ARE CARCINOGENS RESPONSIBLE FOR THE SUPERIMPOSED NEOPLASTIC CHANGES OCCURRING IN MOUSE TUMOR CELLS?

The Effect of Methylcholanthrene and Urethane on Pulmonary Adenomas and of Methylcholanthrene on Mammary Carcinomas

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Plates 55 to 62

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The cells of not a few tumors attain to their worst by further neoplastic changes which are scarcely less significant than the one primarily responsible for their state. Indeed the practical importance of these changes is often greater as meaning death to the patient. Why they come about is not known; man is still in the observational stage as concerns them: but this much has become plain, that they are discontinuous, irreversible, heritable, in that they are passed on from cell to cell, and frequently, perhaps always, are step-like and abrupt. In these ways they resemble primary neoplastic change, and it has been easy to suppose that carcinogens have a hand in them too,—an inference the more reasonable because these agents induce cancers of many kinds first off. But does their responsibility for the superimposed changes actually extend further than the initial conversion of normal cells to tumor cells, with such liabilities as their new state may entail? The present paper is concerned with this question. The work now reported shows that two carcinogens, 20-methylcholanthrene and urethane, which readily induce pulmonary adenomas in mice, possess no power to bring on the further neoplastic changes that tumors of this kind often undergo. Collateral experiments to learn whether methylcholanthrene will cause alterations in polymorphic mammary adenocarcinomas have proved it ineffective in their relation as well.

GENERAL PLAN

Tumor cells of many sorts are so labile as to undergo marked alterations in response to extraneous influences, those of bacterial infection and tissue necrosis for example. We are not here concerned with events thus brought on, but with the changes to which certain neoplasms are prone for deeper, unfathomed reasons, papillomas of the skin or bladder becoming carcinomas for instance. One might think

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that the papillomas induced by carcinogenic hydrocarbons on rabbit or mouse skin would be best for the experimental study of superimposed malignant changes, as providing a classic material; but these growths cannot be successfully transplanted (1), as would be necessary to free them from normal epidermal cells on which the carcinogen might act. The pulmonary adenomas of mice,[1] almost standardized growths which frequently undergo further neoplastic changes when maintained by transplantation, present no such obstacle. Hence they have been used in most of the tests here reported. The definitive paper by Stewart, Grady, and Andervont (2) on the happenings in propagated adenomas has provided an ample background for the work and aided much in interpreting its results.

Methylcholanthrene was the carcinogen used in all save one of the experiments,—in which an adenoma was tested with urethane; and the tumor cells were brought into close contact with it by implanting them in the thigh muscles, together with the carcinogen in solution. This is a highly effective method of inducing neoplastic change in normal cells (3).

Materials and Methods

The Tumors.—The pulmonary adenomas induced with carcinogens completely resemble those occurring spontaneously, and the number of them arising in pure strain mice varies directly with the liability of these strains to spontaneous adenomas,—from which they cannot be told apart. Both have the same tendency to undergo cancerous change. The adenomas utilized for the present work were spontaneous. Using these one knew what one had.

Sixteen adenomas (Ads.)[2] from as many old C mice of our laboratory stock were repeatedly transplanted to obtain those suited to test by reason of their rate of growth. A single small piece was transferred to each new host by trochar, as most likely to yield homogeneous growths and to leave normal pulmonary cells behind. Some of the tumors grew with extreme slowness, and others underwent sarcomatous change; but eventually three proved right for our purposes. On subcutaneous implantation they formed, as usual, big, thin walled cysts containing watery fluid, often blood-stained, with a little solid tissue interspersed; but when placed in the posterior thigh muscles they produced solid masses having only tiny cysts at most until they got so large as to break forth into the subcutaneous tissue, when again big cysts rapidly formed. Hence the thigh was chosen for the implantations.

The two mammary cancers tested, spontaneous growths in old female C mice, were carcinomas forming tubules and acini in some regions while in others appearing anaplastic. On the possibility thus suggested that they contained several neoplastic components, the preliminary transplantations were done with tissue suspensions in order to provide opportunity for any notably malignant constituent to come to the fore. But nothing of the sort happened; the growths were as polymorphic as ever when utilized for the experiments.

Method of Test with Methylcholanthrene.—To obtain representative implants the neo-

[1] These pulmonary adenomas, generally so called because of their benign aspect and habit, exhibit on occasion some of the characters of acinous carcinomas (2); yet they will here be termed adenomas, as is customary.

[2] The following abbreviations will be used: Ad., ad. = adenoma; Mam. T = mammary tumor; s-L = 1 part mouse serum in 19 parts Locke's solution; MC = methylcholanthrene; OO = olive oil; OSS = OO containing Scharlach R (Grübler); OM = OO containing 1 per cent of MC; OSSM = OM containing Scharlach R; U = urethane; TP = test period; T = period of simple transfer in s-L.

plastic tissue was taken in quantity, pooled, finely minced with scalpels, and a small portion of the mince was suspended in serum-Locke's solution (s-L) for injection. Most of the experiments were begun with tissue from a single growth, for the sake of uniformity, and occasionally only one was available for later transfer, but ordinarily material from three to five of the same group was mixed in about equal quantity. Pooling was important because now and then superimposed neoplastic changes take place in some Ads. and not in others of the same implantation (2). The tumor cells were brought into close contact with methylcholanthrene (MC) by implanting them together with a 1 per cent solution of the carcinogen in olive oil (OM). The control implantations were made with olive oil alone (OO). The same olive oil and MC were used as in previous experiments with normal tissues (3 *a*). When the resulting growths had got big their tissue was implanted in new hosts, again with MC or OO, and by repeating the procedure whenever necessary, exposure to the carcinogen was kept up throughout many months. In the exceptional experiment above mentioned, mice carrying a transplanted adenoma received intraperitoneal injections of urethane.

The OM was found to retard the growth of all the tumors so greatly that four to eight times as much neoplastic tissue had to be implanted with it as with OO if it was to produce growths of comparable size. Often the quantitation was done with a 0.25 cc. syringe but usually by eye, with the hashed tissue for exposure to OM separated into four to eight equal portions on a glass plate, and the control tissue utilized to the amount of one portion. Sometimes transfers had to be made without OO or OM, and then the two suspensions consisted of the same amount of tissue, as nearly as could be judged.

The implantation technique was like that employed previously with normal tissue (3 *a*): 0.025 cc. of tissue suspension was drawn up into a syringe, followed by 0.15 cc. of s-L, and this in turn by 0.025 cc. of either OM or OO,—which had been broken up into fine droplets in one-third its amount of s-L. Injection into the muscles was done forcibly to scatter the materials. In a few of the early tests, each thigh of the host mice received an implant, with OO and OM respectively; and occasionally the oil of both preparations had then been saturated with Scharlach R (Grübler) on the possibility that it would attract the adenoma cells as it does epidermal elements (3 *a*); but it had no such effect on them, as the microscope disclosed. Preparations containing the dye are designated OSS and OSSM. In all the later work one thigh only of each animal was used.

Sarcomas sometimes appeared because of the action of MC on the connective tissue (3 *a*) and more or less completely supplanted the tumors under test. None was transplanted and they are omitted from the charts. The first sarcoma was noted 70 days after the implantation, and they became frequent later. They were easily recognized in the gross. Because of their occurrence the test periods (TP's) were usually terminated within 2 months, though sometimes sooner and occasionally much later, as laboratory exigencies demanded. Owing to these latter the tumors had sometimes to be carried along without MC for weeks between the TP's. This was done by the implantation of tissue suspensions merely as such (T). After the last exposures to OM and OO one or two such transfers were regularly made to rid the growths of these materials entirely.

The method of test with urethane is sufficiently given in the protocol of the single experiment done.

Other Technical Matters.—Every growth transplanted was examined microscopically and so too were three to nine of the final tumors of each series. Now and again mice were killed during a TP to learn from their tumors how things were going (Charts I to V). Blocks were taken of these as well. Fixation was always in acid-Zenker's solution, and the sections were stained with eosin and methylene blue and examined as unknowns.

At each implantation the biggest tumors were chosen for pooling, on the assumption that any cell changes capable of asserting themselves would have been in the direction of

greater malignancy and more rapid growth. The tissue for transfer was selected from sagittal slices when possible, with adjacent slices fixed for section. Ulcerated tumors were excluded,—though frequently searched microscopically to learn whether their state was due to some change in neoplastic character. It never was. No metastases originated from the adenomas, but occasionally a few in the lungs resulted from the mammary tumors.

Most of the growths arising where OM had been put were inspected under ultraviolet light, when first cut open, to learn whether MC was still present, a more or less brilliant purple fluorescence demonstrating this. It generally persisted at least 2 months, often much longer, and occasionally was so pronounced that the tissue suspension prepared for the next implantation with OM shimmered in purple. The control tumors never fluoresced.

The mammary carcinomas were known to proliferate actively, and hence very thin suspensions of their tissue were used in the early TP's; but so drastically did the OM retard growth that only an occasional implant with it had given any tumor by the time those with OO had produced huge ones, forcing their transplantation. When even thinner suspensions were used with OO, thus lengthening the available test period to 40 to 60 days, the tumors resulting from the OM implants, even when eight times as rich in tissue, were often too small for transfer by the time this had become imperative for the controls. On such occasions a simple transfer was made of tissue from these latter, thus providing time for the OM growths to get big enough to yield material for the next test period. As is the case of the adenomas, ultraviolet light disclosed much MC in the mammary growths after 2 months, and after they had become several centimeters across later on, widely distributed patches of purple fluorescence could often still be seen in them.

Young female C mice of 16 to 22 gm., paired by weight, were used as hosts in all the adenoma tests save those of TP's 1 and 2 of Ad. MC I, in which animals of both sexes were employed,—without any difference in the results. All the hosts for the mammary tumors were young males.

Any accidental pooling of control and experimental tissues, perhaps occurring after months of successive tests, would have wrecked an entire experiment, so great care was taken to exclude this. The markings of the mice with carbolfuchsin were frequently inspected and renewed; the animals of each group were kept in separate boxes; and the selection of every tumor transplanted, and the labelling of every block for section, were checked by two or three persons. Weekly records of the size of each growth and of any individual peculiarity of it or of its host provided a further safeguard against error. The authors themselves did all the preparation of materials and implantations.

The Effect of Methylcholanthrene on Adenoma Cells

The Findings with Adenoma 4.—Adenoma 4, a typical adenoma with some tendency to hydropic degeneration (Fig. 1), was used in most of the experiments. When the first was begun (Chart I) it had been propagated for 11 months, during which its rate of growth had gradually increased.

Ad. MC I, Test Period 1 (16 mice).[3]—The tissue for suspension was got from an adenoma of the 3rd Gen. (Fig. 1), and was implanted in both thighs, with OSS and OSSM respectively. Tumors arose rapidly from the OSS implants and grew well, whereas none had resulted from those with OM in half of the mice after 11 weeks, and most of the others had only small ones. Several were killed between the 49th and 77th day to learn how the Ad. had fared (Fig. 2). *77 days:* Implantations done for *Test Period*

[3] When more than ten mice were implanted in any group of hosts, their number is given in parenthesis.

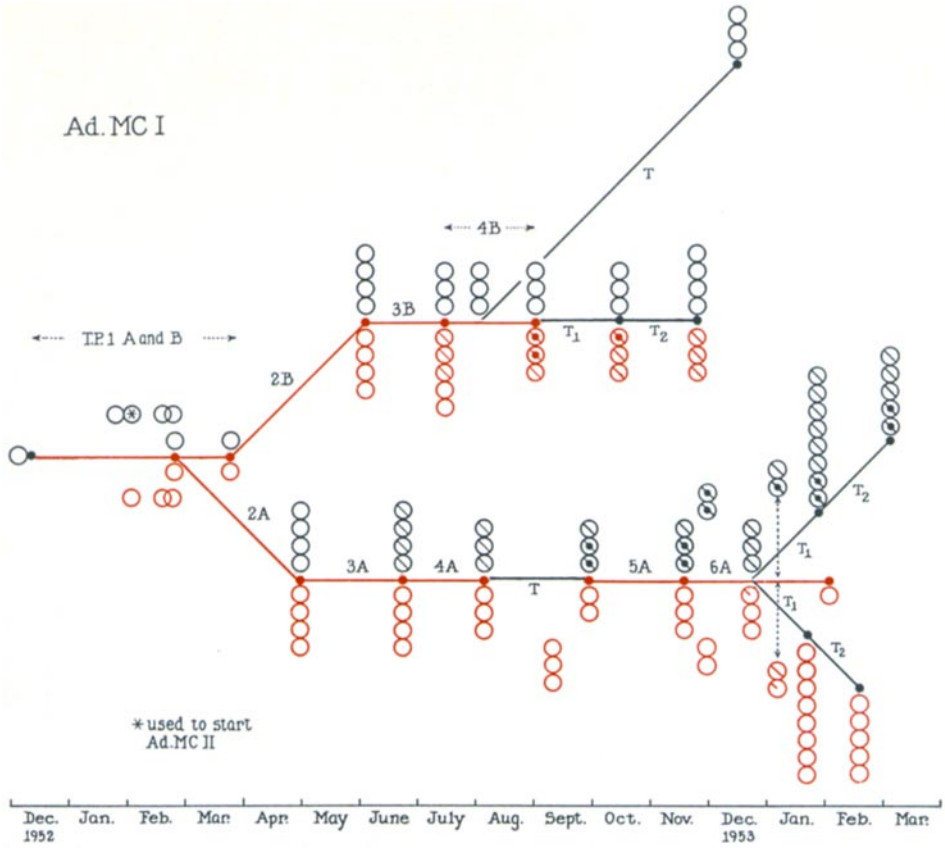

CHART I. Incidence and time of occurrence of the successive neoplastic changes in Adenoma 4 during an experiment (Ad. MC I) to learn whether methylcholanthrene would cause alterations in the growth.

Each circle stands for a tumor, the black for a control growth, the red for one that had been exposed to MC. An empty circle denotes an adenoma that had not changed; an oblique line within it means that adenoma solidum was now present also, little of this when the line is short, more when it crosses the circle. A central black spot tells that carcinomatous tissue had appeared as well.

The lines in red represent the periods of test,—which are duly numbered; and their length, measured transversely from dot to dot, tells that of the periods. The circles ranked vertically just above and below a dot are those for the tumors furnishing the tissue suspension with which the next succeeding period was begun, black for the control growths, red for those of the MC series. The isolated circles at some distance from the lines represent tumors procured merely for microscopic examination.

The lines in black, marked T, T_1, T_2, represent periods of mere transplantation, yet the circles along them are still designated in black or red in order to show whether the growths they stand for were of the control or the methylcholanthrene series.

2 (TP 2), Series A: Pooled tissue utilized from two growths, 18 and 25 mm. across, which had arisen from an OSS implant, and from a single growth 13 mm. across derived from an OSSM implant. *103 days:* Four mice still living with tumors from OSSM implants. Three are sarcomatous, but the fourth, barely palpable on the 80th day, is now 9 mm. across and still an adenoma. It was used, with a 45 mm. OSS tumor, to start *TP 2, Series B.*

(*Series B* is described further on.)

Series A.

TP 2.—Equal amounts of tissue again implanted, together with OSS or OSSM, in one thigh only of each animal. *64 days:* The OSSM growths have lagged far behind; they are 7 to 15 mm. across and still intramuscular, whereas those with OSS are 28 to 38 mm. across and have emerged into the subcutaneous tissue. Four tumors of each sort taken for *TP 3.* The growths with OSSM still contain red droplets of this latter.

TP 3.—OO and OM now first used, and twice as much tissue implanted with the latter, yet the resulting tumors grew more slowly than the controls. Those of both groups were big and cystic after *54 days* though. Tissue from four of each group pooled now for *TP 4.*

TP 4.—Tissue suspension with OM made four times as thick as that with OO. *43 days:* Some OM growths are slightly the larger, but others smaller than any OO's. Simple transfer (T) done now of pooled tissue from three growths of each sort, all big and cystic.

T.—Suspensions of approximately the same cell density injected subcutaneously into new mice. *55 days:* Tumors of the OM series have grown much the faster. Three biggest controls, each 14 mm. across, and two OM's, both of 35 mm., pooled respectively for *TP 5.*

TP 5 (15 mice).—Suspension with OM made four times the heavier; tumors appeared and grew at same rate in both groups, averaging 30 mm. after *50 days.* Tissue from three of each taken then for *TP 6.*

TP 6 (15 mice).—Again the suspension with OM was made four times the heavier. *15 days:* Tumors of each group just palpable; two from each taken for serial section. *35 days:* OO tumors 23 to 29 mm. across; two of the 15 OM mice still negative, others have growths smaller than the OO's. Three of each lot used for T_1. *49 days:* Two OO tumors and three OM's taken as specimens. *77 days:* Last OM host killed; its 22 mm. tumor (only 6 mm. after 49 days) shows MC fluorescence still.

T_1 (15 mice).—Equally thick suspensions used. *29 days:* Growths from OM material are the larger, 26 to 35 mm. Eight pooled for *T2, OM. 36 days:* OO's have now got as big. Eight pooled for *T2, OO.*

T_2, *OM.*—Moderately heavy susp. used. *28 days:* Growths now 21 to 30 mm. across. *Sagittal slices of five tumors taken as final specimens.*

T_2, *OO.*—*35 days:* Growth now 23 to 32 mm. across. *Sagittal slices of five taken as final specimens.*

Series A of this experiment lasted more than 14 months, the test periods themselves occupying more than a year. The tumor grew rather slowly during the first two TP's, but then much faster, necessitating transfers at shorter intervals (Chart I); yet in the gross it appeared in both lines to be the same adenoma to the end, solid where surrounded by muscle, cystic after extension into the loose subcutaneous tissue. The OSSM and OM prevented the forma-

tion of tumors in not a few animals, and by inducing reactive tissue rendered those arising in the others firmer than ordinary until they had become subcutaneous. Then they came to resemble the controls wholly, forming huge cysts with soft, friable tissue interspersed, often mostly necrotic. Since the growths in neither line gave any sign of alteration, microscopic examination of the stained sections was deferred until those of TP 5 were at hand, when the whole accumulation was gone through. To our great surprise carcinomas in wide variety were found in many of the growths, some largely consisting of them.[4]

The cancers were present only in the control tumors (Chart I).

The first difference from ordinary adenoma was encountered in the four OO growths of TP 3 that had provided the tissue pooled for TP 4. The microscope showed all to contain solid areas here and there (Fig. 3), especially at the periphery of lobuli, consisting of masses of cells individually resembling those of the adenoma proper. So closely packed were these cells, if often with narrow rifts amidst them (Fig. 4), and so sparse was their vascularization, that the wonder was they had survived in good condition. They showed many mitoses but so too did the adenomatous tissue,—which had been pushed aside in some places by their increase. The new solid areas deserve the term *adenoma solidum*. Stewart, Grady, and Andervont noted what may have been similar ones, or else areas of carcinoma solidum, in adenomas repeatedly transferred,—"solid sheets" of cells, "closely packed together without any appreciable intercellular substance,"—and reported them to be more common at the periphery of the growths (2).

The patches of ad. solidum were numerous and larger in the control tumors taken at the end of TP 4 for simple transfer (T), and more abundant still in the growths resulting from this, some of them consisting largely of solid tissue (Fig. 5). Now for the first time frank carcinomas were found amidst the general mass. Many of them were merged with, or lay next ad. solidum, as if derived therefrom, and had the form of carcinoma solidum, with cells and nuclei of widely various sizes and shapes. Those which were nearly similar morphologically often stained differently (Fig. 6). But there were other cancers too, consisting of ropes of cells, more or less compacted (see Fig. 7, from a growth obtained later on), while still others were alveolar in pattern (see Figs. 10 and 11 from later instances), often with such crowding as almost to obliterate any spaces. These were manifest adenocarcinomas.

Cancers were present again in the slides from two control tumors of TP 5, but not in any from the three controls of TP 6, pooled for the two final transfers,—though ad. solidum had persisted. Yet they had done well in two nodules removed for serial study 15 days after the control implantations of TP 6, and they were found again in one of two growths of this same TP, taken merely as specimens 2 weeks after the first of the final transfers, as also in some of the tumors resulting from both of these latter (Chart I, T_1 and T_2). Adenoma solidum continued present in all of the final

[4] Stewart, Grady, and Andervont also came upon cancers in transplanted adenomas that had appeared unchanged in the gross.

control growths, and ordinary adenomatous tissue as well. Sometimes the cancer cells were oat-shaped and sometimes spindles (Fig. 8), epithelial alterations which can be regarded as at the ultimate remove from those obtaining in the original adenoma. The many forms of growth had often coalesced (Fig. 9), with result in an almost bewildering picture; yet when studied individually nearly all of them had the aspect of discrete entities, as if each were the outcome of individual cell change (Figs. 10 and 11). The sole exceptions were occasional areas of spindle-cell carcinomatosis; these graded into the surrounding carcinoma solidum as if local conditions might have been responsible for them.

The findings were sharply different in the tumors exposed to MC; they remained ordinary adenomas until the first of the final transfers was to be made. One of the three TP 6 growths taken for this purpose contained a few small areas of ad. solidum (Chart I), and a few were present also in one of two growths of TP 6 taken 2 weeks later, the other having a considerable number; yet none of the many tumors resulting from the final transfers showed any, and the last growth of TP 6,—77 days old and still fluorescing with MC,—was a mere adenoma, like that with which the experiment had been begun (Fig. 12).

The apparent absence of cancers from the three control growths of TP 6 used for final transfer was probably due to failure to include them in the blocks sectioned; for they were present in another big tumor of this group procured later. They had also flourished, as already mentioned, in the two, tiny control growths of TP 6 removed for study when only 15 days old (Figs. 13 to 16), serial sections showing in both instances rounded, discrete masses of carcinomatous tissue, as also small cysts, frequently multilocular, lined with adenoma cells and in some cases with adenomatous extensions into their interior (Fig. 13). Composite islands were not infrequent, consisting of carcinoma with adenomatous cysts in its midst (Figs. 14 to 16). Cancers of more than one type were present and they were often invasive (Figs. 15 and 16).

Only cystic adenoma was found in the nodules resulting from two corresponding implantations with OM 15 days previously (Fig. 17). The carcinogen contained in the scattered oil globules had killed the tumor cells lying nearest it. Yet as time passed some of the OM globules had become incorporated in the adenomatous cysts as these enlarged and the intervening tissue barrier thinned and was lost. The first adenoma cells to extend around the oil had soon perished (Fig. 18), but their successors had survived, probably because protected by the increasing cyst fluid.

Adenoma 4 was the sole growth available when Ad. MC I was begun, and to ensure its success two tumors 103 days old, of its first TP, were utilized to start a second series of tests in parallel. So lengthy had the exposure to OM become by then that only a single adenoma of the OM group still persisted as such, sarcoma having largely replaced the others (see protocol). With this one and a huge control growth of the OO group the new test series was started (Chart I, Series B).

Ad. MC I, Series B.—The initial test period of Series B rates as TP 2, because the tumors utilized had already passed through a TP 1.

TP 2 (12 mice).—The suspension implanted with OSSM contained twice as much

tissue as that with OSS. *48 days:* OSS growths are up to 27 mm. across, whereas the OSSM's are barely palpable. *72 days:* Four OSS tumors (28 to 30 mm. across) pooled for TP 3, and the three largest OM's (12 to 18 mm.).

TP 3 (20 mice).—Use of OO and OM begun; five times as much tissue implanted with latter. *41 days:* OO tumors up to 23 mm. across whereas largest OM 13 mm.; four of each group pooled for *TP 4*.

TP 4 (15 mice).—Suspension implanted with OM twice as thick as with OO. *18 days:* OM tumors barely palpable, whereas OO's measure up to 13 mm. Biggest OO's pooled now for simple transfer, T, as safeguard, while the OM's grow large; exceedingly thin suspension used. (The resulting tumors grew very slowly. They proved unnecessary for the work, but blocks from the three biggest were taken as specimens after 133 days (Chart I).) *50 days:* Many of the OO mice have died of their growths; OM tumors now large. Three of each group pooled for T_1.

T_1, OO and OM.—Suspensions contain same amounts of tissue. *43 days:* Tumors have grown at same rate. Three largest of each pooled for T_2.

T_2, OO and OM.—Suspensions again equally thick. *41 days:* Tumors have grown at same rate. *Four OO's and three OM's fixed as final specimens.*

Series B was discontinued when Series A seemed secure. Its outcome proved diametrically different. This time the OM tumors, not the controls, had undergone carcinomatous changes (Chart I); and these were preceded, as in Series A, by patches of adenoma solidum. This was first noted in the OM growths examined at the end of TP 3; it increased in amount during the later test periods and transfers; and in two of the three OM growths of TP 4 utilized for T_1 carcinomas had arisen as well. Yet in only one of the three growths of T_1 itself and in none of the three of T_2 were cancers visible, though ad. solidum was present in all, amidst ordinary adenomatous tissue. The controls remained mere adenomas throughout.

The ad. solidum of Series B precisely resembled that of Series A, and the carcinomas, as a group, were like those of this series.

Three other safeguarding experiments with Ad. 4, begun somewhat later and involving no exposure to Scharlach R, were terminated at about the same time as Series B of Ad. MC I and for the same reason. As usual, OM delayed tumor growth so markedly that much more tissue had to be implanted together with it than with OO. Chart II sums up the findings, and Chart V tells which generation of Ad. 4 provided the tumors to start the experiments.

A control growth from TP 1 of Ad. MC I was used for Ad. MC II. During three consecutive test periods, totalling 5½ months, and 4½ months more of maintenance by simple transfers, the adenoma underwent no change. In Ad. MC III, on the other hand, a few patches of ad. solidum had appeared in some tumors of both lines by the end of a first TP of slightly more than 2 months; they became frequent later, and after some 7 months of consecutive testing (end of TP 3) carcinomas were present as well in one of three tumors from the OM group. But by this time they had arisen

also in one of three growths derived from the corresponding OO group by simple transfer early in the test period.

In Ad. MC IV adenoma solidum appeared somewhat earlier in the OM group than in the controls, as a few small patches only, though in all four tumors; a month

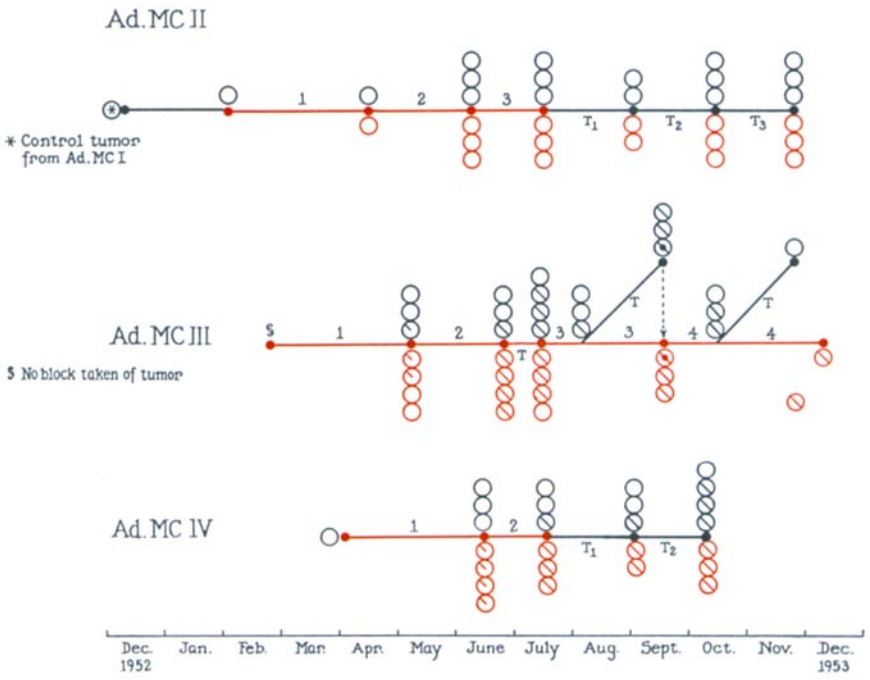

CHART II. Incidence and time of the successive neoplastic changes occurring in further experiments with Adenoma 4 and methylcholanthrene. The symbols have the same meaning as in Chart I.

Ad. MC II was begun with tissue from a control tumor of Ad. MC I, procured after Test Period 1 had lasted 24 days.

In charting Ad. MC III, a vertical line has been inserted with an arrow head pointing to the dot at the end of TP 3, thus indicating that three control tumors of a simple transfer (T), made earlier in this period, were utilized to start TP 4, together with three growths that had been exposed to methylcholanthrene until its end.

later, at the end of a second period of test, it was present in both groups of animals; and most of the final specimens from both, after two periods of simple transfer, contained it. Carcinomas were still wholly absent.

The successive changes occurring in the adenomas of these experiments yielded carcinomas that were morphologically identical, as a group, with those of Series A and Series B of Ad. MC I; and as in the case of these latter they

were regularly preceded by Ad. solidum, this usually persisting to the end, as did ordinary adenoma tissue always.

The Findings with Other Adenomas.—After the experiments with Ad. 4 were well along, two other adenomas, Nos. 10 and 11, became available, and an experiment was begun with each. They were terminated when it became plain that only through sheer good fortune had the lengthy tests of Ad. 4 disclosed the fact that methylcholanthrene exerted no influence upon the neoplastic changes taking place in this tumor. If by chance one or both of the new adenomas were found to have become cancerous after implantation with OM in the single experiment begun with each, and the OO growths in contrast remained unchanged, what then? Then more long term serial tests, like those of Ad. MC I–IV, would be in order, and many might have to be done before proof was forthcoming that OM had really influenced the results. This being so, it seemed logical to discard "sight unseen" all blocks taken of the tumors of the new experiments; but old habit prevailed, all were examined, more specimens were procured from the animals still living, and, as luck would have it, they furnished data that fall in with the findings yielded by Ad. 4.

The new adenomas were still growing so slowly when utilized that each test period with them could last several months if the risk were taken of a complicating sarcomatosis due to the action of MC on the connective tissue at the site of implantation. Accordingly this risk was assumed.

Ad. 10 closely resembled Ad. 4, but lacked its tendency to hydropic change (Fig. 19).

Ad. MC V (Chart III) (15 mice).—A tumor of the 3rd Gen. provided the tissue. Some of the suspension utilized with OM was thinned with three parts of s-L for implantation with OO. *69 days:* Tumors have grown at the same average rate; three of each group pooled for *TP 2.* The OM's all contain much MC. *98 days:* Four OO growths and one OM taken for microscopy. (Ten of the OM group had died early from intercurrent causes.) *112 days:* Remaining tumors of both groups taken for section.

TP 2.—Again the OO suspension was quarter strength, yet the tumors resulting from it grew much the faster. *61 days:* OO's now so big that five pooled for simple transfer. *75 days:* OM's still not so large as the OO's after 61 days; five pooled for transfer; all contained much MC.

Experiment terminated after 30 days more. Five tumors of each transfer taken for section.

One of the three control tumors of TP 1 of this experiment, used after 69 days to start TP 2, was found microscopically to have contained a solitary, discrete carcinoma (Fig. 20), 4 mm. across and hedged about by reactive tissue into which it was extending (Fig. 21). Similar cancers, also tiny and encapsulated, were come upon in two out of five other control tumors of TP 1, taken merely as specimens after 112 days (Figs. 22 and 23). None was found

in the growths exposed to OM, nor in any of the later tumors of either line (Chart III).

The carcinomas found in the three control growths of TP 1 were identical morphologically and sharply different from any of those due to the changes in Ad. 4; they were not preceded by Ad. solidum as in its case, but appeared

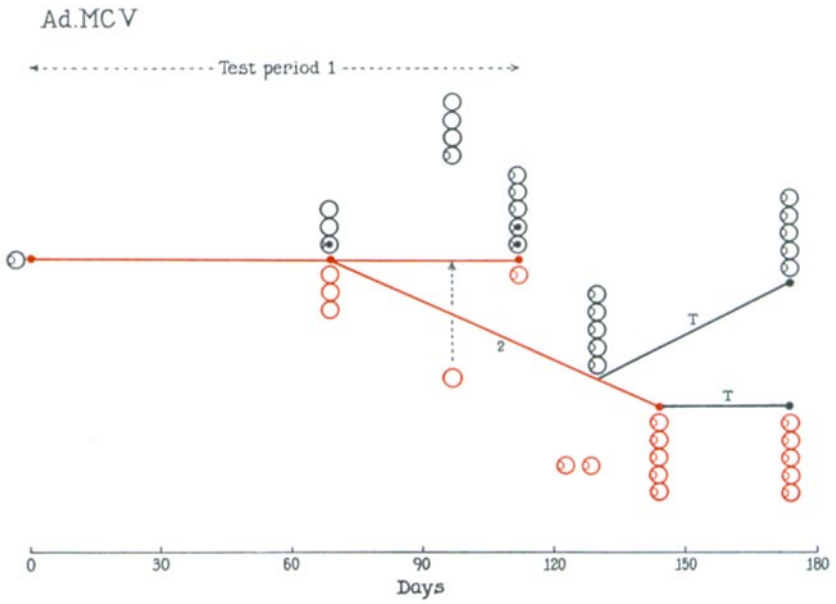

CHART III. Incidence and time of appearance of carcinomatous alterations in Adenoma 10 during its exposure to methylcholanthrene (Ad. MC V).

When utilized for test Adenoma 10 already contained two distinct components,—typical adenomatous tissue consisting of a single layer of tumor cells supported on a delicate stroma, and "lumpy adenoma," termed such because its cells built up into irregular masses on a more substantial stroma (Figs. 24 to 26). The presence of this lumpy component in addition to the ordinary in the growths under test is indicated by a projecting knob inside the circles representing the latter. Central spots indicate that carcinomas were present also.

to have derived directly from the primary adenoma (Fig. 22), retaining some of its cytological characters (Figs. 21 and 23).

The first of the control tumors of TP 1 to show cancer (Fig. 20) contained another unexpected component, namely an adenoma distinct from the typical growth making up the main bulk of the tumor. Instead of having its cells disposed in a single layer on a delicate stroma like the latter, this component had a layer one to three cells thick on a stouter stroma, and at many places its cells had piled up into lumpy masses. In a second tumor of the same TP which

had been let grow longer, the "lumpy adenoma" was found again (Figs. 24 and 25), and renewed search of sections from the 3rd Gen. tumor with which the experiment had been started disclosed in them a small island of it. Later it became frequent, all the tumors of TP 2 and of the final transfers containing it (Chart III). On casting back through the slides of Ad. 10 in the generations before the experiment was begun, it was come upon in several instances (Fig. 26), and it may conceivably have been present in the original Ad. 10, a growth so tiny as to have provided only five small bits of tissue for as many hosts, with none left over for section. Though disorderly in appearance and long exposed to MC, the lumpy adenoma at no time underwent change.

Ad. MC VI was started with a tumor of the 2nd Gen. of Ad. 11, a growth resembling Ad. 4 in general, but calling forth a profuse reactive tissue. During the second test period this tissue became sarcomatous in many tumors of both groups.

Ad. MC VI, TP 1.—45 days: Four times as much tissue had been implanted with OM as with OO, yet the biggest OM tumor is still only 12 mm. across; the OO's are much larger. *105 days:* Growths of both sorts now big; three OO tumors used for TP 2, but only two OM's because the two others taken for pooling proved sarcomatous.

TP 2.—Suspension with OM again four times the thicker. *98 days:* All tumors big, the OM's the bigger; simple transfers made of both for safety. Two of the five OM's taken with this aim had to be discarded because sarcomatous. *118 days:* All remaining growths garnered for section, four OO's and five OM's of TP 2, and five from each of the transfers just mentioned. Two of the OM's of TP 2 proved sarcomatous.

Adenoma 11 persisted as such throughout this experiment, though it was subjected to two consecutive test periods of 3½ and almost 4 months respectively, each of such length that the methylcholanthrene induced several sarcomas.

The Effect of Urethane on Adenoma Cells

A single injection of urethane into mice of strains naturally liable to pulmonary adenomas,—and C mice are amongst the most liable (4),—increases the incidence of these growths and makes them arise earlier, as is common knowledge; and the oftener the substance is administered, the more numerous the tumors become. Accordingly an experiment was done to learn whether repeated injections of urethane throughout many months into a succession of C mice carrying Ad. 4 would bring about changes in the tumor.

Ad. U I (Chart IV).—A suspension in s-L of pooled tissue from three adenomas of the 4th Gen. was injected into the right thigh of 40 young adult males, all receiving 0.15 cc.; and after 15 days, when the animals had developed palpable nodules, they were paired by tumor size and body weight. Two days later a 5 per cent solution of urethane in distilled

water was injected intraperitoneally into one group (0.5 mg. per gm. mouse), and saline solution in equal amount into the other. The injections were repeated each week thereafter (1 mg. of U per gm. mouse, an amount rendering the animal unconscious) until, after eight had been given, the tumors were so large as to require transplantation (Chart IV). The pooled tissue from five of each group was used for the new TP, and 7 days later, to allow for vascularization of the grafts, the injections of U were resumed. But now the Ad. grew faster and fewer injections could be made before the next transplantation, this holding true in subsequent test periods as well. Between the 3rd and 4th TP's, and again between the 4th and 5th, the two lines of tumors were carried for some weeks in uninjected hosts, as also after TP 6, to provide the final specimens. The experiment lasted nearly 13 months in all, and urethane had been injected 30 times.

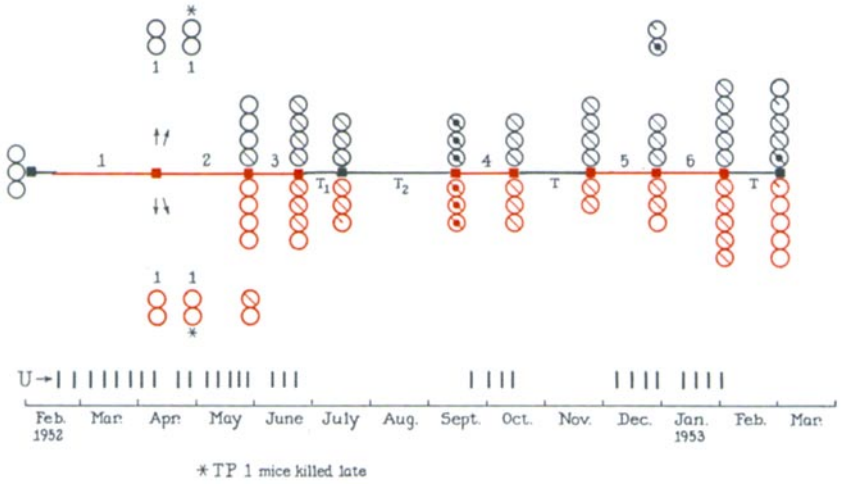

CHART IV. Incidence and time of the successive neoplastic changes occurring during an experiment to learn whether exposure to urethane would induce alterations in Adenoma 4.

The symbols are those used in Charts I and II. The short vertical lines near the base of the chart mark when urethane,—or salt solution for the controls,—was injected into the mice.

No blocks were taken of the TP 1 tumors used for TP 2, but slices from two other growths of the same groups were procured for section at this time and from two more 19 days later (Chart IV). By then the mice receiving U had been given a ninth injection of it, and one of them had at autopsy an adenoma 2.5 mm. across in its lung,—this although it was still a young adult and had first got urethane only 69 days previously. None of the later hosts, which received fewer injections, had any pulmonary growths.

The mice injected many times with U during TP 1 gained weight more slowly than the controls, these averaging 19.8 gm. at the beginning of TP 1, with an increase to 25.4 gm., their growths included, when TP 2 was started, whereas the corresponding weights for the U mice were 20.2 and 22.9 gm. respectively. Nevertheless the adenomas of the two groups did not differ significantly in size, those of the 11 controls that survived to the end of TP 1 averaging 29 mm. across as compared with 28 mm. for the 13 urethanized mice.

Chart IV tells the findings in this experiment. Patches of adenoma solidum were first noted in one of the four control growths of TP 2 that were used for TP 3 but not in any of the four OM tumors also utilized, though it was present in one of two other OM's taken merely as specimens. Because of our impending absence TP 3 had to be terminated after less than a month, and so too with the first of two simple transfers immediately following. Ad. solidum was present in all except one of the 14 tumors fixed for section on these occasions, and frank carcinomas were found as well in all of the tumors of both groups of the second simple transfer (T2), which were pooled for TP 4; yet no cancers were come upon later until the mice of TP 5 were killed, when a single growth of the control series was found to contain them. It was not amongst those used for transplantation. Cancers were absent from then on until the final specimens were obtained, when they appeared again in one of the five controls examined. Adenoma solidum was present in four of the five, as also in one of the five final growths of the urethane series.

It can be seen that urethane did not hasten in the least the change from adenoma to adenoma solidum, nor the appearance later of carcinomas. In this respect the findings resembled those with methylcholanthrene as also in the character of the superimposed neoplastic changes, the ad. solidum (Fig. 27) and the cancers (Fig. 28) being wholly like those of the Ad. MC experiments. The controls served an important purpose in relation to these last, making plain as they did that the same successive changes took place in Ad. 4 when merely transplanted repeatedly in serum-Locke's solution as when olive oil had been added to the suspension.

The Effect of MC on Mammary Carcinomas

The two mammary cancers exposed to MC underwent no significant changes, and hence few details of the tests need be given.

Mam. MC I.—This experiment, begun with tissue from a growth of the 3rd Gen. of Mam. Tumor 1, lasted 14 months in all, the eight test periods totalling 304 days, with two simple transfers between some of them, and two final transfers. A single tumor was used for TP 1, and one or two of each group for TP's 2 and 3, with an increase to five of each group for the later TP's and five for the two final transfers and the end specimens.

Mam. MC II lasted more than 14 months, the nine periods of test for Mam. T2 extending over 323 days, with two intercurrent, simple transfers, and two to end the experiment. The neoplastic tissue utilized for the first test periods came from single growths of the 2nd Gen. of Mam. Tumor 2 and from either one or two for the next two TP's; but later it was procured from four or five animals of each group, and from four of each for the two final transfers and for the end specimens.

When the experiments were begun, the two carcinomas looked closely alike in the gross,—soft, grayish-pink tumors with small hemorrhages here and there, and the usual scattered cysts containing thin, chocolate-colored or blood-stained fluid. As the tests went on they came to have fewer hemorrhages and cysts, and their fine

textured, almost homogeneous tissue underwent more necrosis; but no other gross changes were perceptible.

Mam. T1 proved the more complex tumor microscopically. The 3rd Gen. growth with which the tests were started provided only enough tissue for the implantations, but Figs. 29 and 30 show part of another tumor of the same group. A control growth of TP 1, removed after 48 days with OO, had a wholly similar morphology (Figs. 31 and 32). In this TP, as not in the succeeding ones, both thighs of the test mice were implanted with tumor and OO or OM. The OM tumor opposite the OO of Figs. 31 and 32 resembled it histologically except that it was much smaller (9 mm. as compared with 35 mm.) and was subdivided by reactive tissue elicited by the carcinogen (Fig. 33). Save in these respects the growths remained similar throughout the later tests, but the tumors of the two final transfers, growths long since freed of all OO and OM, were found to differ somewhat histologically (Figs. 34 and 35). At their extending border both consisted of cells massed in solid alveoli, but within the controls differentiation had taken place almost everywhere, innumerable acini having formed, the majority without any lumen, whereas in the growths of the MC series much of the neoplastic tissue was but poorly differentiated, though with scattered acini that were relatively large, many of them with open lumens (Fig. 35). Edema accounted for the loose texture of the growth pictured.

A remarkable difference was encountered in the final specimans of Mam. MC II. When this test was begun, the carcinoma (Mam. T2) was less diversified (Fig. 36) than Mam. T1, though it contained well defined tubules and acini that were mostly open. Structures of both these sorts were found in most of the TP tumors, whether exposed to OM or not, and they were present in all the final transfer specimens of the OM series, whereas in the corresponding specimens of the control series only acini had formed,—not a tubule anywhere in sagittal sections of the five OO tumors (Fig. 37) though in the five OM's they were abundant (Fig. 38). Judged by their morphology, the controls might have been the more malignant cancers. The reason for this singular finding will be dealt with further on.

One fact stands forth from the experiments with the mammary carcinomas, namely that long exposure to MC induced no superimposed neoplastic changes in the cells of these polymorphic, differentiating growths.[5]

DISCUSSION

The Changes Undergone by the Tumors under Test.—The carcinomatous changes occurring "spontaneously" in pulmonary adenomas are multifarious, and the assumption has been general that they are random in character; but this was not true of those witnessed in Adenoma 4. By good fortune our experimentation with this growth necessitated carrying it in twelve parallel lines. In nine

[5] As briefly stated in the *Proceedings of the American Association for Cancer Research* (Dumbell, K. R., and Rous, P., 1954, **1**, 12) the cells of a benign hepatoma of the mouse remained unaltered throughout 10 months of exposure to methylcholanthrene. The findings will be reported in detail after the results of tests with more appropriate carcinogens become available.

of these, new tumors arose in wide variety, in eight of them almost concurrently after more than a year in which the adenoma had undergone no perceptible alteration (Chart V).

The first alteration was always the same, from adenoma to adenoma solidum, a neoplasm of remarkably standardized character, looking as if it had arisen because the proliferative activity of the adenoma cells had newly exceeded their ability to differentiate into acini (Fig. 39). In six lines its presence was soon followed by the appearance of several, or many, highly various carcinomas, all developing at nearly the same time. Though diverse individually, these cancers as a group were similar from tumor line to tumor line.

The assumption had been implicit in our experiments that any adenoma cells undergoing further change in the direction of malignancy would come to the fore and take over the growths. But often this did not happen. Adenoma solidum existed at so many scattered spots, even when first come upon in the growths (Fig. 3), that many of its cells must surely have been present in the pooled suspensions utilized for subsequent transplantations; yet sometimes it was not to be found in the resulting tumors (Charts II and IV), even though large slices of these latter were searched. The same held true of the carcinomas, and much more often. Two of the three control tumors of TP 5, Ad. MC I, Series A, which were used to provide the pooled suspension for TP 6, contained cancerous tissue of several kinds, and this "took" well in its new hosts, proliferated actively, and was anaplastic and invasive, as proven by the findings in 15-day-old nodules (Figs. 13 to 16); yet it was not encountered in any of the large slices from three growths of the same implantation, also controls, that were let grow 35 days and utilized then for the first of the final transfers (Chart I). Cancer was found again, however, in one of two growths of the resulting group, examined after 49 days, and in four of 13 control tumors of the succeeding transfers (Chart I).

The failure of the new tumors to reappear regularly can be readily understood. The rate of proliferation of the adenoma had become rapid before ad. solidum first asserted itself, and, to judge from the relative number of mitotic figures in both, it cannot have grown much the faster. The carcinomas were more aggressive than ad. solidum and certain of them obviously had greater proliferative power, soon constituting most of the tumor in some instances (Figs. 5 and 9). Yet often they had formed only local masses by time of transfer, and since these could not be recognized in the gross they must frequently have escaped inclusion in the transplanted material and in the blocks taken for section. Ordinary adenoma tissue persisted to the last, obviously because cyst formation removed many of its cells from direct replacement. Due allowance must be made for these facts when interpreting the alterations charted.[6]

Chart V shows that adenoma solidum was first come upon in the tumors of both lines of TP 1 of Ad. MC III, examined a year and 5 months after primary transfer of the adenoma. Carcinomas asserted themselves within another 4 months. Early in this interval ad. solidum was found in both lines of the urethane experiment (Ad. U), and again, after 4 months, carcinomas had appeared as well. Meanwhile a similar

[6] Stewart, Grady, and Andervont (2) have already noted that pulmonary adenomas and the derivative carcinomas may grow together throughout many generations, though with the latter gradually taking over.

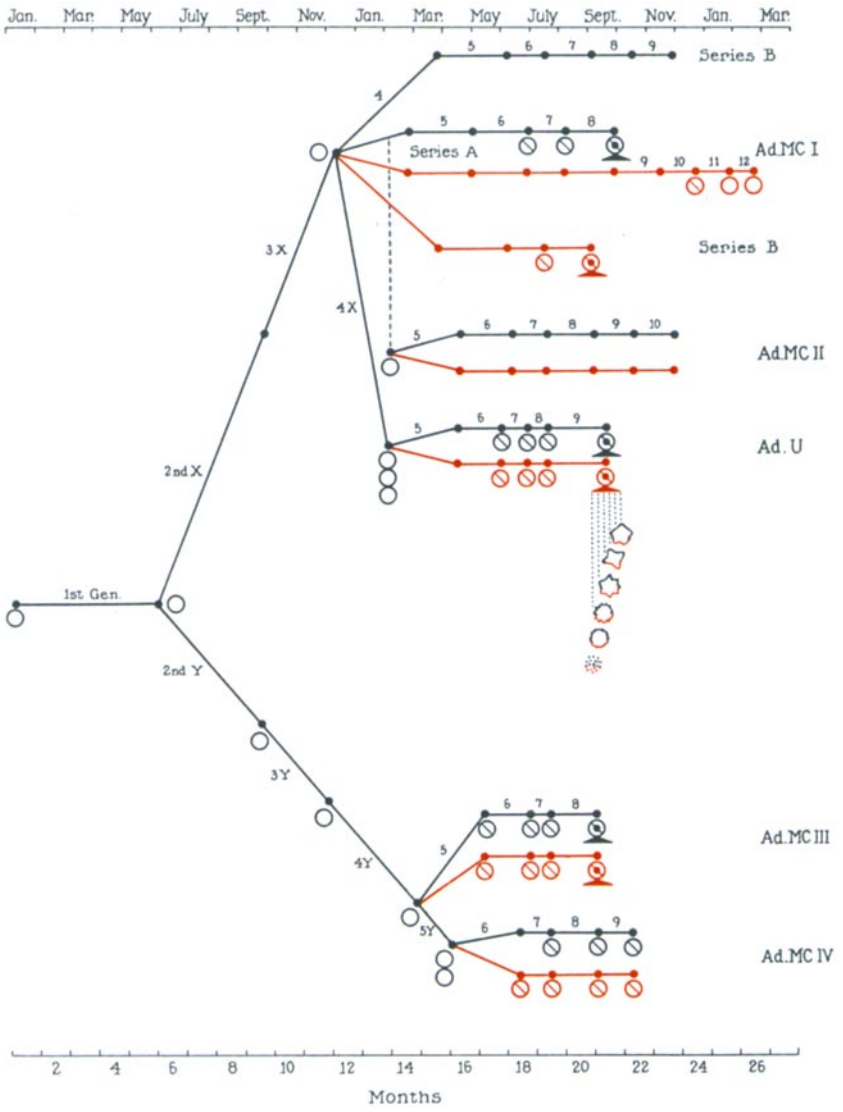

CHART V. Incidence of the successive changes occurring in 12 lines of Adenoma 4 during the experiments of Charts I to IV.—(The course of the preliminary transplantations is shown as well.)

A single tumor of the 1st Gen. was utilized for the 2nd, each mouse receiving a bit by trochar, and two growths (X and Y) of the resulting group were separately transferred after different times, thus providing lines X and Y of the 3rd Gen. A single tumor of the 3rd Gen. X supplied the tissue to start Ad. MC I, and one of the control tumors from the first test period of this experiment was utilized (broken line) to start Ad. MC II. Tissue from three

succession of events was taking place in the controls of Ad. MC I, Series A, and in the line of Series B exposed to MC, cancers developing within 3 months after ad. solidum in the former and after scarcely a month and a half in the latter, ad. solidum in its case having appeared late. Ad. solidum arose concurrently in both lines of Ad. MC IV; but this experiment was discontinued too soon to learn whether cancer would have supervened. In a ninth line of tumor cells (Ad. MC I, Series A, line exposed to MC), ad. solidum was not noted until nearly 2 years after the initial transfer of Ad. MC IV, and then only transiently.

On first view of Chart V, one is inclined to conclude that the occurrence of adenoma solidum at nearly the same time in so many tumor lines must have been the expression of a tendency common to the cells of Adenoma 4. But certain facts rule this out. In every instance the solidum, when first come upon, lay scattered in patches here and there throughout the adenoma (Fig. 3), the patches looking as if they had resulted from sudden neoplastic alterations occurring at many places at once. But if this had been the case, and the changes were consequent on a general tendency of the adenoma cells, why did they never manifest themselves in either line of Ad. MC II and in only one line of Ad. MC I, Series B, and appear only after more than a year in one line of Series A? In all these instances a wide net had been cast for them through transfers of pooled tissue containing many thousand cells; and actually the two lines of Ad. MC I, which remained consistently negative, had been started with the same tumor suspension as the two in which ad. solidum developed, while furthermore a control growth from one of the latter in its first TP (Ad. MC I, Series A) had provided the tumor with which the negative Ad. MC II was begun (Chart I). It seems clear that ad. solidum cells must have been present in the single growth of the 3rd Gen. X from which every line thus far mentioned had stemmed, but so few cells and multiplying so slowly as to escape inclusion in some of the lines. The findings in the experiment with urethane support this inference. Pooled tissue from three growths of the 4th Gen. X was used to start its test lines, thus increasing the chance threefold

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other tumors of the 4th Gen. proper was pooled for the experiment with urethane (Ad. U). Ad. MC's III and IV were begun a little later, with tissue from tumors of the 4th and 5th Gens. Y.

Each pair of symmetrical forkings represents the first test period of an experiment, the black and red indicating respectively the control lines of tumor cells and those exposed to the carcinogen. The symbols for adenoma, adenoma solidum, and carcinoma are the same as in previous charts, but now they represent the general findings at the end of each period instead of standing for individual tumors. A black wedge has been placed beneath the circles that contain central spots bespeaking the presence of cancers, in order to emphasize the morphological spread manifest in these growths; and, to stress their diversity still more, vertical lines of differing length have been extended down from one of the wedges (of Ad. U) toward symbols expressive of the widely various degrees of anaplasia exemplified by the cancers. (These lines and symbols might equally well have been placed under any of the other wedges, whether of the control series or of those exposed to the carcinogen, a fact indicated by making each symbol for degree of anaplasia half black and half red.) After the appearance of cancer in an experimental series, the charting of it is carried no further.

The progressive increase in rate of growth of the adenoma can be inferred from the shortening of the numbered intervals between transplantations.

that the suspension would contain cells of the sort forming ad. solidum; and patches of this soon appeared in both lines of the experiment (Chart III).

It will be noted (Chart V) that a single growth of the 1st Gen. of Ad. 4 provided the tissue to start the 2nd Gen. lines (X and Y) from which respectively the succeeding tumor generations derived that were utilized for the two groups of experiments. Since ad. solidum appeared at nearly the same time in both groups, its cells were probably present in the 1st Gen. tumor just mentioned. They may even have existed in the primary adenoma.

The case was sharply different with the carcinoma cells. Their proliferative power exceeded that of the cells of Ad. 4 sufficiently for some of them at least to have formed visible masses during the many months of propagation preliminary to the tests, had they been present. Furthermore the cancers were always preceded by ad. solidum, not a few of them appearing to have derived from it by very slight alterations. Everything speaks for their origin by superimposed neoplastic changes in ad. solidum cells, —changes occurring before the latter had as yet formed solid masses, in the case of the adenocarcinomas and those forming ropes (Fig. 7).

From all this it seems plain that the concurrent appearance of adenoma solidum in many lines of Adenoma 4, after its propagation throughout more than a year, was not the result of further neoplastic alterations in the ordinary adenoma cells making up the bulk of the tumors, but was due to the gradual multiplication of preexisting solidum cells,—which may even have been present as such in the primary Ad. 4. It was from cells of their kind that the carcinomas derived by superimposed neoplastic changes after many months. Patches of adenoma solidum were always their precursors, and so often did they follow upon these, after nearly the same brief interval (Chart V), that they cannot be ascribed to the influence of extraneous causes but must be laid to an ordered tendency of the solidum cells. They provide indeed type instances of those changes whereby neoplastic cells of certain kinds progress from bad to worse as if by nature.

Adenoma 10 contained two neoplastic components primarily, ordinary adenoma and "lumpy adenoma" (Figs. 24 to 26, Chart III). During the tests the cells of the ordinary adenoma (Fig. 19) underwent change, with result in carcinomas (Figs. 20 to 23) sharply different from any of those which derived in wide variety from the ad. solidum cells of Ad. 4. They were adenocarcinomas of a single sort; they bore the morphological imprint of the parent adenoma, and in one instance they appeared to be taking origin directly from it (Fig. 22). All of them occurred in control growths of TP 1. They were but few and whether consequent on an innate tendency of the adenoma cells cannot be said. Carcinoma cells may have been present in the tumor with which the experiment was begun. But why then did they give rise to growths so episodically?

As will be recalled, no significant change took place in Mammary Carcinoma 1 during its long exposure to methylcholanthrene, and Carcinoma 2 proved

equally obdurate. But the controls in this latter experiment seemed at its termination to have undergone marked change. The reason for this finding becomes plain when one casts back through the literature to Bashford's comprehensive analysis (5) of the changes taking place in "hemorrhagic acinous and alveolar mammary carcinomas" long maintained by transplantation.

Bashford pictured many mammary carcinomas morphologically identical with those of our experiments. At their extending periphery they regularly consisted of alveoli solid with cells, but they underwent differentiation further in, with result in tubules and closed and open acini like those of our Figs. 36 and 38. But this happened only when sufficient time was allowed. In Mam. MC I of the present work the final tumors had grown for the same length of time when procured for section, and those of the test and control series proved essentially alike microscopically. In Mam. MC II, on the other hand, the controls were only 18 days old when examined, as compared with 23 days for the methylcholanthrene series, and these latter had undergone the greater degree of differentiation, in agreement with Bashford's findings.

Circumstances and Length of Exposure to the Carcinogens.—The implantations had been so carried out as to obtain a wide range of exposure to methylcholanthrene, the assumption being that somewhere along this range an optimum would exist for further neoplastic changes to be induced. As already stated, MC killed the tumor cells most closely exposed to it (Fig. 18), and the multiplication of the survivors did not soon remove them from its influence. In consequence, many of the implants failed to yield tumors, and the growths produced by the others were often only a few millimeters across after several weeks, with but slow enlargement later. No fast-growing, outlying nodules appeared such as should have arisen had some of the implanted cells lain beyond the influence of the MC. The control implants, on the other hand, though consisting of relatively little tissue, rapidly gave rise to tumors which soon became big. These findings fall in with previous knowledge of the repressive effect of the polycyclic hydrocarbons on neoplastic cells (6).

The wide distribution of MC in the tumors was strikingly perceptible under ultraviolet light. Growths sliced through when 10 to 12 mm. across after 6 or 7 weeks shone brilliantly purple everywhere, owing to their content of the carcinogen, and still shimmered in purple even on reaching 15 mm. It could sometimes be perceived in large tumors as scattered fluorescing patches after 75 days or more. The occurrence then, or soon after, of sarcomas due to its action on the connective tissue attested to its oncogenic power. There had been reason to fear that the experiments might be prematurely terminated through the development of sarcomas early in some test period because of survival and proliferation of the transplanted stroma of the tumors, with result in continuous exposure of it to MC. For the homogeneity of our C strain mice is such that normal tissues in wide variety can be successfully trans-

planted from one to another of them. But no such untoward happening ended the tests; the minimum time elapsing before the appearance of sarcomas continued to be more than 2 months.

Not a few of the individual test periods of Ad. MC I–IV (Charts I and II) were long enough in themselves to make plain that the adenoma cells were markedly refractory to MC. Nevertheless the exposures were repeated, since it seemed possible that the carcinogen might bring about neoplastic changes eventually through a cumulative effect on the tumor cells. If one assume, as warranted by the findings, that every cell in an intramuscular adenoma reaching a diameter of 15 mm. underwent some direct exposure to MC, the approximate total length of its direct influence during an entire experiment can be calculated. In Ad. MC I, Series A, for example, a single tumor 13 mm. across of TP 1, exposed for 54 days to OM, provided the suspension for TP 2, and when this had lasted 64 days the pooled tissue of four growths, 7, 7, 13, and 15 mm. in diameter, was used for TP 3. Proceeding in this way to the final OM tumor of TP 6,—which had measured only 6 mm. after 49 days,—one arrives at a total of more than 8 months of direct exposure to the carcinogen.

Urethane is so rapidly degraded and removed from the body after intraperitoneal injection that the pulmonary cells are exposed to it for only a few hours (7), yet nevertheless it determines adenomatous change. In our own experience three injections of urethane at weekly intervals into adult C mice of the Institute stock (1 mg. per gm. mouse) are enough to induce multiple adenomas visible in the gross within 3 months; and a single such injection into a mouse far gone in pregnancy will cause swiftly enlarging adenomas to become microscopically perceptible in her young within a few days of birth (8). They appear so rapidly because the increase in proliferative activity due to adenomatous change is complemented by the much greater activity normal to alveolar cells of the growing lung. Knowing these facts, one might suppose that any superimposed neoplastic changes undergone by adenomatous cells already multiplying rapidly (like those exposed to urethane in the present work) would soon come to view. And there was the more reason to think so because of the numerous injections of urethane given during the first test period (Chart IV). Adenoma solidum did indeed appear during the next test period, but to the same extent in the control tumors as in the urethanized ones. This held true also of the later carcinomas.

In sum, the direct exposure of the adenoma cells to methylcholanthrene and urethane was vastly greater than that required for these agents to render normal lung cells adenomatous, and must have immensely exceeded any intercurrent stimulation of carcinogenic sort to which adenoma cells might be exposed under natural conditions. Furthermore the methylcholanthrene and urethane were brought to bear during periods which would seem *a priori* to have been especially favorable to their action, that is to say before and during times

when carcinomatous changes were taking place spontaneously in the adenomas. Yet no alterations occurred that could be referred to these agents.

The growth rate of Adenoma 4 was wholly unaffected by the urethane, a fact which accords with Rogers' conclusion that this substance acts only to initiate neoplastic change, not to promote multiplication of the tumor cells it brings into being (9).

The mammary carcinomas tested were of a kind that our C mice provide not infrequently. They had the morphology of those determined by the Bittner agent, and such growths take origin from mammary adenomas which this factor in some way induces, arising from them much sooner when methylcholanthrene is painted on the skin (10) or insufflated in almond oil (11). Several investigators believe that the Bittner agent may do no more than bring the adenomas into existence, these undergoing cancerous changes for reasons still unknown. Because of uncertainty in this matter the only conclusion warranted by the experiments here reported is that the polymorphic, differentiating cells of the mammary cancers proved refractory to change by methylcholanthrene.

As already stated, methylcholanthrene repressed the mammary tumors even more than it did the pulmonary. Of 12 mice implanted for the OM group of TP 3, Mam. MC I, eight still had no growths when the next TP was started after 41 days, and so too with six of the 15 of TP 8 when this was terminated after 44 days. Such tumors as did arise enlarged slowly. The OM tumor of TP 1, used to start TP 2 after 29 days, was then only 10 mm. across (as compared with 33 mm. for the one providing the control tissue); that of TP 2 measured 11 mm. when utilized after 39 days for TP 3; and the two growths of TP 3, pooled after 41 days for a simple transfer with s-L, were 7 and 12 mm. across, respectively,—all of which is to say that the neoplastic cells were closely exposed to MC throughout three consecutive periods amounting in all to more than 3 months.

On a calculation like that from the data of Ad. MC I, the total direct exposure to MC in Mam. MC I amounted to more than 10 months, and to only a little less in Mam. MC II.

Methylcholanthrene had such an inhibitory influence on all the tumors tested that it seemed possible some selection would take place from amongst their cells, with result in growths unlike the controls. This did not happen, nor was the slowed proliferation of the tumors due to any permanent change in their cells. After the test periods were ended the growths long held back by MC grew as fast, on the average, as the controls.

Some of the tumors exposed to OM, both pulmonary and mammary, were allowed to get big before they were utilized, together with smaller ones, for

the next test period, and much of their recent growth must have gone on at a distance from the oil droplets,—which furthermore were gradually losing the MC within them. Also at times during each experiment transfers were made without OM, the tumor cells proliferating for weeks in a milieu free from it. Here, one might think, were abundant opportunities for them to have escaped from its influence. But it may be recalled that what a carcinogen does toward rendering normal cells neoplastic is passed on to their successors, and in consequence it has a summative effect when reapplied (12). If the tumor cells of the present work failed in this transmission, their difference from normal cells was profound. These latter often continue to multiply for months or years after exposure to a carcinogen has ceased, doing so because of the chronic inflammation it has induced in the supporting tissue, and nevertheless tumors ultimately arise (13).

General Considerations.—The present findings with pulmonary adenomas and mammary carcinomas differ diametrically from those obtained by exposing rabbit epidermis to methylcholanthrene while it is undergoing infection with the Shope papilloma virus. Such exposure, though lasting but a few days, was found to retard the growth of the papillomas caused by this latter, yet to hasten markedly the appearance of carcinomas derived from their virus-infected cells (14). But the conditions obtaining in these cells were singular. The papillomas caused by the Shope virus closely resemble those induced by the carcinogenic hydrocarbons in their general features and often undergo similar alterations to squamous cell carcinomas, yet distinctive differences can be perceived in their finer cytology (15), and mongrel tumors arise after injection of the Shope virus into the blood stream of rabbits carrying tar papillomas (16). Under such circumstances the virus lodges in these growths, and, in addition to changing some of them into carcinomas almost overnight, converts many into exceedingly vigorous tumors composed of cells exhibiting individually some of the morphological traits of the papillomas of both sorts. Furthermore when pieces of the carcinomas induced with chemical carcinogens are exposed to the papilloma virus *in vitro* and implanted in their original hosts, they give rise frequently to cancers which not only are greatly more malignant than those produced by implants of unexposed pieces of the same cancers, but which often exhibit cytological alterations indicative of the influence of the virus (1 *d*). It follows that to expose epidermal cells to methylcholanthrene while they are becoming infected with virus, as in the experiments already mentioned, is to bring to bear on them concurrently two carcinogenic agents acting in ways that differ; and, this being so, the hastening of cancerous changes can be readily understood. No such conditions obtained in the tests here reported with the cells of pulmonary adenomas. These were of a kind readily induced by methylcholanthrene or urethane, and the point at issue was whether exposure to one

or the other of these substances would bring about further alterations in them, such as are often witnessed.

The failure of the carcinogens to induce superimposed changes in adenoma cells does not necessarily imply that they are fundamentally different in character from primary neoplastic changes. The adenoma cells might have been refractory to the influence of methylcholanthrene and urethane merely because of their pathological state. Much more significant, as bespeaking such a difference, is the *spontaneous* occurrence of superimposed neoplastic changes, primary neoplastic change being always an induced phenomenon, so far as present knowledge goes. The carcinomas arising after adenoma solidum asserted itself in Adenoma 4 were the expressions of a new diversity: the solidum was a growth of narrow morphological type, yet the cancers deriving from it were highly various. Similar instances, if less comprehensively exemplified, are familiar to those studying tumors widely. Any hypothesis on the essential nature of the neoplastic state must take them into account.

Foulds has lately reviewed what is known of the successive neoplastic changes undergone by tumor cells (17) and has inferred that "the capacity for progression is probably inherent in the nature of tumors" and is independent of specific carcinogenic stimuli. The superimposed carcinomatous changes that took place during the tests here reported were certainly independent of such stimuli. And to judge from the findings with Adenoma 4, they were independent as well of the rate at which the neoplastic cells divided, appearing no sooner in control tumors growing rapidly than in those that enlarged very slowly because of the repressive effects of methylcholanthrene. In this respect, as in some others mentioned in our introductory paragraph, the superimposed neoplastic alterations resembled primary neoplastic change. Investigators have been much concerned until recently with whether the initiation of neoplastic change in normal cells is influenced by the rate of their proliferation. The bulk of the evidence now accumulated indicates that it is devoid of effect (18).

In a previous paper facts have been reported (13) which seem to show that the exposure of normal cells to a carcinogen often does no more than start a train of cellular events which culminates in the neoplastic state after this agent has ceased to act, sometimes long afterwards. The later, superimposed, neoplastic changes whereby tumor cells progress from bad to worse may well represent further steps in a sequential process begun primarily by carcinogenic stimulation but taking place independently of this thereafter.

SUMMARY

Three spontaneous pulmonary adenomas of C mice, morphologically resembling those induced by methylcholanthrene or urethane, were propagated in host after host under conditions such that the neoplastic cells were directly exposed, while proliferating, to one or the other of these agents. The successive

periods of test lasted for more than a year in some instances, the total exposure to the carcinogens far exceeding that required to change normal pulmonary cells into adenoma cells. One of the adenomas remained unaltered, and the others underwent cancerous changes; but these took place with equal frequency in the control growths, and their occurrence was neither hastened nor delayed by the carcinogens.

Two polymorphous mammary carcinomas of "milk-factor" type, with the characteristic tendency to form acini and tubules, were exposed to methylcholanthrene in the same way as the pulmonary adenomas and for periods quite as long. Their cells continued to differentiate, and in other respects underwent no significant change.

Urethane had no influence on the rate of growth of the adenomas exposed to it; methylcholanthrene, on the other hand, markedly retarded the enlargement both of them and of the mammary tumors. Its inhibitory influence was not passed on from cell to cell however; when freed of the carcinogen by further transplantation, the retarded tumors grew as fast as the controls. Furthermore the retardation caused no evident delay in the occurrence of cancerous changes in the adenomas.

One of the adenomas was maintained in twelve parallel lines while under test and new tumors arose in nine of them, the earliest appearing more than fifteen months after initial transfer of the growth. Always it was an adenoma solidum, this appearing almost concurrently in eight of the nine lines. In six of them it was soon followed by carcinomas, the sequence of events and the morphological findings both indicating that they had derived from it. Individually the cancers were widely various, but they were similar on the whole from line to line. Carcinomas of a wholly different aspect arose from the other adenoma undergoing cancerous change, and they were not preceded by adenoma solidum. In both instances the character of the superimposed neoplastic alterations seemed to have been determined by some inherent trait of the adenoma concerned.

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EXPLANATION OF PLATES

The sections were all stained with eosin and methylene blue.
(Figs. 1 to 18 are from the same experiment.)

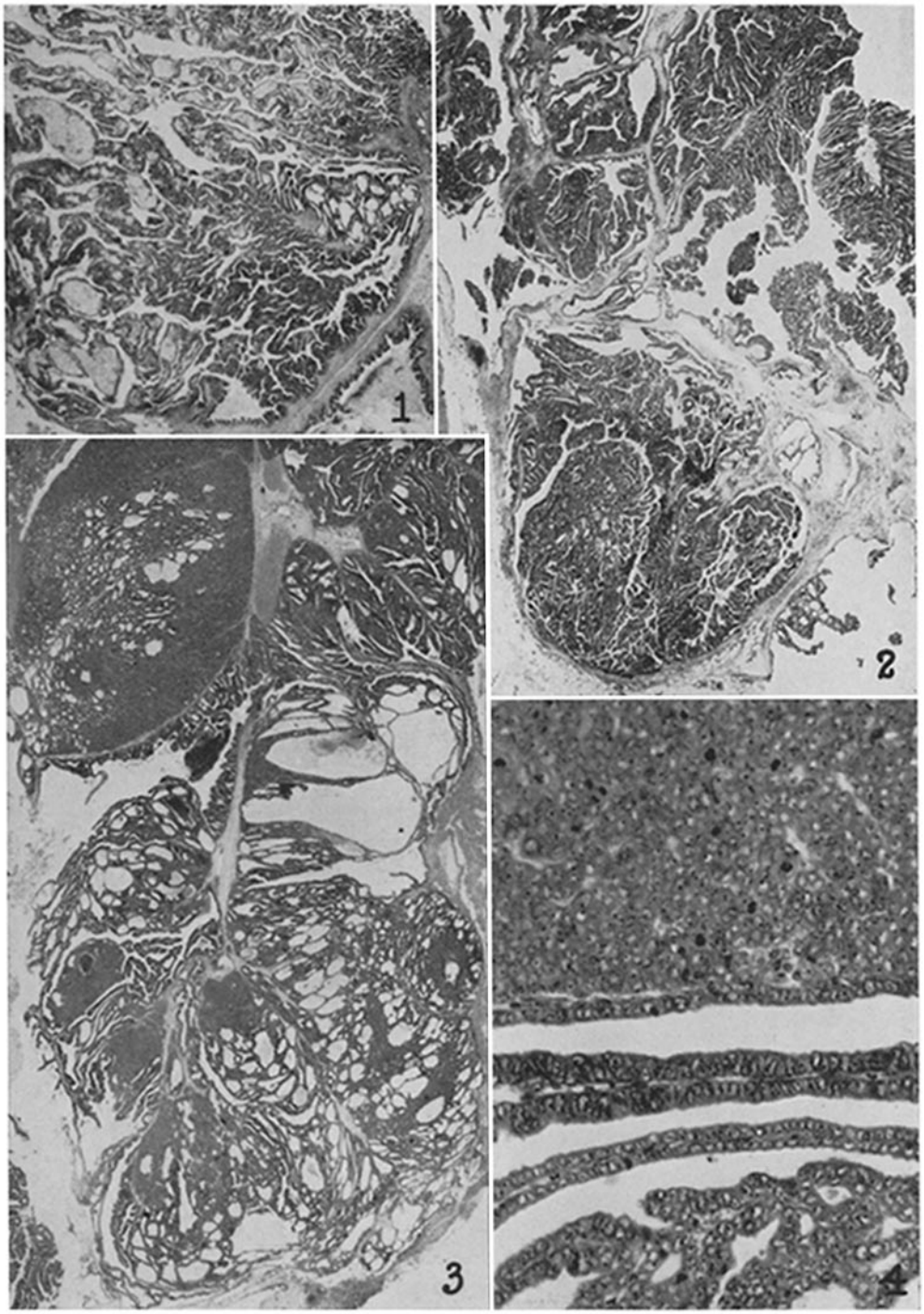
PLATE 55

FIG. 1. Tumor with which Ad. MC I was begun, a 3rd Generation growth of Adenoma 4. It shows some hydropic degeneration. × 14.

FIG. 2. A control tumor of Test Period 1, Ad. MC I (Chart I), removed 49 days after an implantation of tissue suspension together with olive oil (OO). The adenoma is unaltered, but in better condition than that of Fig. 1. × 14.

FIG. 3. Part of one of the control growths of TP 3, Ad. MC I, Series A, which furnished the tissue for TP 4. Numerous patches of adenoma solidum can be seen. They are situated mostly at the periphery of subdivisions in the tumor. × 17.

FIG. 4. Part of the tumor of Fig. 3 at higher magnification, to show ordinary adenoma and adenoma solidum next each other, their cells looking nearly alike. The solidum is not yet wholly compacted. Mitoses are numerous in both growths. × 308.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

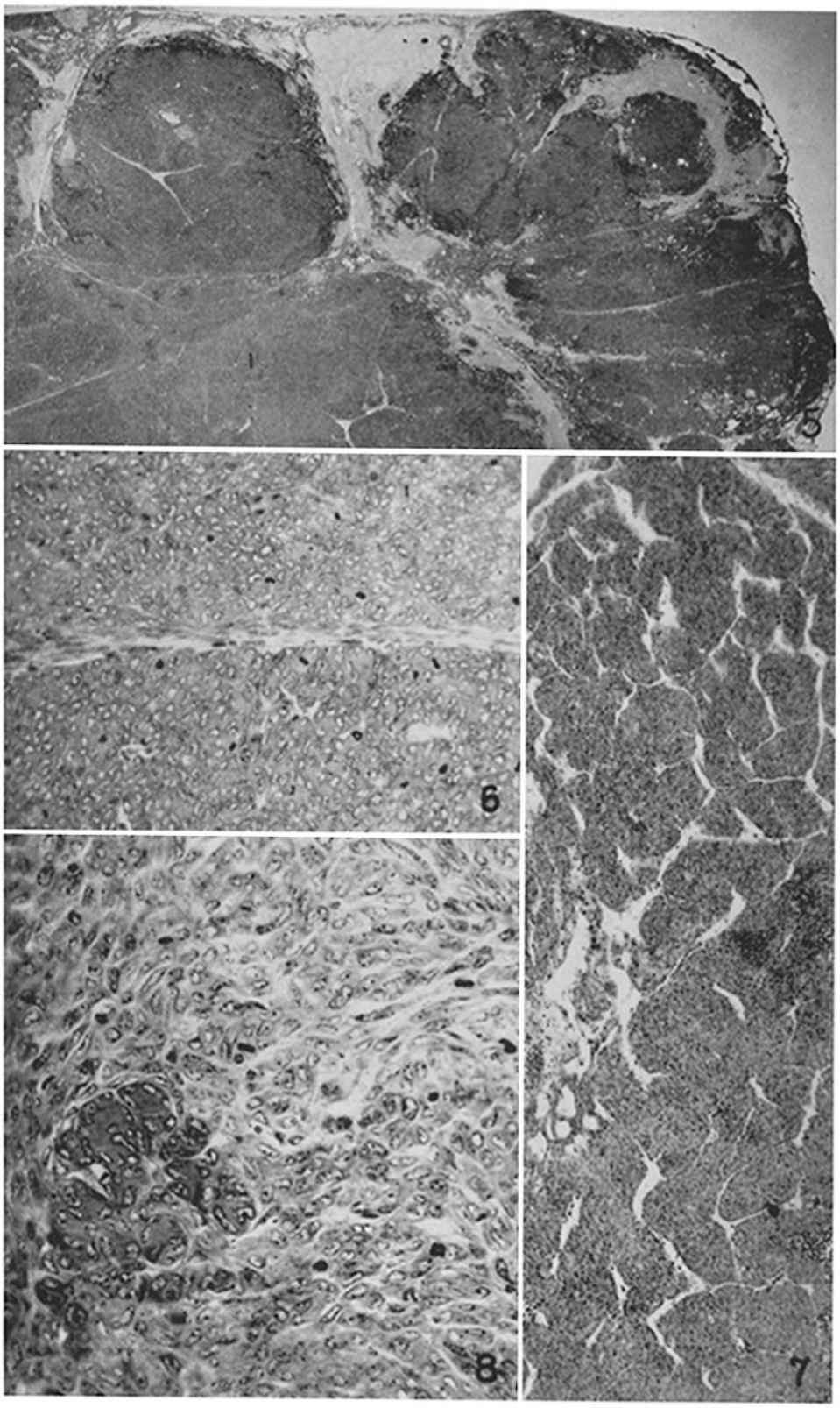
PLATE 56

FIG. 5. Part of a 60 day old control tumor resulting from the first transfer, T, made after TP 4, Ad. MC I Series A. The growth looks almost solid, but in some spots ordinary adenoma can be discerned. Higher magnification showed the solid areas to consist of adenomasolidum, adenocarcinomas, and carcinoma solidum. × 13.

FIG. 6. Part of the same tumor: adjoining cancers of differing tinctorial value, but of nearly similar cytology. The darker one is a rifted carcinoma, whereas the lighter is a carcinoma solidum. The nuclei of both have lost the uniformity of adenoma solidum. Mitoses are very numerous. × 229.

FIG. 7. Carcinoma consisting of thick ropes of cells; part of a control tumor of T_1 OO after TP 6 of Ad. MC I, Series A. × 90.

FIG. 8. Another region in the same tumor; carcinoma with fusiform cells and an included island of adenocarcinoma. × 395.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

PLATE 57

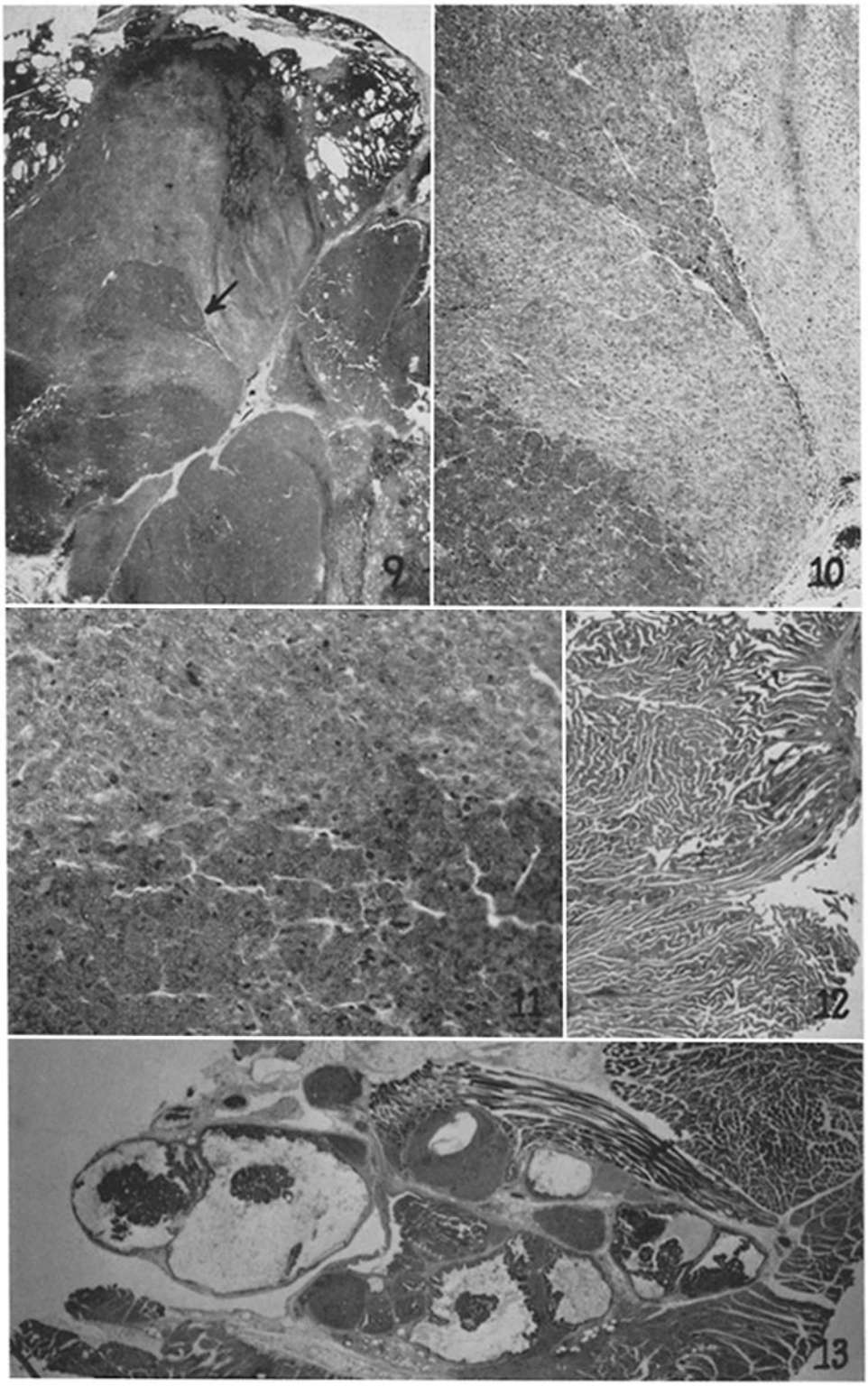
FIG. 9. Part of a tumor of the final control group of T_2 OO, Series A. Five carcinomas at least are present in the mass shown, but much ordinary, cystic, adenomatous tissue is also present, with patches of ad. solidum amidst it. × 13.

FIG. 10. The wedge of compacted adenocarcinomatous tissue to which the arrow points in Fig. 9. It has stained more deeply with methylene blue than the cancers to either side,—one solid, the other rifted,—as has also another adenocarcinoma at the lower corner of the picture. × 60.

FIG. 11. Border of the adenocarcinoma shown at the lower corner of Fig. 10, and an adjacent area of carcinoma solidum. × 192.

FIG. 12. Part of the tumor from the last surviving animal of the TP 6 group, which was exposed to methylcholanthrene in olive oil (OM). The adenoma has not changed since its first exposure to OM 14 months before. × 14.

FIG. 13. A control implant of TP 6, removed from the thigh muscles 15 days after implantation. It consists of small, discrete masses of carcinomatous tissue (see also Figs. 14 to 16) and of cysts lined with adenoma cells, many of them containing papillomatous ingrowths covered with these latter. Numerous small, round lacunae along the periphery of the nodule mark where droplets of olive oil were present. × 14.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

PLATE 58

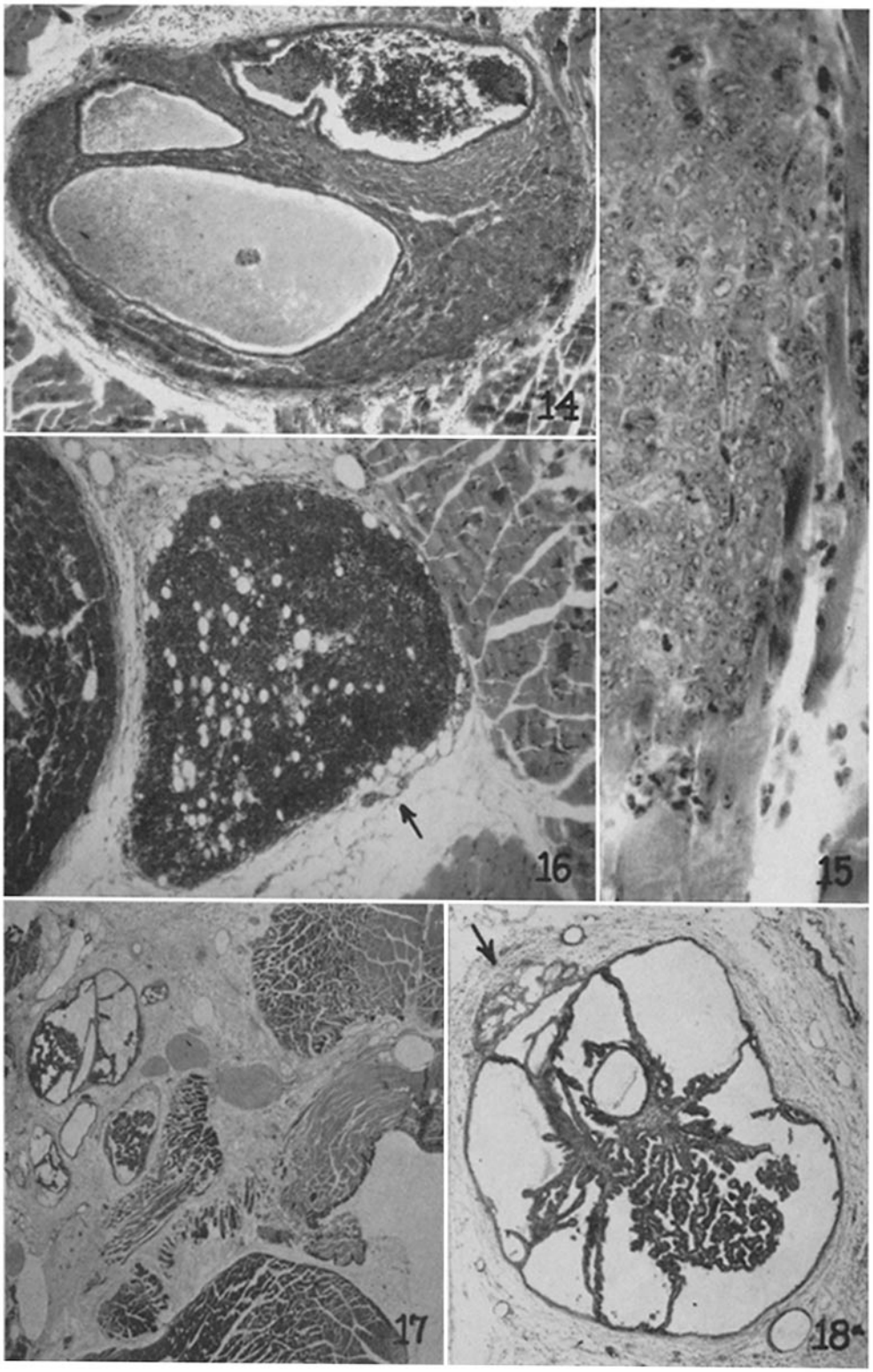
FIG. 14. One of the cancerous masses of Fig. 13, containing three cysts lined with a single layer of adenomatous epithelium. One has many red blood cells within it. The cancer itself is a partially compacted adenocarcinoma; most of its enlargement has been expansile. × 58.

FIG. 15. One of the cancers of Fig. 13, invading the adjacent muscle by extension between its fibers and replacement of them. Several mitoses can be seen. There is almost no cellular reaction round about. × 364.

FIG. 16. Another cancerous mass in the same implant. It consists of carcinoma solidum lying amidst adipose tissue and penetrating between the individual fat cells (arrow),—of which it has already engulfed many, as attested by the small holes of almost uniform size in its midst. The larger holes in the connective tissue along the top of the photograph mark where olive oil droplets were present. Part of another mass of carcinomatous tissue can be seen at the left. × 69.

FIG. 17. A TP 6 implant removed after 15 days' exposure to methylcholanthrene in olive oil: for comparison with the control implant of Fig. 13. The nodule, scarcely one-third the size of the control, consists of several relatively small, adenomatous cysts. The light gray areas near by are sectioned nerves. Round holes mark where droplets of OM lay scattered in the muscle and intermuscular connective tissue. The two larger, irregularly oblong holes at the left are those of cysts partly lined at their upper ends by dead or dying adenoma cells, as higher magnification showed. × 14.

FIG. 18. Adenomatous cyst from another of the 15-day implants with OM. The enlargement of the cyst has brought it into contact with several small OM globules (arrow), and the adenoma cells have extended around and between these, only to succumb to the methylcholanthrene they contained. Other near by droplets of OM have narrow, dark zones around them of polymorphonuclear leucocytes. × 33.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

PLATE 59

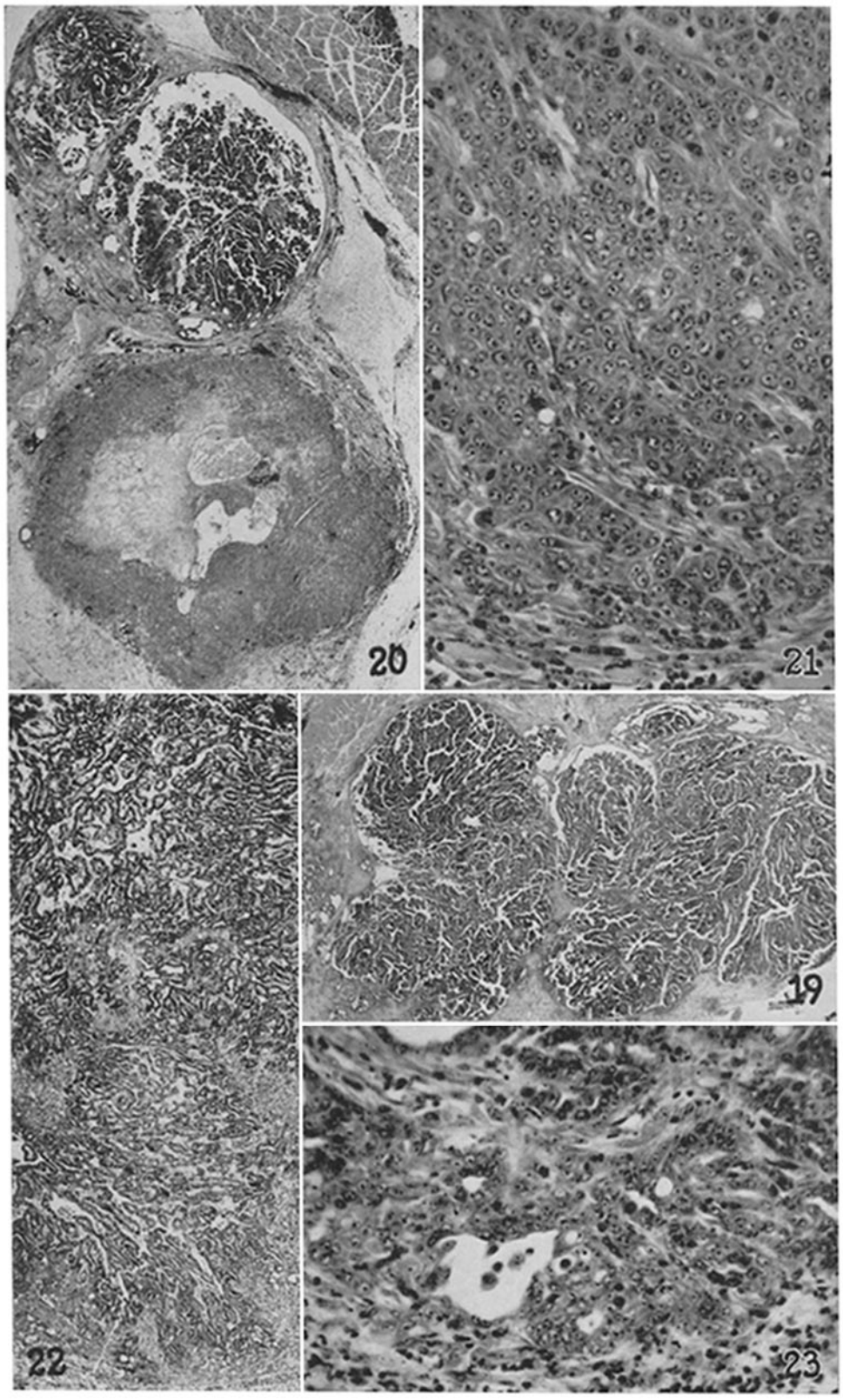
FIG. 19. Adenoma 10; part of the 3rd Gen. tumor providing the tissue for Ad. MC V (Chart III). The growth closely resembles Adenoma 4 (Figs. 1 and 2) save that it has no tendency to hydropic change. × 14.

FIG. 20. Part of a control tumor of the first test period of Ad. MC V, procured after 69 days to start TP 2. The round, gray area consists of carcinomatous tissue, necrotic and cystic at its center. Near-by are two nodules of ordinary ad. All are separated by reactive tissue. × 14.

FIG. 21. Border of the carcinomatous mass of Fig. 20, at a spot where invasion of the reactive tissue is taking place. The neoplastic cells still much resemble adenoma cells, but lie in columns or oblong masses. × 280.

FIG. 22. Similar cancerous change in another control tumor of the same group, now 112 days old. All gradations can be seen between ordinary ad. and carcinoma. This latter is invading along its periphery (bottom of picture). × 36.

FIG. 23. Edge of the carcinomatous growth of Fig. 22. Its cells have adenomatous features, and are like those of Fig. 21 × 224.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

PLATE 60

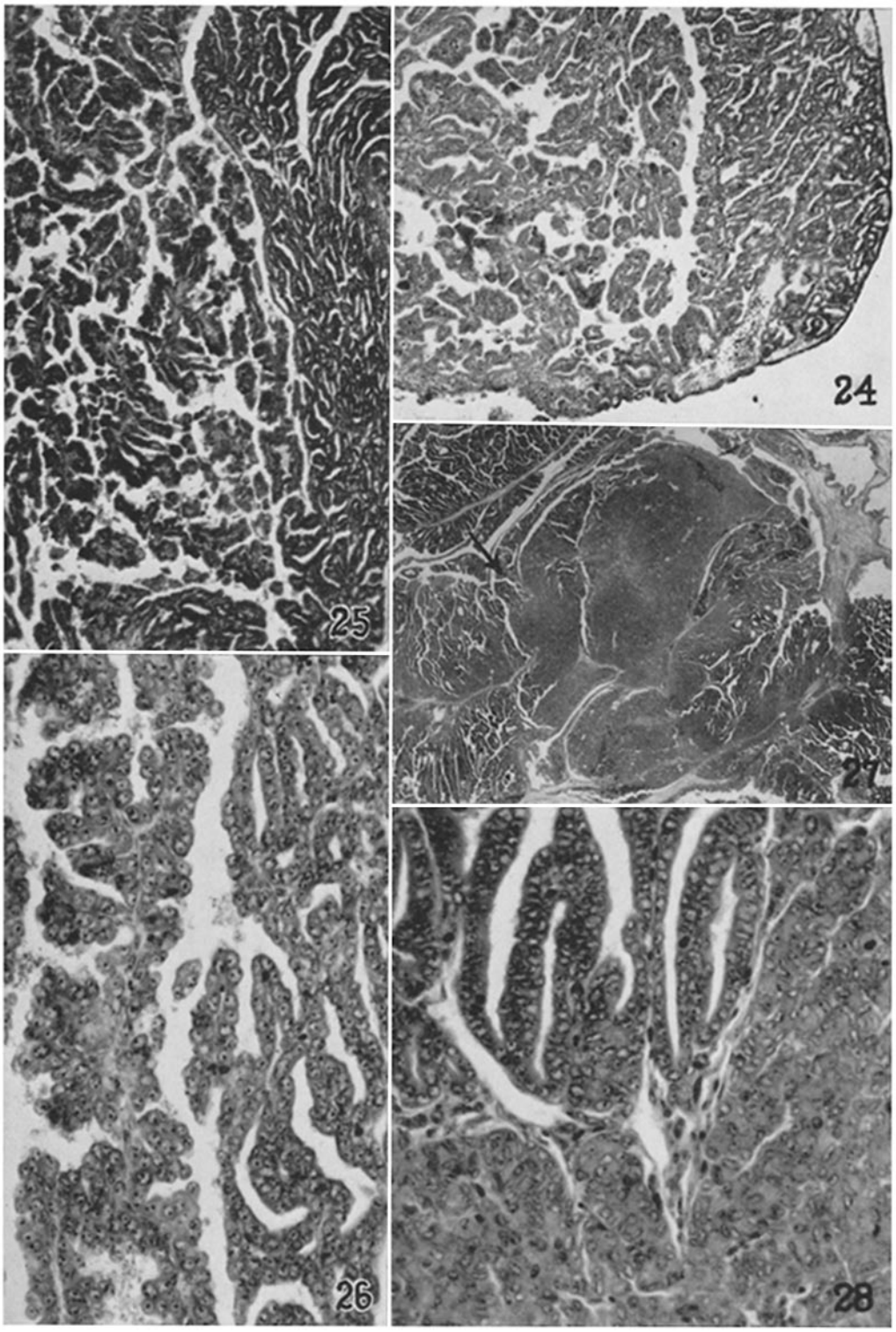
FIG. 24. Adenoma 10. A tumor of the 1st Gen., to show its two components. On the right is ordinary adenoma tissue, more close textured than that of Ad. 4. It connects near the bottom of the picture with the adjacent "lumpy adenoma." × 51.

FIG. 25. Part of a control tumor of Ad. MC V, from a mouse killed after 97 days of TP 1; to show ordinary adenoma and lumpy adenoma, separate though next each other. × 60.

FIG. 26. Part of the section furnishing Fig. 25 at higher magnification. The cells of the two adenomas are similar. Mitoses are rare in both. × 280.

FIG. 27. Part of a final control specimen of the experiment with urethane (Chart IV); from a tumor provided by a simple transfer after six test periods. The large solid mass consists of adenoma solidum and several adenocarcinomas; around it is much ordinary adenoma tissue containing patches of ad. solidum. × 13.

FIG. 28. Higher magnification of the spot toward which the arrow points in Fig. 27; adenocarcinoma with ordinary adenoma next it. × 90.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

PLATE 61

FIG. 29. A 3rd Gen. growth of Mam. Tumor 1. Another tumor of the same transplantation was used *in toto* to provide the tissue for the primary implantations of Mam. MC I. The cancer is characteristically polymorphic. × 10.

FIG. 30. Higher magnification of a part of the same tumor, to show an area consisting of "closed acini." × 120.

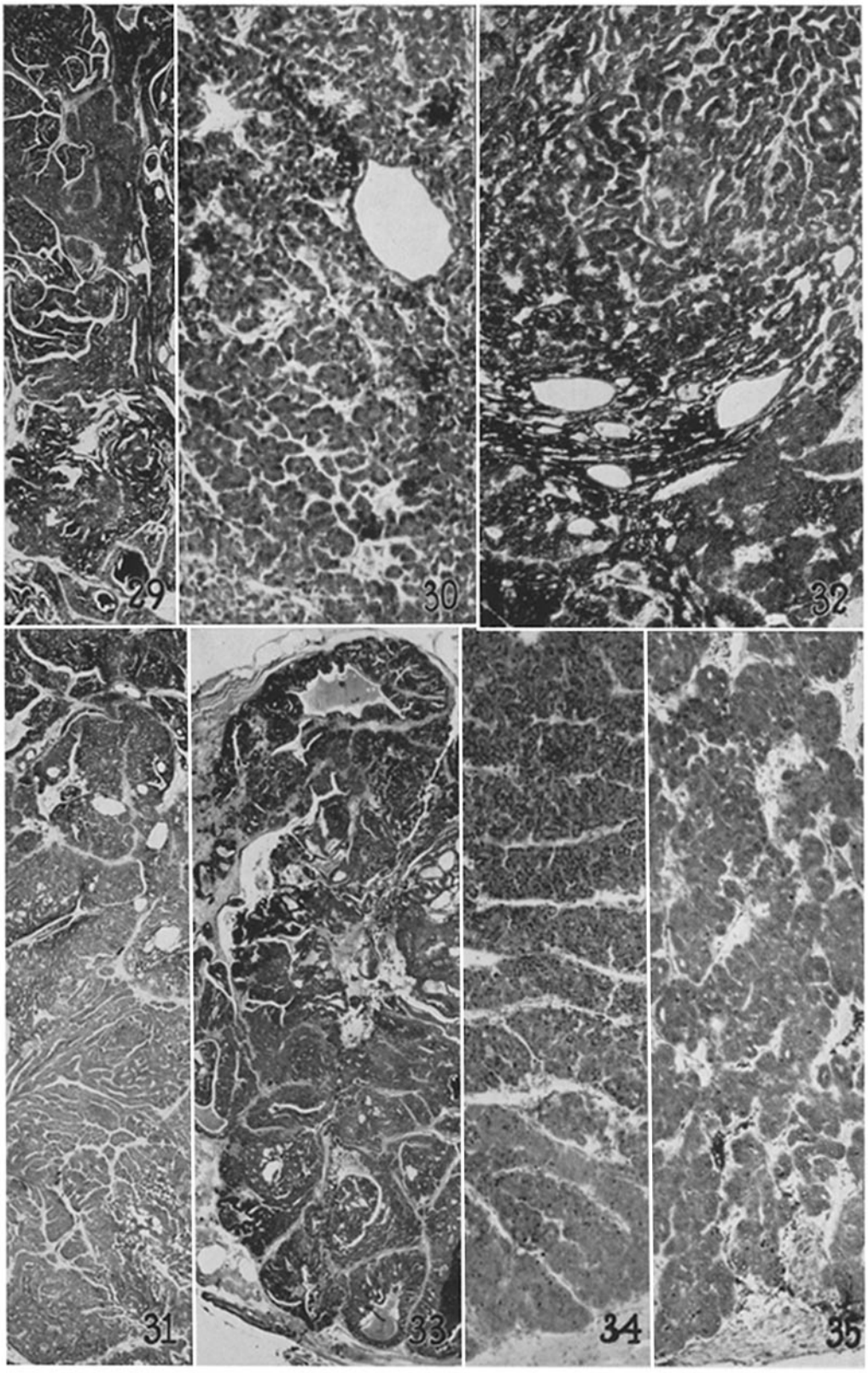
FIG. 31. Part of a control tumor of TP 1, as utilized for TP 2, OO, Mam., MC I. It was 49 days old, 35 mm. across, and resembled the growth of Fig. 29 in its polymorphic character. × 11.

FIG. 32. Part of the tumor of Fig. 31 at higher magnification; to show solid alveoli, acini, and tubules. × 56.

FIG. 33. Tumor from the opposite thigh of the animal providing Fig. 31, after 49 days of exposure to OM. The growth was only 9 mm. across, wholly surrounded by muscle, and was split up and toughened by reactive connective tissue. The holes just outside it (lower left) mark where globules of OM were present, and it fluoresced brilliant purple in ultraviolet light. At high power it had the same polymorphism as the control growth of Figs. 31 and 32. × 11.

FIG. 34. One of the final control tumors of Mam. MC I, a growth of the second transfer after eight test periods with OO. Along its border are solid alveoli, which differentiate into acini further in. The acini are mostly closed, like those of the carcinoma when it was first subjected to test (Fig. 30). × 85.

FIG. 35. Border of a tumor from the corresponding group of the second transfer after eight periods with OM. Its loose structure is due to edema. Again one finds solid alveoli along the border of the growth, and acinar differentiation further in, but many of the acini are open. The bright dots in the alveolar region are nuclei. × 90.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)

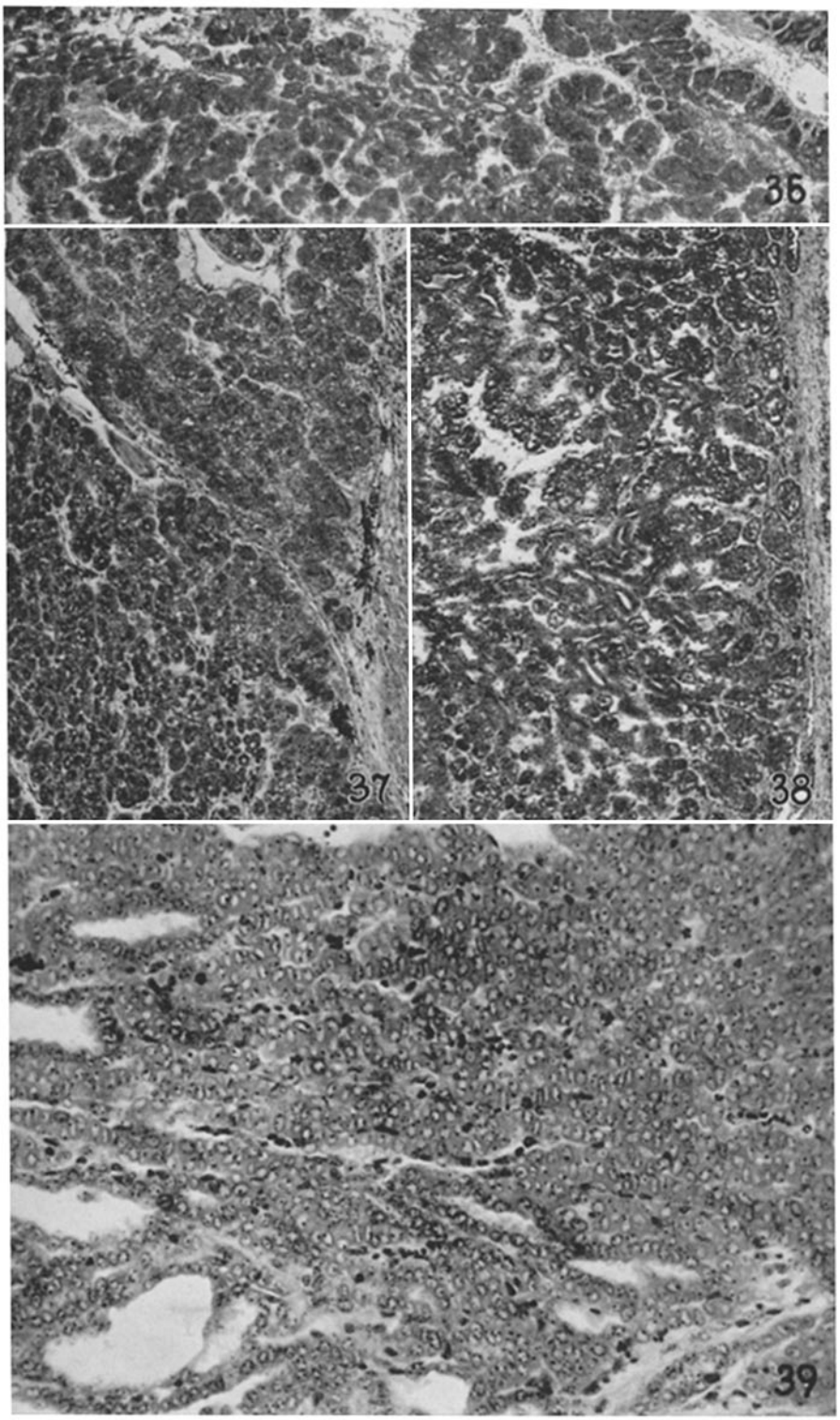
PLATE 62

FIG. 36. Tumor of the 2nd Gen., Mam. T 2; a suspension of its tissue was used for the primary implantations of Mam. MC II. The growth consists of solid alveoli, acini, and tubules. × 60.

FIG. 37. Periphery of a final control tumor of Mam. MC II, after 9 periods with OO and two subsequent transfers without it; the growth is 18 days old. Along its edge (right) are solid alveoli and differentiated acini lie further in. Nowhere can tubules be seen. × 60.

FIG. 38. Periphery of a corresponding final tumor from the series submitted to OM; growth 23 days old. Not only have the cells of the marginal alveoli differentiated into acini further in, but into tubules as well. × 60.

FIG. 39. Early stage in the formation of an adenoma solidum; border of the mass,—in which there are some clefts still. Its cells have the ordinary adenomatous aspect, save where laterally compressed in consequence of their proliferation. There they have elongated nuclei. Some ordinary acini like those round about are undergoing inclusion in the mass. From a control tumor of TP 2, Ad. MC IV. × 253.



(Dumbell and Rous: Carcinogens and superimposed neoplastic changes)