidermis to *MC* and *9:10* at time of virus infection had enduring effects on the cells of the resulting papillomas, effects falling short of the immediately neoplastic yet persisting throughout long periods of proliferation and eventually hastening cancerous change. Evidence has recently been obtained, both in man (26) and the rabbit (12), which indicates that the exposure to chemical carcinogens sometimes merely starts cells on the way toward becoming neoplastic, a process going on to conclusion during later months or years. One is reminded in this general relation of the phenomenon lately discovered by geneticists, "phenotypic lag," in which mutations assert themselves only after the cells exposed to the mutating agent have undergone one or more divisions (27). But to draw attention to this similarity is not to subscribe to the mutation hypothesis of tumor causation.

The neoplastic viruses and the generality of other carcinogenic agents have been viewed as lying in categories scarcely to be reconciled, as alike only in their end products, the cancers ultimately resulting from their action. But it now seems plain that some functional basis for reconciliation must exist in the case of the Shope virus and the carcinogenic hydrocarbons.

SUMMARY

Areas of rabbit skin previously rendered hyperplastic with turpentine were scarified, inoculated with the Shope papilloma virus, and covered with a

dressing that contained 20-methylcholanthrene (*MC*) or *9:10*-dimethyl-1:2-benzanthracene (*9:10*). The dressing was left on until healing had been well completed, a matter of 5 to 7 days. The papillomas which subsequently arose often appeared later, were fewer, and remained less vigorous than those due to the action of virus alone, but throughout several months they appeared to differ from these in no other ways. Then, more or less abruptly, the large majority became carcinomatous, frequently at several situations, whereas with few exceptions the control growths underwent no such alteration. The cancers were of the sorts ordinarily deriving, by secondary change, from epidermal cells infected with the virus.

Collateral data have made plain that the hydrocarbons acted in their carcinogenic capacity to bring on the cancers. Indeed in control tests *9:10* sometimes conferred latent neoplastic potentialities on uninoculated epidermis exposed to it while healing after scarification, a fact disclosed months later by painting these expanses with chloroform until hyperplasia occurred. Under the promoting influence of this agent papillomas formed which had the distinctive morphology of those induced by the chemical carcinogens.

So strong and enduring were the effects of *MC* and *9:10* as to cause cancers to arise from many virus papillomas which were retrogressing after months of proliferation, that is to say under circumstances ordinarily unfavorable to malignant change.

The facts justify the conclusion that the virus and the hydrocarbons acted jointly and in their carcinogenic capacities.

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EXPLANATION OF PLATES

PLATE 33

Early cancerous changes in virus papillomas influenced by *MC* or *9:10*.

One of the photographs of each rabbit has been printed with the back of the negative against the paper, for the readier comparison of growths at corresponding situations. Hence the animals appear to have two left sides. × about 1/4.

FIGS. 1 and 2. D. R. 3-33 of Experiment 2; 146th day.

The growth furthest to the right in Fig. 1 was on an area inoculated with virus and covered with a dressing which contained 1 per cent *MC*. The original pap. mass has now been wholly replaced by a much larger, raised, foul, ulcerated cancer with an intradermal extension in the direction of the groin (Chart 2). The epidermis over this extension,—2 cm. high, falling abruptly to the skin level,—is tense, and shows dark areas of sanguineous ulceration. The middle growth, again where 1 per cent *MC* had been put, is everywhere cancerous save at its anterior ventral end, where some outlying, sooty tissue has persisted. The growth on the left, where the dressing had contained 0.5 per cent *MC*, is covered with melanotic keratin except where a vertical fissure has opened in its midst, exposing the creamy keratin of a pearl. The growth appears to be wholly papillomatous, but actually it was underlain by a cancer, as yet unulcerated, which had extended out laterally into the skin (arrow A). The bulge next the upper margin of the mass (arrow B) is due to a large keratinized pearl of pap. tissue.

The control growths on the opposite side of the animal are still mere paps., dry to the base, but with several subepidermal pearls along their edges, testifying to vigorous previous growth. Most of the bright flecks on the surface of the masses are reflections from the dark keratin, but some of this that is creamy protrudes here and there.

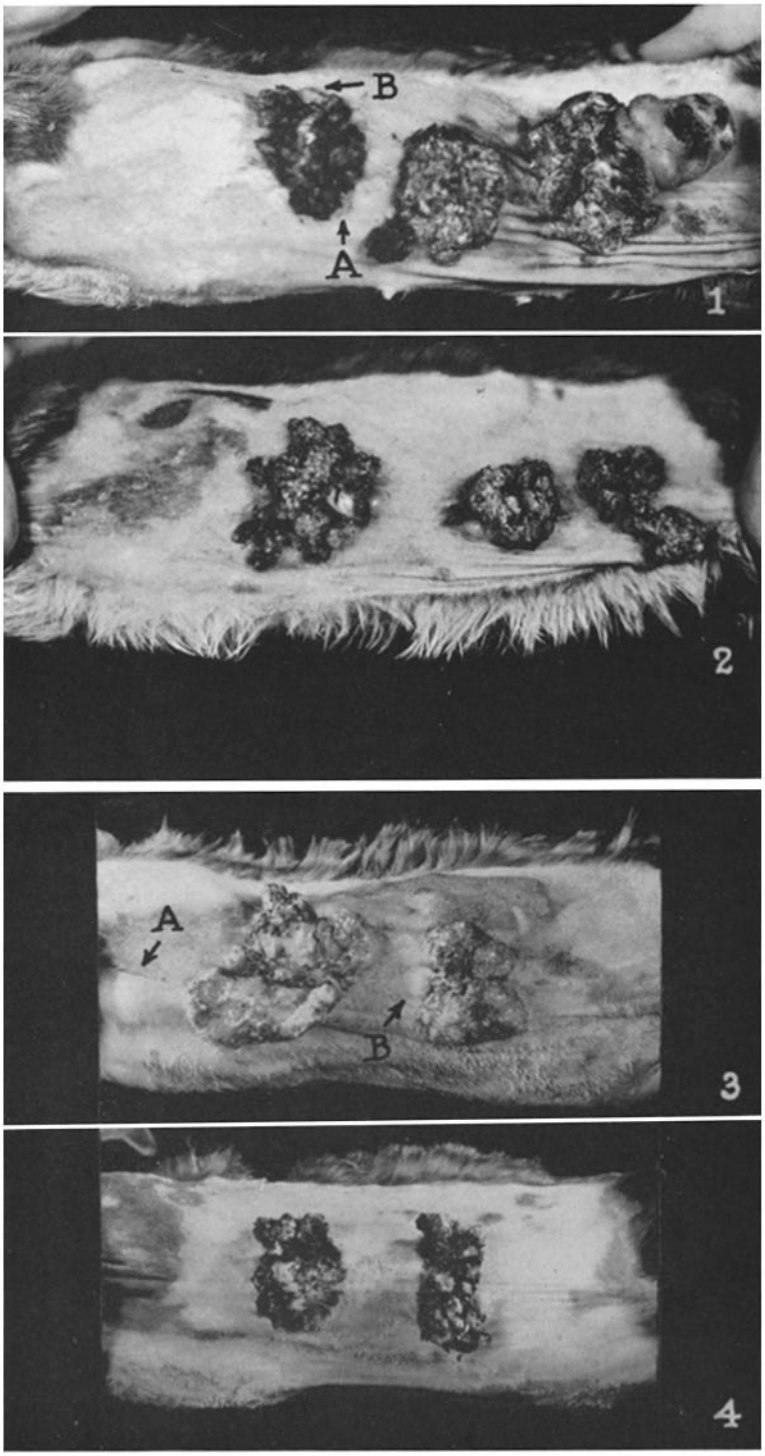
When the experiment was begun, a square of skin on each side of the animal, amidst the large anterior expanses now clipped bare, was scarified and dressed with 1 per cent *MC* but not inoculated. The squares could no longer be discerned when chloroform was first applied after 122 days to the whole region where one of them had been. The applications, repeated thrice weekly, caused the skin to become thickened, hyperplastic, irregularly gray, and somewhat scurfy (Fig. 2); but no tumors arose on it, nor did any on the opposite expanse of untreated, apparently normal skin (Fig. 1).

FIGS. 3 and 4. The growths of D. R. 3-26 (Experiment 3, Chart 3); photographed after death (167th day).

The tumor on the left in Fig. 3, almost entirely cancerous, has extended far beyond the limits of the papillomas it replaced and is ulcerated to the skin level at several situations. A large axillary metastasis can be seen (arrow A). The discoid growth on the right is wholly cancerous, patched with dark blood; arrow B points to an extension into the skin. The cancers had arisen from papillomatous masses on areas dressed with 0.5 and 1 per cent *9:10* respectively. The skin below them is rough with dried exudate.

The control growths of Fig. 4 were mere papillomas still, and had lately begun to dwindle.

The gray pigmentation on a large area seen in part only at the extreme left in Fig. 4 was due to the repeated application of chloroform to skin long previously scarified and exposed to 1 per cent *9:10* but not inoculated. Neither here nor on the corresponding area of Fig. 3, also exposed to *9:10* but left undisturbed thereafter, did any tumors arise.



(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 34

FIG. 5 and 6. D. R. 3-28, another animal of Experiment 3 (Chart 3), photographed on the 168th day, 2 months before death. × about 1/4.

The cancers (Fig. 5), which have in large part replaced the pap. masses where 0.5 and 1 per cent *9:10* had been put, together with virus, enlarged mostly by expansion, exerting such lateral pressure on the surrounding pap. tissue as to turn their marginal, keratinized peaks sideways, almost parallel with the skin; hence the masses appear much larger than they really were.

The control paps. on the opposite side of the rabbit (Fig. 6) had for some while been retrogressing.

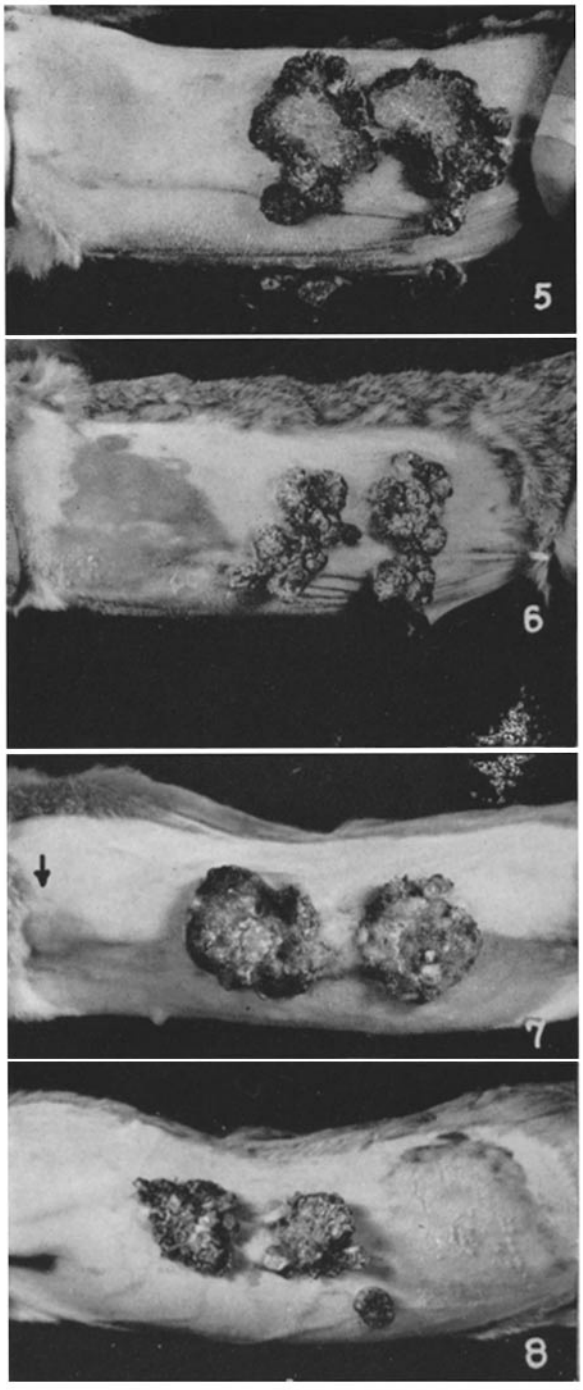
The chloroformed area at the left in Fig. 6 had become markedly hyperplastic, gray, and scurfed in some regions, as can be seen, but neither on it nor on the corresponding area of the opposite side,—also exposed to *9:10* long previously but receiving no chloroform,—did growths appear.

FIGS. 7 and 8. D. R. 3-48, Experiment 4, (Chart 4), photographed on the 166th day, 31 days before the animal was killed. × about 1/4.

Fleshy, discoid cancers have almost entirely replaced the virus paps. derived from cells exposed to *9:10*, and have extended much beyond the limits of these latter (Fig. 7). An axillary metastasis can be seen (arrow). Later on the growths fused into a single huge mass of malignant tissue (Chart 4), with a raised, intradermal extension toward the groin, very like that of Fig. 1.

The control growths are mere active papillomas, now largely unpigmented (Fig. 8). They never became cancerous.

Chloroform called forth no tumors on the expanse at the right in Fig. 8, where was an area of skin previously exposed to *9:10*, though it caused marked hyperplasia, with scurfing and slight pigmentation. A swollen lymph gland draining the hyperplastic area can be dimly seen; it proved devoid of cancer.

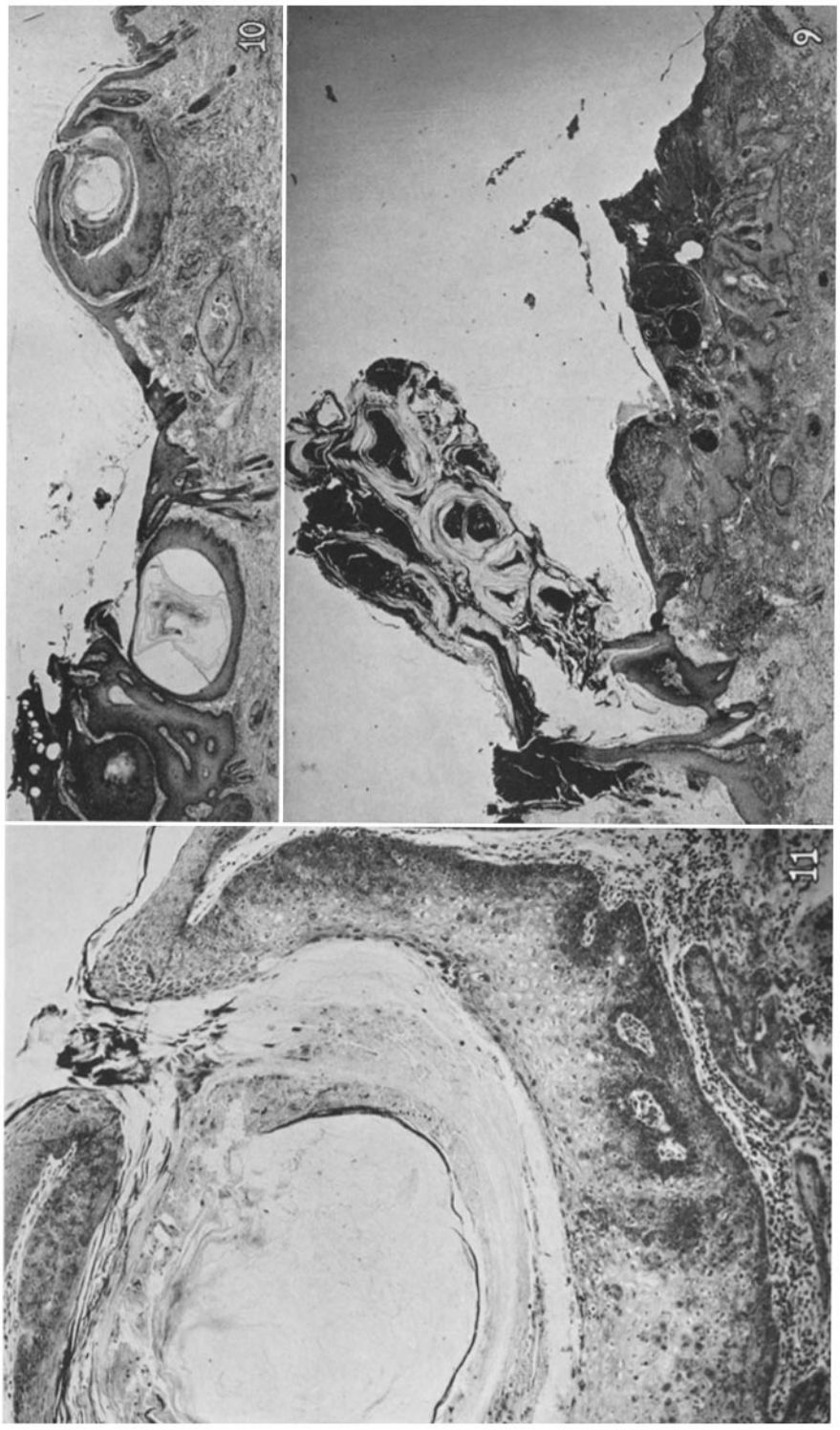


(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 35

FIG. 9. The "peninsular cancer" of D. R. 3-46, Experiment 4, a squamous-cell carcinoma. Between its ulcerated edge and the virus pap. mass on the left the skin surface is covered with a thick, flat layer of epithelium, which, on higher magnification, was found to consist of keratinizing, virus papilloma cells. The reactive tissue underlying the cancer contains numerous lymphocytes, and some can be seen under the papilloma nearby. × 15.

FIGS. 10 and 11. The "pustule" cancer of D. R. 3-46, Experiment 4. It consists of a subepidermal cyst lined with a thick layer of obviously malignant, keratinizing cells which have extended in tongues into the underlying reactive tissue (Fig. 11). The cyst, which has a narrow opening on the skin surface, contains concentric layers of keratin, together with a little recent cell debris. It was separated from the adjacent mass of virus papillomatosis (on the left in Fig. 10) by an expanse of markedly hyperplastic, ordinary epidermis, from which hair follicles extended deep, and by another subepidermal cyst, also opening on the surface and full of keratin, but lined with the epithelium characteristic of Shope papillomatosis. × 15 and 94 respectively.

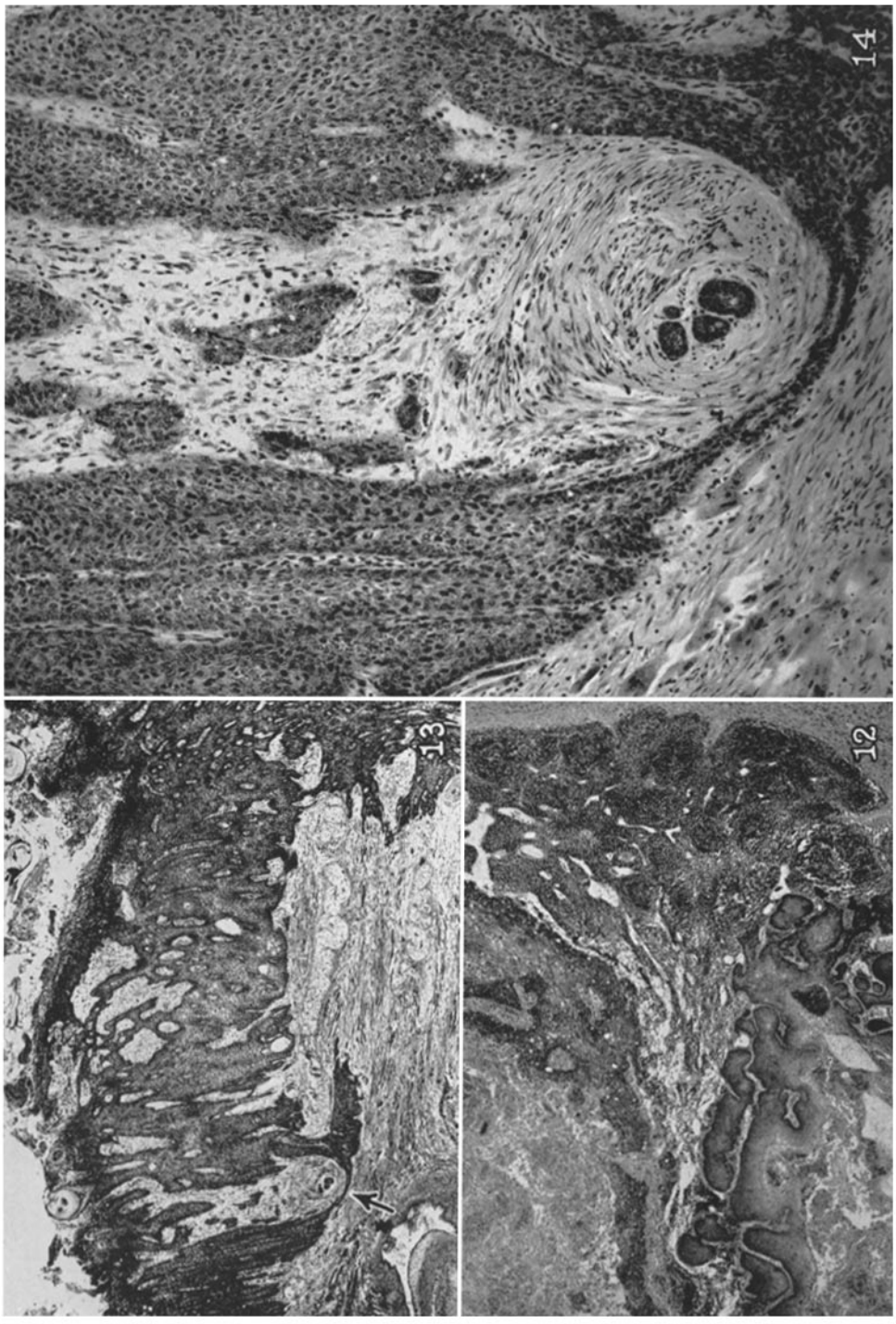


(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 36

FIG. 12. Metastases of two widely differing carcinomas in the same axillary gland (D. R. 3-26, Experiment 3). Only the periphery of the nodules is shown, amidst the gland tissue. × 17.

FIGS. 13 and 14. A carcinoma of peculiar morphology, derived from a papillomatous mass of Experiment A, which had arisen where virus had been put, together with crystalline *MC*. Its aspect recalls that of the "anomalous" tumors sometimes resulting from secondary infection of tar papillomas with the Shope virus (1). Fig. 14 shows the region indicated by an arrow in Fig. 13. × 20 and × 113.

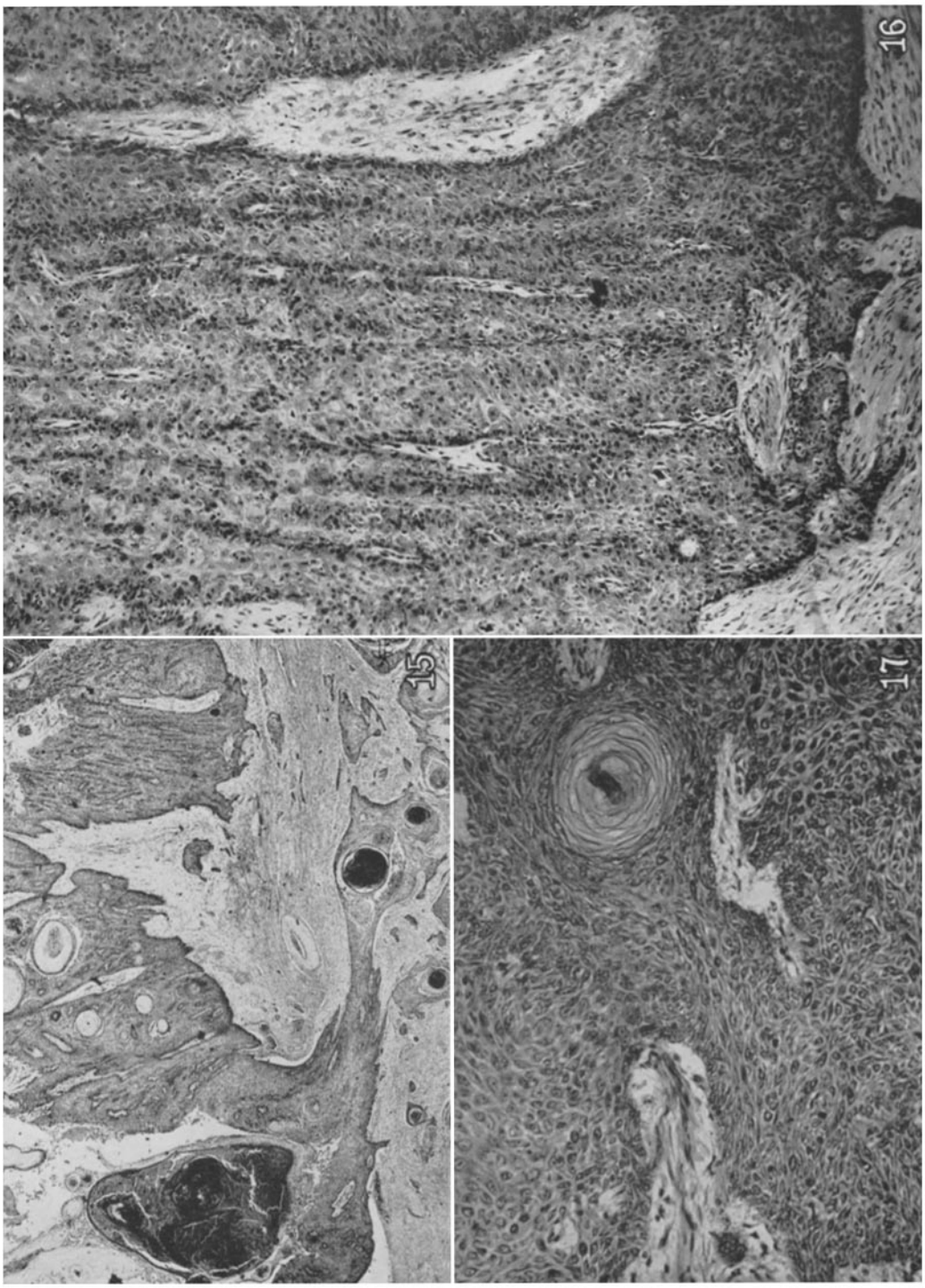


(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 37

FIGS. 15 and 16. Another carcinoma very similar to that of Figs. 13 and 14, which developed in D. R. 3-21 of Experiment B,—again where crystalline *MC* had been put, together with virus. × 16 and × 106.

FIG. 17. Carcinoma with fusiform cells, from D. R. 3-48 of Experiment 4. × 170.



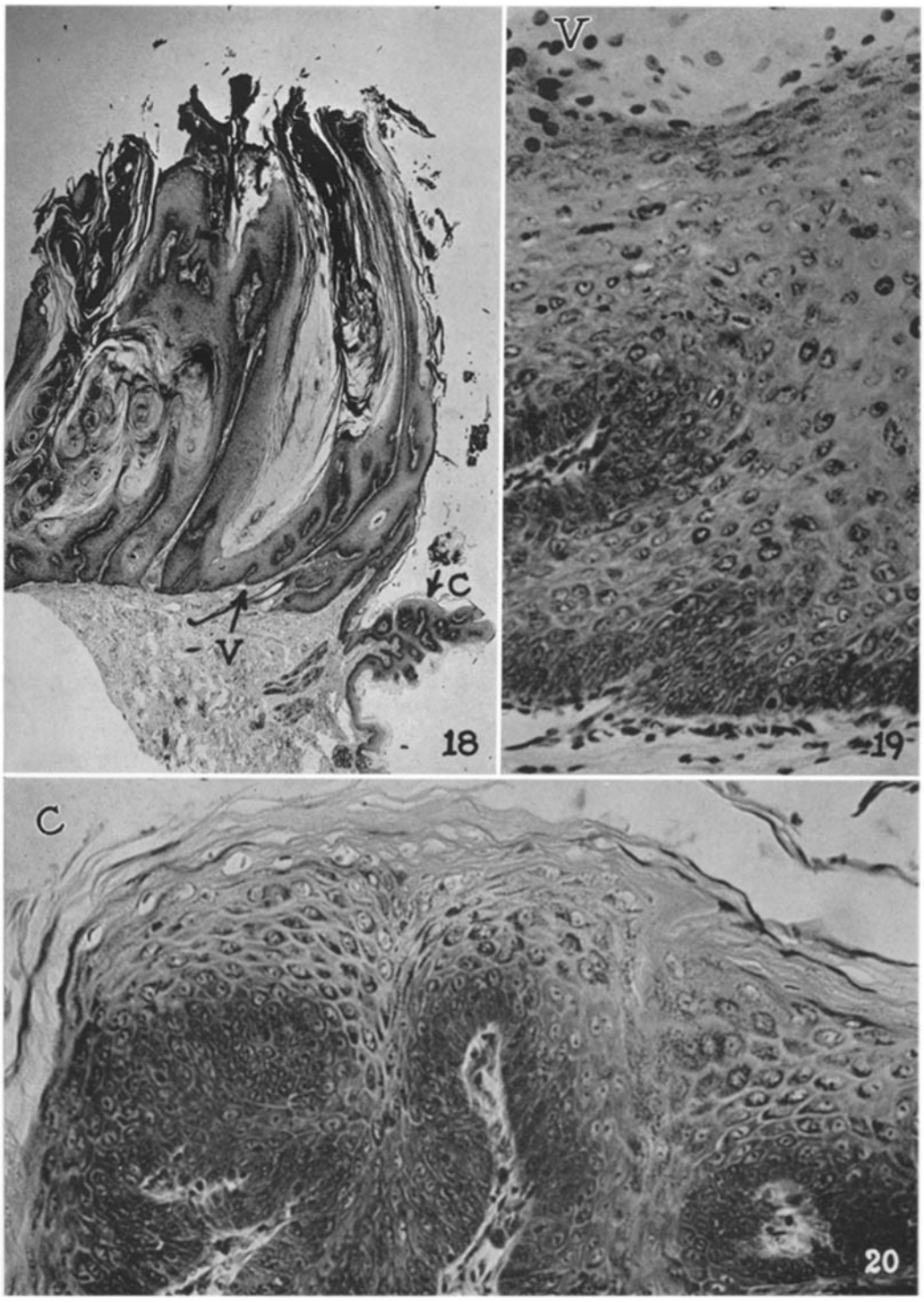
(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 38

FIG. 18. Tiny papilloma (C) situated on the hyperplastic epidermis in the angle next an overhanging mass of virus papillomatosis (D. R. 3-50, Chart 5). When the block of tissue was fixed the angle was obliterated by pulling the skin around so that it lay as seen, almost vertical to the pap. mass, and thus the independence of the little growth next the latter was brought out. It was situated in the uninfected marginal zone of an expanse inoculated with virus and dressed with *9:10*. × 17.

FIG. 19. Higher magnification of the epithelium of the virus pap. of Fig. 18 (region indicated by the arrow V). × 287.

FIG. 20. The epithelium covering the little growth next the virus pap. mass of Fig. 18. It is very different from that of Fig. 19, showing as it does changes such as are found in the papillomas the carcinogenic hydrocarbons produce. × 287.



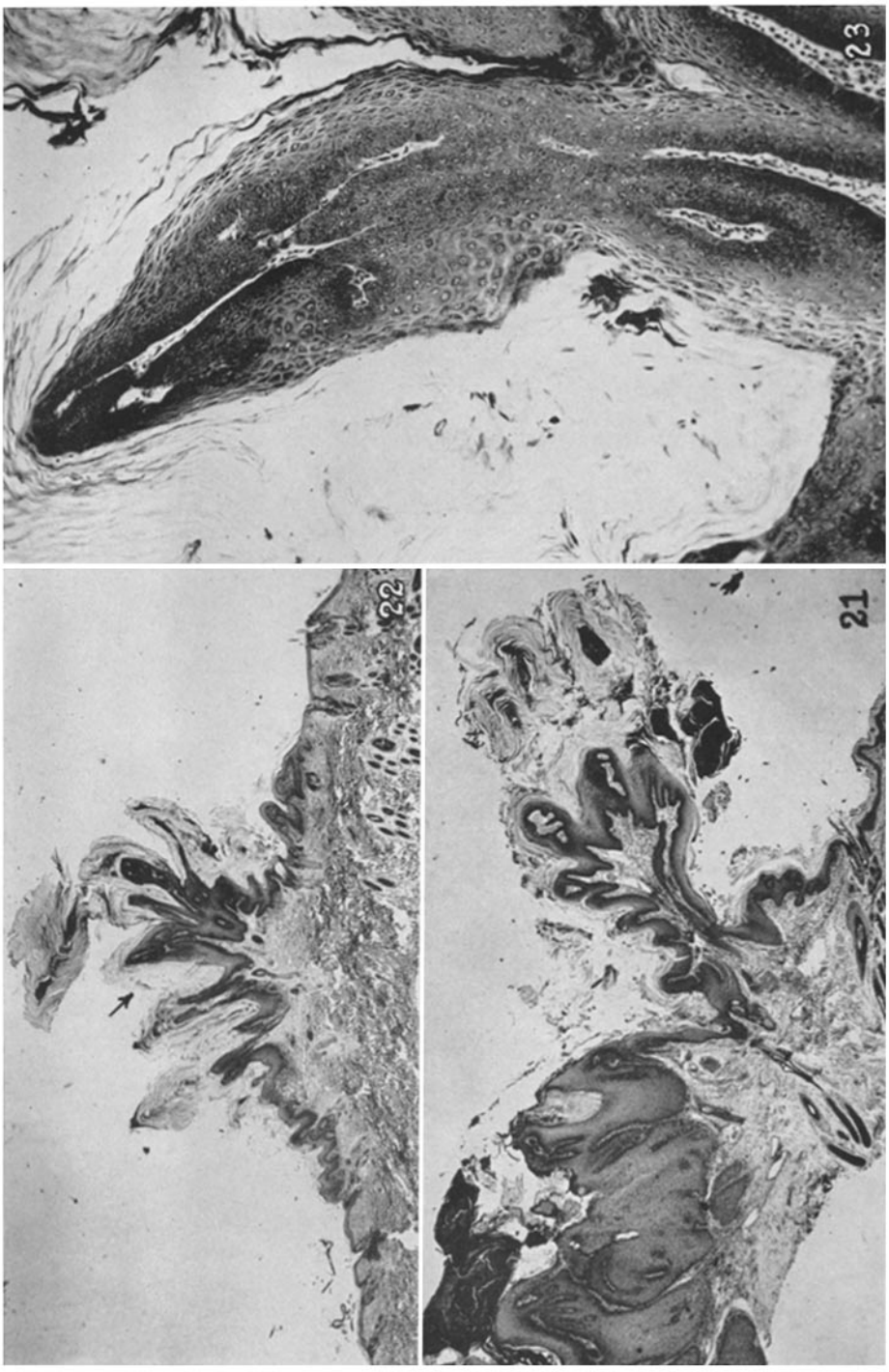
(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 39

FIG. 21. Another, larger papilloma in the angle next a virus pap. mass of D. R. 3-50 (Chart 5). The mass is low because lately gnawed down, and hence less distortion of the relations has been needed to make plain that the adjacent papilloma has arisen where a pronounced local epidermal hyperplasia exists. This fades off towards the right. × 15.

FIG. 22. Broad-based papilloma elicited by the repeated application of chloroform to skin exposed to *9 : 10* while healing after scarification. The applications were not begun until nearly 5 months had elapsed, the growth was first noted 44 days later, and it was excised after 34 days more. The chloroform has rendered the surrounding skin moderately hyperplastic. × 17.

FIG. 23. Epithelium of the papilloma of Fig. 22 at higher magnification; from the region indicated by the arrow. The growth shows the same cytological changes as that of Fig. 20. × 113.



(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

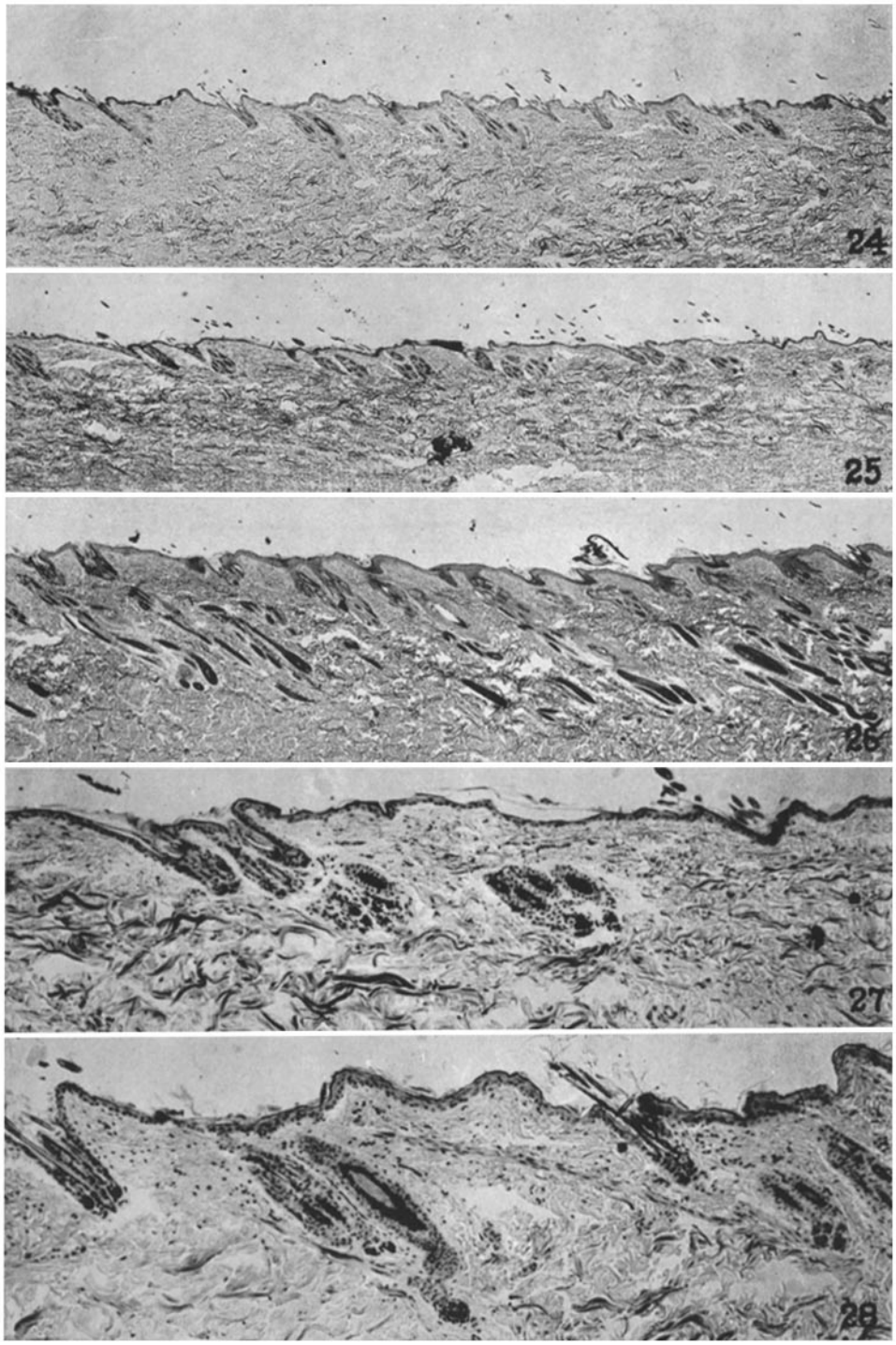
PLATE 40

FIG. 24. Skin from an area on the side of D. R. 3-47, Experiment 4, which had been scarified nearly 6 months previously and covered until healed with a dressing that contained *9:10*. × 20.

FIG. 25. Adjacent normal skin. Even at so low a magnification the epithelium of Fig. 24 appears thicker and its follicles deeper. × 20. See also Figs. 27 and 28.

FIG. 26. From another rabbit of the same experiment (D. R. 3-46). To show the hyperplasia induced by repeated paintings with chloroform. The skin had long previously been exposed to *9:10* while healing, and the paintings were kept up for 62 days. The surface epithelium is only moderately hyperplastic in this instance, but the hair follicles have penetrated far down. × 20.

FIGS. 27 and 28. Portions of Figs. 24 and 25 at higher magnification. × 78.



(Rogers and Rous: Joint action of a chemical carcinogen and a virus)

PLATE 41

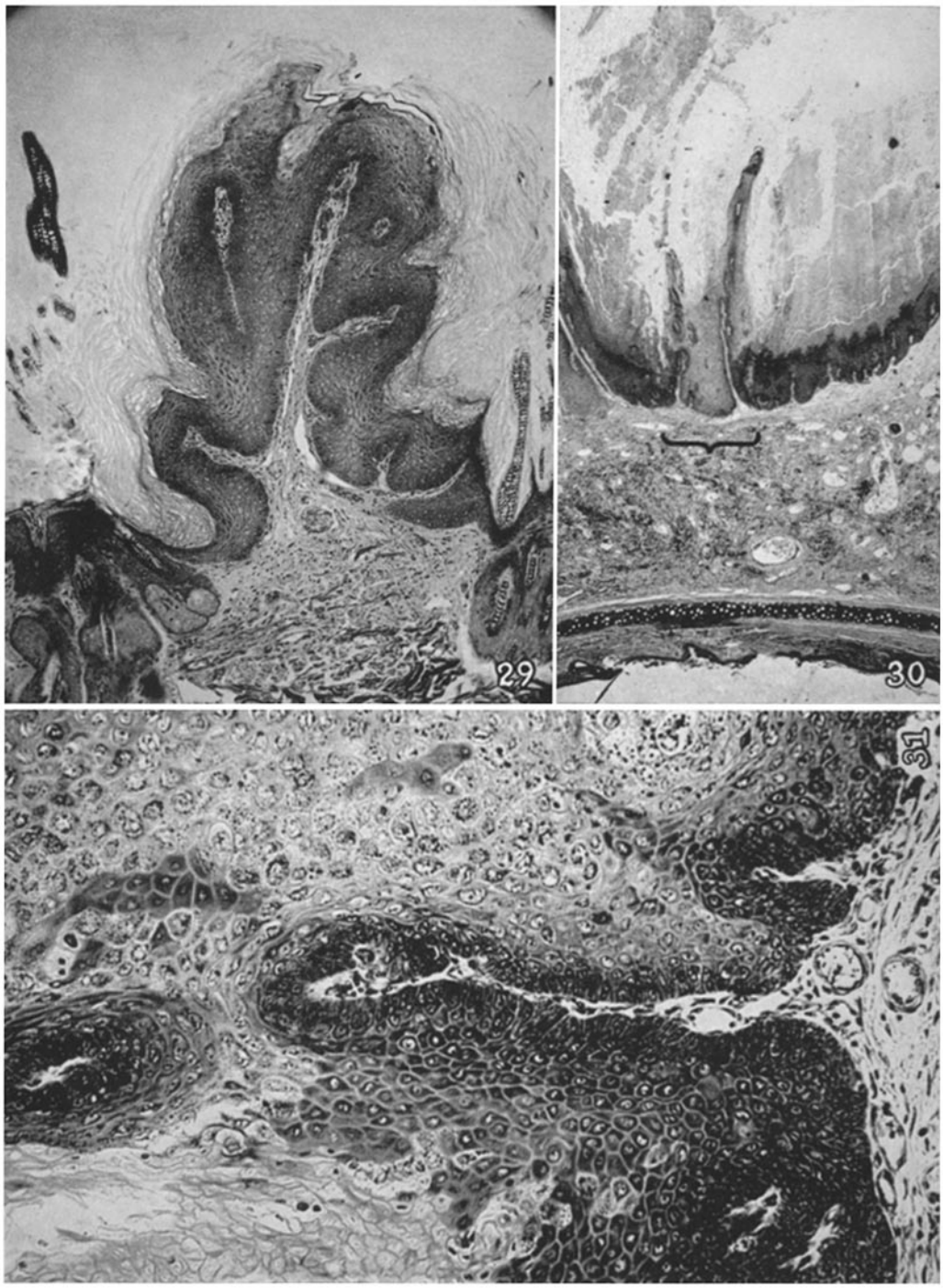
FIG. 29. A papilloma elicited with chloroform on the skin of D. R. 3-53, Experiment 5; the area on which it appeared had been scarified months previously and covered with a dressing that contained *9:10*. The chloroform soon induced an exceedingly pronounced epidermal hyperplasia, the growth arose within 16 days, and on the 23rd it was removed. It has the morphology of those due to the hydrocarbons. × 72.

FIG. 30. Changes in the living base of a frill horn as result of secondary infection with the papilloma virus. Where this latter has acted (bracketed area along the base of the growth) the epithelial layer is thickened and pale, the overlying keratin has failed to stain, and two papillary protrusions have formed. Such protrusions are never encountered in ordinary frill horns, but are a regular feature of virus papillomas. × 21.

The inner expanse of the rabbit's ear had been tarred twice a week during a period of 89 days, and 8 days after the last tarring 15 cc. of a 0.5 per cent suspension of virus in saline was injected into a vein. The frill horn was one of three growths present at this time (rabbit 21 of reference 2). The animal was killed 109 days later; no more tarrings had been done.

FIG. 31. Part of the bracketed region of Fig. 30 at a higher magnification. The cells proper to the frill horn are relatively small and dark, with small, bird's-eye nuclei, whereas those influenced by the Shope virus are larger, paler elements with big vesicular nuclei in which the chromatin is marginated,—typical virus papilloma cells in short. × 205.

(The changes occurring in tar papillomas secondarily infected with virus have been figured sufficiently in previous papers (1–3).)



(Rogers and Rous: Joint action of a chemical carcinogen and a virus)