THE NEOPLASTIC POTENTIALITIES OF MOUSE EMBRYO TISSUES

V. The Tumors Elicited with Methylcholanthrene from Pulmonary Epithelium

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Plates 6 to 11

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Benign and malignant tumors arise soon and with great regularity from the epidermis and the gastric epithelium of mouse embryos after fragments of these tissues have been implanted, together with methylcholanthrene, in the thigh muscles of adult animals of homologous strain (1–3). The present paper describes experiments, already reported in brief (2, 4), which were carried out with fetal lung. Methylcholanthrene induces remarkable changes in the transplanted pulmonary tissues, and tumors in wide variety quickly derive from it.

Method and Materials

The technique was essentially that already described (1, 3). For most of the tests mice of C strain have been used as previously, though some tests have been made with Webster-Swiss animals and those of the A breed. Embryos 18 days along, as determined by the vaginal plug method, were used in the main; they averaged 18 mm. in length. The youngest embryos employed were 15 days old (13 mm. long). The pulmonary tissue of smaller ones is difficult to procure in the necessary quantity without inclusion of the cells of other organs.

To expose the lungs, an apron-shaped flap in the body wall, extending down and across the abdomen, with its base on the upper thorax, was freed with the cautery, reflected over the neck, and pinned down. The thoracic viscera were torn out in a mass with fine curved forceps, washed in Tyrode's solution, and under a binocular dissecting microscope at × 17, the lungs were cut away and washed again. After those of the entire litter had been assembled in a little of the salt solution, they were chopped into small fragments and the resulting small quantities of tissue suspension were implanted through skin slits into the posterior thigh muscles of young adult C males. Olive oil saturated with Scharlach R and containing 1 per cent of methylcholanthrene (*OSSM*) was ordinarily introduced with the tissue, but many control implantations were made with Locke's solution or Tyrode's solution only, or else with olive oil saturated with Scharlach R (*OSS*). Just prior to use the oil preparations were shaken in a mechanical shaker with one-third as much Tyrode's or Locke's solution to make an emulsion, and this emulsion was maintained throughout the period of the injections by drawing it in and out of a syringe through a needle. Each implantation consisted of 0.025 cc. tissue suspension—containing often but a few fragments of tissue—followed by 0.075 cc. salt solution, and 0.025 cc. of oil. The oil was drawn first in the injecting syringe, then the salt solution, and lastly the tissue suspension, the interposed column of Tyrode preventing the fragments from becoming covered with oil and these latter blocking escape of the oil back along the needle track. More than 200 implantations were carried out, in 13 experiments,

each with the pooled lungs from a separate litter of embryos. The injected thighs were palpated for nodules 3 weeks after implantation and any found were drawn to size on charts, as they were also at each of the frequent later examinations prior to sacrifice. Autopsy findings were usually sketched as well as recorded, and blocks were taken in acid Zenker solution for sectioning and staining with eosin and methylene blue. Many were cut serially. When tumors were to be propagated, the transplantations were carried out with trocars, through skin slits, into the thigh muscles of young male adults of the same strain. As in the case of epidermis and stomach, special care was taken when the tumors were transplanted to exclude the possibility that any methylcholanthrene was transferred with them. Scharlach R remains even longer in olive oil than the carcinogen, as ultraviolet light has shown, and the neoplastic tissue utilized was regularly devoid of any pink coloration with the dye.

EXPERIMENTAL

Findings in the Control Implants in C Mice

The lung tissue of embryo mice of the C strain, in the latter half of gestation, regularly established itself after intramuscular implantation in Locke's or Tyrode's solution, and some of it was still living in animals kept 143 days, the longest period of observation. Occasionally the bronchiolar tissue outlasted the alveolar.

Within a few days after implantation the fragments coalesced into a grayish, translucent layer 1 to 3 mm. across, sometimes flattened to a skim by the pressure of the muscle, but in occasional instances as much as 1.5 mm. thick. It could not ordinarily be felt between the fingers. Not infrequently it was stippled with red or brown, owing to fresh or old blood pigment, or a blood-filled cyst was found in its place. There was no reactive proliferation or accumulation of small round cells about the implants, which were still doing well even after 143 days. As a rule they were separated from the muscle fibers by a few fibroblasts only (Fig. 1).

The pulmonary tissue, as obtained from near-term embryos (20 mm. long) is exceedingly cellular, its alveoli lined with cuboidal elements, and its smaller bronchi and bronchioles little differentiated (Fig. 2). Within a few days after implantation in salt solution the fragments lying next one another united to form a mass in which they could no longer be discerned individually, and maturation and differentiation went on so rapidly that within 3 weeks characteristic bronchi had formed, some with cartilage about them; the alveoli had become moderately distended with fluid, presumably of bronchial origin, and the lining cells had undergone flattening,—though this and the distention were less considerable than in the functioning lung. Often the alveoli were compressed, and there the epithelium was cuboidal, as in the atelectatic lung of the adult. Large alveolar macrocytes were frequently present ("heart failure cells") and these cells sometimes became numerous and were heavily engorged with erythrocytes or brown pigment. A few of the grafts disappeared eventually, owing, it would seem, to destructive hemorrhage into them. No tumors arose from any of the implants in salt solution, nor were any structures suggestive of them encountered microscopically.[1]

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[1] Weddell (*Arch Path.*, 1949, **48**, 227) has recently transplanted fragments of mouse embryo lungs to the subcutaneous tissue of adults. "Cyst formation, bronchiectasis and bizarre epithelial overgrowths" occurred frequently.

There was good reason to suppose that the lining cells of the bronchi and bronchioles of ung fragments might respond to Scharlach R in olive oil by proliferating and becoming metaplastic; for the dye stimulates and attracts the cells of adult (5) and embryo (1) epidermis, causing them to take on a carcinomatous appearance, and it has a similar though less marked effect on the epithelium of the squamous portion of the embryo stomach (3). Yet only occasionally did it induce noteworthy changes in the lung tissue though the identical dye specimen (Grübler) was employed. These changes took the form of a mild metaplasia of the lining of the bronchi and bronchioles, where they had been cut across and a droplet lay next them, the columnar epithelium altering within a few weeks to a transitional layer and then to one stratified and squamous. The changes were never widespread nor accompanied by active proliferation, and the state of the alveolar tissues differed not at all from that when it had been introduced together with Locke's or Tyrode's solution. However in one implantation nodule with *OSS*, (out of 37 examined microscopically in serial sections), a characteristic pulmonary adenoma was found. The implant had been in place 64 days, as had another of the same tissue with *OSSM* in the opposite thigh of the same animal. This latter also contained an adenoma.

The Early Changes Due to Methylcholanthrene

Very different were the findings when methylcholanthrene was present in the olive oil in addition to Scharlach R. The pulmonary tissue proliferated actively and had usually formed a firm nodule within 3 weeks, of sausage or football shape as a rule, and 3 to 5 mm. or even 8 mm. long, but rarely more than 2 mm. thick.

Alveoli formed as usual, but the *OSSM* did not attract the lining cells as it had the epithelium of stomach and skin, none extending out to surround adjacent oil droplets. Many of the latter underwent inclusion within the pulmonary tissue as the implanted fragments coalesced, but others remained lying free, and in consequence sarcomas were a relatively frequent complication though fortunately a late one.

The epithelium of the bronchi and bronchioles was strongly attracted to the *OSSM*. Wherever the cut-across lumen of these structures lay near oil droplets the lining cells extended out in tongues and surrounded then, even when they were large, just as embryo epidermis does under similar circumstances (1). Striking metaplastic changes took place concurrently. The columnar epithelium of bronchi and bronchioles built up into a pseudostratified, transitional layer which soon became stratified and squamous, the cells shedding off at first but later forming keratinized lamellae. No squamous epithelium exists in the normal lung of the mouse.

These changes could usually be seen within 14 days after the implantation. Already a thick, keratinizing, squamous layer was covering the surface of the neighboring large oil droplets; and tongues of epithelial cells were extending to those a little further off. By the 21st day the picture was more complicated. Occasionally the epithelium of a bronchus had undergone a redundant proliferation, forming a papillomatous intrusion into its lumen (Fig. 12). More often the bronchial epithelium had a carcinomatous aspect and had actively invaded the alveolar tissue, filling its spaces and sometimes replacing it *in toto* (Fig. 3), though in other instances leaving its pattern intact (Fig. 5). Invasion occurred especially where the pulmonary tissue contained many scattered, minute oil droplets. Sometimes reactive connective tissue had formed about these latter, and the cells invaded this also.

The changes simulated cancer but after transplantation of the apparently malignant tissue to other mice, in tests made on the 51st, 64th, and 77th days, respectively, it failed to give rise to tumors (Fig. 4). The sequence of morphological events nearly resembled that reported by Passey as taking place in rats with bronchiectasis due to bacterial infection (6), but the epithelium proliferated far more profusely, gave rise to more diversified pictures, was often invasive, and in many instances extended into and largely replaced the alveolar tissue.

The changes in the alveolar elements while less dramatic were considerable. Alveoli formed as usual but the lining cells exposed to the methylcholanthrene remained cuboidal instead of flattening; there was much desquamation; and frequently after some weeks the cells situated at the periphery of the mass became close packed, increased considerably in size, and occasionally pushed out a little way into the reactive connective tissue which by now had formed about the mass. Yet it seemed unlikely that they had become neoplastic since the changes were not focal but affected the cells all along the periphery of the nodule.

A singular phenomenon was squamous keratinization of the alveolar epithelium. Sometimes an entire alveolus became filled with stratified lamellae as result of differentiation of the cells lining one side (Fig. 5). On this side the living cells usually heaped up two or three deep prior to keratinization and occasionally they formed tufts. Serial sections showed that the affected alveoli often were situated at a distance from any bronchioles, the stratified squamous layer having no direct connection with bronchial epithelium.

The proliferative and metaplastic activities of the bronchial epithelium continued during later weeks, though at a diminishing pace. In not a few instances the adjacent alveolar tissue became compressed or was largely replaced by the accumulating mass of living or keratinized metaplastic elements. The histological picture was rendered still more various as time went on by neoplastic changes superimposed on the metaplastic.

The Neoplastic Changes

It was found that the complications introduced by metaplasia could be largely avoided if the tissue for implantation were taken from along the wedged-shaped edges of the pulmonary lobes, from regions that is to say where only small twigs of the bronchial tree existed. The alveoli of the resulting nodules were then much less frequently encroached upon by the proliferating bronchiolar epithelium. Yet metaplasia of this latter was so frequent that it was still impossible to tell whether metaplasia had not preceded carcinomatosis or when this latter had superseded it. Tumors of another type arose though, which the bronchial metaplasia did not simulate, namely pulmonary adenomas. These appeared early (Figs. 7 and 8), were frequently multiple, and sometimes numerous (Fig. 9). Serial sections disclosed eighteen discrete adenomas in a single small nodule measuring $3 \times 2 \times 1.5$ mm. that derived from an implantation made 28 days previously. The lungs of the adult hosts, in contrast, very seldom showed the growths, and then they were nearly always solitary.

The adenomas which develop in adult mice as result of the action of carcinogens are similar in morphology to those occurring spontaneously (7) and have been many times described. Grady and Stewart (8) have studied the early stages of those due to methylcholanthrene. The injection of urethane into C strain animals in late pregnancy is followed by such prompt development of adenomas in the lungs of the young they carry that characteristic growths are sometimes present within 3 days after their birth and are frequent and much bigger after 10 days (9).

The earliest stages of adenoma formation in the implants of the present experiments could not be perceived because of atelectasis and because the lining of the alveoli exposed to *OSSM* tended to remain cuboidal; but the growths proliferated so actively as soon to form sizable spheres amidst the alveolar tissue (Figs. 7 to 9), becoming recognizable in not a few instances toward the end of the 3rd week. For some while the cells composing the spheres were markedly basophilic, with round vesicular nuclei and scanty cytoplasm, and lay close packed, undergoing almost no differentiation, in which respects they resembled many of the adenomas encountered in the suckling offspring of urethanized mothers (9). But as time went on they formed the characteristic acini lined with cuboidal elements, and then by all morphological criteria they resembled the adenomas of adults. Occasionally one of them was situated next a terminal bronchiole, but the large majority were wholly surrounded by alveolar tissue.

As time went on, the growths increased in number and enlarged, compressing the pulmonary tissue, and replacing it more or less completely (Fig. 9), though individually they were never more than a few millimeters across. Several that were successfully transplanted merely established themselves (Fig. 6) as did the non-neoplastic pulmonary tissue which in some instances accompanied them. But one did more. It slowly yet steadily proliferated, retaining the adenomatous morphology; and, though almost non-invasive, within 4 to 6 months formed masses up to 50 mm. across (Fig. 10). This tumor has been carried through 6 successive groups of new hosts thus far. Mitoses have been very rarely seen. In growths of the second and subsequent "generations" spicules of bone were often laid down amidst the epithelium (Fig. 11). The deposition of bone was in some instances so extensive that the whole tumor mass had a rock-like hardness. This phenomenon has already been described in adenomas transplanted from adult mice (10).

A sudden enlargement of an implantation nodule during the first 3 months, with progressive growth thereafter, generally meant that a carcinoma had arisen, sarcomas developing later as a rule. On incision of the nodule soon after this change was first noted there was usually found a solitary cyst, full of watery fluid, in which were ruddy droplets of *OSSM*, and some lumps of

TABLE I

Outcome of the Transplantations

Growth No.	Embryo length	Tumor: First noted after	Tumor: Transplanted after	Character of growths transplanted	No. of hosts	Total implants	Fate of implants: Grew	Regressed	Survived	Failed	Character of the Transplanted tumors
	mm.	*days*	*days*								
1	15–17	37	51	5 mm. nodule: *squam. metaplasia or squam. carc? Adenoma*	4	4			1	3	The one successful transplant was wholly *adenoma*
2	18–20	59	64	6 mm. nodule: *squam. metaplasia, adenoma*	4	8				8	
3	18–20	77	77	12 mm. mass: *squam. metaplasia, adenomas, sarcoma*	4	8	6			2	*Sarcomas*
4	18–20	77	77	6 mm. nodule: *squam. carc.? Adenomas*	3	6	2	3	1		*Adenomas. Sarcomas*
5	15–17	70	81	12 mm. thick-walled cyst: *squam. carc.*	3	4	1	1		2	The successful transplant was a *squam. carc.* which grew with great vigor and regularity in a 2nd and 3rd generation of new hosts
				Three discrete 1 mm. nodules, *adenomas*	2	2			2		*Adenomas*, 1 mm. across
6	18–20	85	91	5 mm. cyst: *squam. metaplasia or squam. carc.?*	4	8				8	
7	15–17	70	93	10 mm. solid mass: *3 transitional cell carcs.*	4	4	4				*Transitional cell carcs.* transferred through 3 generations of new hosts
8	17–19		102	5 mm. cyst: *squam. carc.*	3	3	1				Slow growing *malignant papilloma*
9	17–19	102	102	8 mm. cyst: *anaplastic carc. and 2 squam. carcs.*	4	4	1			3	Slow growing *anaplastic carcinoma*
10	13	94	108	10 mm. cyst: *transitional cell carc.*	4	4	4				*Transitional cell carc.* transferred through 3 generations of new hosts
11	15–17	115	123	10 mm. cyst: *alveolar cell carc.*	4	4	3			1	*Carc.* like original. Transferred through 2 generations of new hosts
12	20–23	139	188	5 mm. solid mass: *adenomas*	3	3	3				*Adenomas*, which became huge. Transferred through 6 generations of new hosts

Squam. = squamous.
Carc. = carcinoma.

alics indicate microscopic diagnoses.)

keratin or pultaceous material stained pink with Scharlach R. The wall of the cyst was not infrequently several millimeters thick and its inner surface rugose, but more often it was thin and one or more discrete cauliflower growths or rugose discs protruded inwards. These were the cancers; often they were multiple. As time passed the neoplastic tissue extended into the adjacent muscle though the mass continued to be cystic. When the animal was let live the tumors became huge, surrounding the femur, extending below the knee, and up over the pelvis, and frequently rupturing through the skin with liberation of much pultaceous, pink or creamy material. Eventually they proved fatal. The course of events was in other words identical in the gross with that of the carcinomas originating from the epidermis or the squamous portion of the stomach of mouse embryos (1, 3). From these latter tissues benign papillomas arose occasionally, but no such tumors took origin from the implants of pulmonary tissue.

Most of the animals were killed early, for the better study of the tumors in relation to the tissue from which they had arisen. Transplantation was frequently carried out to test whether the growths were autonomous neoplasms. Results of the transplantations are summarized in Table I. Growths described in the text can be identified in the table by reference to the day on which transplantation was carried out.

The carcinomas, though diverse in morphology, had no peculiarities suggestive of a derivation from embryo tissue. Many were papillomatous (Figs. 13 and 14), and not a few were composed of transitional epithelium (Figs. 15 to 18). A single tumor was obtained (Fig. 19) which had the aspect that in the case of human growths is usually held to betoken an origin from alveolar epithelium (11). The generality of the growths were manifestly of bronchial origin and most were of squamous cell type, though differing considerably in detail. Some bore a close resemblance to the tumors of this sort which arise from implants of the gastric lining of embryos in response to methylcholanthrene (3). Usually their cells keratinized, but in not a few cases desquamation took place before this could happen. The cancers were frequently multiple, indeed sometimes a potpourri of malignant entities, and transplantation occasionally yielded neoplasms quite different from what microscopic examination of a slice from the original growth had led one to expect. With continued transplantation, one or another of the neoplastic components eventually came to predominate as has been the general experience with other mixed tumors. For example, three widely differing neoplasms were present in the first and second generations of the tumor shown in Fig. 15, but in the third generation there remained but one, a transitional cell carcinoma (Figs. 17 and 18). In the earlier passage a singular intracanalicular growth had been prominent, com-

posed of thick connective tissue septa covered with a shallow layer of neoplastic epithelium which was desquamating into irregular spaces (Fig. 16).

After 70 days spindle-cell sarcomas occasionally arose and after 90 days they were frequent. Serial sections of the small sarcomas showed in their midst depots of *OSSM* that had escaped encystment by epithelial cells. The results of a typical experiment will be described.

The lungs of ten embryos 15 to 17 mm. long (about 16 days old) were chopped fine in Locke's solution, and 0.025 cc. of the resulting suspension was implanted, together with *OSSM*, in one thigh of twelve adults and, as control, with the same quantity of Locke's solution but without *OSSM* in the other thigh.

The first mouse was killed after 51 days. No trace was found of the implant with Locke's solution, but that introduced with *OSSM* had given rise to a nodule 5 mm. long which was sectioned serially. Fig. 4 shows it in cross-section. It consisted of a central cyst partly lined with stratified squamous epithelium with scattered large and small islands of alveolar tissue about it. The cyst had contained ruddy oil at autopsy. It opened directly into a large bronchus ramifying amidst pulmonary parenchyma, and there was more oil amidst this latter, as evidenced in the section by rounded lacunae. Two characteristic pulmonary adenomas were present (arrows) amidst the alveoli. At the other end of the oblong nodule was what appears to be an active squamous cell carcinoma. Pieces were transplanted to the thigh muscles of four young adults, which were killed 91 days later. Only one had any nodule then, and it was only 1 mm. across. Serial sections showed it to be adenomatous in character (Fig. 6), the presumptive carcinoma having failed to survive.

A second mouse was killed 57 days after implantation. Again no growth was found in the leg where the tissue had been implanted with Locke's solution; but where it had been put with *OSSM* there was a 3 mm. mass in which 8 discrete adenomatous nodules were found in serial sections amidst hemorrhagic alveolar tissue (Fig. 9).

Mouse 3 was killed after 71 days. Where the control implant with Locke's solution had been placed there was a layer 3 mm. long and 1 mm. deep of grayish tissue speckled with brown spots. It consisted of distended pulmonary alveoli containing many macrophages laden with brown pigment. There was nothing to suggest adenomatous growth. In the thigh injected with *OSSM*, only a trace of lung tissue had survived and sarcomatosis had begun.

The next mouse, No. 4, was killed after 81 days. Where the lung tissue in Locke's solution had been implanted there was a shallow, gray layer, brown-speckled, of about the size of that found in mouse 3 and of similar composition (Fig. 1). In the opposite thigh amidst the muscle there was a cystic mass measuring 12 by 7 by 5 mm. The cyst contained pink, semisolid, necrotic material and its wall was 2 to 3 mm. thick. Pieces of the wall were implanted in three young adult mice, and the rest taken for section. The microscope showed that the cyst had contained jumbled masses of keratin, and its wall was lined with what appeared to be a squamous cell carcinoma of papillomatous tendency (Fig. 13). Transplantation proved that a cancer was indeed present, for the graft succeeded in one of three new hosts and the resulting tumor was passed to a second and a third group of mice in turn before it was discarded, the carcinoma growing in all three hosts of the third generation. It was still cystic but the cysts were now lined with almost filiform papillomatous protrusions covered with living, keratinizing epithelium only a few cells thick (Fig. 14).

Mouse 5 was killed on the 93rd day. A thin layer of lung tissue 5 mm. across was found where the control implant had been put, while in the other leg there was a sarcoma 15 mm. in diameter with a cyst in its midst from the squamous lining of which a growth had arisen that appeared microscopically to be a carcinoma.

Mouse 6, also killed after 93 days, showed a filmy patch of lung tissue as result of the control implantation. There was another patch where *OSSM* had been put with the embryo fragments, but it was nodular because of adenomas scattered through it; and nearby in the same thigh there was a spherical cystic nodule, 10 mm. across, its cavity pink with Scharlach R and full of dead and living papillomatous ingrowth from a thick wall. Histologically the nodule was highly diverse: in some regions what appeared to be a desquamating, squamous cell carcinoma was present, in others a cancer of transitional cell type, and in yet others the rifted, intracanalicular growth already mentioned in the general account of the tumors. Its spaces were lined with proliferating epithelium, cuboidal or more or less flattened, and one to three cells deep.

Pieces of the cyst wall quickly yielded growths on transfer to new hosts, and these were carried through two further groups of mice. In most animals a tumor of transitional type, having a minimal amount of connective tissue, eventually predominated or became the sole entity (Figs. 17 and 18), but some of the growths continued to be complex (Fig. 15), the rifts in them became larger, and the connective tissue between them took on a fibromatous aspect (Fig. 16).

Mice 7, 8, 9, and 10 were killed after 123 days. The embryo fragments implanted in Locke's solution had given rise to shallow patches of lung tissue in excellent condition, yet no larger than in the animals killed earlier. Where *OSSM* had been added to the implants only sarcomas could be found in three instances, but in the fourth a cystic nodule 10 mm. across was present, with a wall consisting of tumor tissue of two widely differing types, ordinary squamous cell carcinomatosis, and a papillomatous neoplasm with narrow connective tissue cores supporting an epithelium one to two cells deep, consisting of cells which differentiated to a pear shape and then came away while still to all appearances in excellent condition (Fig. 19). Bits of the wall gave rise to growths on transplantation and these were propagated in two successive groups of hosts. The same tendency of the supporting connective tissue of the tumor to increase in relative quantity and take on a fibromatous aspect was noted as in mouse 6. During the successive transfers of the tumor the epithelium tended to lose the tall pear shape and become flattened and layered. The tumors of the second generation had nearly the same transitional character as the final tumors of mouse 6. The two remaining mice were killed after 128 days. There were tiny patches of pulmonary tissue at the site of the control implantations, and where *OSSM* had been put a large sarcoma in one instance and in the other a nodule of pulmonary tissue containing two characteristic adenomas.

In sum, sarcomas were more frequent than in the case of epidermal or gastric tissue. The only benign epithelial tumors were pulmonary adenomas, benign papillomas being notably absent. Pieces of two adenomas were transferred to adult hosts, but after 51 and 91 days, respectively (Fig. 6) had done little more than survive. A presumptive carcinoma, found in an implant after 51 days did not succeed on transplantation; it may have been merely metaplastic bronchial epithelium. Three other growths looking like carcinomas, and transferred after longer periods, all did well in new hosts and were carried through several successive groups of them.

The lung tissue from the youngest embryos employed (13 mm. long, 15 days old) yielded growths no different from those derived from the lungs of older embryos.

The First Occurrence of Neoplastic Change

Many of the implants with methylcholanthrene were removed very soon, sectioned serially, and searched for signs of neoplastic change. The alveolar epithelium was found to remain cuboidal during the first 2 weeks, or even longer, but as the alveoli gradually became distended with fluid it flattened and now the adenomas stood forth as discrete spherical entities (Figs. 7 and 8). They remained nearly spherical despite the compression exerted by the thigh muscles, and slowly increased in size. The first one encountered was in an implant 20 days old. Methylcholanthrene had a stimulating effect upon them, as evidenced by the larger size and occasional mitoses in those situated near oil droplets containing the carcinogen.

So completely did the metaplastic activities of the bronchial epithelium simulate malignancy that it proved impossible to tell precisely when carcinomatous changes began. According to the literature mitoses are infrequent in adult lung tissue undergoing metaplasia, but they were numerous when it was taking place in the embryo implants. Where fine droplets of *OSSM* lay scattered amidst the nodules, the metaplastic cells actively invaded and replaced the alveolar tissue and tongues of them frequently extended to outlying droplets situated in the surrounding muscle (Fig. 3). Only when serial section showed that such extension had taken place where there were no droplets could one assume that the cells were independently aggressive and hence presumably malignant. This was first noted after 51 days. Successful transplantation of the growths yielded decisive evidence of their neoplastic character, but when it was unsuccessful one could not be sure that cancer had been absent; for previous tests with the growths arising from epidermis have made plain that some carcinomas which are highly malignant in the original host fail to establish themselves in other hosts of the same homogeneous C strain (1). The longer the time elapsing before transplantation is attempted, the greater seemed to be the likelihood of success, an observation already made as concerns the spontaneous mammary tumors of rabbits (12) and those obtained with methylcholanthrene in embryo epidermis (1). A growth that was morphologically a carcinoma, in an implant 51 days old (Fig. 4) failed on transfer, and so too did another tested after 77 days, whereas success was had with a tumor procured after 81 days and 6 out of 7 succeeded that were tested after 91 to 188 days (Table I). Four of these 6 cancers were carried into further groups of mice in series. On three occasions transfers were made of tissue which the microscope showed to have been merely metaplastic. It gave rise to no nodules.

Effect of Methylcholanthrene on the Implanted Pulmonary Tissue of Adult Mice of the C Strain

In three experiments a suspension of fragments of the lungs of a young adult C mouse was injected, together with *OSSM*, into the thigh muscles of other animals of the same strain and of about the same age. Only the peripheral lung tissue was utilized, to minimize the possibility of carrying bacteria into the graft.

The same technic of injection was employed as with embryo tissue. Serum had been added in one instance to the Locke's solution in which the tissue was suspended, with a view to minimizing injury to the cells, but no better results were obtained than with Locke's solution as such. The 12 implanted mice were killed after 21 to 205 days. Most of the grafted tissue failed to survive, but in animals killed after 21, 28, 44, and 65 days some small patches of it were found. It had not enclosed the *OSSM*, and metaplasia was only once observed. Adenomas were not encountered in serial sections of the 21, 28, and 65 day specimens, but two of them were present in a tiny patch of pulmonary tissue removed 44 days after the implantation. None were present in the lungs of the hosts. Cancers failed to arise in any of the animals though 5 of them were allowed to live for more than 65 days. Seldom was any trace of the implants found. Sarcomatosis had often supervened.

It seems reasonable to ascribe the failure to elicit cancers in these tests to insufficient exposure of the pulmonary cells to the carcinogen. Though some lung tissue survived for more than 2 months, it did not proliferate sufficiently to enclose the *OSSM*, metaplasia did not occur, and the only sign that the carcinogen had influenced it was the development of adenomas. Mice of the C strain usually develop such growths spontaneously as they become old, as is well known (13), and their appearance can be greatly hastened and their number increased in breeds liable to them merely by painting the skin repeatedly with methylcholanthrene (14). Their failure to appear in any considerable number in the lungs of mice receiving *OSSM* together with embryo tissue can be laid in part to the fact that the hosts were still young when killed and in part to the small amount of the carcinogen introduced.

Results with the Pulmonary Tissue of Embryos of the A Strain

Spontaneous adenomas are far more frequent in adult A mice than in those of the C strain (15). Hence the lungs of A embryos were utilized in several experiments. A colony had been raised from individuals supplied by the Roscoe B. Jackson Memorial Laboratory, and random sampling had shown adenomas to be fairly frequent in aging adults.

The general technique was the same as with C embryos, the litters furnishing the lung tissue being about as far along toward birth as those from C females which had been successfully utilized. As usual, some of the implantations were made with Locke's solution only, and some with *OSSM* in the opposite leg of

the host. The control implants did well at first, but later they often became surrounded by great numbers of round cells, and soon after were absorbed. When the tissue had been injected together with *OSSM* it usually enclosed droplets of this latter, metaplasia followed, and adenomas or carcinomas, or both, arose in some instances; but the findings were rendered irregular by local round-cell accumulation and death of the implanted tissue. This phenomenon had been noted previously when the epidermis of A strain embryos was exposed to *OSSM*.

From one of the implants with *OSSM* a cystic nodule had developed by the 125th day and in this there was a malignant papilloma with an invasive base. The growth had a core consisting of large cells resembling ganglion cells (Figs. 20 and 21) which showed mitoses and were obviously neoplastic. They wholly resembled those making up the core of a benign epithelial papilloma induced with methylcholanthrene in an implant of fragments of the squamous portion of the stomach of a C strain embryo (3). Ganglion cells are amongst those elements which do best in ordinary grafts of embryo tissue (16), and it would appear that they had been rendered neoplastic in both the instances now described.

RÉSUMÉ AND DISCUSSION

The lung tissue from C embryos established itself and differentiated to a remarkable extent after transfer to adult hosts of the same strain. It had little capacity for growth, as compared with the fetal cutaneous and gastric tissues, yet sufficient for the implanted fragments to coalesce into a mass within a few days, and after 2 weeks a parenchyma had developed having alveolar spaces of considerable size, lined with flattened epithelium,—as result of distension with fluid, elaborated presumably by the elements lining the bronchi. The spaces were not so large though, nor the cells so flat, as in normal mouse lungs just after birth. No reactive proliferation occurred about the graft but frequently hemorrhage took place into it,—owing perhaps to the trauma of palpation,—and occasionally this was so extensive as to convert it into a little cyst full of blood. More often mere ecchymoses occurred which found expression later in "heart failure cells" plumped out with erythrocytes or brown pigment. Horning has encountered such cells in implants of adult lung tissue with methylcholanthrene crystals (17).

Usually the graft persisted almost unchanged for a long period but occasionally it disappeared after a few months, owing apparently to local accidents, hemorrhage for instance, not to incompatibility of the host. In the case of A mice and Webster-Swiss animals incompatibility was often evident, the grafts dying early amidst a profuse accumulation of round cells; and even in the absence of such accumulation many fared badly. Mice of the Webster-Swiss

breed gave the most irregular results, as might have been expected from their mixed inheritance. The present findings further demonstrated the superiortity of C material. In previous experiments by the same technique the epidermis and gastric tissue of C embryos regularly did well after transplantation, whereas that of A, C3H, and I strain mice often died amidst a profusion of round cells, and successful grafts proliferated poorly as compared with those from C animals (2).

These were the findings after the injection of lung fragments suspended in Locke's solution, with or without Scharlach R in olive oil. In but a single instance did a tumor arise,—an ordinary small adenoma in a graft containing Scharlach R. This dye is known to be weakly carcinogenic, eliciting hepatic adenomas (18), and hence the pulmonary neoplasm cannot be deemed spontaneous. Scharlach R markedly stimulates epidermal cells and under its influence they often grow invasively as if carcinomatous (5), yet it only slightly encouraged proliferation of the lung elements.

Very different were the findings when methylcholanthrene had been added to the olive oil. Considerable growth of the grafted tissue ensued and sizable nodules of it resulted. Reactive connective tissue proliferation took place about these, as also about any outlying droplets of oil. Widespread metaplasia occurred also, mostly of the bronchiolar epithelium but not infrequently of the alveolar lining cells, sometimes of those distant from any bronchioles (Fig. 5). These changes took place irrespective of whether Scharlach R was present. Passey has reported upon a similar alveolar metaplasia in the lungs of rats with chronic bronchiectasis (6), and they provide strong indications, as he remarked, that the cells lining the alveolar wall are epithelial in character, a point long debated. The differentiation taking place secondarily in the cells of many pulmonary adenomas, such that they come to resemble the epithelium of the bronchioles (9), constitutes further evidence to the same effect, for these growths originate from the alveolar lining (8, 9, 19).

The metaplastic changes took the same course as those occurring in the adult lung of several species during pulmonary infections or after the injection of injurious substances (20), and the end result, as in such instances, was a stratified squamous epithelium; but the amount of metaplastic tissue was exceptionally great and the changed cells proliferated actively and were aggressive, often replacing a large proportion of the lung parenchyma (Fig. 4). Sometimes they extended in tongues to oil droplets lying in the muscle round about the graft, and not infrequently they replaced individual muscle fibers. Methylcholanthrene has similar stimulating, attracting and alterative effects on the cutaneous and gastric epithelium of embryos (1, 3), and the findings as a whole suggest that its carcinogenic influence may be exerted on the cell surface and perhaps is physical in nature. So closely did the metaplastic changes

in the pulmonary tissue mimic the carcinomatous that it was often impossible to tell when cancer had put in an appearance and serial sections, even of what appeared to be established squamous cell cancers, were often necessary to determine whether malignancy was really present.

Pulmonary adenomas are not infrequently found in ageing C mice, usually as solitary growths. Animals of the A strain are notably liable to them, and they can be made to appear sooner and in greater number in such animals by painting the skin repeatedly with methylcholanthrene (14). The mere injection of urethane into pregnant C or A mice during the latter half of gestation results in an immediate development of adenomas in the young, these sometimes reaching perceptible size within 3 days after birth (9). In view of these facts there is no ground for surprise at the early development and multiplicity of such growths in grafts of C embryo lung tissue directly exposed to methylcholanthrene. Unlike the carcinomas they were recognizable almost at once after they had begun to form, and hence they appeared to arise earlier than these latter; but whether they really did so is uncertain. None of the cancers seemed to have derived from them, although in adult mice spontaneous malignant adenomas differing but little morphologically from the benign are now and again encountered and the benign ones usually become cancerous after repeated transplantation (21).

Horning has procured tumors of the adult mouse lung by wrapping pieces of the pulmonary tissue around crystals of methylcholanthrene and implanting them in the subcutaneous tissue, with the addition on occasion of stilbestrol (17). Many adenomas and squamous cell carcinomas resulted, as did also two growths which he termed adenocarcinomas, and an anaplastic carcinoma with oat-shaped cells. The growths arose late as compared with those of the present work,—a finding which can be accounted for by the indolent state of the adult pulmonary tissue and by less extensive contact with the carcinogen. There was considerable variation within the primary tumors and an over-all tendency to squamous metaplasia. Horning concluded that all were bronchogenic.

Many of the malignant tumors obtained during the present work were obviously the outcome of a secondary fusion of several carcinomas of very different aspect. Not infrequently they became intermingled and a morphological potpourri resulted. Some of the growths of this sort continued to be complex after transplantation, but when the transfers were kept up one or another of their components eventually outgrew the others and dominated. The carcinomas were remarkably various as a group.

It has been the general assumption that all pulmonary cancers of stratified squamous character, in whatever species, must have arisen from the epithelium of the bronchial tree; but the fact that cells altered by metaplasia not infre-

quently undergo neoplastic change secondarily, when taken in conjunction with the ability of the alveolar lining of the rat and mouse lung to undergo squamous metaplasia, gives cause to doubt this.

Carcinomas of transitional type not infrequently arose from the implants of embryo lung with methylcholanthrene, and on transplantation they retained their character in host after host (Fig. 17). Their occurrence was the more noteworthy because no transitional epithelium exists in the normal lung of the mouse, although it appears temporarily,—but never extensively,—where the cells lining the bronchioles are undergoing metaplasia from the cylindrical to the stratified squamous form. In the cancers just mentioned the course of metaplastic events was abruptly checked and rendered permanent at the transitional stage, as result of the intercurrent neoplastic change.[2]

The prime object of the present experiments, as of the previous ones with embryo skin, stomach, and lung has been to learn how soon in the life of the individual its cells possess the ability to become neoplastic, and how widely distributed and diversified in expression this ability is. Pulmonary tumors have been elicited less consistently than were gastric or cutaneous growths, but this is readily understandable since the olive oil globules containing methylcholanthrene were seldom encapsulated in bulk, but instead were included in the implant only incidentally in most instances, as small globules caught amidst the coalescing lung fragments. Whenever a considerable droplet lay where a bronchiole had been cut across, as now and then happened, the lining epithelium of the latter extended out and around it, just as cutaneous and gastric epithelium is prone to do; and then, as in their case, cancer arose from the wall of the resulting cyst.

The findings stress a fact already made plain by the work with the skin and stomach, that cells procured from mouse embryos in the latter half of development are at least as responsive to the carcinogenic influence of methylcholanthrene as those of adults and undergo neoplastic changes in as wide variety. The results with each of the successive tissues tested have made it more difficult to suppose that these changes can be due to the action of viruses *reaching the cells from without after birth and having effects as narrowly specific as those of the tumor viruses thus far discovered.* As pointed out previously a great multitude of such viruses, all circulating together in the adult host implanted with embryo tissues, would be required to account for the multifarious growths arising from these latter. There would even have to be a virus causing and maintaining transitional cell tumors of the mouse lung, growths never yet encountered under natural conditions. In the case of the lung adenomas ap-

[2] In previous work with the glandular portion of embryo stomachs as affected by methylcholanthrene a transitional cell tumor was also obtained incidentally to induced metaplasia (3), but it was so small as only to be discovered with the microscope.

pearing in the new-born young from mice injected with urethane while pregnant (9), the potentiality to undergo neoplastic change would appear to be possessed by the cells while still *in utero*.

SUMMARY

The lung tissue of mouse embryos of the C strain proliferates to some extent after implantation in adult hosts of the same breed and rapidly differentiates, forming a parenchyma remarkably like the normal. The grafts persist long. When methylcholanthrene dissolved in olive oil has been introduced with them much more growth of them occurs. The carcinogen induces a pronounced metaplasia of the epithelium of the bronchial tree, and the altered cells are often aggressive, multiplying, invading, and largely replacing the parenchyma about them. So closely do they resemble malignant elements in aspect and behavior that it is frequently difficult to tell whether carcinomatous change is not actually present. Genuine tumors soon arise, multiple benign adenomas sometimes appearing within 3 weeks, and indubitable carcinomas a few weeks later. Not a few of the cancers are of transitional cell type, that is to say are expressive of an intermediate stage in the metaplasia.

Under the influence of methylcholanthrene the cells lining the alveolar spaces of the graft sometimes undergo metaplasia also, with result in stratified squamous epithelium. It follows that there is reason to doubt the current assumption that all squamous cell carcinomas of the lung necessarily arise from the bronchial tree. The findings, taken with others previously reported, make it difficult to suppose, furthermore, that the generality of lung tumors can be due to neoplastic viruses entering the organism in postnatal life and having no broader scope than those thus far discovered.

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EXPLANATION OF PLATES

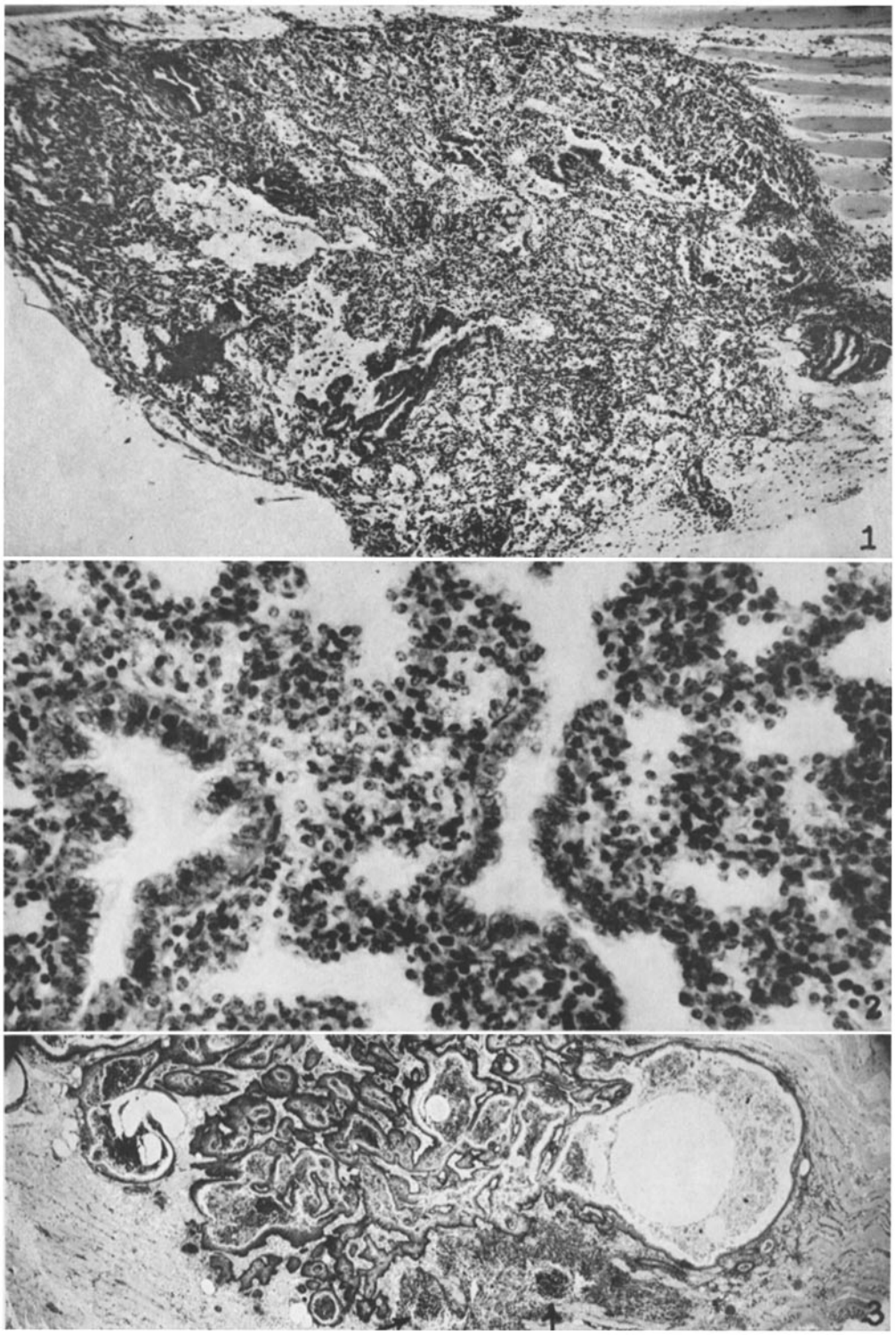
All the sections were stained with eosin and methylene blue. Mr. Joseph B. Haulenbeek made the photographs.

PLATE 6

FIG. 1. Growth found 81 days after implantation of pieces of mouse embryo lung tissue in the thigh muscles of an adult mouse along with Locke's solution. The implanted fragments have proliferated and united to form a tissue with the architecture of adult lung. The several large bronchial structures were lined with tall ciliated columnar epithelium. The rest of the growth is composed of thin-walled aveoli distended with fluid, with some spaces containing large free cells filled with brown pigment ("heart failure cells"). The nodule is wholly unencapsulated, muscle and pulmonary elements lying directly juxtaposed. × 60.

FIG. 2. Section of lung from a mouse embryo near term (20 mm. long). Two bronchioles can be seen lined with columnar epithelium. The alveolar elements are cuboidal. See Fig. 1 for the differentiation occurring after implantation. × 425.

FIG. 3. Growth removed from the muscle 53 days after implantation of embryo lung tissue with methylcholanthrene and Scharlach R in olive oil (*OSSM*). It illustrates the extreme metaplasia induced by the carcinogen. The many rounded lacunae show where the oil lay. The implanted tissue has undergone extensive metaplasia and most of the nodule consists of stratified epithelium necrosing prior to keratinization. The largest oil depot had been encysted by such epithelium. The arrows point to two spherical growths,—which were benign adenomas as higher magnification showed. They lie amidst compressed alveolar tissue. × 25.



(Smith: Neoplastic potentialities of mouse embryo tissues. V)

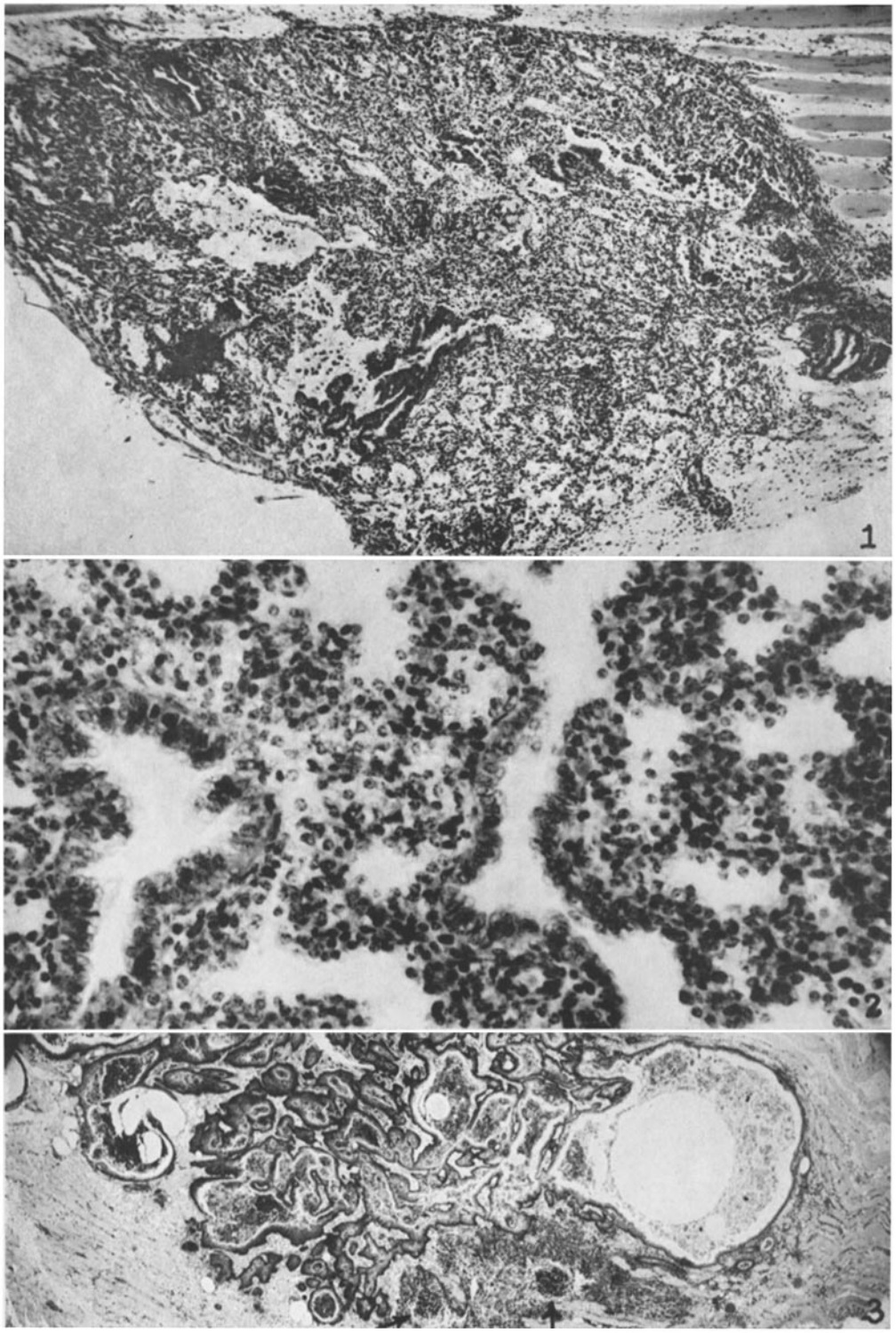
PLATE 7

FIG. 4. Mass resulting from the implantation of embryo lung tissue and *OSSM* 51 days previously. Two adenomas can be seen (arrows) lying amidst alveolar tissue. On the left there is a nodule of carcinomatous aspect which may have been merely metaplastic. Transplants failed to survive in new hosts, though one of the adenomas established itself (Fig. 6). × 30.

FIG. 5. Another section through the mass of Fig. 4 to show tufts of squamous epithelium which have originated from the alveolar wall. Lamellated keratin has been produced. The squamous tufts had no direct contact with the bronchioles, as serial sections showed, and often were situated far from them. A typical adenoma lies at the lower right (bracket). × 230.

FIG. 6. Adenoma resulting from the transplantation 91 days previously of a fragment of the growth found in the implant furnishing Figs. 4 and 5. The tumor has done little more than establish itself, but fluid from it has given rise to a small cyst. × 55.

FIG. 7. Adenoma in an implant of embryo lung with *OSSM* examined after 20 days. × 220.



(Smith: Neoplastic potentialities of mouse embryo tissues. V)

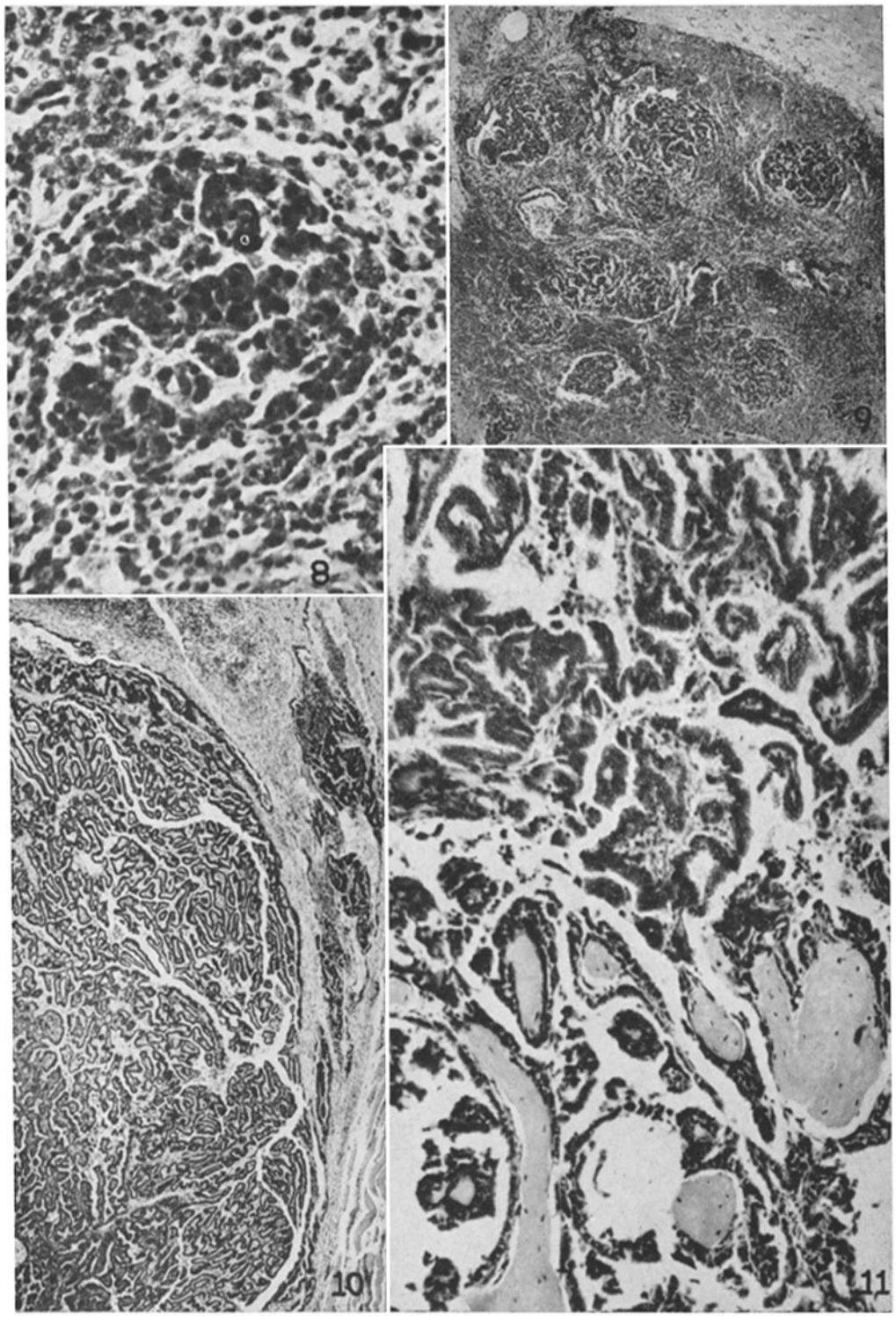
PLATE 8

FIG. 8. An adenoma in an implant of embryo lung with *OSSM* examined after 23 days. × 350.

FIG. 9. Nodule of lung tissue removed 57 days after implantation with *OSSM*. Six large adenomas and several small ones are present in the single slice. × 35.

FIG. 10. Section of tumor resulting from the second successive transfer of another adenoma in new hosts. The original growth had been induced in embryo lung tissue implanted in the muscle of an adult mouse along with *OSSM*. The neoplasm is in its sixth serial passage. It has grown progressively in every host, although very slowly, the animals dying after 4 to 6 months with tumors 30 to 50 mm. in diameter. They are for the most part solid and well circumscribed with only small invasive outgrowths into the surrounding tissue, as the present photograph shows. Occasionally they are honeycombed with fluid-filled cysts, and very commonly they contain bone. × 30.

FIG. 11. Higher magnification of the same tumor, in another transplant of the second generation. The cuboidal or columnar cells rarely show mitoses. In this and subsequent generations numerous spicules of dense bone were found. × 125.



(Smith: Neoplastic potentialities of mouse embryo tissues. V)

PLATE 9

FIG. 12. To illustrate various stages of metaplasia. At the center of the photograph the columnar bronchial epithelium is thrown into redundant folds. On the right there is a shallow layer of stratified squamous epithelium, as yet not keratinizing, while on the left there is a thicker layer of transitional epithelium. × 125.

FIG. 13. Part of a nodule 12 mm. across which formed where embryo lung tissue together with *OSSM* had been implanted 81 days previously. An area of alveolar lung tissue lies just below the center of the photograph. The nodule owed most of its size to a cyst full of cellular debris and lined with a carcinoma of papillomatous form. Part of this cyst is shown. The cancer grew rapidly in the 3 successive groups of new hosts to which it was transferred, forming big cysts. × 25.

FIG. 14. Wall of a cyst resulting from third serial transplantation of tissue from the specimen of Fig. 13. The cancer has retained its original morphology. × 35.

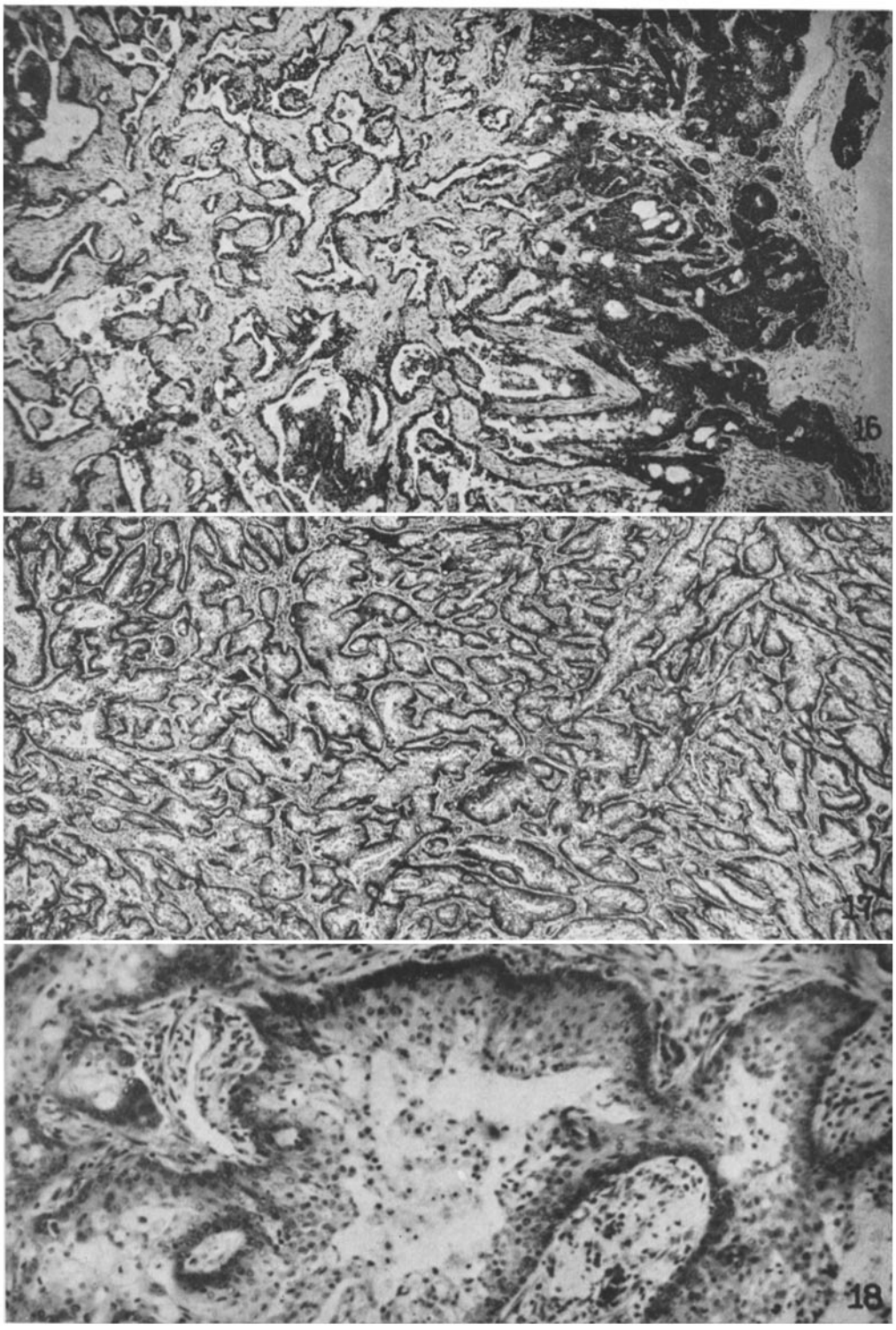
FIG. 15. Cross-section through a 10 mm. mass resulting from the initial transplantation of a tumor induced with *OSSM* in embryo lung tissue. Sections of the original growth showed carcinomas of three distinct morphological types. The same three can be seen in the present specimen. The growth occupying the middle of the slice consists of thick septae of fibromatous appearance covered with cuboidal epithelial cells, as higher magnification showed. The clefts between the septae are small. Toward the left there is a carcinoma composed of transitional epithelium heaped up into a layer many cells deep. On the right there is a squamous cell carcinoma. × 17.

PLATE 10

FIG. 16. A tumor resulting from further transplantation (2nd tumor generation) of the growth seen in the middle of the slice of Fig. 15. In the older part of the mass there are numerous clefts separated by thick septae of connective tissue covered with non-keratinizing cuboidal cells in a layer 1 to 2 deep. The vigorously invasive edge of the growth consists of undifferentiated epithelial cells. The tumor was 20 mm. in diameter when its host was killed 38 days after implantation. × 50.

FIG. 17. The transitional cell carcinoma of Fig. 15 growing in an animal of the 3rd tumor generation. Everywhere the neoplasm has the same character. × 30.

FIG. 18. Higher magnification of the transitional cell carcinoma shown in Fig. 17. × 175.



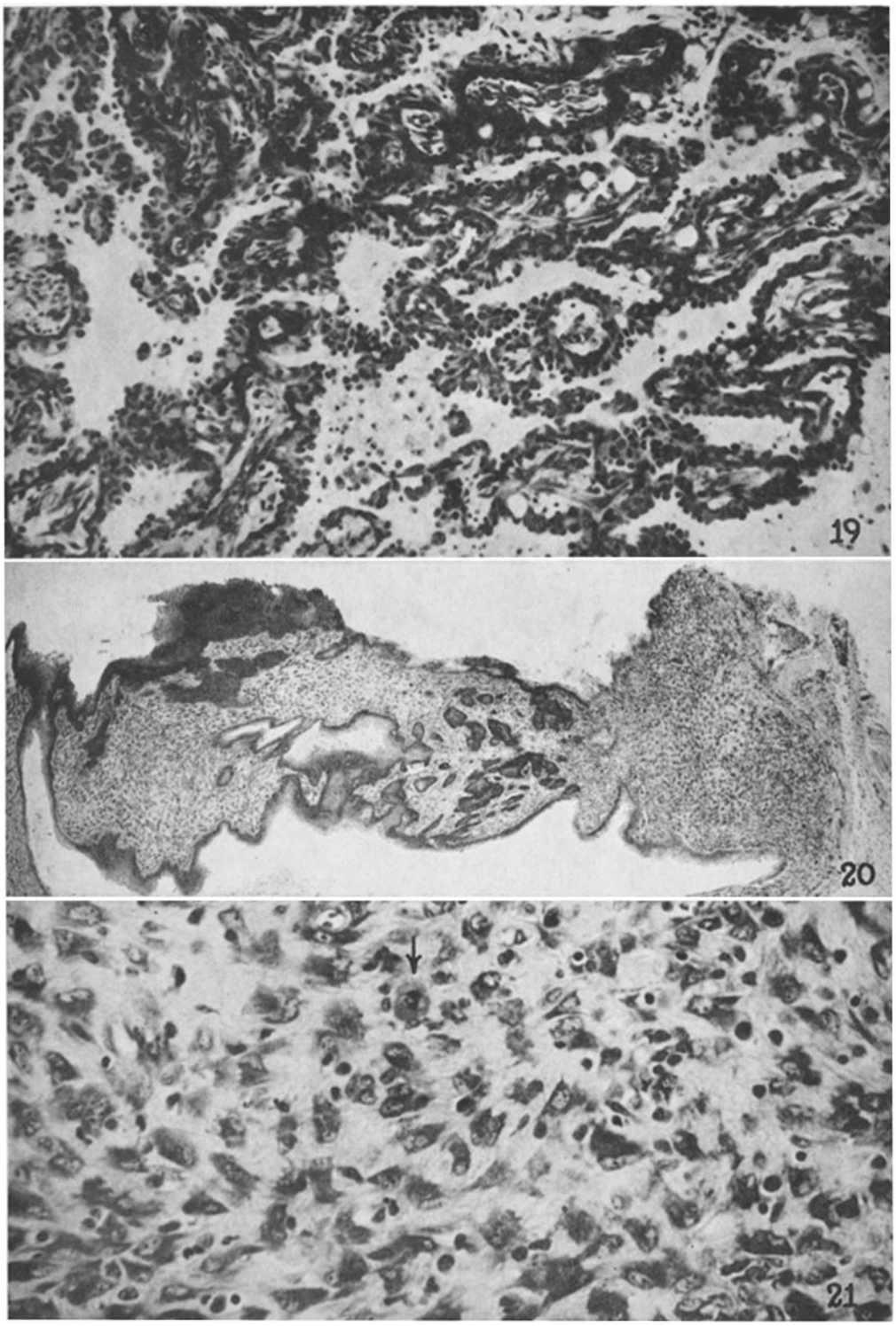
(Smith: Neoplastic potentialities of mouse embryo tissues. V)

PLATE 11

FIG. 19. Alveolar cell carcinoma resulting from the implantation 123 days previously of embryo lung tissue together with *OSSM*. The tumor succeeded on transplantation, but on repeated transfer was eventually encroached upon and destroyed by a transitional cell cancer present with it in the grafts. × 70.

FIG. 20. Section of malignant papilloma, having a core of what appear to be ganglion cells. The growth resulted from the implantation of embryo lung tissue with *OSSM* 125 days previously. × 30.

FIG. 21. Higher magnification of the core, showing the neoplastic character of the cells and a mitosis (arrow). × 350.



(Smith: Neoplastic potentialities of mouse embryo tissues. V)