THE DETERMINING INFLUENCE OF TAR, BENZPYRENE, AND METHYLCHOLANTHRENE ON THE CHARACTER OF THE BENIGN TUMORS INDUCED THEREWITH IN RABBIT SKIN

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Plates 10 to 15

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In an accompanying paper (1) the ability of benzpyrene, methylcholanthrene, and a carcinogenic tar to produce benign tumors on the skin of domestic rabbits has been analyzed experimentally and found due not only to the power of these agents to convert normal cells into neoplastic cells but in more or less considerable degree to their influence to promote tumor formation. The growths obtained with the three carcinogens will be compared here at some length as bearing on the question of how far such substances act to determine the character of the neoplasms they induce, a matter of searching theoretical importance.

The tumor-producing viruses exert a remarkably precise determining influence upon the growths they cause, each bringing about neoplastic changes of a definite kind in the cells upon which it acts, whereas the chemical and physical carcinogens thus far studied have exhibited but a dubious influence at most on tumor character. True, some of them call forth growths more or less special to themselves because, by reason of chemical affinity or biological circumstance, they act upon special tissues, as *e.g.* ergot upon nerves, with result in neurofibromas (2), and aniline dyes on the wall of the urinary bladder, with papillomas and carcinomas as a consequence (3); but it has been the general experience that widely different agents often engender tumors of identical type, as further that a single one may produce the most heterogeneous growths if cells in great variety are exposed to it. So completely have these and other observations made for a belief in the non-specificity of carcinogenic action, save in so far as this is conditioned by the affinities or circumstances just mentioned, that the custom has arisen of testing new chemical carcinogens upon either the connective tissue or epidermis at the will of the investigator, with appraisal of the results as if these were interchangeable. Yet one cannot take for granted that this is the case. Indeed, parallel tests on both tissues indicate that it is not always so (4).

Needless to say, carcinogens must be brought to bear upon cells of identical kind in any attempt to find out whether they influence the type of neoplastic change. Only when this is done can one assume that the growths produced are truly expressive of qualitative differences in oncogenesis. When tar is applied to rabbit skin benign tumors of two sharply distinct sorts arise from the basal cells of the epidermis (5). We have tried to find out whether other carcinogenic agents when brought to bear upon cells of the same kind may not give rise to different neoplasms.

Materials and Methods

It was manifestly desirable to employ test agents of widely diverse character, but the practical possibilities were few. Several investigators have tried unsuccessfully to induce skin tumors in rabbits by means of ultra-violet light, and they have been obtained with the Roentgen rays only after many exposures throughout a period of several years (6). Tar, the polycyclic hydrocarbons, and arsenic have seemed by exclusion to be the best available agents for our purpose and we have employed them all.

The Benign Tar Tumors

The benign tumors obtained in our laboratory by tarring the inner surface of the ears of domestic rabbits have been described and figured in several previous papers (5, 7). They tend to be fleshy and many enlarge rapidly as the applications are continued, some becoming huge; yet all are dependent not only for growth but for survival on the encouragement which tarring provides (7). They are of three easily recognizable kinds, papillomas, carcinomatoids and frill horns.

Rabbit epidermis, like that of most laboratory animals, does not consist of superimposed cell layers expressive of stages in differentiation, such as are present in man, but is only one or two cells thick, keratinizing abruptly. When cell division is so stimulated however that it occurs more rapidly than does the change to the keratinized state a characteristic stratified squamous epithelium results, with a Malpighian stratum and a stratum granulosum nearly resembling the human. The benign tar tumors take origin from an epidermis which has been brought to this condition.

The commonest growth is a *papilloma* deriving from the basal epidermal layer of the skin surface or hair follicles. If the papilloma proliferates actively its cells form a differentiating stratified sheet much thicker than that due to ordinary hyperplasia, with a much more coarsely and irregularly granulated stratum granulosum; and the keratinized material often holds together tenaciously and builds up into peaks or masses. Under such circumstances the growths are discrete entities sharply different from the hyperplastic epidermis about them. They are dependent though on favoring factors for continued activity, as above remarked, and if these are no longer operative they regress and while doing so, unless they have persisting connective tissue papillae or a core, they take on the appearance of mere local areas of especially pronounced hyperplasia. At length no histological sign of them may be discernible, yet even then cells with the capacity to form papillomas may remain present for half a year at least, as shown by the prompt reconstitution of some of the growths in their previous form when non-carcinogenic agents which stimulate proliferation are painted on the skin (8).

Many of the papillomas form dry crags or cones as time goes on while others become fleshy; for continued tarring renders the connective tissue of the corium very excitable and it then readily provides a profuse stroma to the proliferating tumor cells. Under such circumstances big growths frequently develop, of hassock, or cauliflower, pear, or onion shape. When such tumors regress the bulky core of connective tissue persists, overlain by epidermis which, though hyperplastic for a while, may eventually become thin and normal looking. Indolent growths that lack the papillomatous form are sharply demarcated patches of thickened epithelium microscopically.

Not a few papillomas have cells so responsive to stimulation that under its influence they

behave for some while as if cancerous, invading, destroying, and replacing the structures about them. Such growths are to be found on skin continually tarred or stimulated to hyperplasia in other ways (5) and in the past they have generally been taken for carcinomas. Sometimes the cells along the base of typical papillomas grow down irregularly amidst reactive connective tissue but in the most pronounced instances the tumors have the aspect of primary squamous cell carcinomas from the first, both in the gross and histologically. They form ulcerated discs or mounds, invade and undermine the skin next them, penetrate into lymph vessels, and not infrequently extend through lacunae in the ear cartilage to appear on its other side, where again they may ulcerate. We have called such seeming cancers *carcinomatoids* (8). Occasionally they reach a diameter of 2–3 cm., yet after a while they either take on the form of ordinary benign papillomas, or round up into keratinized cysts, sometimes lined with stratified squamous epithelium,—as happens when tongues extend downwards from ordinary papillomas,—or else they vanish to all appearance, like many of the latter. While tarring is going on carcinomatoids may be even more common than papillomas but within a few weeks after it is stopped they all undergo one or another of the changes mentioned and are no longer recognizable for what they were. Non-carcinogenic stimulation, as *e.g.* that incidental to wound healing, may cause them to reassume the malignant aspect (8).

The third kind of benign tar tumor, the *frill horn*, has only of late been discriminated as a type[1] though a drawing of such a growth is to be found in a paper by Halberstaedter (9) published in 1923. Frill horns can usually be distinguished in the gross, being cylindrical or strap-shaped keratinized horns, often dark gray with melanin, striated horizontally, not vertically like papillomas, and always dry to the base where they are surrounded by a raised collar of connective tissue. They are seldom more than 2 mm. in diameter, though perhaps as much as 2 cm. long, and their nearly even thickness bespeaks a slow rate of proliferation. They are sharply different microscopically from papillomas though like these they derive from the basal epidermis,—either of the skin surface or the hair follicles,—and undergo keratinization. Instead of stratifying their irregularly polygonal cells with bird's-eye nuclei lie higgledy-piggledy, and fail to granulate or flatten before they keratinize, a process relatively abrupt. The keratinized material is dense on section and usually colors red on eosin-methylene blue staining, whereas that of papillomas appears reticulated and ordinarily takes the blue or remains unstained. The living epithelium pushes down in an irregular frill, as viewed laterally, slowly replacing the corium, and only rarely does it extend up into the dry stuff of the horn and then as one or two short fingers.

The invasive habit of frill horns suggests that they may be malignant yet they do not burrow deep, are wholly dependent upon favoring factors for existence, and never,—at least in our experience,—undergo secondary cancerous change, as happens now and again with papillomas, most of the familiar tar cancers being consequent on such change. When regressing they retain their peculiar histological character, gradually becoming smaller from the periphery, keratinizing wholly and flaking off.

The Benign Tumors Due to Benzpyrene and Methylcholanthrene

The benzpyrene we employed was mostly in 0.3 per cent benzene solution but sometimes in mineral oil. The methylcholanthrene (0.3 per cent) was either in benzene or in Crabtree's solvent (12) (ether and mineral oil in a 49:1 proportion). The animals were those of an accompanying paper (1), namely domestic rabbits of agouti breed, and in said paper details of method will be found and a description of the gross character and course of the tumors.

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[1] Since the recognition of frill horns (5), Flory (10) and Brunschwig, Tschetter, and Hamann (11) have reported finding them on tarred rabbits.

Benzpyrene and methylcholanthrene produce benign cutaneous tumors of identical sorts, as we have found, and hence the findings with them will be considered together. The literature contains no detailed report on the growths produced by benzpyrene, though Schürch and Winterstein (13) state that they lack the "polymorphous character" of those due to tarring. The tumors caused by methylcholanthrene in the domestic rabbit seem not to have been studied at all.

Careful records have been kept (1) of all growths arising. Many were excised for study when first noted, and others after known times, with no attempt ordinarily to select special kinds. They were fixed in acid Zenker solution and often were sectioned serially. The stains were eosin and methylene blue.

The first growths to arise on ears painted with a benzpyrene or methylcholanthrene solution usually appeared when the skin was as yet only slightly changed, whereas it was already much thickened and hyperemic when the tar tumors arose. In the gross the benzpyrene growths differed strikingly from these latter (1). Instead of arising as fleshy mounds, often originating deep in the cutis and several millimeters across when first noted, or as fleshy, more or less rapidly enlarging verrucosities or hassocks, they were minute, often punctate, and would have been overlooked had not the search for them been careful. Most of them failed to enlarge later and those which did become a few millimeters across eventually vanished though new ones arose. Those which persisted were low mounds, plaques or spicules, tiny dry verrucosities, discrete, keratinized scabs, or exceptionally big keratinized plugs protruding from distended hair follicles. Occasionally small hemorrhagic mounds formed which vanished later or became papillomatous. As the applications of carcinogen were continued however and the skin became increasingly inflamed some of the long existing growths began to enlarge, new ones appeared in increasing number and showing more tendency to grow, and a few became fleshy and assumed the shapes of the tar tumors.

Microscopically the benzpyrene and methylcholanthrene growths nearly all proved to be papillomas, carcinomatoids, or frill horns. Yet histologically they differed in significant ways from the tar tumors of these kinds. The papillomas (Figs. 1, 2, 4, 5) were in general far less vigorous than tar papillomas[2] and though those which were active had the characteristic cytology (Fig. 1), many showed merely a hyperplasia and keratinization greater than that round about, as is usual with indolent growths. The carcinomatoids,—these being the small, hemorrhagic, subepidermal mounds above mentioned,—were sometimes invasive on first arising (Fig. 6), but only for a brief period. Instead of becoming ulcerated, discoid tumors and perhaps penetrating to the other side of the ear like the carcinomatoids due to tarring, they soon failed to enlarge further and changed to papillomas, became cystic (Fig. 7), or vanished while the carcinogen was still being applied. The frill horns (Figs. 8–14) were usually minute, seldom formed horns, and those that they did form were tiny in most instances, usually mere spicules, though occasionally filiform and as much as a centimeter long. Only when the growths were exceptionally active was there a collar of reactive tissue about their base (Fig. 8). Most of them had striking cytological peculiarities, to be described further on.

The gross differences from the tar tumors of the same types have proved due in the main to the slowness with which benzpyrene, or methylcholanthrene, bring about local conditions favorable to the proliferation of the neoplastic cells, such as result from tarring (1). The

[2] No pictures of large and active papillomas will be presented since they have already been figured (5, 8).

microscopic sections show that for a long while the cutaneous connective tissue was little altered, remaining inert and failing to provide more than the scantiest support for the papillomas, instead of cooperating actively in the formation of tumors. In consequence many of the growths were mere shallow saucers (Fig. 2) or plaques almost flush with the surface (Fig. 4), overlain in most instances by a low scab of keratin. When such scabbing was lacking they sometimes escaped notice even though several millimeters across. When the condition of the connective tissue was slightly more favorable the papillomatous epithelium was raised on low folds or fingers, though with the thinnest of cores (Fig. 5). Almost never did the provision of scaffolding and vessels seem commensurate with the capabilities of the neoplastic cells, and no excrescences arose, plump, papillomatous, and consisting mostly of connective tissue, such as tarring calls forth. Melanosis was frequent, as with indolent tar tumors, the growths then appearing gray or black.

The lack of preparation of the connective tissue for tumor needs was an evident reason why the carcinomatoids showed little aggressiveness. It found expression also in the morphology of the frill horns. These, as already stated, had only occasionally a collar of reactive tissue about the base, and their neoplastic epithelium seldom formed the characteristic invasive frill (pictured in Figs. 8 and 9) and indeed sometimes remained superficial (Figs. 2, 3), though usually it extended downwards in blunt protrusions or along a broad bulging front (Figs. 9, 11–13). Now and then when the skin was more than usually inflamed it grew down in thin tongues having a malignant aspect (Fig. 14) which was belied by the later failure of the tumors to progress. Similar but larger growths, crateriform and active, though only briefly so, have been called forth occasionally by tarring.

The frill horns were remarkable in other ways. A considerable proportion of them underwent peculiar parakeratotic changes as differentiation took place. The cytoplasm immediately about the nucleus became denser and stained red instead of pink with eosin and methylene blue; the dense region broadened, becoming sharply defined; and soon the rest of the enlarging cell was involved, with result in a dense, somewhat refractile, spherical body which stained an increasingly deep purple as differentiation proceeded, the nucleus for some while remaining visible in its midst (Fig. 13). The end result was a homogeneous bullet-like body which stained almost black (Figs. 3, 11–13). In most instances these lay interspersed amidst cells undergoing the usual frill horn keratinization, but occasionally nearly or quite all of the cells became "bullets" (Fig. 3). Not infrequently some polymorphonuclear leucocytes migrated into the tumor, dying and becoming eosinophilic lumps, but they could readily be discriminated from the "bullets" present with them.

As already stated, the cells of the frill horns due to tarring usually differentiate into a compact, tenacious mass of keratin, thus forming the horns (5). This was seldom the case with those due to benzpyrene or methylcholanthrene. Instead some cells died before differentiation was well along and often the majority came loose from one another as it took place, the "bullets" lying free amongst them (Figs. 3, 13). Often in fixed preparations the cells above the basal layer appeared to lie separate with a gap between them and this layer (Fig. 13). Generally they built up a low, flaking scab but sometimes they came away so soon that a cup-shaped depression resulted (Fig. 3).

The frill horns due to tarring show no such peculiarities as these, and it has taken the study of a large, graded series of the growths due to the polycyclic hydrocarbons to convince us that essentially they are of identical sort. Fortunately such a series has been available since these agents call forth frill horns in abundance. The singularity of the tumors can scarcely have been due to the direct influence of the carcinogens, for it was evident in growths appearing nearly 3 years after methylcholanthrene was last applied (14). Frill horns are so sharply different microscopically from ordinary hyperplastic epidermis that they can be recognized when exceedingly small, and the "bullets" were often present in the tiniest ones (Figs. 12, 13).

The "bullet bodies" here described would seem to have been the outcome of a dyskeratotic process, yet their aspect led us to search our specimens for evidence of the action of a virus. All of the frill horns were examined with this possibility in view, as also many papillomas and some carcinomatoids. Nuclear inclusions were found in only one tumor, a frill horn, but in this they were very abundant.

A 0.3 per cent solution of methylcholanthrene in benzene had been applied to the ears of rabbit 1–63 over a period of 140 days when the tumor in which the inclusion bodies were found was excised for study as a routine procedure. It had been noted 2 weeks previously, and was a mound 1.5 mm. across, drying at the top and with no evident peculiarities. Elsewhere on the ear 13 growths of the usual benign sorts were present, some of them also mounds, and there were 5 on the other ear from which 5 more had been removed previously. These last had proved microscopically to be ordinary frill horns, papillomas, and a carcinomatoid. The animal was discarded without further scrutiny.

Microscopically the tumor was a frill horn lying almost entirely beneath the surface (Fig. 15). Serial section showed it to consist of three cuppings lined with the characteristic epithelium which was extending downwards in a blunt, coarse frill. There were occasional melanoblasts amidst and immediately beneath it and the cups contained an eosinophilic debris, the result of death of the tumor cells prior to differentiation. Many frill horns of similar structure have been encountered in other animals, but none which showed as this did aggregates of tiny, discrete, eosinophilic dots within most of the nuclei of its living cells (Figs. 16, 17). They were not visible in the basal layer but a few were to be seen in some of those just above, and they became frequent in the differentiating elements three or four cells further toward the surface. At first there were only one to three of the bodies in each nucleus, discrete, rounded dots, all of about the same size, staining a bright ruddy pink with eosin and methylene blue; but as differentiation went on they became numerous, forming a cluster at the center of the nucleus. Its chromatin lay amidst them in dark, irregular lumps or as a short, thick rod. Eventually they occupied two-thirds of the nucleus or even more, this latter enlarging moderately and clearing so as to appear empty except for the pink dots and the central chromatin. Some of the cells, both those with and without the dots, took a purple stain now and on further differentiation became "bullets" of the usual sort; but the majority stained light blue until they became necrotic or underwent abortive keratinization, in either case coloring ruddy pink. The nuclei were often nearly filled with the pink dot-like elements prior to cell death, but never ruptured and let them escape, and the chromatin underwent pyknosis as usual. After the cells had died the dots could no longer be distinguished, either in the necrotic debris or in the keratinized scales. The epidermis round about the growth was wholly devoid of them and was only moderately hyperplastic.

The nuclear inclusions were so numerous and distinctive that they cannot be dismissed as artifacts or cellular accidents. They had many similarities to the minute eosinophilic bodies which appear within the nuclei of liver cells during yellow fever in man (15). Like them they bore a resemblance to intranuclear colonies and were clustered at the center of the nuclei, which became empty round about them. But no margination of the chromatin took place, the nuclei enlarged instead of shrinking, and did not rupture and let the bodies out,—all of which happens in yellow fever. According to Cowdry and Kit-

chen (16) the inclusions in this disease represent an unusual kind of oxychromatic degeneration due to the action of the virus.

The inclusions had no evident relation to the "bullets" present in small numbers in the affected frill horn, and the latter had no other special abnormality. They were of a sort which is generally assumed to bespeak the presence of a virus. We are inclined to believe them the outcome of infection with a "passenger virus" (Andrewes) which had by chance become associated with the tumor cells without significantly affecting the behavior of the growth. Many instances of the sort have been described (17).

Occurrence of Tumors of a Type Which Tarring Does Not Produce

Very occasionally benzpyrene and methylcholanthrene evoked growths which were wholly unlike anything that tar calls forth on rabbit skin. They were sebaceous adenomas, low, subepidermal mounds never more than 3 or 4 mm. across, appearing during the period when the other benign tumors did. Like these others they were conditional neoplasms, dependent upon favoring circumstances for development and persistence, as their incidence and course showed. They consisted of aggregates of sebaceous glands (Figs. 18, 19), proliferating at the periphery yet differentiating in the ordinary way, and they were connected with the surface by ducts widely distended with keratin. They wholly resembled the benign sebaceous adenomas of man (18) and like the latter sometimes showed signs of glandular obstruction.

Amongst the many tumors which arose where punch holes in the rabbit ears were healing, one proved to be a sebaceous adenoma, and the circumstances of its origin and growth ruled out obstruction as its cause.

The ear of the rabbit had been painted with benzpyrene in mineral oil for not quite 11 months and it carried two tiny growths when a hole 8 mm. across was punched in it where no tumors were visible. As repair took place a narrow, discrete mound, raised only a little above the general level, formed along a radius on the new tissue growing in toward the center of the hole. It was excised before healing was complete, together with some of the ear tissue outside the original rim of the hole, and was cut in serial sections along its length. The microscope showed it to be made of huge, sebaceous glands (Fig. 20), many times the size of the normal ones present in the tissue outside of the region of healing, and resembling those of which adenomas are composed. Ordinary sebaceous glands are frequently present at the periphery of healed punch holes but they never extend in as a radial series of giant glands while closure is taking place.

Relative Frequency of the Tumor Types

The tumors called forth by benzpyrene and methylcholanthrene differed from those of the same kinds due to tarring, not only in behavior and structure but in relative incidence. It was to have been expected that the papillomas evoked by the polycyclic hydrocarbons would exhibit the carcinomatoid form much less frequently than those due to tar since these agents have but a slight pro-

moting effect upon the cells which they render neoplastic; and this proved to be the case (Table I). But there were other differences. Benzpyrene and methylcholanthrene caused sebaceous adenomas to appear and tar did not. Frill horns, neoplasms which tarring only occasionally evokes, arose often in the rabbits receiving the other agents, and in some animals made up the majority of the growths obtained, a fact not brought out by the table. Indeed in one

TABLE I

*Relative Incidence of the Various Kinds of Benign Tumors Appearing in Response to Tar, Benzpyrene, and Methylcholanthrene**

(The growths included all appeared during the applications or shortly afterwards.)

Carcinogen	No. of rabbits	No. and character of the tumors					Remarks
		Papillomas	Carcinomatoids	Frill horns	Sebaceous adenomas	Total	
Tar (*vide* ref. 5)	18	50 (39%)	74 (57%) 60%‡	5 (4%)		129	
Benzpyrene in benzene	21	87 (62%)	16 (11%) 16%‡	36 (25%)	3 (2%)	142	
Benzpyrene in mineral oil	6	10 (58%)	1 (6%) 9%‡	3 (18%)	3 (18%)	17	Only one ear submitted to treatment
Methylcholanthrene in benzene	9	27 (47%)	10 (18%) 27%‡	20 (35%)	§	57	
Methylcholanthrene in mineral oil and ether	4	11 (57%)	2 (11%) 15%‡	6 (32%)	§	19	Only one ear submitted to treatment

* Growths called forth by wound healing have been excluded.

‡ Percentage of carcinomatoids in the combined total of papillomas and carcinomatoids.

§ Amongst the growths removed some weeks after discontinuance of the applications several proved to be sebaceous adenomas.

rabbit painted with benzpyrene in benzene, incidentally to certain experiments in which Dr. Ian MacKenzie collaborated, the ears became rough with a multitude of minute frill horns, several hundred of them, in the absence of any discernible papillomas or carcinomatoids. Often the frill horns were so small (Figs. 12, 13) as to come to attention only during the microscopic examination of random slices of the ears, cut where no growths had been perceived during life. It follows that the actual frequency of these tumors was much more considerable than the table would indicate since this is concerned with neoplasms visible in the gross.

Selective Evocation of Tumors by the Carcinogens

Mention has just been made of the reason why carcinomatoids are much more frequent on tarred ears than on those receiving the polycyclic hydrocarbons. The absence of sebaceous adenomas remains to be accounted for and the rarity of frill horns.

The collateral effects of tar will sufficiently explain the failure of adenomas to appear. It very soon does away with the sebaceous glands as Guldberg noted (19) and as we have had abundant opportunity to observe. The induced hyperplasia of the epidermis rapidly involves the ducts of the sebaceous glands, which become lined with a thick, many-layered differentiating epithelium like that on the ear surface, the gland cells persisting for but a brief period as small islands amidst it.[3]

The reason for the scarcity of frill horns on tarred skin is not so immediately evident. As already mentioned they spring from the same elements as do papillomas, both deriving from the basal cells of the surface epithelium or that lining the distended keratinizing cysts into which the hair follicles are converted. It follows that the differing yield of frill horns to tar and the polycyclic hydrocarbons cannot be laid to any selective influence of these agents upon epithelial elements of differing types. To account for it one might suppose either that the carcinogenic agents differ in ability to initiate the various kinds of benign neoplastic change possible to cells of a single sort, or that they resemble each other in this respect but differ as concerns which neoplastic cells they encourage to multiply, with result that tumors of the same kinds arise in differing proportionate number. Test has shown this latter to be the case. It is known that each of the carcinogenic agents renders several times as many cells neoplastic as manifest themselves by forming tumors (1).

The ears of eight rabbits were painted on the inner surface with benzpyrene in benzene for from 8 to 10 months, that is to say until benign tumors of the usual sorts had appeared, and then tarring was substituted. Sometimes only one ear was tarred while treatment of the other was discontinued so that it might serve as control,—a purpose which it served imperfectly since most of the animals soon transferred tar to it. The growths were charted in the usual way at the start of the tarring and frequently thereafter, and it was kept up for 1 to 2 months.

Within 2 to 3 weeks the ears became heavily scurfed or macerated under the tar layer and new tumors appeared (Charts 1 and 2), in much greater number than when normal ears are tarred and far sooner on the average, some of them growing fast and becoming fleshy as ordinary tar tumors do when the applications are kept up. A few came from deeper in the skin than any previously produced by the benzpyrene, raising the surface only when they were several millimeters across, with most of their bulk well beneath it as palpation made plain. Those near the thin edge of the ear were not infrequently embedded spheres when first noted,

[3] Some gland elements must occasionally remain, for tar carcinomas with a morphology indicative of a sebaceous gland origin have been reported now and again by previous workers.

projecting on both surfaces and erupting on one or both as papillomas or active carcinomatoids. Growths of the latter sort were frequent (Chart 1) and some of the largest became ulcerated

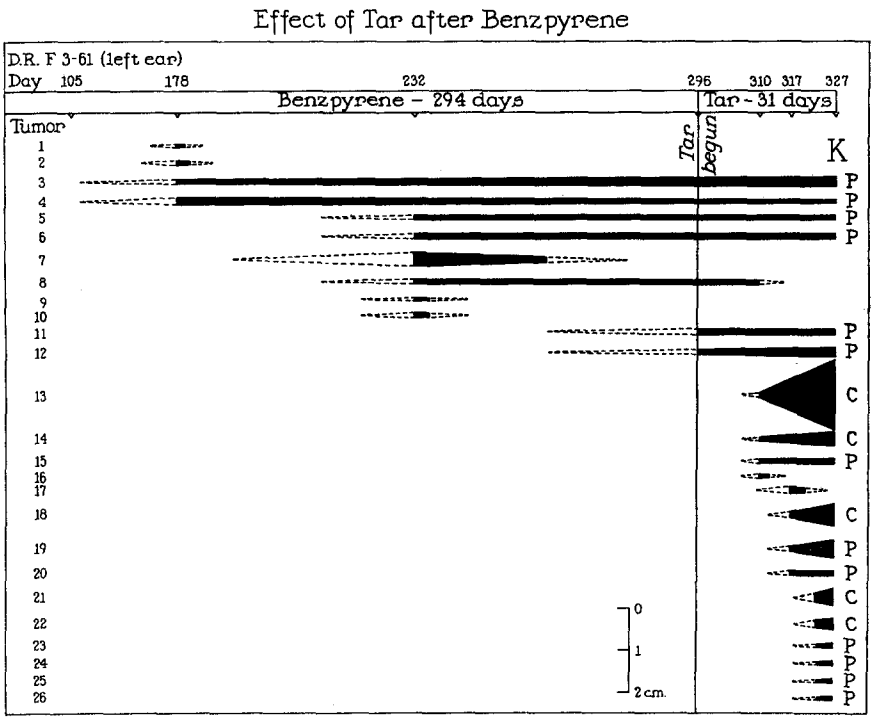

CHART 1. *The Effect of Tar after Benzpyrene.*—Each black bar or triangle represents a tumor on the inner surface of the left ear of rabbit 3–61. Its situation and length tell when the growth was first noted and its thickness is the diameter of the latter as charted on the days indicated by the arrows. The hatched lines preceding or following the bars are introduced merely to indicate that the enlargement or disappearance of the growths in the intervals between the chartings was doubtless gradual. K = rabbit killed on the 327th day; P = papilloma, and C = carcinomatoid, as determined by microscopic examination.

The instance was exceptional in the persistence of the benzpyrene tumors while the tar treatment was going on. The first tumors were noted on the 178th day of the benzpyrene applications, and though others appeared later they remained few, showing almost no tendency to grow, and nearly half of them soon disappeared. After tar was begun they underwent no enlargement which can be attributed to it nor did they alter from frank papillomas to carcinomatoids, although the tarring caused many tumors of both sorts to appear within a few weeks, some of which grew rapidly, the carcinomatoids in special.

discs more than a centimeter across, one of them extending to the outside of the ear and forming a mound there. Frill horns arose but rarely. Occasionally growths appeared on the control ears at situations where tar had been transferred to them.

The longer the previous treatment with benzpyrene, the more numerous in general were the growths produced by the tar; and the animals which developed most of them in response

to one agent usually did so to the other as a rule. Yet the tumors which the one called forth fared badly as a rule under the influence of the other, those produced by benzpyrene mostly vanishing as the applications of tar were continued. A few enlarged (Chart 2) and very occasionally one became a carcinomatoid.

In some cases tarring was left off after a while and the treatment with benzpyrene was resumed. Under its influence even the biggest tar carcinomatoids totally disappeared (Chart 2) or became papillomas.

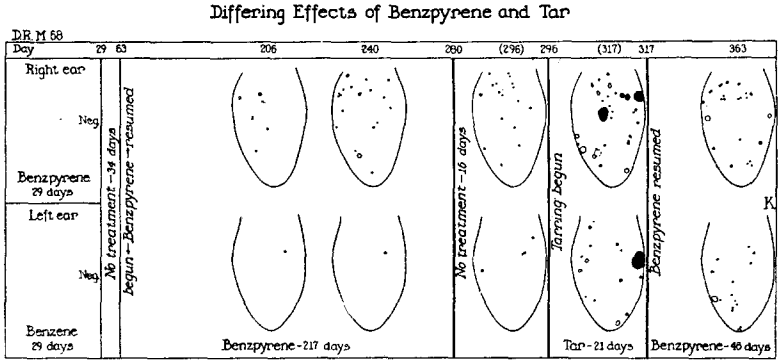

CHART 2. *The Neoplastic Effects of Benzene, Benzpyrene in Benzene, Tar, and Benzpyrene in Benzene, Applied Consecutively.*—The tumors are shown as charted on tracings of the ears. in black were ulcerated carcinomatoids.

Benzpyrene in benzene was applied to one ear of the rabbit for 29 days and benzene alone to the other. No growths resulted. After an interval of 34 days both organs were painted with the benzpyrene solution, with result that one tumor appeared on the ear previously benzened and many on that which had previously been exposed to benzpyrene. After a further short interval tarring was begun. It called forth numerous new tumors within 3 weeks, some of which rapidly became large carcinomatoids. A few of the growths due to benzpyrene enlarged concurrently but most of them either underwent no change or vanished. With the resumption of benzpyrene the tar carcinomatoids all quickly disappeared despite the circumstance that some new small tumors arose.

In this experiment the tar evoked many growths yet failed to cause preexisting benzpyrene tumors to enlarge and indeed seemed prejudicial to them. The observation becomes the more significant when one recalls that the renewed tarring of neoplasms due to tar generally causes them to grow fast (7). The tumors that it called forth in the present instance were like those ordinarily developing in response to repeated applications of it, fleshy, vigorous, and in not a few instances carcinomatoid in type and aggressive. They appeared in abundance so early that most of them must have resulted from the encouragement of latent tumor potentialities (1) already present in the tissue as result of the action of benzpyrene but not stimulated by this latter to form growths.

The effect of benzpyrene in benzene upon tumors which had arisen upon ears tarred to begin with was tested in eight rabbits. The tarring of some of the

animals had just been stopped, after 2 to 3 months, but in other cases an interval of 4 to 5 months had elapsed and the growths were retrogressing and small when the new treatment was instituted.

When one ear only was painted with benzpyrene, and tarring had just been left off, the skin returned toward the normal at about the same rate as that of the ear which received no further treatment. The fact that benzpyrene had but a negligible influence to main-

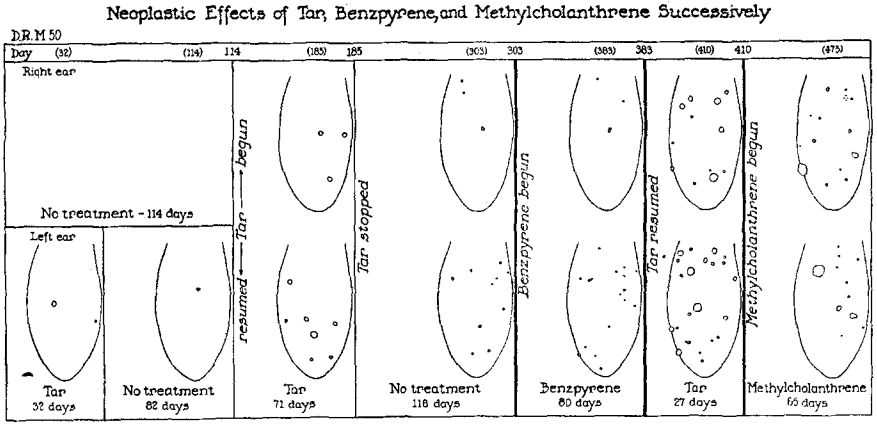

CHART 3. *The Neoplastic Effects of Tar, Benzpyrene, and Methylcholanthrene Applied Successively.*—One ear of a rabbit was tarred for 32 days, an interval of 82 days was allowed to elapse, and then both were tarred. The organ previously exposed to the carcinogen developed the larger number of tumors. After another interval, during which the growths dwindled while a few new ones came in, treatment with benzpyrene in benzene was begun. It had no evident effect to maintain the existing tumors and the new ones arising may or may not have been attributable to it. Now tarring was begun again and within 27 days it called forth numerous growths, some of them big and not a few of them carcinomatoids,—of which, unfortunately for the charting, no sufficient record was kept. A methylcholanthrene solution was then substituted for the tar, with the result that most of the tumors elicited by the latter soon disappeared although a few enlarged and some new ones arose which grew fairly well.

tain the pathological *status quo* was further made plain by the changes which took place in the tar carcinomatoids, these now either becoming papillomas or rounding up into cysts or disappearing, just as happens when all treatment is discontinued. The frank papillomas keratinized and dried to the base. Yet while these involutionary changes were taking place on the benzpyrened ear new tumors appeared there as not on the control organ and long before benzpyrene would have called them forth had the skin been normal previously (1). The new tumors were minute, dry, and indolent like those which arise first under such circumstances.

In the instances in which tarring had been discontinued for several months the skin had assumed nearly the normal gross aspect by the time benzpyrene was begun, but it soon became slightly scurfed and inflamed and new tumors arose although those due to the previous tarring were dwindling for the most part. In a few cases the benzpyrene was discontinued after some

weeks and tarring was begun again. It caused growths to start forth rapidly (Chart 3); yet while they were doing so many of the tumors called forth by benzpyrene vanished.

In a single instance (Chart 3) methylcholanthrene, 0.3 per cent in benzene, was eventually applied to ears which had been alternately tarred, benzpyrened, and then tarred again. It failed to maintain most of the tumors evoked by the last previous tarring, though a few enlarged; yet it called forth growths where none had previously been present.

From the results of these tests (Charts 1–3) it is clear that tar and benzpyrene must have promoted the multiplication of different neoplastic cells from amongst the latent hosts of those already present, and that in consequence they elicited and maintained different tumors. Under the circumstances of successive application of the two carcinogens the second to be painted on may very well have completed in some instances a conversion to the neoplastic state already got well along by the first. Yet the likelihood of a summation of effects does not diminish the importance of the fact that in the main the agents promoted the multiplication of different tumor cells from amongst the hidden horde.

The Benign Tumors Arising on Skin Exposed to Arsenic

According to Raposo (20) 10 per cent arsenic trioxide in vaseline produces papillomas within a few months when rubbed into the skin of rabbit ears. We applied such a preparation to the inner surface of one ear of 12 rabbits, and vaseline[4] to the other ear 6 days out of 7 for nearly 6 months without obtaining more than a superficial cutaneous inflammation and small erosions. The vaselined control ears remained unchanged. A mixture composed of arsenic trioxide with quinine (U.S.P.) and acetone[5] was now substituted,—the quinine to prevent the animals from licking off the preparation,—yet after a further 13½ months no tumors had arisen either on the treated ears or on the control organs receiving the acetone and quinine. Then because of the shortage of this last substance 1.8 per cent of arsenic trioxide dissolved in a 6 per cent watery solution of gum acacia was utilized, and at last after 22 months six small tumors were visible on the ears of 3 of the 7 remaining animals,—which still appeared healthy after exposure to arsenic for 2 years in all. The growths were minute verrucosities or discrete, keratinized mounds. Three have been excised thus far.

The ears treated with arsenic are now slightly scurfed in some cases but not noticeably thickened. One of the animals with most scurf died of intercurrent causes, and sections of the treated skin showed changes like those caused by benzpyrene and methylcholanthrene (Fig. 21),—epidermal hyperplasia as manifested in a stratified squamous epithelium, moderate hyperkeratosis with widening of the hair follicles and cystic dilatation, an increase in the number of sebaceous glands, and a corium slightly more cellular than the normal. The outer, untreated side of the ear showed the same changes in less degree.

The growths were minute hassocks or low verrucosities, with little or no tendency to enlarge. Some were gray. The three removed for section proved to be papillomas, two of them with the histology indicative of the stationary condition (Figs. 22, 23), the third appearing fairly active (Fig. 24). Because of the long duration of the applications and the various impure media employed we are inclined to write off the findings as not certainly the result of arsenic.

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[4] White Petroleum Jelly of the Chesebrough Mfg. Company, New York, New York.

[5] 50 cc. of a 2 per cent (warm, saturated) solution of arsenic trioxide in water was mixed with 100 cc. of reagent acetone (Mallinckrodt) containing 3.5 per cent of quinine (U.S.P.).

There is the more reason to do so because one of the tumors arose on a control ear,—which in other respects appeared normal in the gross.

DISCUSSION

Orr has reported that six related carcinogenic hydrocarbons all gave rise to cutaneous tumors of the same sorts in the mouse (21). The benign growths which result from tarring rabbit skin and those due to methylcholanthrene and benzpyrene differ much more than might have been predicted from this finding and from the evidence that tar owes its carcinogenicity to the presence of benzpyrene.[6] The differences fall into several categories.

The benign tumors due to tar often enlarge rapidly and become fleshy as the applications are continued whereas those of the same types induced by the pure hydrocarbons nearly all remain tiny and dry during long periods (1). These differences are referable in the main, if not wholly, to collateral effects of the carcinogenic agents on the corium, tar rendering this tissue excitable and abnormally vascular with result that it cooperates actively in the formation of tumors whereas it remains inert during months of painting with benzpyrene and methylcholanthrene. This state of affairs finds striking expression in the saucer or plaque shape of many of the papillomas, in the infrequency and lack of vigor of the carcinomatoids, and in certain peculiarities of the frill horns, these pushing downwards along a bulging front instead of invading in a jagged frill, and failing in most instances to build up horns, the keratinized cells flaking away instead.

A second sort of difference is to be seen in the cytological changes taking place in frill horns due to benzpyrene and methylcholanthrene, changes which are obviously the consequence of a perverted differentiation of individual tumor cells and which culminate in the formation of "bullet bodies." We have never come upon any such bodies in the frill horns which tar calls forth. It may be that when frill horn cells are tarred they divide too rapidly for the bodies to be formed. They cannot be due to any direct influence of the polycyclic hydrocarbons, for they have been found in frill horns arising several years after these were last applied (14).

The "bullets" closely resemble those characteristic of a rare malady of human skin, *hyperkeratosis follicularis vegetans* (23) (Darier's disease), and they are the outcome of very similar morphological changes in the differentiating epidermal cells, which first form *corps ronds* and then *grains* (in the terminology of Darier) as the cytoplasm becomes wholly involved in the parakeratosis. Some of the other peculiarities of frill horns are also like those of Darier's disease. More will be said on this theme in a succeeding paper.

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[6] Berenblum and Schoental have just reported that tar contains other carcinogens besides benzpyrene (22). The number and nature of the new agents have still to be determined.

A third difference is in actual tumor kind. The tarring of rabbit skin does not result in sebaceous adenomas, and when causing them benzpyrene and methylcholanthrene indubitably determine the kind of neoplasm they produce. Yet on looking further into the matter one finds that tarring could scarcely give rise to the adenomas since it does away at an early period with the gland cells from which they derive, whereas benzpyrene and methylcholanthrene bring about a large increase in their number.[7] The difference resolves itself into one of cellular opportunity for the carcinogens, and it serves to reemphasize a principle basic to any comparison of the effects of these agents, namely, that such a comparison is only valid when they are tested, not merely on animals of the same species and strain, and on the same tissues, but upon cells of identical character.[8] One might liken a tissue with its various cell components to a musical instrument capable of emitting this or that fixed tone when one or another string is plucked. According as the carcinogen plucks differing strings the response in types of tumor produced will be diverse; but the diversity will be less if some of the strings are missing,—as when tar has destroyed all of the sebaceous gland cells. This analogy makes no provision for the emission of different tones when strings of a single sort are plucked,—as when a carcinogen calls forth in some instances papillomas and in others frill horns by its action on the basal cells of epidermis. Nor is the curious observation covered that strings ordinarily emitting a tone may remain silent no matter how hard they are plucked,—as when mice of "low-tumor" strains, as concerns growths of certain internal organs, which are common in other strains, fail to develop such growths on drastic carcinogenic stimulation. These are phenomena which propound anew the riddle of tumor causation.

The observed differences in proportionate yield of tumors of the same kinds to tar and the pure hydrocarbons respectively are due in part to the differing amounts of stimulation that these agents provide,—tar, which provides most, calling forth relatively large numbers of active carcinomatoids,—and in other part to the selective encouragement of certain cells amongst the multitude of latent neoplastic elements.[9] But neither of these facts will account for the fact

[7] Simpson and Cramer (24) have lately noted that a single painting of mouse skin with methylcholanthrene causes all of the sebaceous glands to disappear. Rabbit skin is much more slowly affected by the hydrocarbons and may conceivably be so impervious to them that only a stimulating amount reaches the gland cells, with result in the observed multiplication.

[8] Kirby-Smith, Blum, and Grady (25) report that the majority of the cutaneous tumors arising in mice submitted to ultra-violet rays are sarcomatous, not of epithelial origin like nearly all those induced in human skin, and they refer the difference to the failure of the rays to penetrate deeply into the human cutis.

[9] In experiments with non-carcinogenic agents which cause tar tumors to reappear (1, 8) we have had occasion to note how tricky is the encouragement of neoplastic proliferation. Turpentine, for example, may or may not call forth tumors from skin previously tarred and encourage or repress growths already present, according to the amount of inflammation it en-

that the few frill horns which tarring calls forth do well, whereas the many which appear in response to benzpyrene and methylcholanthrene do poorly.

The findings emphasize a point already stressed in a preceding paper (5), that the epidermis of the rabbit seems capable of forming benign tumors of only a very few types. Those we studied took origin from the stratum germinativum, yet no tumors of basal cell type, as histologists define this, ever arose.

The presence in a frill horn due to benzpyrene of nuclear inclusion bodies of a type that would seem to indicate the presence of a virus is of no large import since the tumor did not differ significantly in other ways from the generality of growths of its kind. So many viruses have been found to persist in tumors without significantly affecting them that it is rather surprising, and certainly fortunate for students of tumor etiology, that intercurrent virus infection of neoplastic tissues is rare under natural conditions.

SUMMARY AND CONCLUSIONS

The benign tumors of rabbit skin which appear in response to benzpyrene and methylcholanthrene are nearly all of the same kinds that tar produces, namely frill horns, papillomas, and carcinomatoids; but the collateral effects of these agents render many of them very different from the tar tumors. The connective tissue of the corium is so slowly excited by the pure hydrocarbons that for a long while it furnishes to most of the growths only a scanty stroma, when any, and in consequence they remain small,—low, dry, indolent, bas-relief affairs, made up almost entirely of the neoplastic epithelium,—not florid, fleshy excrescences with a large connective tissue component, such as tarring calls forth. The frill horns usually desquamate instead of building up like those due to tar, and some of their cells undergo a dyskeratotic change with result in spherical, homogeneous, deep-staining bullet-like elements, which give to the growths a singular aspect. In a frill horn due to benzpyrene numerous inclusion bodies were come upon which would seem to have been the result of intercurrent infection with a "passenger virus."

Benzpyrene and methylcholanthrene produce papillomas, carcinomatoids, and frill horns in very different proportionate numbers from those obtaining when tar is the carcinogen. Tar gives rise much more frequently to carcinomatoids, —papillomas urged on to mimic cancers,—as would follow from its pronounced stimulating influence; yet it seldom produces frill horns whereas the pure hydrocarbons do so frequently. All three agents cause many more cells to become potentially capable of forming tumors than do so ordinarily, but the

genders. Many of the elements rendered neoplastic by tar fail to form visible growths despite its great compulsive influence, yet they will do so in response to the stimulus of wound healing (8). One must think of cells which have been rendered capable of forming tumors, even tumors of a single kind, as differing considerably in their requirements, some multiplying in response to conditions which fail to stimulate others.

latent neoplastic elements on which tar exerts a promoting influence, causing them to form visible growths, are in general not the same as those which respond to benzpyrene and methylcholanthrene. Yet the relatively rare occurrence of frill horns due to tar cannot be wholly explained in this way and it becomes necessary to suppose that tar seldom changes normal cells into frill horn cells.

Benzpyrene and methylcholanthrene give rise now and again to sebaceous adenomas, as tar does not. But tar does away with the sebaceous glands at an early period whereas the other two carcinogens cause them to increase notably in number.

No evidence has been obtained that tar, benzpyrene, methylcholanthrene, arsenical preparations, or benzene,—which produces tumors of rabbit skin occasionally (1),—bring about any neoplastic changes peculiar to them individually when they act upon cells of a single sort, those of the stratum germinativum of rabbit epidermis. Yet the experimental findings make plain that these agents exert no inconsiderable influence on the morphology of the benign cutaneous growths they call forth and on the frequency with which this or that kind occurs.

In appraising the action of carcinogens one must take into account not only the capacity of these agents to induce neoplastic change and to promote, or perhaps suppress, tumor growth but an ability to condition to no inconsiderable extent both the kind of tumor arising and its structure.

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EXPLANATION OF PLATES

The sections were stained with eosin and methylene blue.

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

PLATE 10

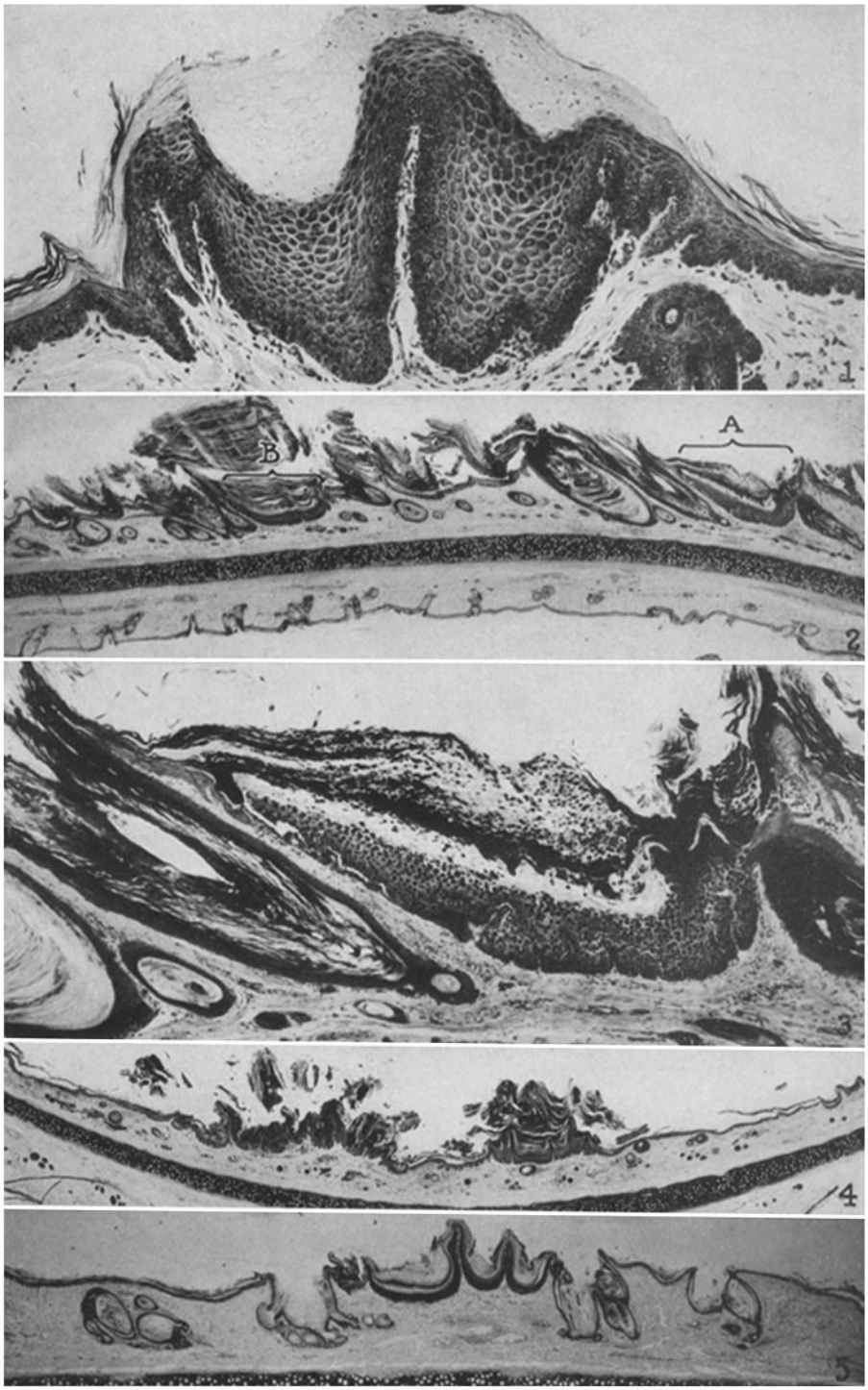
FIG. 1. Early stage of active papillomatosis,—to show the characteristic cytology. The growth was first noted after benzpyrene in benzene had been applied to the skin for 45 days, and it was removed at once. The epidermis round about it is hyperplastic. × 114.

FIG. 2. Saucer-shaped growths on skin which had become exceptionally scurfy as result of treatment with benzpyrene in benzene for 14½ months. The tumor *A* is a frill horn which has desquamated instead of building up. The growth *B* is a papilloma (as seen at higher magnification) and keratin has accumulated over it. Like *A* it is less than 2 mm. in diameter. × 10½.

FIG. 3. The frill horn *A* of Fig. 2. It has extended down scarcely at all and its cells have mostly undergone the change to "bullets" as they differentiated. × 46½.

FIG. 4. Papillomas from an ear which had received benzpyrene in benzene for 15 months. The neoplastic epithelium is active as proven by its thickness and foldings, but almost no stroma for it has been forthcoming, and the growths are mere plaques overlain by keratin. They had been present more than a month. × 11.

FIG. 5. Another papilloma with very little stroma. There are a few short, narrow foldings of the thickened epithelium. The skin had been exposed to benzpyrene in benzene for slightly more than 9½ months, with result in the usual hyperkeratosis and distension of the hair follicles. × 17.



(Friedewald and Rous: The determining influence of carcinogens)

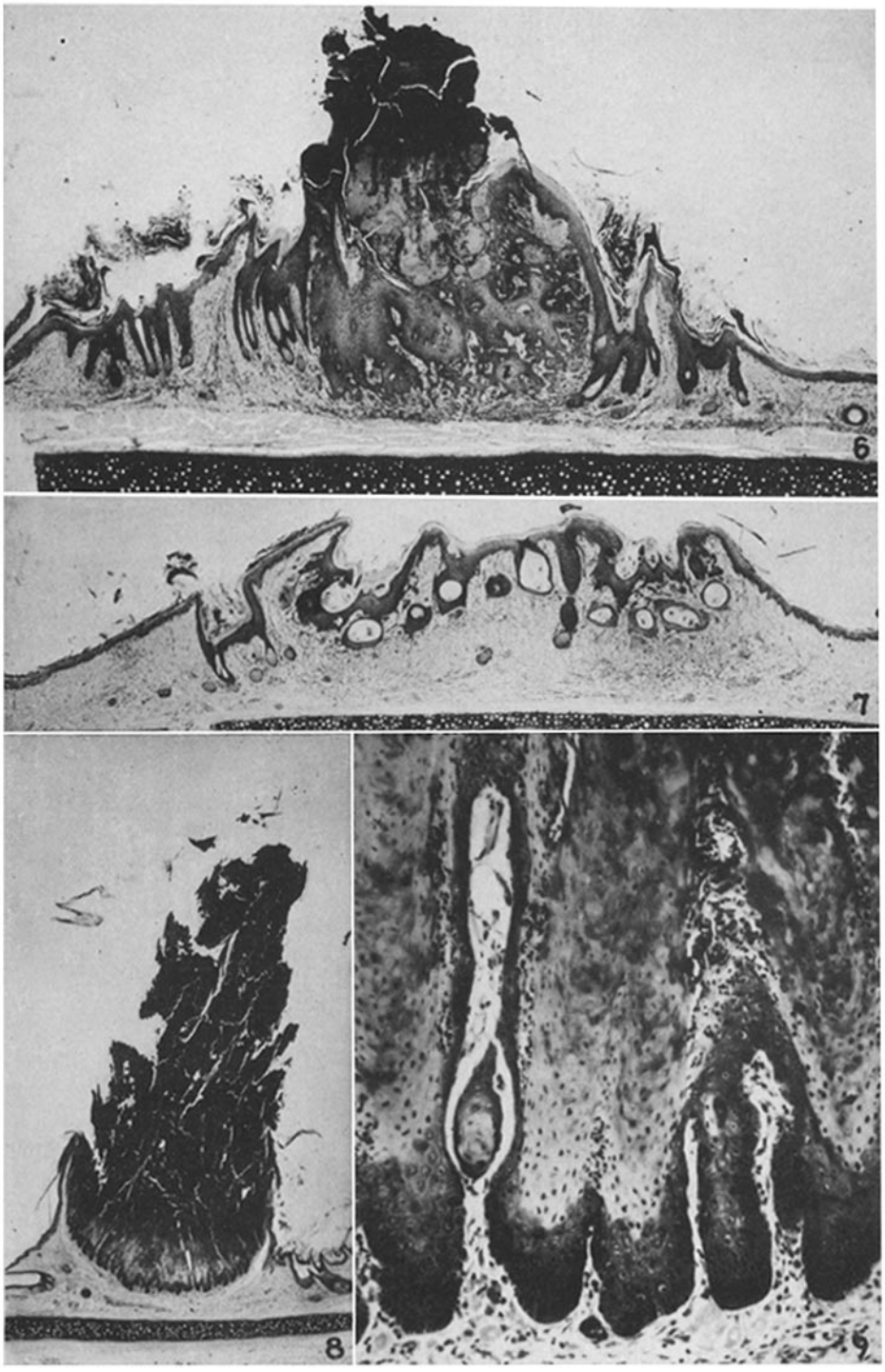
PLATE 11

FIG. 6. An active carcinomatoid elicited by methylcholanthrene in benzene. It is capped with dried blood. The solution had been painted on the skin for 4½ months when the growth appeared, and it was removed within a few days. The neighboring epidermis is markedly hyperplastic. × 27.

FIG. 7. Inactive carcinomatoid from an ear painted with methylcholanthrene in benzene for 11½ months. The thickened, cellular connective tissue toward the right under the growth contains embedded lumps of keratin resulting from the differentiation and death of tumor cells which had penetrated deep. The magnification is not sufficient to show this dead stuff. Toward the surface the tumor has rounded out into cysts containing keratinized pearls. × 20.

FIG. 8. Frill horn from an ear treated with methylcholanthrene in benzene for slightly more than 3 months. The tumor had grown much faster than usual with result that the short horn is conical. The epidermal frill along the base of the growth is characteristic of active neoplasms of the sort, as is also the connective tissue collar. × 12.

FIG. 9. Part of the frill along the base of the growth of Fig. 8,—to show the typical morphology and abrupt differentiation into compact keratin. The numerous black dots in clefts amidst this last, which look like lymphocytes, are actually the fragmented nuclei of polymorphonuclear cells which had wandered in. × 150.



(Friedewald and Rous: The determining influence of carcinogens)

PLATE 12

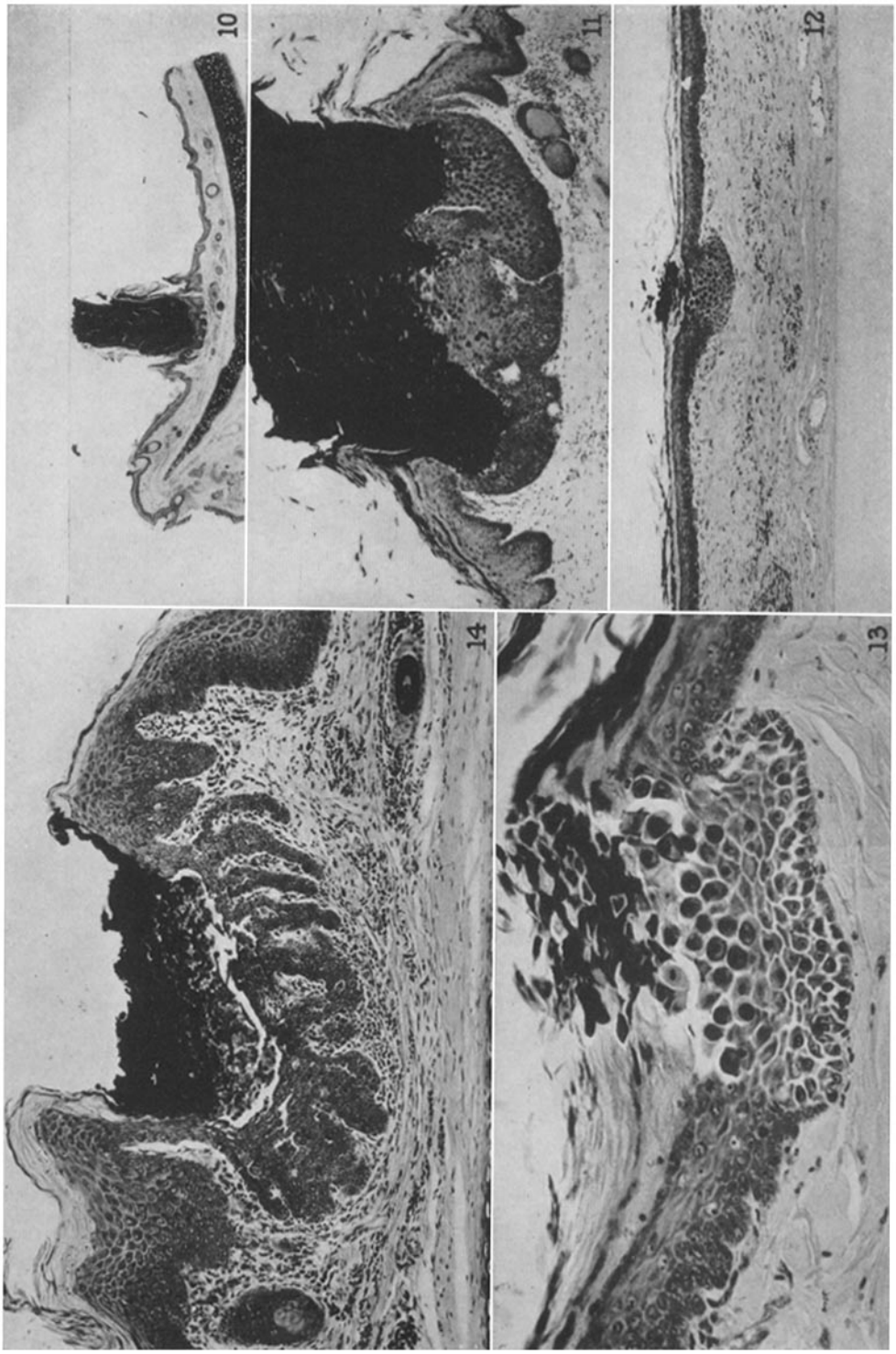
FIG. 10. A less active frill horn which arose near the thick edge of an ear painted with benzpyrene in benzene for not quite 10 months. The growth had been present more than 3 months but was still not a millimeter across. Most of the horn has been cut away to facilitate sectioning. × 13.

FIG. 11. Inactive frill horn from an ear treated with benzpyrene in benzene for 9½ months. It had a horn, now mostly cut away, but no frill and its epithelium contains many "bullets." × 57.

FIG. 12. Tiny frill horn which escaped attention during life because the keratinized material desquamated. The skin had been painted with benzpyrene in benzene for more than 10 months. The differentiating cells appear to lie free. There are many "bullet bodies." × 57.

FIG. 13. Another minute frill horn,—to illustrate the formation of "bullet bodies:" from skin receiving methylcholanthrene in benzene 4½ months. There is a gap all along above the basal epithelium, the differentiating epithelial cells seem to lie free, and they desquamate soon instead of heaping up. × 253.

FIG. 14. Frill horn extending downwards in narrow tongues as if malignant. Its cells are dying early and coming away instead of differentiating, and the result is a crater instead of a horn. There are many macrophages and some lymphocytes in the corium. The skin was more than usually affected by the benzpyrene in benzene, which was applied for slightly less than 8 months. × 101.



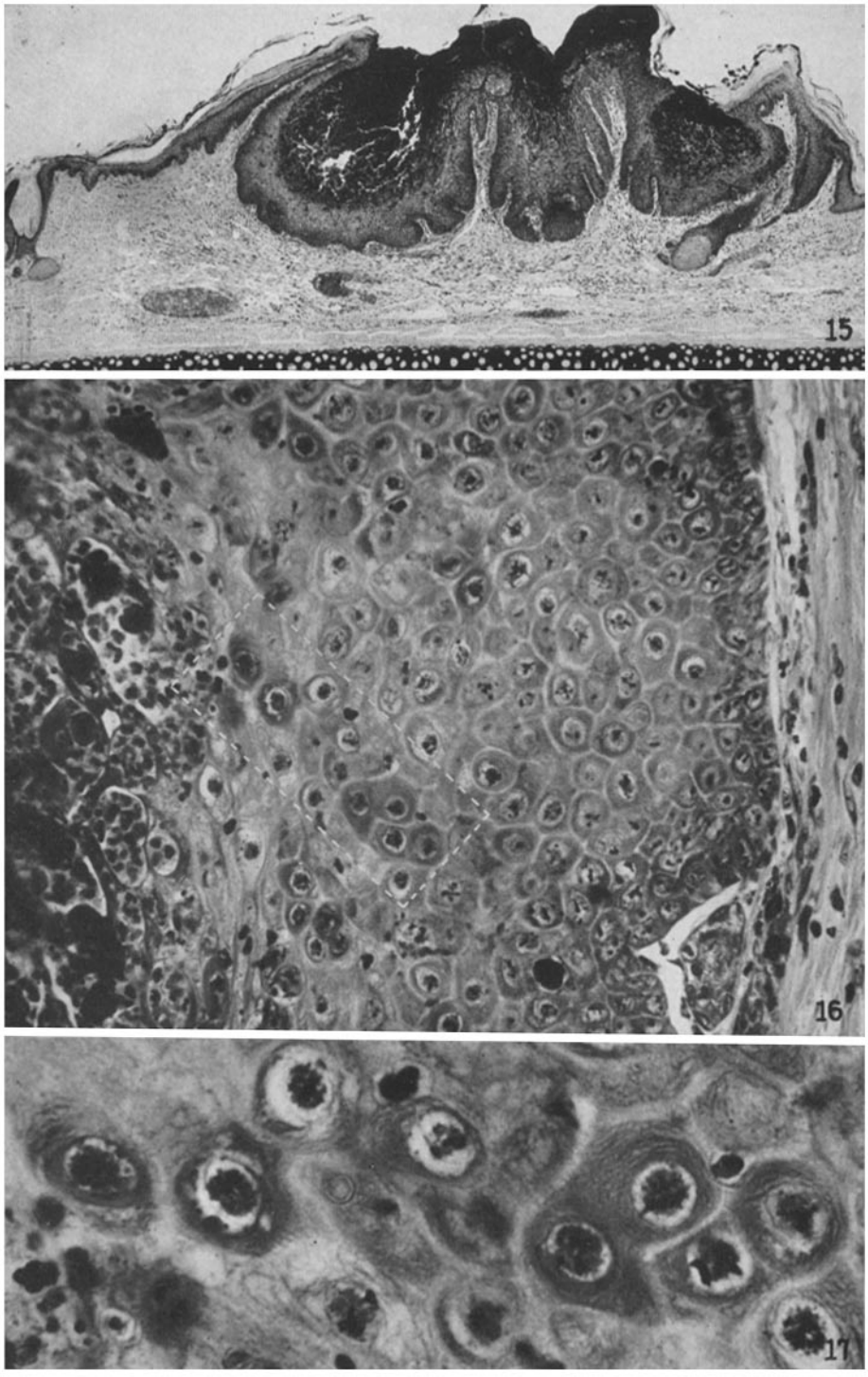
(Friedewald and Rous: The determining influence of carcinogens)

PLATE 13

FIG. 15. The frill horn in which nuclear inclusions were found (see text and Figs 16 and 17). The growth had the same general character as that of Fig. 11, was inactive that is to say, but its cells died early (as in the growth of Fig. 14) and formed no horn. The ear had been painted with methylcholanthrene in benzene for 140 days. × 35.

FIG. 16. Section through the base of the frill horn of Fig. 15,— to show the inclusion bodies. They are most clearly visible in the Malpighian layer where rounded aggregates of them occupy the central region of every nucleus, the rest of it appearing empty. The chromatin is also centrally placed, as dark rods or coarse lumps. A few of the bodies can be seen in cells next the basal layer (which lies to the right), and as these differentiate they become numerous and the clumps of them sharply defined. There are many polymorphonuclear leucocytes in lacunae amidst the keratinized material on the left. The cells included in the broken square are shown in Fig. 17. × 411.

FIG. 17. Some of the nuclear inclusions of Fig. 16 at a higher magnification. × 1027.



(Friedewald and Rous: The determining influence of carcinogens)

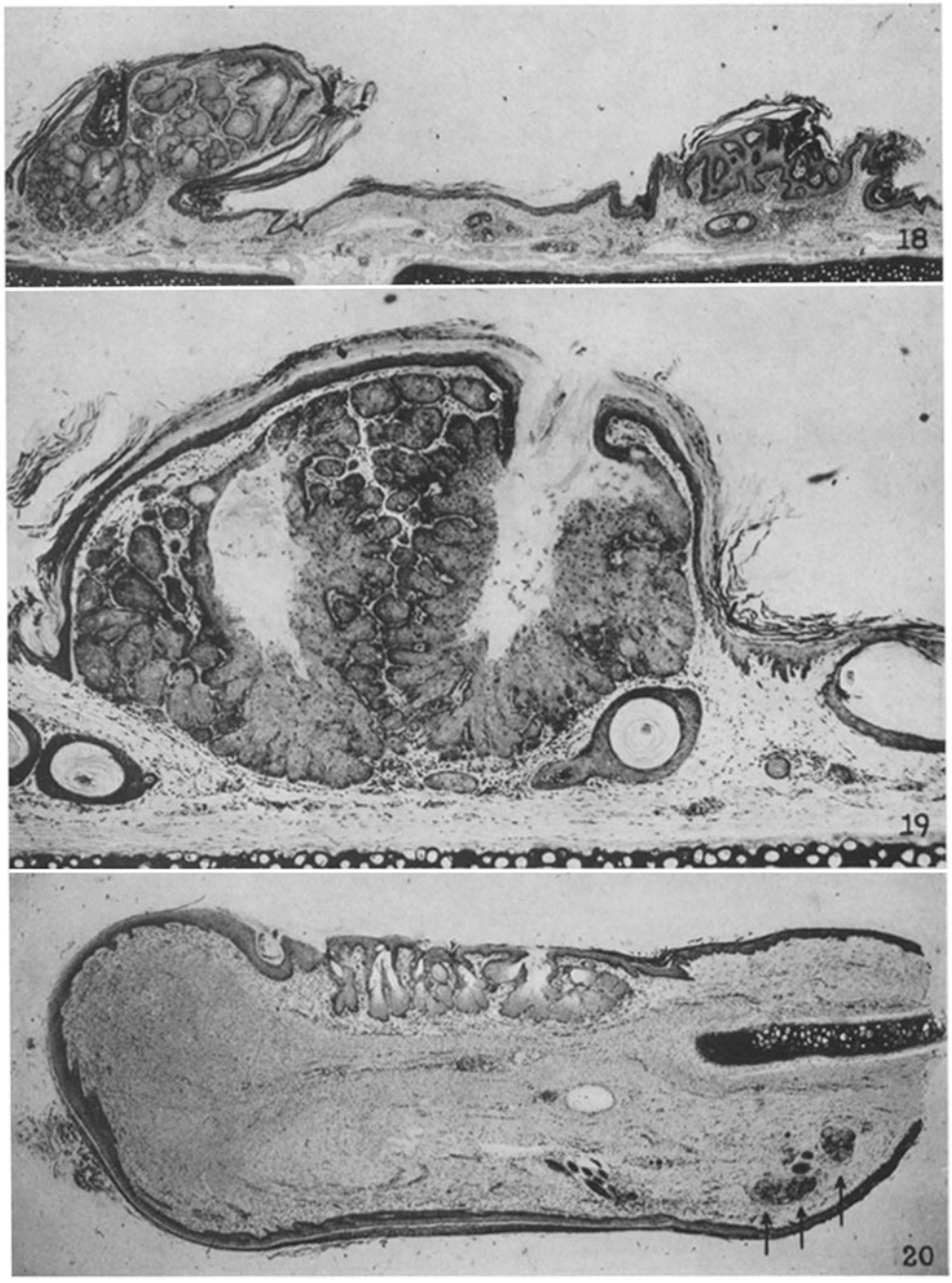
PLATE 14

FIG. 18. A sebaceous adenoma and a retrogressing papilloma on skin which had been painted with methylcholanthrene in benzene for more than 8 months. The adenoma did not appear until 4 months later and it was removed after about 6 more months had passed.

In most regions the growth is inactive but in some spots new glands are in process of formation, and here high magnification showed mitoses to be not infrequent. Serial sections disclosed several gland ducts, distended with keratin and lined with stratified squamous epithelium, one of which can be seen in part. There are many macrophages and lymphocytes in the connective tissue under the papilloma. × 13.

FIG. 19. Another sebaceous adenoma from an ear treated with benzpyrene in benzene for 14 months. It had been present for more than 3 months when removed. × 60.

FIG. 20. Section through a growth which appeared on the tissue growing in toward the center of a punch hole. Healing was incomplete when it was removed together with a piece of the adjacent normal tissue. The original margin of the hole is at the junction of old and new cartilage, to be seen on the right, and a curving layer of epithelium on the left covers the new tissue which has extended into the hole. The growth is a sebaceous adenoma. Its giant glands should be compared with the normal sebaceous glands (arrows) situated outside the original rim of the hole. × 15.

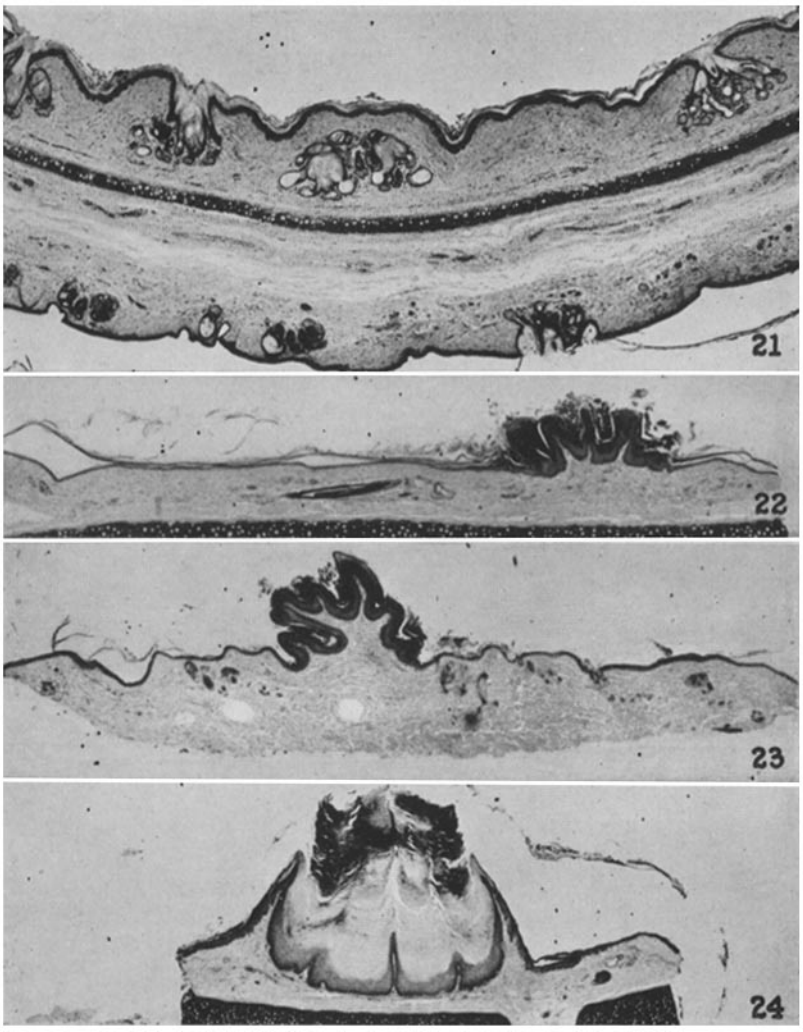


(Friedewald and Rous: The determining influence of carcinogens)

PLATE 15

FIG. 21. Skin changes resulting from the application of arsenic trioxide over a period of not quite 2 years. It was put on the inner surface of the ear, and here there is epidermal hyperplasia, hyperkeratosis, cystic distention of the hair follicles with keratin, a pronounced increase in the number of sebaceous glands and chronic inflammation of the corium. Most of these changes took place to some extent on the outer side of the ear,—which is on the lower side of the section. They closely resemble those due to the polycyclic hydrocarbons. × 21.

FIGS. 22–24. Papillomas first noted after the application of arsenic trioxide for 22 months. × 18½.



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