THE NEOPLASTIC POTENTIALITIES OF MOUSE EMBRYO TISSUES*

II. Contributory Experiments; Results with the Skin of C3H and Webster-Swiss Embryos; General Considerations

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Plates 40 to 42

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The work of an accompanying paper (1) has shown that epidermal tumors rapidly arise from mouse embryo skin after it has been implanted in adults of homologous strain (C strain mice) together with methylcholanthrene. The experiments were done as a step toward learning whether the potentialities of epidermal cells for neoplastic change are inherent or acquired, and they were rendered necessary by observations which seemed to exclude the possibility that such potentialities are conferred during postnatal life. These observations will now be described as also certain additional findings.

Do Epidermal Cells Acquire the Liability to Neoplastic Changes after Birth?

Recent findings have stressed the possibility that neoplasms as a class may be due to viruses; but it is obvious that the generality of growths cannot be referable to directly pathogenic agents of this sort (2). There are reasons though for the supposition that widely distributed, harmless precursor viruses exist, which, reaching the cells, enter into an association which does not affect them unless they are rendered abnormal by one of the various agents termed carcinogenic. Then, owing to the changes induced in the cellular environment of the virus, this may undergo alteration here or there in the affected tissue, and reacting upon the elements with which it is associated, cause them to be tumor cells. All that is known about the "milk factor,"—which confers on suckling mice the liability to mammary cancer in adult life,—falls in with this conception (3). The factor, which is non-pathogenic though possessed of the physical attributes of a virus,—at least so far as it has been studied,—is ingested with the milk and reaching the mammary tissue, lodges there and determines the occurrence many months later of carcinomas, which, failing its presence, do not arise despite contributory favoring conditions. Exposure of the mice to a chemical carcinogen hastens the occurrence of the tumors and much increases their number (4). These facts lead one to ask whether there may not be other agents which enter the body after birth and confer on other tissues the liability to tumors. The experiments which follow were carried out as bearing upon this possibility.

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The epidermis of mice was utilized for the work. No breeds are known which have cutaneous growths spontaneously in any considerable number, but on the other hand there are none which fail to develop them eventually upon repeated application of the carcinogenic hydrocarbons. The yield differs widely from individual to individual however, even when they have been closely inbred for many generations, some developing many growths soon while others have few and sometimes only after long carcinogenic stimulation (4). It has seemed possible to us that these differences might be due to the transfer in greater or lesser quantity to the suckling young of some agent associated with the epidermal cells of the mother mouse, which, desquamating in the ordinary way, would be swallowed with the milk. According to this notion the agent might reach the skin of the young from the gut, just as the milk factor reaches the mammary gland, and lodging and persisting in the epidermis, determine the occurrence of tumors there when a provocative carcinogenic agent is applied. An experiment to test the conception was carried out by one of us with Dr. W. F. Friedewald,—who has generously consented to the reporting of it here. Methylcholanthrene, which has a very powerful influence to bring on mammary tumors due to the milk factor (5), was utilized as the carcinogen.

Experiment A.—A colony of Webster-Swiss mice (Institute stock) was raised, and the new-born young were fed (*a*) a suspension in salt solution of skin scrapings from normal adult mice; or (*b*) suspended scrapings of epidermis rendered hyperkeratotic by tarring some while previously; or (*c*) a suspension of the epithelium of papillomas which had been produced by painting the skin of adult mice with methylcholanthrene in benzene. A few drops of one of these suspensions was fed with a pipette to every mouse of a litter twice during the first 24 hours after birth, and the animals of every fourth litter were set aside as controls. Several hundred animals in the four categories were assembled during the course of 3 months and nothing further was done to them for 7 to 10 months more (according as they had been born early or late in the feeding period). Each group now consisted of about 200 mice apiece and they were painted over the entire back twice a week with 0.3 per cent methylcholanthrene in benzene until tumors had appeared in the majority. Individual charts were made of the time of appearance, number, and size of the growths. The findings differed widely from individual to individual but no group differences could be perceived.

This test negated the idea that the liability to epidermal tumors is passed on to the young by way of desquamated and ingested squamous cells, but it did not dispose of the possibility that an agent ultimately responsible for such growths may reach the skin at some postnatal time. The factor responsible for mammary cancer can be found on occasion in the blood stream and doubtless attains to the breast tissue by way of it. Furthermore, substances of large molecule are known to escape from the blood into inflamed areas, sometimes persisting there, and the epidermotrophic viruses when circulating, *e.g.* vaccinia, are prone to localize in such areas. Hence, an attempt was made, by keeping the skin inflamed, to obtain an exceptionally abundant localization

out of the blood of a presumptive agent conferring the liability to skin tumors. Sucklings were used, both because the milk factor reaches the mammary tissue during the first weeks after birth and because the wall of the gut is then most permeable to large molecules in general.

Experiment B.—Litters of Webster-Swiss mice from our colony were painted all over the back twice a week with a mixture of turpentine and acetone (equal parts) from the day of birth until they were about 2 months old. Every third litter born was set aside as a control. The treatments soon rendered the skin hyperemic and thick, and sometimes when the inflammation was great they had to be left off for a few days. Nothing more was done to the animals for 6½ to 9 months (according to time of birth), and then the backs of them all were painted three times a week with 0.3 per cent of methylcholanthrene in Crabtree's medium (ether containing 2 per cent of mineral oil) (6). The skin had long since become apparently normal. The carcinogenic applications were stopped after 51 days when tumors had begun to appear. These increased in number thereafter and were duly charted until the 107th day when more than half of the animals had them,—61 per cent (49 of the 80 surviving turpentined mice) and 60 per cent of the controls (27 out of 45 animals). There were 4.86 tumors per turpentined mouse on the average as compared with 4.12 per control. Most of the growths were papillomas, but there were 0.51 cancers per turpentined animal and 0.67 per control.

The slight differences found in the two groups of this experiment fall within the realm of chance. As many as 20 tumors developed on a few of the mice and the occurrence in the control group of one more animal carrying them in such profusion would have brought the average number of growths up to that for the turpentined individuals. *A priori* it had seemed not improbable that there would be large differences in the two groups, consequent upon changes residual to the turpentining,—changes rendering the skin more liable to become inflamed by the methylcholanthrene. For methylcholanthrene produces cutaneous inflammation, and this plays no inconsiderable rôle in the effectiveness of the carcinogen by furthering proliferation of the cells it has rendered neoplastic (7).

In both of the foregoing experiments the inflammation was maintained during the first weeks after birth. For the test which follows adult mice of another strain were employed.

Experiment C.—The hair was clipped close with an electric clipper over the entire right side, almost to the backbone, of 46 young adult, hairy male heterozygotes from a colony of the Crew hairless stock, bred from individuals procured from the Carworth Farms through the generosity of Mr. C. N. W. Cumming and Mr. F. G. Carnochan. Thrice a week thereafter for 9 weeks the denuded area was painted with a turpentine-acetone mixture like that of the preceding experiment, and clipping was done whenever necessary to keep the skin bare. Care was taken that none of the irritant spread beyond the dorsal line. It caused marked cutaneous inflammation and thickening, with some eventual epilation, and many of the animals succumbed to it. Nothing was done afterwards to the survivors for 24 weeks, and long before the end of this time the skin had come to resemble that on the other side. Now both sides of the body were clipped and biweekly applications of 0.3 per cent of methyl-

cholanthrene in Crabtree's solvent were begun on both, with charting of the individual growths when these began to appear. In the tabulation of them as on the right or left all were excluded that were situated within 5 mm. of the backbone. The applications were kept up for 9 weeks.

Only 17 mice lived to the end of the test. After 78 days 5 tumors had appeared on the right side of the animals and 4 on the left; by the 103rd day there were 24 on the right and 25 on the left; on the 127th day 34 on the right, 33 on the left; and on the 140th day, when the observations were discontinued, 51 on the right side and 50 on the left. The growths were the usual papillomas and carcinomas.

The observations were now extended to domestic rabbits since tumors can be readily produced on their skin with methylcholanthrene. Previous observations (7) had shown that when both ears are exposed to it to the same extent, approximately the same number of tumors arise on them. The results of experimental and control procedures can therefore be directly compared on the same animal.

Experiment D.—Seven new-born litters of Dutch belted rabbits were utilized. One ear of each suckling was painted all over, inside and out, with the mixture of turpentine and acetone during the first 24 hours after birth, and the treatment was repeated at intervals of 2 or 3 days during the next 10 to 19 days. Inflammation and induration rapidly developed, the treated ear becoming ruddy and swollen to twice or three times the thickness of its control; and sometimes the changes were so pronounced that one or more of the applications had to be omitted. Nothing was done afterwards for 4 to 6 months, according as the litters were amongst the last or first to be turpentined. By the end of that time the ears all looked normal save for some small persisting scars left from the inflammation.

Now painting was begun three times a week of both sides of both ears with 0.3 per cent of methylcholanthrene in benzene, and it was kept up for 145 days. By then all of the 15 rabbits had tumors. The yield varied from one animal to another, but the total number of growths present when the test was discontinued was 84 on the ears that had been turpentined and 83 on the controls. The tumors were all papillomas or frill horns (8).

This experiment, like those upon mice, failed to provide evidence that any agent circulates in the blood of young animals that lodges in the skin and determines the later liability to epidermal tumors. It may be objected that the agent might not have stayed permanently in the tissue,—unlike the milk factor after it has lodged in the mammary gland,—or it may be urged that so much of the agent might have reached the epidermis everywhere during the months elapsing between the inflammation and the methylcholanthrening as to mask any early local accumulation. But this latter supposition is difficult to maintain in view of the practically identical findings in the control and experimental animals. Furthermore the fact that chemical carcinogens produce cutaneous tumors as abundantly in mice 2 to 3 months old as in those 12 to 13 months of age (9) is against the supposition that precursor virus entities conferring the liability to neoplastic change assemble gradually in the epidermis during the course of life.

Neoplastic Potentialities of the Transplanted Skin of Webster-Swiss Mouse Embryos

Because of the negative results of the foregoing experiments attempts were made to learn whether the cells of the transplanted embryo epidermis possess the capacity for neoplastic change. It seemed unlikely that the grafted tissue would survive for any considerable time in Webster-Swiss mice, since the breed is not entirely homogeneous; and hence resort was had to animals of the C strain, with the positive results reported in Paper I. It would have been well to follow up with tests for the localization of a precursor virus in areas inflamed with turpentine, but the prevalence of a debilitating pulmonary disease (1) in the stock of C animals rendered it impossible. So despite the *a priori* objections to the use of Webster-Swiss animals for the transplantation of embryo epidermis this was attempted with them. Sucklings were employed as hosts, both because they had been used in Experiment B, involving local inflammation, and because the adverse reaction to grafts of foreign tissue is known to be delayed in very young animals.

A technic with sucklings was first worked out on C mice.

Bits of the skin of embryos 13 to 14 mm. long were transplanted to one thigh of 2 three day old mice, together with an equal amount (0.025 cc.) of olive oil containing 1 per cent each of methylcholanthrene and Scharlach R (*OSM*); and half as much of each material was injected into the peritoneal cavity of 9 other individuals,—where nothing was found after 81 days. Much of the implanted material had been forced out through the needle track. But in one mouse implanted in the thigh a small nodule promptly formed, and between the 39th and 46th days it suddenly became round and grew larger, an alteration usually bespeaking the presence of a tumor (1). The enlargement did not continue however, and when the animal was killed after 83 days the nodule was still only 4 mm. across, a spherical cyst containing pultaceous matter pink with Scharlach R. The microscope showed an invasive squamous cell carcinoma at one spot on the cyst wall, and elsewhere the epidermis was in the preneoplastic state. The pattern of the keratinized cyst contents (1) indicated that the cancer had long been present (Fig. 42).[1]

An implant with *OSSM* was put into 1 thigh of 12 more mice 2 days old, but the squirming of the animals forced the materials out through the needle wound within the next few minutes and no trace of them was found after 3 months.

Because of the difficulty of implanting very young animals mice 9 days old were next utilized as hosts. Equal quantities of *OSM* and of a suspension in Locke's solution of the skin of embryos 11–13 mm. long were injected into the muscles of one thigh of the individuals of litter A, 0.05 cc. in all, whereas those of litter B got from 0.01 to 0.015 cc. of *OSM* followed by 0.025 cc. of skin suspension. The implantation was done with a needle of the smallest feasible caliber, through a slit in the skin made far up next the shoulder. The syringe had such a narrow bore that when the oil was drawn up after the tissue suspension it did not rise above this but was forced out first into the new host, with result that the embryo fragments, following after, blocked backward escape of it. At autopsy the oil was in most instances some distance away, and often they had lodged in the subcutaneous tissue.

[1] The numbering of the figures is consecutive to that of Paper I.

A nodule developed in all 11 animals of litter A. The one which arose in mouse 2 was situated within the aponeurosis of the thigh muscles. It began to enlarge rapidly after 38 days and by the 55th was 12 mm. in diameter, a somewhat flattened sphere which had extended into the groin secondarily and become attached to both the abdominal wall and skin. The animal lagged behind the others in growth. It was killed now and the nodule proved to be a cyst full of dye-stained pultaceous matter, with several discrete carcinomatous masses on its wall (Fig. 43). The lining epithelium between the masses was everywhere carcinomatous and of such varied morphology as could only be accounted for by the occurrence of multiple neoplastic changes. The larger tumors had extended beyond the encapsulating reactive tissue at several places.

In another animal of litter A the nodule, which was subcutaneous, suddenly enlarged after the 45th day but altered little thereafter. An invasive squamous cell carcinoma was found in it on the 81st day. A third animal also killed then had a large sarcoma at the injection site. The other 8 mice either had nothing when killed after 80 days or else small, creamy opaque nodules like those which form where skin has been implanted alone.

A nodule in the thigh of one of the 6 mice of litter B suddenly enlarged between the 31st and 38th days, and when the animal was killed on the 45th day it had become a thick-walled cyst 8 mm. across, attached to the abdominal muscles as well as to those of the thigh. A benign papilloma with a fleshy stalk (Fig. 31 of Paper I) protruded into the cyst, and there were 3 carcinomas elsewhere on its wall. Pieces of the papilloma were transplanted to one or both thighs of 11 mice of C strain (15 sites in all). It failed to grow in any (Tumor I, Table I of Paper I) though from 2 of the grafts an anaplastic carcinoma promptly arose, which had been present at one spot in the papillomatous mass as serial sections showed. The other mice of litter B had only small creamy cysts when killed on the 81st day.

These findings warranted the view that tumors could be produced during the first 2 months of the life of Webster-Swiss mice implanted when 9 days old, within the period, that is to say, of the turpentining for localization of a virus (Experiment B). The animals were generously supplied by Dr. H. A. Schneider.

Three litters of 9 day old Webster-Swiss animals were implanted in one thigh by the technic to block the escape of oil. The 5 mice of litter A received equal quantities (0.025 cc.) of *OSM* and the skin of 13 mm. embryos. All were killed on the 70th day. At autopsy one had a sarcomatous nodule on the wall of a dye-stained, dead epithelial cyst 2 mm. across, another had no tumor but a similar cyst 3 mm. across, while a third had a tiny necrotic nodule. In the remaining 2 nothing was found.

The 7 mice of litter B had received similar implants, but the 13 mm. embryos providing the skin were from a different mother. All of the injected animals had small dead cysts after 70 days, in one case colored with Scharlach R.

The 8 mice of litter C were treated like the others except that they received the skin of 20 mm. embryos. A nodule which formed early in one mouse, dwindling afterwards, suddenly enlarged again between the 67th and 70th days, and autopsy then disclosed a sarcoma lying next a tiny, keratinized dead cyst, with some globules of *OSM* amidst it. In another mouse there was, on the 70th day, a similar dead pink cyst with a sarcoma newly arisen from its wall. Of the other 5 animals 4 had small dead cysts which were creamy and the 5th had one pink with Scharlach R.

In a collateral test 21 male mice 26 days old (weight 9–14 gm.) were injected with the same materials as the younger individuals and with the same amounts, but in both thighs. The needle was introduced through a skin slit as usual.

Group D, consisting of 6 animals, got in the left thigh the same materials as litter C, and

4 received in the right thigh the same materials as litter A, while the remaining 2 got that of litter B. On the 46th day an enlarging nodule was noted in the left leg of one of these last and it was killed on the 55th day. The nodule was a cyst with epithelium everywhere dead except at one spot where a squamous cell carcinoma was extending out from the wall (Fig. 44); and elsewhere in the nodule there was a sarcoma containing droplets of *OSM*. When the other animals were killed on the 70th day, one had a sarcoma and the rest small, dead keratinized cysts, some of them dye-stained.

The 15 mice of group E had received portions of the same skin of 20 mm. embryos that was used for litter C. Two were killed on the 55th day because each had a nodule that had suddenly enlarged. The enlargement was due to sarcomas which had taken origin from the wall of small cysts, one with a small, dying patch of epithelium, the other with it wholly dead. The remaining 11 animals were killed after 70 days. Five had sarcomas in one or both legs, associated in some cases with small dead cysts; the remaining 6 had only these latter, creamy or dye-stained.

In these tests the skin formed small cysts but died later, even where it had not come in contact with the carcinogen. In one of the 3 mice killed soonest, 55 days after implantation, the cyst lining was everywhere necrotic, and so too in another animal, save at one small spot where it was dying, while in a third instance the cells had survived only where they had become carcinomatous (Fig. 44). In the mice killed after 70 days the epithelium had wholly succumbed.

The findings contrast the more with those in animals of the C breed because the implanted hosts were in excellent physical state, free from the chronic pulmonary disease that often pulled C mice down. And they were not peculiar to implants in young animals as the following experiment attests. It was done with the aim of learning whether the papillomas induced with methylcholanthrene on the skin of adult Webster-Swiss mice contain an agent capable of conferring on embryo epidermis the liability to neoplastic change.

The minced skin from a litter of 15 mm. Webster-Swiss embryos was separated into 2 equal portions, and to one of them twice its bulk of Tyrode's solution was added and to the other the same amount of an extract of epidermal papillomas which had arisen some weeks previously as result of painting the skin repeatedly with methylcholanthrene in Crabtree's solution. To procure the extract the tumors were ground with Tyrode's solution, two successive centrifugations of it were done, with transfer of the supernatant fluid, and before this was finally pipetted off—through a long needle inserted into its midst—a heated metal disc was held just above the meniscus to kill any cells that had risen to it (10). The mixtures with skin fragments were let stand at room temperature slightly more than an hour, with occasional agitation, and then 0.025 cc. of them was implanted in the muscles of the right and left thighs respectively of 10 young adult male mice, together with equal quantities of *OSSM*. The same procedures were gone through with the skin of 10–11 mm. embryos, but to the portions of fragmented tissue five times their bulk of Tyrode's solution and papilloma extract respectively were added, prior to standing. Again 10 animals received both mixtures.

The implants gave rise at first to nodules of about the size of those forming in C mice but after a few weeks they had disappeared. One nodule in a mouse killed after 19 days contained skin which had died without proliferating, and the other consisted of a small epidermal cyst,

also dead. In a second animal, killed after 26 days, the implantations had resulted in cysts with very little epidermis still surviving on their walls. The remaining hosts were kept for 91–119 days. No cysts were found in any of them, with a single exception, though some had small lumps of concentrically lamellated keratin where once they had been. In the exceptional case a retrogressing small nodule had begun to enlarge again at about the 101st day, and when the animal was killed on the 115th day a solitary spherical cyst was found 1.1 cm. across, with a pultaceous dye-stained content and a living epidermal lining which was carcinomatous at 2 spots and in the preneoplastic state elsewhere. The connective tissue surrounding the cyst was everywhere sarcomatous. In the opposite thigh only red oil droplets remained.

Sarcomas had developed in 11 of the 18 animals living 91 days or longer, and in 5 were present at both implantation sites.

In a further experiment, 8 adult Webster-Swiss mice were implanted in one thigh with 0.025 cc. of hashed skin from embryos 20–22 mm. long, followed by the same amount of *OSSM*, while in the other thigh the skin was followed by Locke's solution. Small nodules formed during the first weeks but dwindled soon after and had almost or quite vanished when the animals were killed after 91–92 days, save in one instance in which a sarcoma had arisen with *OSSM* amidst it.

It will be seen that the implants in Webster-Swiss mice did badly in almost every animal; only in a few can they have lived long enough for the neoplastic potentialities of the embryo epidermis to have been tested. Possibly it was less resistant to the injurious effects of methylcholanthrene than that of C mice, but there was another, less problematical, reason for the early death of the epidermis, namely the heterogeneity of the animals. Transplants of the hashed embryos of market-bought albino mice become necrotic after flourishing for a few weeks (11), and those of the skin of Webster-Swiss animals generally die even when nothing more than Locke's solution has been introduced with them. Now and then some epidermal tissue survived in association with *OSSM* and in these instances a carcinoma occasionally appeared, though so late as to suggest that but little methylcholanthrene had been in contact with the tissue.

Sarcomas arose much more frequently in the Webster-Swiss animals than in mice of C strain and perhaps somewhat more rapidly. The death of the epidermal lining of the cysts had removed a barrier between the methylcholanthrene and the surrounding reactive tissue.

The Findings with C3H Mice

Pulmonary disease so often complicated the tests with C mice that attempts were made to utilize another breed. Accordingly C3H mice were procured from Dr. L. C. Strong and a colony started. The animals proved unsuited to the transplantation work.

The skin was procured, minced, and implanted in the usual way. In one experiment it came from embryos 15 days old (13 mm. long), in 2 from litters 16 days old (14–15 mm.

long), in 2 more from slightly older litters (16–17 mm. long), and in a 6th test from a litter close to term (embryos 24 mm. long).[2] The skin was placed in both thighs of 44 young male mice in all, with an equal quantity (0.025 cc.) of *OSSM* at 47 of the 88 sites, *OSM* instead at 8 sites, oil containing 1 per cent of methylcholanthrene (*OM*) at 13 sites, and Tyrode's solution at 8 sites, Locke's solution at 3, and oil supersaturated with Scharlach R (*OSS*) at 11. The implants of the last three types served as controls and they were placed in one thigh only of the animals, with skin and *OSSM*, *OSM*, or *OM* in the opposite leg.

All of the host mice remained plump and healthy until killed after 11–175 days. The epidermis of the control implants soon gave rise to tiny cysts, but these seldom lived for more than a few weeks. Even under the most favorable conditions the implanted skin formed no fatty layer with embedded hair follicles such as develops in C mice; yet during the first days some differentiation of the follicles did take place and pigmented hairs developed in many instances. Death of all the epithelium soon supervened though, and in individuals killed after 11–34 days the cysts were small as a rule and completely keratinized, with a wall consisting merely of reactive tissue. Sometimes the nodules had a gray hue because of pigmented hairs amidst the keratin.[3]

The skin implanted with methylcholanthrene proved unable to tolerate it in quantity and either failed to form cysts or succumbed soon after. The residual keratin usually contained no hairs, proof that the follicles had never functioned. There were instances however in which only a little of the carcinogen was encysted and then islands of epithelium sometimes survived. Of 34 implants with *OSSM* in hosts autopsied after 49–175 days, 27 had no surviving epidermis, 3 had formed cysts with some living epithelium (in the precancerous state in 2 cases), while in 4 instances epidermal tumors had arisen. The implants with the other carcinogenic materials yielded similar findings after 47–112 days. Of 7 implants with *OSM* 6 had died and 1 become carcinomatous, while of 10 with *OM* 8 had died, 1 had some living epithelium in the preneoplastic state, and in the tenth a large cyst had developed with 2 malignant papillomas on its wall.

The earliest growths encountered were 3 microscopic carcinomas on the walls of cysts procured after 47, 49, and 57 days from mice receiving *OM*, *OSM*, and *OSSM* respectively, together with the skin of embryos 16–17 mm., 13–15 mm., and 14 mm. long. The first tumor large enough to be recognized as such on palpation derived from a nodule due to 14 mm. embryo skin together with *OSSM*, which began to enlarge rapidly soon after the 47th day and by the 75th was 15 mm. across. It proved to be a cyst containing a benign papilloma (Fig. 45) with a stalk of sarcomatous tissue. Nearby on the cyst wall were 2 minute, actively invasive carcinomas.

Sarcomas arose soon and often. One was found with the microscope after 34 days, 2 were perceptible in the gross after 57 and 61 days respectively, and after 70 days they became very frequent. Careful slicing sometimes disclosed dead cysts or residual lumps of keratin amidst them, but more often globules of ruddy oil only. As in C mice and Webster-Swiss animals their presence was frequently signalized either by the sudden enlargement of a nodule long stationary, by its reappearance, or by the development of one where none had ever been.

Three of the sarcomas were transplanted to both thighs of young male C3H mice by the routine trocar method (1). One grew fast in the legs of 2 animals but failed at both sites in 2 others; a second grew fast in both legs of 2 animals, slowly in those of another, and failed

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[2] C3H mice are bigger at birth than C mice and much larger when adult.

[3] Greenstein and Andervont (13) implanted the minced tissues of 14 day C3H embryos at subcutaneous sites in adult hosts and obtained progressive growth during several months. The mince contained all of the embryo tissues.

entirely in 4 more. The grafts for the third transplantation were from a slightly thickened cyst wall which had undergone sarcomatous change in a small area, as the microscope later showed, and the results of transfer differed from graft to graft as well as from host to host, tumors arising in 4 of the 5 animals, but in one thigh only.

These findings made plain that the stock of C3H mice was far less homogeneous than that of the C strain. The control injections of embryo epidermis with one of the salt solutions or with *OSS* mostly died within a few weeks, and the sarcomas which appeared where the tissue had been placed with *OSSM* failed to "take" in some hosts on transplantation, whereas the sarcomas of C animals succeeded in them all (1). But there were other reasons why papillomas and carcinomas seldom arose from the implants and only after a relatively long time. Even in the most favorable hosts the embryo epidermis showed but slight ability to grow and to withstand methylcholanthrene. So little of it lived for any considerable time that the opportunity for neoplastic changes must seldom have existed. In those instances in which the epithelial cells survived, their proliferation was much less active than in the case of C animals, and it is known that the rate at which epidermal tumors form in response to the chemical carcinogens varies in general with the collateral stimulation of the layer from which they take origin (12). Yet despite these adverse circumstances a few papillomas and carcinomas did arise.

Sarcomas appeared much sooner than in C mice.

The Response to Methylcholanthrene of Suckling and Adult Mice of C Strain

The rapidity with which growths were induced in the embryo skin of C mice led us to set up comparative tests with new-born animals and young adults.

OSSM could not be used since new-born animals were almost at once cleansed of it by the mother. Hence a 0.3 per cent solution of methylcholanthrene in Crabtree's medium was employed. This dried practically at once when painted on, and inspection in ultraviolet light 4 days later showed the skin still to be brilliantly fluorescent with the carcinogen. The applications were made twice weekly,—on the entire back of litters of C mice, beginning on the day of birth, as also on the backs of 50 males about 2½ months old. The dorsal hair of these latter was clipped away in approximation to the hairless state of the young. The mother mice were painted with the carcinogenic solution on the day they delivered, as also when next their sucklings were so treated, with the aim of lessening the chance that they would kill these because of the odor of the solution. They seldom did so.

Out of the 10 litters receiving methylcholanthrene 39 young mice were still living at the end of 6 weeks. At first the carcinogen caused no perceptible inflammation, and the animals grew as well and hair came in as rapidly and thickly on their backs as on those of control sucklings. Only after about 2 weeks did a difference from the latter become perceptible. Keratin flakes had by then appeared amidst the dorsal hair, and by the 24th day this was matted and was coming away in patches or entirely, baring a ruddy skin. After little more than another week though, the animals were hairy once more. The methylcholanthrene was discontinued on the 25th day (3 litters), 28th day (1 litter), 31st day (3 litters), 32nd day (2 litters), and 35th day (1 litter), and neither then nor in the next weeks did any tumors arise.

The hair of 3 animals was removed with barium sulfide after the first 2 weeks to learn whether it hid growths, but none were found.

Very different were the results in the mother mice, which had received only 2 applications of methylcholanthrene. Within 9 or 10 days the back was bare of hair from head to tail, markedly inflamed and glazed or scurfy, irrespective of whether or not the hair had been clipped away prior to the first application. Only after about 18 days did the inflammation wear off and hair begin to come in again. By the 28th day 3 of the 10 mothers had characteristic papillomas where the methylcholanthrene had caused most change and 2 more had them by the 35th day. The findings with the adult males were nearly similar. By the 14th day only 9 of the 50 had any hair left on the back, what remained was thin and patchy, and there was much cutaneous inflammation. On the 21st day hair was coming in again on a few individuals and 3 had definite papillomas,[4] as proven by later enlargement and course. The applications were continued for 42 days. Thin hair had reappeared on most of the animals by the 31st day and 8 of the 50 had papillomas then. On the 39th day many were so hairy as to have to be clipped anew and 10 had papillomas, the total number of these growths amounting to 18.

Next, 4 litters were painted three times a week. During the first 10 days the skin of the 21 animals did not become inflamed, hair came in as rapidly as on control litters, and the mice grew as fast, but by the 12th day they were smaller than the latter and by the 21st much smaller and with much less hair. The observations were now discontinued because the appearance of cutaneous tumors in response to the chemical carcinogens is greatly retarded and often prevented by an ill-nourished state of the animal (14). The 4 mother mice (painted twice in all) showed the early and pronounced skin changes already described and by the 21st day one had 2 papillomas. No tumors had arisen on their young nor did any appear within 42 days. By then all were growing fast and in excellent condition.

In further attempts to elicit tumors in new-born animals 2 litters and their mothers were painted twice only with 0.6 per cent of methylcholanthrene in acetone containing 2 per cent of mineral oil. (1 per cent of the carcinogen could not be used because it fell out in a powder on drying.) By the end of 11 days the backs of both mothers were hairless and inflamed but the young were as hairy as those of control litters, though lagging behind them in growth. On the 37th day each of the mothers had several papillomas whereas the thriving young had none.

Acetone containing 0.6 per cent of methylcholanthrene and 2 per cent each of mineral oil and quinine was painted on 4 litters thrice weekly from the day of birth for 15 days. The quinine had been added to prevent any possible licking off of the carcinogenic material from the young by the mothers. These latter received only 2 applications, as in the previous tests. The sucklings remained free from inflammation during the first 2 weeks and developed hair promptly but in the 2nd week became sick and cyanotic and some died later, whereas the mothers, though well nourished and vigorous, became hairless all over the back, this time within 7 days, and their skin was scurfy and much inflamed. By the 33rd day 3 of the 4 mothers had numerous papillomas but the 4th was still negative in this respect. No tumors had developed in any of the 8 surviving young, which were now in excellent state.

These observations disclosed a surprising fact:—The skin of new-born C mice tolerates methylcholanthrene solutions far better than does that of adults.

[4] It seemed possible that tumors could be perceived very early if the skin was examined under a binocular dissecting microscope, and consequently a magnification of 17 diameters was for some time used in searching for them on both adults and young. But it disclosed none that were not perceptible in the gross, though it did give information on their aspect.

In these it became hairless and inflamed within 7 to 11 days, after only 2 applications of the hydrocarbon, whereas no changes whatever were perceptible in the skin of the young for about 2 weeks, the hair coming in as rapidly as on control sucklings although the applications of methylcholanthrene were kept up. Only after some 20 days was the skin partially denuded and inflamed.[5] Tumors failed to appear on any of the young animals during 5 to 6 weeks of observation whereas some of the mother mice had them within 3 to 4 weeks, as had also some of a group of young adults which, like the sucklings, received many applications.

In seeking for reasons why the methylcholanthrene had so little effect on sucklings it may be recalled that the skin of C embryos near to term when transplanted together with *OSSM* goes on differentiating during the first days and forms hairs unless the carcinogen is present in such strength as to cause death of nearly all of the epidermis. The encystment of the methylcholanthrene by the latter results in a continual exposure of the epithelial cells to it in high concentration. Under the circumstances of our experiments with newborn litters it was not possible to bring enough of the carcinogen to bear on the skin to check primary follicular development and hair formation to any perceptible extent; and indeed the amount required to cause secondary epilation soon reduced the animals to such a poor physical state that tumors could scarcely have arisen promptly even if neoplastic changes had occurred.

In an early experiment, 5 new-born litters of Webster-Swiss mice were painted twice weekly for 74 days with 0.3 per cent methylcholanthrene in Crabtree's solution in an effort to learn whether neoplastic potentialities were present within the first 2 months after birth, the period during which other sucklings had been turpentined (Experiment B). The 11 surviving animals of 2 of the treated litters were first examined after they had been methylcholanthrened for 33 days; one already had a big papilloma. The 6 animals of a third litter were negative after 34 days although by then the mother, though only painted twice, also had a big papilloma. The remaining 2 litters (13 mice), not examined until the 46th or 47th day of treatment, had growths in 9 instances (13 papillomas and 1 carcinoma), confirmed as such microscopically. The suckling mice had withstood the methylcholanthrene far better than the young C mice and it was kept up for a longer time. Nearly all of them had developed tumors before the last of the treatments, and some of the growths were cancers.

In this test most of the mother mice were painted only on the snout.

The fact brought out by this experiment, that epidermal tumors can be readily induced in Webster-Swiss mice during the first 2 months after birth, suffices to exclude one possible explanation of the failure to obtain an especially large yield of such growths on the skin of methylcholanthrened adults which had been turpentined throughout this period (Experiment B). Since tumors can be induced within the first weeks after birth it cannot be supposed that a hypo-

[5] In the case of some litters not mentioned heretofore, the sucklings were painted but twice, with no evident effects, although the mothers treated to the same extent not only became hairless, with skin markedly inflamed, but in some instances developed papillomas on the back of the neck or between the shoulders.

thetic agent conferring neoplastic potentialities circulates only at a later period.

The Earliest Occurrence of Neoplastic Changes in the Embryo Skin of C Mice

The implants of embryo epidermis in C mice (1) were closely scrutinized to find out how soon neoplastic changes took place in the cells exposed to methylcholanthrene. A carcinoma 3 mm. across developed within 31 days in one animal receiving the tissue with *OSSM*, and cancers up to half a centimeter across had become frequent by the end of the 7th week, and they were often multiple. Papillomas had also developed, but they were much fewer. Despite this early gross yield no indubitable neoplasms were disclosed by the microscope until 26 or more days had elapsed, though the implants in most of the animals killed between the 7th and 30th days were searched in serial sections.

During the first 3 weeks the wall of the cysts containing methylcholanthrene showed the hyperplasia, increased basophilia, irregularities of cell size and disposition, and gradual accumulation of lymphocytes already described (1). Even where globules containing methylcholanthrene were passing through the epidermal layer and in direct contact with its cells or actually within them, as occasionally happened, they seldom elicited any immediate morphological changes. The cells next the globules conformed to their contours, and no surface bulging of the keratinized layer took place over them (Fig. 24 of Paper I) but instead the living layer of cells was shallower to the extent of their diameter, as if proliferation were here slowed down. More considerable abnormalities could not perhaps have been expected, since methylcholanthrene is known to act gradually and the elements in direct contact with it keratinized very soon and were cast off into the cyst contents. Yet more there was. Occasionally one of the cells next a droplet or containing it (Figs. 46 and 47), or in the layer lining a cyst in which the carcinogen was present (Fig. 51), became a giant element, undergoing polyploid mitosis with result in huge cells containing big nuclei. And there were other spots at which individual cells underwent a parakeratotic change culminating in pyknotic "bullet bodies" (15) (Figs. 48 and 49). This latter change, found as soon as the 6th to the 10th day, affected only occasional cells and has never been encountered in the controls. In the earliest specimens to show bullet bodies these were situated near the free surface of the epidermis, or in the recently desquamated keratin (Fig. 48), as if the cells had been well along toward differentiation when they first underwent the parakeratotic change; but as time passed and the methylcholanthrene continued to influence the encysting layer of epidermis, some of its basal elements showed the curious alteration, dividing and giving rise to other elements which in turn displayed it (Fig. 49). Bullet bodies were a prominent feature of some of the well established cancers present later (Fig. 50), and for this reason it seems probable that those found in the early days were the manifestation of a neoplastic change.

By the time the implants had been *in situ* 2 weeks extensive local alterations had taken place in the epidermis in some cases. The cells at certain spots were now different from the generality, much smaller or larger, with many more mitoses than elsewhere; and sometimes they lay crowded higgledy-piggledy, and were very basophilic. Yet even where aggregates of them bulged into the connective tissue (Fig. 25 of Paper I), one often could not be wholly sure that they were tumor cells. And where the epithelium had invaded the connective tissue to reach oil globules during the first days after implantation and was now proliferating wildly amidst and around these latter (1) the uncertainty was still greater. Here what appeared to be active squamous cell carcinomas were frequently present before the end of the 3rd

week; yet one could not conclude that this was the case, for the reason that the epithelial cells individually had no characteristics definitely bespeaking the neoplastic state and thus distinguishing them from elements merely stimulated and rendered abnormal. Only when they pushed out into the normal tissues and replaced them as time went on (Figs. 6 and 9), or continued anaplastic and actively multiplying (Fig. 8) instead of differentiating into an orderly epidermal layer, or exhibited distinctive features (Figs. 9 and 10), could one conclude that here was a tumor. All of which is to say that it was usually impossible to recognize a neoplastic change until it had become overt. Cells undergoing polypoid mitoses, like those pictured in Figs. 46 and 51, are known to appear in the methylcholanthrened skin of adult mice weeks before any neoplasm arises (16).

GENERAL CONSIDERATIONS

The conditions obtaining in the experiments of the present papers gave assurance that the epithelial tumors induced in the implants of embryo epidermis derived therefrom. In view of this fact it seems well to cast back through the literature in search of previous instances.

Borst (17) reported in 1919 upon the work up to that time. Its outcome had been essentially negative.

Askanazy (18), experimenting during nearly thirty years, to 1926, implanted large numbers of rats with minced embryos and obtained 3 carcinomas. One arose in the mediastinum at a distance from where the implant had been placed after treatment with ether. Another appeared where embryo tissue exposed to chloral hydrate had been injected beneath the skin of the abdomen with result in a lump. This suddenly enlarged after 14 months and ulcerated, causing death after $1\frac{3}{4}$ years; sections showed bone, cartilage, glia, dermal, and endodermal cysts and a squamous cell carcinoma along the base of the ulcer. In the third instance a minced embryo had been placed in the peritoneal cavity of the mother, together with ground-up cockroaches.[6] Seventeen months later a nodule consisting of differentiated tissues of divers sorts was found in the belly wall at the wound site, and amidst them was what appeared to be a squamous cell carcinoma of "pepper-corn" size.

Petroff and Krotkina (19) obtained a squamous cell carcinoma in 1 of 78 rats implanted in the peritoneal cavity with minced embryos and an arsenic solution, and given Fowler's solution by mouth later. The growth arose amidst an intraperitoneal mass of teratoid tissue, and it was successfully transplanted, proving it a true neoplasm. It may well have arisen from the embryo tissue.

Askanazy added Scharlach R to some implants to stimulate them, but obtained only injury (20), and so too did Petrow (21) who likewise worked with rat tissue.

Sorour (22) minced the embryos and placentas of mice of mixed breed and implanted the entire mass in the subcutaneous tissue of the male parent's back, afterwards dropping a benzene solution of benzpyrene on the skin between the shoulders twice weekly for 75 days. Here cancer developed on 24 out of 72 animals but not in any of the implants, 22 of which disappeared while some of the others gave rise to ordinary teratoid growths.

The report of Witschi (23) that he obtained tumors in frogs by implantation of overripe ova still awaits documentation (24).

No previous attempt has been made to expose embryo tissues to the powerful synthetic carcinogens of the present day. The rapidity with which these agents

[6] Fiebiger had reported shortly before that the presence in the stomachs of rats of nematodes from cockroaches caused gastric tumors to arise, including carcinomas.

produce sarcomas of the connective tissue has been a strong *a priori* deterrent from work of the kind since the implants would presumably be destroyed by these growths in the lack of any method to prevent them. Despite recourse to such a method they arose so soon in our C3H mice[7] as to complicate the findings, but in animals of this sort and in the Webster-Swiss stock a more serious difficulty existed,—early death of the epidermis. Yet a few tumors of the embryo skin of C3H and Webster-Swiss embryos were procured despite the adverse conditions, enough to demonstrate that the epidermis transplanted to adult hosts possessed potentialities for neoplastic change.

Developmental State of the Cells at the Time of Neoplastic Change.—The carcinomas and papillomas which methylcholanthrene elicited in implants of the skin of C mice had no embryonal characters such as are manifest in certain human growths deriving from fetal remains, notably malignant teratomas of the ovary, but were like those arising from adult epidermis or from the long-differentiated and matured embryonal cysts of man. There is no reason to assume that the epidermal cells of the implants were still in an embryonic state at the time when they underwent neoplastic change.

The epidermis of control implants with Locke's solution matured rapidly, sebaceous glands soon appeared, and the follicles formed hairs before they would have done so in suckling animals. The happenings were very different in the presence of methylcholanthrene (1). This kept the epidermis in a hyperplastic condition, preventing differentiation, with result that it continued to appear much like what it had been when taken from the embryo. One might have thought that the carcinogen had actually prevented the tissue from ageing, and with the more reason because Beskrovny (26) has reported that when crystals of methylcholanthrene are introduced into incubating hens' eggs the development of many tissues is inhibited. But other facts make plain that what the carcinogen really did was to injure the skin and thus suppress differentiation. When painted on adult mice it destroys sebaceous glands almost at once (27) and hair follicles after some weeks, as is well known, and causes the epidermis, normally only one to two cells thick, to become hyperplastic and form a stratified squamous layer. When much of it was encysted by the embryo skin the same end result was somewhat differently attained; the carcinogen increased the hyperplasia of an epidermis already in the stratified squamous state, did away with the rudimentary follicles, and prevented the sebaceous glands from developing. When less was present it failed to check differentiation during the first days, the follicles quickly forming hairs; but later on changes ensued like those produced in adult skin, and the cyst lining in consequence came to look as if it were still in the embryonic state, only the hairs amidst the keratin telling of its previous differentiation.

Quite possibly experiments with very young embryos will yield tumors of a truly embryonic sort, even tridermal growths if the fertilized egg proves capable of surviving exposure to methylcholanthrene.

The observation that methylcholanthrene dissolved in olive oil has the same influence as Scharlach R to cause the cells of the embryo epidermis to simulate

[7] Andervont rated the C3H breed as having the greatest liability to induced sarcomatosis of the 8 strains he tested, with the C strain ranking next (25).

cancerous elements raises an important question. Perhaps the action of the chemical carcinogens is physical, and is exerted on the surface membrane of cells; many have thought that this is how the dye acts. Whether Sudan III or indole or skatole,—substances resembling Scharlach R in their effect on epidermal cells,—have any carcinogenic power is uncertain. Scharlach R fails to cause epidermal neoplasms,[8] but it does produce other tumors, notably of the liver (29). We intend to compare the early effects of the carcinogenic hydrocarbons on transplanted embryo epidermis with those of nearly related substances not having this character.

Relative Liability of the Cells of Embryos and of Young and Old Mice to Neoplastic Change.—Tumors were so readily induced in the implanted skin of C embryos as to suggest that the embryonic state entails a special liability thereto, but tests with sucklings and young adults failed to support this conception. Growths arose as rapidly from the epidermis of some adults as from that of the most responsive embryo implants. No tumors were obtained on sucklings during the period of observation, although these should presumably have occupied an intermediate position between fetuses and adults in their liabilities; but there were obvious reasons for this discordance. It is questionable whether the methylcholanthrene reached the basal epidermal cells of sucklings in effective amount, at least for some while. Certainly it failed to produce any perceptible cutaneous changes within 2 weeks, although in much less than this time it had brought about marked inflammation and complete loss of hair on the backs of treated adults. Perhaps the failure was due to the great metabolic activity of the skin, its abundant vascularization, or to some other local circumstance;[9] but whatever the reason it is clear that the tests provided no valid comparison of the liabilities of the epidermal cells of young and old animals to neoplastic change.

In the implants of embryo tissue the conditions were notably favorable to the action of methylcholanthrene and to rapid enlargement of any growths it induced. It was tolerated in high concentration by the implant, and not enough of it was present to affect the health of the host; it came into direct contact with many of the embryonal cells during encystment and later exerted its influence continually on the lining of the cysts; the cells were multiplying actively when they first came under its influence, and it stimulated them further, as did any Scharlach R that was also present. Chance had little to do with the fact that the first tumors to arise were mostly situated at the recently completed ends of the oblong implantation cysts, where the epithelium was still actively proliferating amidst and round about oil globules containing the carcinogen and the dye.

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[8] The rabbit reported by Bungeler (28) as developing a cutaneous carcinoma after the application of Scharlach R for 16 months had received arsenic as well.

[9] The recent observations of Cramer and his associates on the distribution of methylcholanthrene to the tissues after a single application to the skin (30) may give clues to the problem here presented. In adult mice the carcinogen mostly enters by way of the hair follicle shafts, and, accumulating in quantity in the sebaceous glands, spreads therefrom to the other cutaneous elements. In new-born animals such portals of entry and depots are lacking.

The widely different behavior of sucklings and adults when exposed to methylcholanthrene provides a commentary on previous efforts to discover whether old or young animals are the more responsive to chemical carcinogens. The young selected for the tests have generally been some weeks old, since it has seemed unlikely that new-born creatures would survive the applications. When a carcinogen is painted on individuals of different ages one is really testing primarily the influence of local circumstances to affect exposure of the epidermal cells, and coming only secondarily at whether there are age differences in the liability of the cells to neoplastic change. The same holds true, if in less degree, of experiments involving the subcutaneous injection of carcinogens in old and young.

The question comes up of why neoplasms are so rarely present at birth if the cells of human embryos possess any such capacity for neoplastic change as the transplanted skin of mouse embryos. In human beings tumors not infrequently take origin from fetal remains during postnatal life, yet Gideon Wells (31), scrutinizing all the recorded prenatal instances one by one, found not a case of genuine carcinomatosis, and only about 50 true sarcomas,—most of them associated with congenital anomalies, *e.g.*, Wilms' tumor.

Our experiments go some way to solve this riddle. The epidermis is amongst the most responsive of all mouse tissues to the action of methylcholanthrene, and in the embryo implants it was exposed to this in a strength close to the effective maximum and certainly approximating the limit of the tolerable. Yet the first tumor perceptible in the gross was encountered only after the lapse of a longer interval than the total period of gestation in the mouse. The inference seems warranted that the reason why tumors have not been encountered in new-born mice, and only very rarely in human beings at birth, is that even if carcinogenic stimulation took place during intrauterine life the fetus would nearly always be born before enough time had elapsed for neoplastic changes to manifest themselves.

Conditional or Autonomous State of the Tumors Derived from Embryo Skin.—As mentioned in Paper I, the tumors arising from the embryo skin of C mice differed in two respects from those appearing on adult skin in response to methylcholanthrene,—the great majority were primary carcinomas, not benign papillomas, and even the most malignant of them failed to metastasize.

It is conceivable that pressure within the implantation cysts may have prevented the formation of papillomas which would have developed on a free skin surface, and yet not have interfered with proliferation of the malignant cells that many of these growths have been shown to contain (32). Several circumstances account for the absence of metastases. Few of the growths were let run their entire course; the mice were always killed when ulceration threatened;[10] and the experimental conditions were such that infection of the neoplastic tissue with pus-producing organisms never occurred. Thus happenings were avoided which are often attended by an increased aggressiveness of cancer cells. Possibly the intramuscular

[10] In a recent test a large pulmonary metastasis has been found in a C mouse dead of a squamous cell carcinoma due to implantation of the skin of 17–18 mm. embryos together with *OSSM* 125 days previously. The growth had been allowed to ulcerate.

situation is less favorable to metastasis than the cutaneous; for local circumstances greatly alter neoplastic cases in such respect.

During the first weeks after encystment of the methylcholanthrene by the embryo epidermis lymphocytes collected and formed a crowded zone beneath the epithelial layer wherever this became markedly affected by the carcinogen. Orr (33) and Cramer (34) mention no such accumulation in adult skin nor have we encountered it. Lymphocytes are prone to gather wherever transplanted normal or neoplastic tissues are doing badly, and during the work with C3H mice they have at times been found in great number beneath the epidermis of control implants which were dying after cyst formation. Hence it seems probable that the zone of lymphocytes now in question formed merely in response to cell injury as such, not in response to any peculiar metabolic product resulting from the action of methylcholanthrene. Yet this will not explain why the cells fail to gather in adult skin painted with the carcinogen; for its epidermis exhibits the same signs of injury as that of the embryo.

Previous workers have successfully transplanted several induced carcinomas of the adult epidermis, but no comparative tests have been made with the various other cutaneous tumors arising in response to the chemical carcinogens. The availability of uninfected growths in large number as result of the implantations in C mice led us to undertake this task (see Table I of Paper I).

Mottram (35) could not get tar papillomas to grow in the subcutaneous tissue of their hosts, though some formed tiny cysts lined with stratified squamous epithelium. One of the papillomas we transplanted failed to live or barely survived at the new situation, and the other failed consistently, though at 2 spots an anaplastic carcinoma appeared which a later search showed to have been present in the original material. These results are the more significant because the normal epidermis of C embryos always does well in new hosts. They stress a fact already proven (12), that the benign papillomas induced in the mouse and rabbit by the chemical carcinogens are mostly conditional growths, dependent on favoring local conditions for success. These conditions the carcinogens provide through the collateral changes they bring about in the skin.

The fate of the malignant papillomas possesses great interest. They proliferated rapidly after transfer, only to retrogress and die in most instances later. This cannot have been due to genetic differences in the host animals, since the normal epidermis regularly succeeded after transfer, nor can it have resulted from pressure within the cystic tumors, for these often invaded the loose subcutaneous tissue during the first weeks and no mechanical impediment existed to a continuation of this process. It might be thought that the growths had merely simulated malignancy in the original host, as some benign rabbit papillomas do when stimulated by the chemical carcinogens (12), but the mouse papillomas, unlike these growths, did not revert to the benign state when the methylcholanthrene was no longer present (as after the transfer to new hosts) but remained malignant in all obvious characters so long as they lived (Figs. 37 and 38). Many workers have noted that a considerable proportion of the frank carcinomas induced by the chemical carcinogens on the skin of adult mice disappear spontaneously, even though the applications are kept up which first called them forth. The transplanted malignant papillomas of the present tests may well have been conditional tumors,—if with more impetus to proliferate than those which were benign; and yet one is tempted to ask whether such of them as did well at first only to become necrotic later may not have elaborated some constituent crucial to their success, against which the host mice reacted. The retrogression of the virus-induced papillomas of rabbits seems due to such a happening (36), and the Brown-Pearce carcinoma has lately been found to contain a sub-

stance inducing the formation of an effective specific antibody in rabbits to which the tumor is transferred (37).

The squamous cell carcinomas arising from embryo skin fared better after transfer than the malignant papillomas, yet 2 which were anaplastic retrogressed or failed entirely (Tumors 8 and 10 of Table I). The conception that favoring local conditions may have determined their progressive course in the mice in which they first arose gains support from the results of transplanting composite tumors, those due to the coalescence of several neoplasms. Each component of these succeeded, as if when acting together they established conditions favorable to them all.

As a whole the transplantations demonstrate that neoplastic epidermal cells are often far less capable of maintaining themselves than normal elements. The fact that the neoplastic state sometimes entails decisive disabilities has been pointed out in previous papers (12).

The transplanted tumors regularly formed cysts containing fluid, though in some instances these were so packed with crowded papillomatous ingrowths as to appear solid in the gross. No such fluid accumulation is mentioned by the workers who have transferred epidermal cancers of the adult mouse, though Mottram has pictured tiny cysts full of lamellated keratin as forming where tar papillomas had been grafted to the subcutaneous tissues of the animals in which they originated (35).[11]

Induced Neoplastic Changes in Implants of the Embryo Stomach and Other Organs.—Not infrequently squamous cell carcinomas were found in implants of the minced tissues of 7–8 mm. C strain embryos (1), which were very unlike those deriving from the skin of older fetuses. It seemed probable that they had arisen from some other organ than the epidermis, and there was the more reason to think so because various other epithelia had encysted the methylcholanthrene. Following upon this lead we have implanted fragments of many embryo organs together with the carcinogen and have thus procured tumors in wide diversity, notably of the stomach, lung, ovary, and bile passages. Gastric carcinomas, which can be elicited only after months when ordinary carcinogenic technics are applied to adult mice, arose as rapidly and regularly from the implants as they did from embryo epidermis. The findings will be reported in detail later. Fig. 52 shows one of several squamous cell carcinomas which had grown out after 44 days from an implant of the minced gastric tissues of 20 mm. C embryos together with *OSM*. The cancer was actively invading the voluntary muscle. Figs. 53 and 54 depict an adenoacanthoma which appeared where fragments of the secretory portion only of the stomachs of 20 mm. embryos had been placed together with *OSM*. The growth, which had become large when transplanted on the 65th day, grew with immense rapidity in all of the 10 animals to which it was transferred, and the figures are from a

[11] In our laboratory 3 squamous cell carcinomas of the rabbit derived from virus-induced papillomas (38) have been successfully transferred to the muscles of other individuals, and they have all formed cysts containing much fluid.

transplantation nodule 8 mm. across when removed on the 11th day. It may be possible to analyze the neoplastic potentialities of the organs of some strains of mice much more rapidly by utilization of embryo implants than by ordinary methods of test. Incidentally the implantation technic provides growths free from destructive bacteria and hence in a state suited to propagation.

Theoretical Implications.—No evidence was obtained in the work with C mice that the skin of embryos of some litters possessed potentialities for neoplastic change which that of others lacked. Not only did tumors arise very rapidly when the requisite exposure to methylcholanthrene took place but they occurred with remarkable regularity. In Experiments 14 and 15 (Tables II and III of Paper I), involving implantation of the skin of 18 mm. embryos, epidermal tumors arose in every animal kept long enough for the carcinogen to act. Similar results have been obtained recently with the skin of somewhat smaller embryos.

Tumors arose as soon from the tissues of minced 12 day old embryos (7–8 mm. long) as from the skin of those near term (20 mm. long), and with significant frequency considering how unfavorable the conditions were to the encystment of the methylcholanthrene in the case of the smaller embryos. The local conditions in the experiments with embryos $10\frac{1}{2}$ days old (3–3.5 mm. long) were unfavorable to neoplastic change in several crucial respects (1). Nevertheless cancer appeared in one instance. The findings provide no reason for the assumption that the epidermis of the youngest embryos tested was any less liable to neoplastic change than that of fetuses about to be born. More must be done on this point however.

Were the neoplastic potentialities of the embryo cells inherent, or acquired either *in utero* or during sojourn in the animals to which they were transferred? The presence in some epidermal cysts of multiple foci of proliferating neoplastic cells within about 4 weeks after the implantations, and the regular appearance of tumors later on make it seem at first wholly unlikely that the potentialities mentioned can have reached the tissue from the new hosts; one would have to suppose that an agent conferring liability to the growths circulated in every mouse receiving an implant and that it localized in each of these. But it may be recalled that the "milk factor" determining the liability of certain strains of mice to mammary cancer is passed on to the suckling young within a few days and establishes itself in the mammary tissue of practically them all. Furthermore there is evidence that the factor circulates in the blood of adults. This knowledge renders it possible to think that the tendency to neoplastic change is conferred on the embryo tissue by some agent localizing in it after implantation. But there are facts against the assumption which must also be considered. The experiments described in the present paper, to obtain

localization from the blood into inflamed areas of an agent conferring the liability to skin tumors, have given consistently negative results. True, the mice employed were mostly Webster-Swiss animals; but methylcholanthrene produces skin tumors as readily in adults of this breed as in those of the C strain, and sucklings painted with the carcinogen from birth have developed tumors within less than 2 months, that is to say within the period that the skin of other sucklings of the breed was kept inflamed with the object (unattained) of causing localization of an agent conferring the liability to tumors. These findings provide no reasons for the supposition that this liability in Webster-Swiss mice rests on any different basis from that in C animals. The implanted skin of Webster-Swiss embryos only occasionally lived long enough in association with methylcholanthrene for tumors to develop from it, but sometimes tumors did occur.

A second, more substantial, obstacle to the view that the neoplastic potentialities of the implants were acquired in the new hosts is to be found in the singular specificity of those agents which are known to confer such potentialities, namely the tumor-producing viruses and the "milk factor" responsible for mammary tumors. They all act only upon cells of a single kind, no matter how wide a distribution they undergo in the body, naturally or experimentally, and each gives rise to growths of a single sort, with but slight individual differences at most.[12] To account by means of agents of similar specificity for the multifarious tumors occurring "spontaneously" or in response to experimental carcinogenic stimulation it has been necessary to suppose that at some time during the course of life, one or another of these agents reaches the tissue for which it has an affinity, with result in this or that sort of growth after the requisite disturbance of the cell providing its milieu has taken place. Most tumors arise after such an interval of time that there has seemed to be ample opportunity for the necessary localization of the hypothetical agents. But now that it has proved possible to induce the formation of a wide variety of tumors in transplanted embryo tissue during the course of a few weeks, what is one to think? Can one suppose that during these weeks, or for that matter while the embryo providing the grafts was still in the uterus, a host of differing agents, each conferring the liability to one sort of tumor only, were circulating in the blood and localizing in the young tissues? Or can the assumption be warranted that under natural conditions a non-specific precursor agent is distributed widely to the cells, and that secondarily, if and when one or another of them is acted upon by a carcinogen, the associated agent undergoes such alterations as to bring about a neoplastic change of special kind, a kind determined by the character of the cell? These are far flung speculations.

[12] According to Kirschbaum and Bittner the "milk factor" is responsible for mammary adenocarcinomas, not for the other mammary tumors which can be called forth by methylcholanthrene (39).

It would seem easier to explain the findings of the present papers by supposing that the generality of tumors result from intrinsic cell changes. But what then is one to infer from the existence of tumors indubitably due to viruses? Must such growths be written off as mere curious exceptions, although their traits are typical of the neoplasms as a class, and despite the serological and histological evidence for the existence of causative viruses in certain tumors from which they have not yet been obtained? This seems the more unwise because there is no factual alternative to the viruses at present; they are the only actuating causes for tumors thus far demonstrated.

Can it be that animal cells, when brought to the abnormal state in which neoplastic change occurs, elaborate self-reproducing substances which make them into tumor cells and maintain them as such? Very occasionally the substances might prove separable from the cells in a condition to render others neoplastic, and in this way attain to the status of viruses in the current conception of the term. These thoughts will have occurred to everyone familiar with tumor phenomena, with the newer work on cell capabilities, and with the prevailing uncertainty concerning how viruses have come to exist.

SUMMARY

Experiments were carried out to learn whether the widely differing liabilities to induced epidermal tumors of individual mice and rabbits are due to a previous localization out of the blood of an agent capable of undergoing change when the skin is exposed to carcinogenic influences, and of producing tumors in consequence. On the assumption that such an agent would localize in increased quantity where cutaneous inflammation exists, like various inert substances of large molecule and the epidermotrophic viruses when circulating, skin areas on adult and new-born animals were for some weeks kept inflamed, and months later, when the areas appeared normal, methylcholanthrene was applied to them and to control areas on the same or other individuals. No differences were observable in tumor incidence.

These results led to attempts to test whether embryo epidermis is capable of undergoing neoplastic change, and the work of Paper I was done which showed that epidermal tumors arise with great rapidity and regularity from embryo skin transplanted to adults of homologous strain (C strain) together with methylcholanthrene. Webster-Swiss mice proved unsuited to experiments of the sort owing to heterogeneity of the breed, the transplanted embryo skin dying in most instances before the methylcholanthrene introduced with it could have been carcinogenic. The skin of C3H embryos also did badly, as if from incompatibility in some instances but mostly because its epidermal cells proliferated less vigorously than those of C embryos and did not tolerate methylcholanthrene nearly so well. Despite these difficulties, epidermal tumors were occasionally induced, as also in the transplanted skin

of Webster-Swiss embryos, and the growths appeared quite soon, all things considered.

The effect of methylcholanthrene on the skin of sucklings, their mothers, and young adult mice of the C strain was studied in order to find out whether the rapid rate of neoplastic change in the transplanted epidermis of embryos is indicative of some liability connected with its period of development. The skin of new-born animals proved very refractory to the carcinogen, hair coming in at the same rate as on control litters and no perceptible inflammation occurring for about 2 weeks, although within this period the mothers of the treated animals and the young adults became hairless where the methylcholanthrene had been put and their skin was much inflamed. Later on, as the applications were kept up, similar changes took place in the sucklings, but none of these developed tumors during some 6 weeks of observation whereas growths appeared within 3 weeks on more than half of the mother mice and on some of the young adults. The failure to produce tumors in the sucklings seems to have been due to cutaneous conditions preventing the necessary exposure of the deeper epidermal cells to methylcholanthrene. In any projected correlation of age differences with the response of cells to carcinogens allowance must be made for such factors. The present findings give no ground for the supposition that embryo skin has any special liability to neoplastic change.

The results of transferring the tumors derived from embryo epidermis to new hosts have made plain that the neoplastic state not infrequently entails disabilities which are crucial, the tumor cells failing to succeed unless aided. This holds true of some carcinomas as well as of papillomas.

By transplanting pieces of the organs of C embryos together with methylcholanthrene tumors of many sorts besides the epidermal have been obtained. As yet only those of the stomach have been worked with extensively. They can be elicited as quickly and regularly as those of the epidermis and can be as easily transplanted.

The findings as a whole render it impossible to suppose that the neoplastic potentialities possessed by transplanted embryo tissues are due to the lodgement in them of tumor-producing viruses as specialized in their effects as those now known, or of precursor agents conferring neoplastic liabilities specialized to the same degree. Some other possibilities are mentioned.

The rarity of neoplasms at birth is due to the circumstances of intrauterine life and to its brevity, not to any lack of capacity of the cells of the embryo to undergo neoplastic change.

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EXPLANATION OF PLATES

PLATE 40

The numbering of the figures is consecutive to that of Paper I.

All of the sections were stained with methylene blue and eosin.

The photographs were made by Mr. Joseph B. Haulenbeek.

FIG. 42. Cyst resulting from the implantation 83 days previously, in a 3 day old C mouse, of skin fragments from C embryos 13–14 mm. long, together with *OSM*. The cyst was cut open at one end during excision, with result that part of its contents escaped. At its other end (to the right), where it has pushed out into the connective tissue it has no epithelial lining. Elsewhere much of its wall is covered with epidermis which was in the preneoplastic state, and this has elaborated stratified keratin, but in the bracketed region there is a small squamous cell carcinoma, and here jumbled squamous elements have been cast off in such quantity as to provide the greater part of the cyst contents. Evidently the carcinoma had long been present. × 16½.

FIG. 43. Cross-section showing half of a cyst formed as result of the implantation 55 days previously, in a C mouse 9 days old, of skin from embryos 11–13 mm. long, together with *OSM*. The cyst had suddenly enlarged just after the 46th day, was 12 mm. across at death, and had become attached to the skin and abdominal muscles which can be seen above and below the portion shown. Several growths project inwards from the lining epithelium, and between them this was everywhere carcinomatous as higher magnification showed. × 10½.

FIG. 44. Carcinoma derived from the lining of an epidermal cyst due to implantation in an adult mouse 55 days previously of the skin of 20 mm. Webster-Swiss embryos together with *OSM*. The cancer grew out beyond reach of injury by the carcinogen, but this destroyed the epithelium everywhere else on the cyst wall. A stretch bare of it can be seen at the lower left hand corner of the photograph. × 70.

FIG. 45. Papilloma due to the implantation of skin from 14 mm. embryos together with *OSSM* in an adult C3H mouse 75 days previously. The core and stalk of the growth consisted of sarcomatous tissue,—a fact not perceptible at the magnification presented,—and there was more of this tissue next its base. Elsewhere around the cyst wall a dark zone can be seen, of lymphocytes lying amidst ordinary reactive tissue. × 5.

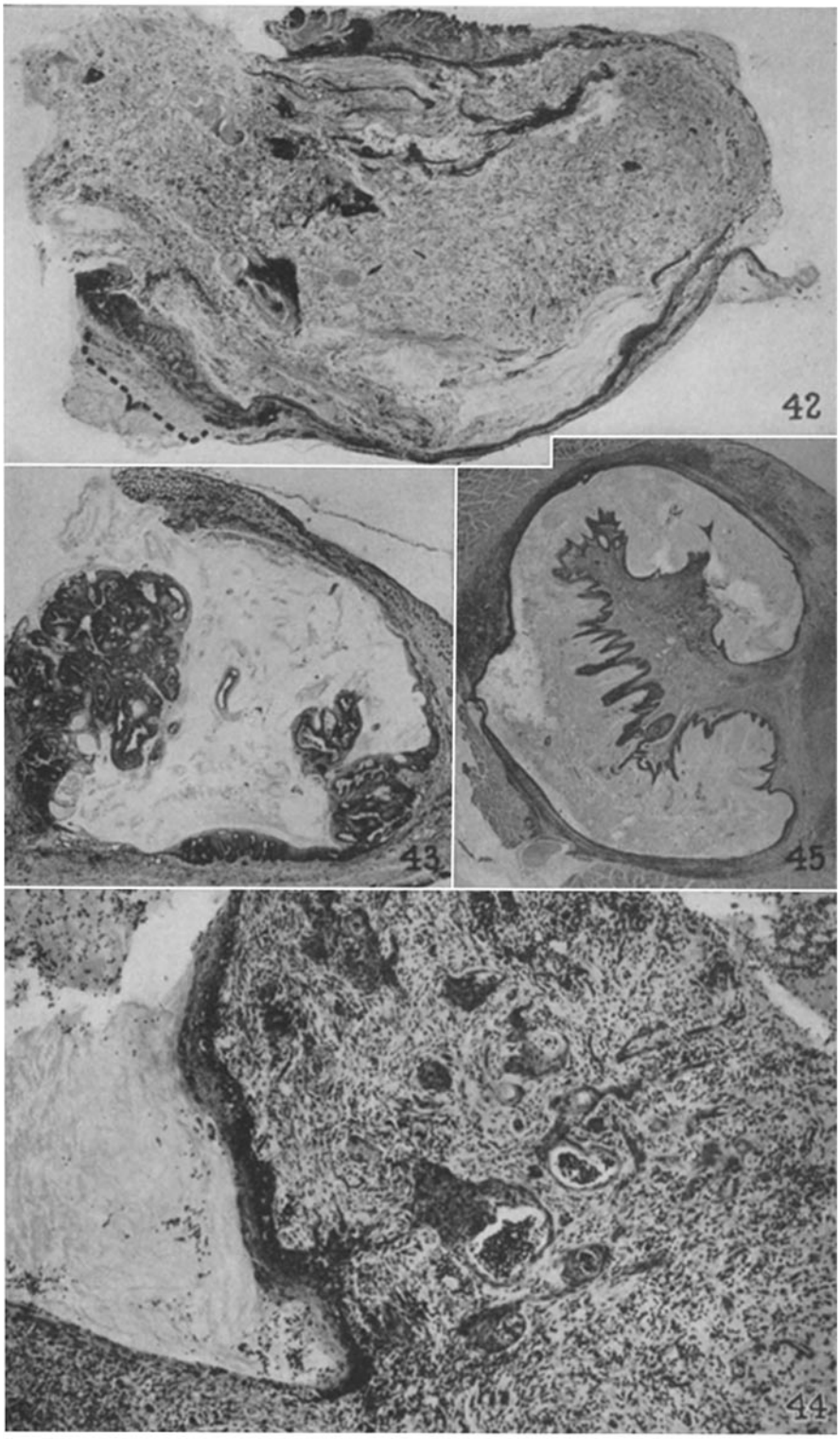

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PLATE 41

FIGS. 46 and 47. Cellular abnormalities due to the action of *OSSM* on the implanted skin of 18 mm. C embryos; specimen procured after 6 days, when cyst formation was still incomplete. The many rounded lacunae tell where droplets of *OSSM* were present. In Fig. 46 at A what appears to be a huge, rounded, polyploid epidermal cell is to be seen, but Fig. 47, from the next section, makes plain that it was merely part of a much larger element containing a coarse mass of chromatin and some scattered small lumps of it. There was a globule of *OSSM* in the cell protoplasm, poorly shown in Fig. 46, more clearly in Fig. 47,—where also part of the dividing chromatin of Fig. 46 is visible. The photographs have been so mounted that these identifying features lie at a slightly lower level in Fig. 47. At B a binucleate cell with huge nuclei can be seen, probably the result of a previous division of the element A. Many of the neighboring epidermal cells are small, their morphology highly various, and their arrangement irregular. The situation of the big cells immediately next the reactive tissue (which contains some polymorphonuclear leucocytes, as so often when *OSSM* is present) proves that they were situated along the base of a much damaged epithelial layer. × 250.

FIG. 48. Part of the wall of a cyst from another animal of the same experiment; implant removed after 10 days. The hyperplastic epidermal layer appears to be already in the precancerous state. Its basal cells have stained deeply with methylene blue, they differ much in size and shape, and their arrangement is disorderly. Several rounded, pyknotic bullet bodies can be seen (arrows) amidst the recently desquamated keratin. (The serial sections to either side of the preparation failed to show how they came to arise.) The very cellular connective tissue contains many lymphocytes. × 230.

FIG. 49. The formation of bullet bodies in an epidermal layer which had been acted upon for a longer time by *OSSM*; from a cyst due to the implantation 29 days previously of the skin of 18 mm. C embryos together with *OSSM*. The basal epithelial cells are implicated, one of them having undergone the parakeratotic change which resulted in the bullet bodies. Further stages of this change can be seen in the more superficial elements (arrow). At two nearby spots the epidermis seems on the verge of malignancy, if not actually cancerous. A papilloma and 2 frank carcinomas were found elsewhere in the implantation nodule. × 300.

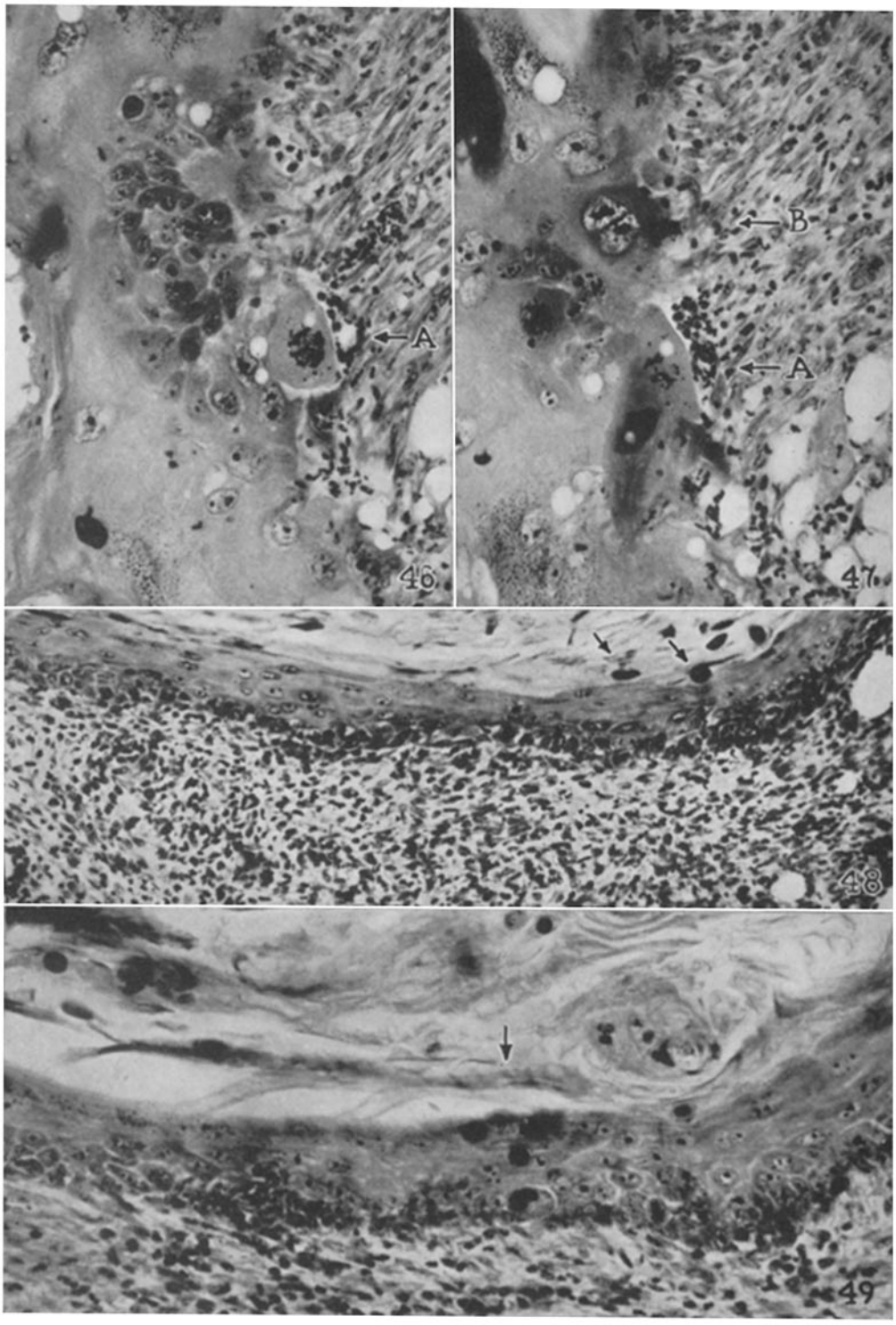

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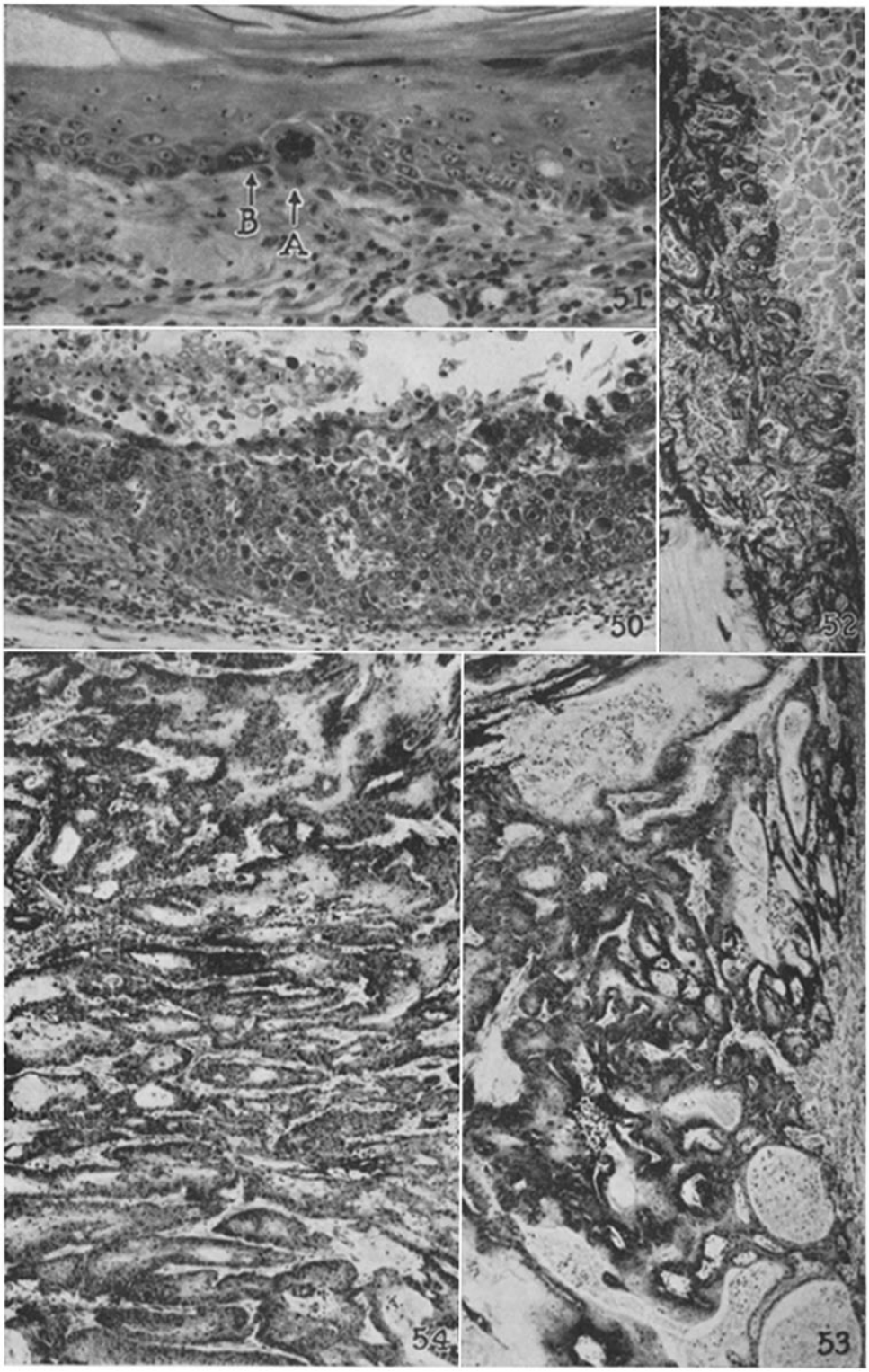
PLATE 42

FIG. 50. Anaplastic carcinoma in the wall of a cyst: from an implant of the skin of 20 mm. C embryos together with *OSSM*, removed after 84 days. The growth contains many bullet bodies. Several other carcinomas had arisen. × 156.

FIG. 51. Polyploid cell (A) in the wall of a cyst from another animal of the experiment furnishing Figs. 46 and 47. Though the implant had been in place 10 days *OSSM* is still being passed on into the interior of the completed cyst, as witness the oval lacuna toward the right in the epidermal layer. Immediately next the polyploid element, as if resulting from a previous division, are 2 large cells (B) with nuclei at least double the normal in diameter and coarsely stippled with chromatin. × 250.

FIG. 52. Carcinoma resulting from the implantation of fragments of the minced stomachs of 20 mm. C strain embryos together with *OSM*. The cancer was one of several found when the implant was removed after 44 days. It differs much in detail from the squamous cell carcinomas derived from epidermal cells and it is actively replacing the adjacent muscles. × 55.

FIGS. 53 and 54. Adenoacanthoma which appeared where bits of the glandular portion of the stomachs of 20 mm. embryos had been implanted together with *OSM*. The figures come from a growth resulting from the transplantation of the primary tumor on the 65th day. The new nodule was 8 mm. across when removed after 11 days. Fig. 53 is from its invasive edge. × 65. Fig. 54 shows its tubular structure. × 83.



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