Induction and Differentiation of an Epithelial Tumour in the Newt (Triturus cristatus)

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WITH SEVEN PLATES

INTRODUCTION

WADDINGTON (1935) and later Needham (1936) have suggested that the autonomy of tumours originates in a ‘morphological escape’ of tumour cells from the controlling influence of an individuation field which has locally become weak or has vanished. The persistence and strength of such an individuation field can be seen in its ability to induce its parts to regeneration (Ruben, 1955). This theory might be supported by the observation that in animals which are capable of regeneration, spontaneous tumours are rarely observed. Gersch (1951) compiled a list of all reports of spontaneous tumours throughout the whole animal kingdom and noted that in animals which regenerate well spontaneous tumour occurrence was low. Waddington and Needham suggested that degraded, that is ‘escaped’ cells, might be brought under control again if they were exposed to the influence of a particularly strong individuation field, e.g. the regeneration field of a regenerating urodele limb.

The first experimental attempts to examine these speculations were performed by Rose & Wallingford (1948). They implanted anuran cancer (Lucké carcinoma of Rana pipiens) into regeneration blastemata of limbs of adult Triturus viridescens. The authors described differentiation of the tumour cells into cartilage, muscle, and connective-tissue cells. Although these results seem to support the speculation of Waddington and Needham, no definite evidence could be given that the differentiated cells were actually the Rana cells. The only available difference used to distinguish Rana and Triturus cells was the smaller size of Rana nuclei.

Ruben (1955, 1956) implanted the same carcinoma into limb regeneration blastemata of Amblystoma larvae and adult T. viridescens and could not observe any alteration of the tumour tissue except degeneration.

In both of these previous experimental attempts the method used does not seem to be adequate to permit definite statements. There was always a foreign

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tumour implanted into the regeneration blastemata of *Triturus* or *Amblystoma*. In all experiments these foreign implants showed only a survival without noteworthy growth, even when implanted into non-regenerating limbs. If the tumour cells failed to be affected by the strong individuation field, this could be due to heterogeneity between individuation field and implant as well as to the degeneration of the implant. Furthermore, the Lucké carcinoma is caused by a virus. The permanent carcinogenic influence of the virus might prevail in spite of the controlling influence of the regeneration field.

We have been interested in the concepts suggested by Waddington and Needham, and in the studies reported on here we have induced epithelial tumours in newts by chemical carcinogens. These induced tumours avoid the previous problems of permanent carcinogenic effect of a virus. Furthermore, we applied these carcinogens to animals which were capable of regeneration so that problems of tumour transplantation were entirely avoided. Using this chemically induced tumour system we examined the problem whether differentiation processes occur in the tumours of such animals.

The results of these studies appear to support the earliest speculative opinion of Waddington (1935) and Needham (1936).

**PART 1**

*The development of a chemically induced epithelial tumour in *T. cristatus*

Our first problem was to produce a typical tumour, comparable with tumours in mammals, which can clearly be distinguished from other proliferative processes such as inflammation and regeneration.

Only in exceptional cases have tumours been produced in urodeles by application of chemical carcinogens (Koch, Schrieber, & Schrieber, 1939; Breedis, 1951); Leone (1957) succeeded in producing metastatic neoplasms in *T. cristatus* by subcutaneous implantation of methylcholanthrene crystals. Numerous other attempts have failed. Nevertheless, a few spontaneous epithelial tumours in urodeles have been reported: a melanoma of the axolotl (Scheremetjeva-Brunst & Brunst, 1948), a transplantable epithelial carcinoma of *T. alpestris* (Champy, & Champy, 1935), and a carcinoma of the epithelial mucous glands in *T. cristatus* (Murray, 1908).

Since a spontaneous mucous gland carcinoma has been reported in *T. cristatus* we attempted to induce tumours at this same site by the application of carcinogens.

**MATERIAL AND METHODS**

Investigations have been made on the newt *T. cristatus*, which were all collected in the same district (Neusiedler See, Burgenland). A total of 1,800 newts were used in these studies. The animals received subcutaneous injections as shown in Table 1.

Since it will be shown that the formation of epithelial growths depends upon
the entrance of the carcinogen into the mucous glands, care was taken to be sure that the carcinogenic solution permeated from the surrounding tissues into the base of the glands. We could be sure of this if the olive oil could be seen extruding from the mouth of the gland pores.

If not stated otherwise all results in this paper refer to subcutaneous injections. Other groups of newts were treated by painting different parts of their bodies with a 2 per cent. solution of benzpyrene in benzene. The injections and painting were made on different parts of the body of the newts. Groups received injections into or painting on: the dorsal trunk, the lateral trunk, the ventral trunk, the tail, and the neck pouch.

After injection or painting, all the animals were observed for the appearance of epithelial tumours which first appeared as small depigmented white spots in the area of injection. The newts were killed at various times between one week and one year after injection. Most sacrificed animals were fixed in formalin and serial tissue slides prepared. The slides were stained with haematoxylin-erythrosin-Orange G, Mallory, or mucicarmin. A total of approximately 600 slides from 85 newts were studied. Since animals had on average three tumours each the behaviour of a total of 250 tumours was studied histologically.

First the normal integument of *Triturus* will briefly be described. It consists of an epithelium, 2–4 cell layers thick. On the outside it is limited by a thin cornified layer of flattened epithelial cells which is desquamated at regular intervals. The epithelium is limited proximally by a basement membrane. Under this basement membrane lies a layer of pigment cells containing the blood supply (arteria and vena cutanea). Beneath this layer, holocrine mucous glands with their outlet ducts are regularly distributed. A thin layer of subcutaneous connective tissue and musculature lies innermost (Plate 1, fig. 1).

**RESULTS**

*Tumour induction by application of carcinogenic substances*

It was found that the epithelial reactions were quite identical following the application of any of the carcinogens used in our studies. The description of tumour development is valid for all carcinogens listed in Table 1. Whereas the histological pictures were identical for all carcinogens we found that the mixture of 2 per cent. DBA+0.2 per cent. BP yielded the highest frequency of tumours. Therefore we preferred this solution in our studies.

All tumours observed began as multicentric growths, each originating in a separate mucous gland. These growths then coalesced to form a large tumour area. Cell changes always started in the basal part of the gland pocket with an excessive proliferation of the cells of the germinative layer of these holocrine glands.

The genesis of epithelial growths can be subdivided into five stages: (1) proliferation inside of a gland pocket; (2) expansively growing tumour; (3) infiltrating tumour; (4) penetration into the peritoneal cavity; (5) metastasis.
Stage 1. Cells of the basal germinative layer of the mucous glands begin to proliferate (Plate 1, figs. 5, 6). While the gland pocket progressively fills with small cells, its distal part and the duct remain open and functional (Plate 1, fig. 7). Compare with a normal mucous gland, shown in Plate 1, fig. 2.

Simultaneously the epithelium also shows an increased mitotic activity, as revealed by experiments with tritiated thymidine.

Stage 2. Not only does the gland pocket fill with tumour cells, but the growths perforate the gland wall and coalesce with the epithelium leaving no boundary (Plate 1, fig. 7, right gland pocket). In this way many small growths originating from mucous glands fused into a continuous mass of cells. This tumour grew expansively without perforation of the basement membrane (Plate 2, fig. 8) even though it penetrated into the musculature, forming long projections. In the region of the tumour, all mucous glands, vessels, and the pigment cell layer are eventually destroyed (Plate 2, fig. 8). Later, connective tissue and muscles are also destroyed. In some cases the destruction of the musculature was more intensive than the growth of the tumour, so that the whole tumour area sank in. In extreme cases tail tumours reached the spine after destruction of all the muscles of one lateral half of the tail. In some instances, such expansively growing tumours formed large projections into the musculature (Plate 2, fig. 9).

Stage 3. In some cases the tumour became an infiltrating growth (Plate 2, figs. 10–13). Infiltration took place either in large areas of the tumour's periphery or infiltrative rod-like projections sprouted from the epithelial tumour (Plate 2, fig. 12).

Stage 4. Some infiltrating tumours (65 per cent.) of the dorsal and lateral trunk were seen to have penetrated into the peritoneal cavity and formed there an epithelial layer which covered large areas of the peritoneum (Plate 3, figs. 14, 15) and continuously desquamated cornified cell layers into the peritoneal cavity.

Stage 5. Metastasis. All primary tumours induced in the sacral region metastasized extensively. A few (slowly growing) tumours induced in the tail

<table>
<thead>
<tr>
<th>Compounds injected subcutaneously into newts</th>
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<tr>
<td>Compounds injected</td>
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<tr>
<td>Carcinogens (in olive oil)</td>
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<tr>
<td>benzpyrene (BP) (0·1–2·0%)</td>
</tr>
<tr>
<td>methylcholanthrene (MCA) (0·1–2·0%)</td>
</tr>
<tr>
<td>dibenzanthracene (DBA) (2%)</td>
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<tr>
<td>benzpyrene (2%) and methylcholanthrene (1%)</td>
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<tr>
<td>dibenzanthracene (2%) and benzpyrene (0·2%)</td>
</tr>
<tr>
<td>Controls: non-carcinogenic, irritating materials (aqueous solutions)</td>
</tr>
<tr>
<td>trichloroacetic acid (2%)</td>
</tr>
<tr>
<td>hydrochloric acid (0·4%)</td>
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<tr>
<td>lactic acid (10%)</td>
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<td>sodium hydroxide (0·4%)</td>
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Each animal received approximately 0·01 ml–0·02 ml of the solution.
TEXT-FIG. 1. Diagrammatic representation of tumour stages. Stage 1: a, the basal germinative layer of a mucous gland begins to proliferate excessively (Plate 1, figs. 5, 6). b, the gland pocket has been filled with tumour cells (Plate 1, fig. 7, left gland). Stage 2: Expansive growth. a, in two tumourous mucous glands the gland-wall has been perforated and the multicentric growths coalesce together with the epithelium (Plate 1, fig. 7, right gland). b, transverse section through the tail with an expansively growing tumour which arose from numerous mucous glands. Two normal mucous glands are drawn in to show the size of the tumour (Plate 2, fig. 8). Stage 3: Infiltrative growth. a, large tumour with partial peripheral infiltration. b, infiltrative rod-like tumour projections sprouting from the epithelial tumour (Plate 2, fig. 12). Stage 4: The tumour has penetrated into the peritoneal cavity and there forms an epithelial pavement (Plate 3, figs. 14, 15). Stage 5: Metastases. a, a metastasis between 2 mucous glands, close to the vessels of the arteria cutanea (Plate 3, fig. 17). b, the metastasis has coalesced with the epithelium (Plate 3, fig. 18). c, large metastasis which had coalesced with the outer epithelium and now spreads between epithelium and musculature but does not penetrate into the musculature (Plate 3, fig. 19).
also were metastatic. Numerous metastases were formed beneath the epithelium in the region of the arteria cutanea. Metastases were seen on the dorsal head, neck, and trunk, also on the tail, and on the extremities (Plate 3, fig. 16). In contrast to the primary tumour, these arose without relation to the mucous glands (Plate 3, figs. 17, 18). The metastases always coalesced with the epithelium and grew only expansively; they did not penetrate through the subepithelial layer into the musculature (Plate 3, fig. 19), as did the primary tumour. The musculature appeared to be impenetrable for these epithelial metastases, and the metastases spread only between the musculature and the epithelium, sometimes attaining considerable size. Here, too, mucous glands, vessels, and the pigment cell layer were destroyed (Plate 3, fig. 19) as was seen in the growth of the primary tumour.  

In a few cases numerous metastases were found in the lung, in the musculature, and in the kidney, but the epithelium always remained the most involved region. Apparently the tumour cells were spread in the newt epithelium by their highly developed and extensive network of arteria and vena cutanea, which represents the most important respiratory system of the newts. In these vessels and also in the vessels of the lung, numerous tumour-cell emboli were found.

These five stages of tumour development are also shown in Text-fig. 1.

It was remarkable that these five stages of tumour development were not represented at the same frequency in different parts of the body; 93 per cent. of tail tumours did not pass the stage of expansive growth, and only 7 per cent. became infiltrative. This percentage has been determined on 100 tail tumours studied histologically. On the other hand, 90 per cent. of the tumours on the trunk soon became infiltrative and only 10 per cent. remained at the stage of expansive growth (here 50 tumours were examined histologically). Metastases arose from a very high percentage (100 per cent.) of primary tumours situated in the sacral region (200 animals). Only 6 per cent. of tail tumours metastasized (300 animals). These differences might be explained by the decreased tendency to infiltration of the tail tumours as compared to those of the sacral region, i.e. only 7 per cent. of tail tumours, but all sacral tumours were infiltrative. Only a few tumours of the trunk metastasized, although 90 per cent. of these tumours were infiltrative. Metastasis always occurred only after a rather long period of tumour growth, and, since the tumours on the trunk were lethal after only a few days, the most probable reason for the very low frequency of metastasis from trunk tumours was the premature death of the animals.

Cytologically, the tumour cells were very similar to epithelial cells, but they had rather spherical or oval shapes while the epithelial cells were more flattened. Tumour nuclei appeared slightly enlarged, the scanty cytoplasm stained strongly with haematoxylin and surrounded the nucleus as a thin border. Numerous mitoses were found.

1 In later stages a secondary parasitic infection of these epithelial metastases sometimes seemed to be involved.
All the phenomena described here have only been observed in animals treated with one or other of the carcinogens used in these studies. Such changes were never seen after application of the irritating, non-carcinogenic substances listed in Table 1.

Concentrations of 0.1 per cent. and 0.2 per cent. of BP or MCA did not produce tumours; only carcinogenic solutions above 0.2 per cent. were successful. For this reason control injections with olive oil alone were omitted.

Tumour induction was most successful on the dorsal and lateral trunk and on the tail. On the neck pouch, only 1 of 40 injections yielded tumour formation, and on the ventral trunk 50 subcutaneous injections of 2 per cent. BP did not produce any tumour at all. These results might be due to the sparse distribution of mucous glands in these areas.

Sixty animals have been painted with benzpyrene. This manner of application was only successful on the tail (in about 50 per cent. of cases). No other body region showed any tumourous reaction following painting of carcinogens. The reason may be related to the abundant distribution of mucous glands on the tail.

It was also found that hibernating animals were most susceptible to tumour formation, whereas animals treated in the spring were most resistant.

Control injection with non-carcinogenic, irritating materials

These injections were performed in exactly the same manner as the injections of carcinogenic substances. We used trichloroacetic acid, hydrochloric acid, lactic acid, and sodium hydroxide as irritating materials. Trichloroacetic acid was found to be especially irritating (Table 2).

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<thead>
<tr>
<th>Material</th>
<th>Number of animals injected</th>
<th>Animals with inflammatory reactions</th>
<th>Died</th>
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<tbody>
<tr>
<td>2.0% trichloroacetic acid</td>
<td>20</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>0.4% hydrochloric acid</td>
<td>20</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>10.0% lactic acid</td>
<td>20</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>0.4% sodium hydroxide</td>
<td>20</td>
<td>8</td>
<td>2</td>
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Most of the animals showed inflammatory processes. These inflammations declined and disappeared after 5–10 days. In the histological sections from these animals no epithelial growth could be seen in the centre of inflammation except a thickening of the epithelium (Plate 1, fig. 3) like that found during normal regeneration. The basement membrane was observed to be temporarily dis-integrated and the epithelium had coalesced with the subepithelial tissues. This same epithelial behaviour has also been observed in normal limb regeneration in Triturus (Rose, 1948).

In the margin of the inflamed zone epithelial proliferation was observed. The
epithelium was seen to have migrated through the outlet ducts into the damaged mucous glands. The gland pockets were filled from without by epithelial cells and only occasionally remnants of the basilar gland epithelium remained (Plate 1, fig. 4). The proliferation stopped when the whole gland pocket was filled; a perforation of the gland pocket never occurred. Finally, these growths degenerated by necrotic disintegration beginning in their centre.

TEXT-FIG. 2. Diagrammatic representation showing the difference between an inflammation process and early tumour formation. A. Inflammatory process. 1, the epithelium begins to encroach from without into the damaged gland pocket. 2, almost the entire gland pocket is filled with epithelial cells (Plate 1, fig. 4, left gland). B. Tumour formation. 1, the basal germinative layer of the mucous gland begins to proliferate and starts to fill the gland pocket from below with tumour cells (Plate 1, figs. 5, 6). 2, the entire gland pocket had been filled with tumour cells. Now the wall of the gland pocket is perforated and the growth coalesces with the epithelium (Plate 1, fig. 7, right gland). Altered areas are depicted by parallel lines.

It should be noted that in the case of inflammatory reactions the epithelium migrates from without into the gland pocket whereas the basal parts of the gland maintain their normal histology for a long time. In contrast, a proliferation following an injection of carcinogenic solutions starts in the basal part of the gland pocket and then migrates through the outlet ducts and coalesces with the epithelium (Text-fig. 2). The further stages of tumour development or similar formations could never be observed after injection of non-carcinogenic substances.

DISCUSSION

Part 1

The major question which arises from the results shown in Part 1 is the following: can we be sure that the growths which were produced in our experiments are real tumours, comparable with the cancer of mammals?

The relatively short time between injection and the appearance of tumours, a minimum of 8 days, may be considered unusual. However, some of these same tumours arose 2 or 3 months after injection. In mice a 0·1 per cent. solu-
tion of benzpyrene yielded a carcinoma after 200 days; 0.5 per cent. benzpyrene resulted in carcinoma after 70 days (Wintersteiner, 1936). After application of 1 per cent. benzpyrene to mice, the first papilloma arose as early as 30 days afterwards (Maisin & Liegeois, 1933). But this concentration of benzpyrene was highly toxic and the majority of the mice died. Newts, on the other hand, can tolerate as much as 2 per cent. or even 3 per cent. similar carcinogenic solutions. Since we used carcinogenic solutions of such high concentration in our studies, the average incubation time of 15–20 days for tumour formation can reasonably be compared with the incubation time in mice treated with lower concentration of carcinogenic solutions. Furthermore, it should be noted that the same carcinogen which causes tumours to appear in mice after a few weeks, produces tumours in monkeys only after one or more years. Thus, there appears to be a high variability in time of tumour appearance between species.

The epithelial growths which were produced in these experiments in Triturus share certain important characteristics with mammalian tumours.

Destructive growth was seen in several instances. In the tail, especially, the whole musculature and connective tissue of one lateral half was often totally destroyed. Extensive destruction was also found in the trunk. Infiltration was a common feature in these growths, and the histological picture resembled infiltrative and destructive carcinoma in mammals.

Above all, the appearance of metastases from these tumours was a characteristic which did not leave us any doubts about the cancerous nature of this epithelial growth in Triturus.

It perhaps should also be mentioned that these tumours arose only after the application of carcinogenic substances and that they were identical for all the carcinogens tested. The tumours arose even following painting where all effects of injury were excluded.

A similar genesis of tumour, i.e. multicentric origin from glands of the skin, has also been observed in man. Boyd (1943) described a precancerous stage in the skin as multiple malignant foci arising in a limited area, apparently deriving from sebaceous glands.

In control experiments with non-carcinogenic but irritating substances an epithelial growth appeared which could easily be distinguished from the post-carcinogenic tumour processes. The detachment of single epithelial cells into the subepithelial tissue in the inflamed zone is very different from infiltration, seen in the tumours. No stage of tumour development was found in any inflammatory lesion.

Regeneration perhaps might also be difficult to distinguish from tumourous growth. Regeneration in urodeles starts with dedifferentiation and increased proliferation of epithelial cells. These cells form a blastema by interaction with the subepithelial tissue. If this interaction is disturbed (e.g. by hypophysectomy) the blastema cone continues to proliferate, and tongues penetrate into the tissue beneath (Schotte & Hall, 1952). However, the small size of such regenerative
tongues is very different from the formations of large projections in the expansively growing carcinogen-tumour of *Triturus*. Furthermore, in regeneration, the proliferation never starts in mucous glands and destructive infiltration and metastases are not observed.

For these reasons we have no doubt that this epithelial tumour of the newt is reasonably comparable with malignant carcinoma in mammals.

During preparation of this manuscript we received the paper of Arffmann & Christensen (1961) who performed similar injections of carcinogens on *T. cristatus*. The authors observed epithelial proliferations, which were most frequent following injection of DBA, as in our study. But real tumours could not be produced, probably because the injections were into the musculature and not, as in our material, subcutaneously, so that the carcinogenic solution could permeate into the gland pocket. Moreover, the authors made the injections into the tail, a very unfavourable location, since we could obtain infiltrating tumours on the tail only in 7 per cent. The epithelial hyperplasias of Arffmann & Christensen regressed after 20 to 30 days. No definite histological description of the origin and the regression of these hyperplasias has been given.

**PART 2**

*Differentiation processes in the epithelial tumour of* *T. cristatus*

It has been demonstrated in Part 1 that the epithelial growth which was produced in *T. cristatus* is a real tumour and that it may be compared with carcinoma in mammals. With the ability to produce tumours in newts we were now able to investigate if this tumour would show differentiation phenomena as predicted by our earlier speculations (see introduction).

In fact the tumour showed a pronounced tendency to spontaneous regression, even without any experimental influence. The frequency of tumour regression appeared to be highly dependent upon the anatomical site of the tumour and various biological conditions of the animals such as age, seasonal rhythm, &c. These differences in the frequency of tumour regression will be presented in a later paper. In every case in which no such spontaneous healing occurred the tumours led to the death of the animals.

Our attention was addressed to the manner in which this spontaneous healing took place. We wanted to see if differentiation phenomena occurred and if tumour cells were perhaps reincorporated into the normal tissues of the organism. Furthermore, we wanted to investigate if the different types or stages of tumours (expansive, infiltrative tumours, and metastases) showed differences in their manner of spontaneous healing.

**RESULTS**

Numerous serial histological sections of healing or already healed tumours showed that differentiation had occurred during spontaneous healing. Tumour cells were seen to have differentiated into apparently normal cells of *Triturus*
and to have become organized into tissues, normally found in this newt. In some cases these newly differentiated tissues had formed abnormal but not cancerous structures.

The differentiation of the cancer into normal cells depended upon the initial type of tumour growth observed. Expansively growing tumours which had differentiated yielded structures quite different from infiltrative tumours or metastases. The differentiation fate of each of these tumour types will be considered separately.

**Differentiation of expansively growing tumours**

The differentiation of such expansively growing tumours took place in various ways, e.g. differentiation into continuous tissues or differentiation by single cells without tissue continuity. Furthermore, abnormal cell types could also be observed.

Differentiation into continuous tissues usually took place by pigmentation and simultaneous cornification. This differentiation began by the appearance of an epithelial arrangement of the tumour cells on the margin of the tumour (Plate 4, fig. 20). This process did not set in simultaneously all over the tumour but started largely in those parts of the tumour which had most deeply penetrated into the musculature. In some cases, the beginning of this differentiation could be observed in areas of the tumour which were adjacent to blood-vessels. Those cells which were now epithelially arranged began to lengthen and were continuously supplemented by additional elongated cells also derived from the tumour (Plate 4, fig. 21). Subsequently, the formation of pigment granules was observed in these cells. This pigmentation became more intense and the cells began to take on dendritic shapes (Plate 5, fig. 26). Finally, a dense layer of pigment cells was formed at the periphery of the tumour (Plate 5, fig. 27).

Simultaneously, in the centre of the growth, a cornification process set in. Single cells rounded off and then became enveloped by flat pavement cells (Plate 4, fig. 22). By this process continuously new layers of squamous epithelium were formed (Plate 4, fig. 23). These layers, built up by former tumour cells, were histologically identical with the cornified external layer of the normal epithelium which is periodically desquamated. In this way, an onion-like multilayered structure was formed which was cornified in its centre. This structure continuously enlarged and therefore approached the peripheral pigment cell layer of the tumour. This process of cornification stopped at a distance of two to three cells from the pigment layer.

The resulting formation became stable. This final structure then consisted of an onion-like cornified centre which was enveloped by epithelium and pigment cell layers (Plate 4, fig. 24). This envelopment was very similar to the normal integument.

This similarity with a normal integument became further evident by the differentiation of mucous glands from the tumour (Plate 4, fig. 24). Numerous
small tumour-cell groupings were formed at the outer margin of the tumour (Plate 4, fig. 25). A swelling of cytoplasm could be observed in these cell groups, and, finally, the central cells of these groups disintegrated to form mucus. The resulting mucous glands were usually enveloped by the pigment cell layer (Plate 5, figs. 26, 27).

These mucous glands oriented their ducts to the centre of the cornified ‘onion’ where they formed thickened ends, filled with degenerated cells. This orientation of the gland ducts suggested that the cornified layers in the centre were also physiologically identical with the external layer of normal epithelium.

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**Text-fig. 3.** Scheme of the differentiation types in expansively growing tumours. A. *Simultaneous cornification and pigmentation process.* 1, beginning of central cornification and peripheral pigmentation (compare with Plate 4, figs. 20, 21, 22, 23). 2, final stage. An epithelium remains between the cornified and pigmented tissues. Mucous glands are not drawn in this scheme (Plate 4, fig. 24). B. *Cornification without pigmentation.* 1, beginning of cornification is seen in the centre of the tumour; no pigmentation occurs at its periphery. 2, final stage: the entire tumour is cornified (Plate 5, fig. 28). C. *Pigmentation without cornification.* 1, beginning of pigmentation at the periphery of the tumour is shown; no cornification in its centre takes place. 2, final stage: the entire tumour is melanized (Plate 5, fig. 29).

In such structures, the system of vessels of the arteria and vena cutanea, as well as the subepithelial connective tissue, were notably absent and in distinct contrast to the normal integument. The pigment cell layer of these structures was always in immediate contact with the musculature (Plate 5, figs. 26, 27). Once such a structure formed it remained stable for the entire period of observation, which was more than 9 months. Ninety per cent. of the expansively growing tumours in these newts showed this type of differentiation.

Other variations in tumour differentiation were cornification without pig-
mentation or pigmentation without cornification. If no pigmentation took place at the periphery of the tumour, the cornified onion-like structure enlarged until it reached the margin of the tumour. Therefore in these cases no epithelium and no mucous glands were formed and the whole tumour was cornified (Plate 5, fig. 28). Cornification without pigmentation was found to occur in deeply penetrating tumours. Several of these deep tumours arose following external application of the carcinogen.

On the other hand, if pigmentation without cornification occurred, the entire tumour differentiated into pigment cells. Pigmentation began at the periphery of the tumour and in this type of response, progressed centrally until the entire structure was pigmented.

This variation of tumour differentiation could only be observed in 4 cases: all of these were in tumours which followed injection of dibenzanthracene + benzpyrene. In this type of differentiation there was also no epithelium formation. The final stages of these responses resulted in a pigmented tongue or nodule which represented the former tumour (Plate 5, fig. 29).

These three different variations of differentiation seen in expansively growing newt tumours are shown in Text-fig. 3.

If tumour differentiation occurred without tissue continuity, the same differentiation processes were found as above but occurred in a less-organized form. Throughout the distal parts of the tumour differentiation of single cornified or pigment cells took place. The differentiated cells were scattered between the unchanged tumour cells (Plate 5, fig. 30). Cornification was very rare and difficult to see because of simultaneous degenerative processes. Pigmentation was imperfect; pigment cells contained only few pigment granules. These abnormal pigment cells did not show the oblong and dendriform shape of melanophores; usually they maintained the spherical shape of melanoblasts. Sometimes several pigment cells formed irregular clusters.

In conclusion, it can be stated that expansively growing tumours of the tail (stage 2 of tumour development) may differentiate into cornified, pigment, glandular, and normal epithelium cells. Differentiation occurred either into continuous tissues or only in single cells. In the latter case, the differentiation was less perfect. Necrosis of tumour cells could only exceptionally be observed. It occurred either in the centre of large expansive tumours or during the differentiation phase of large tumours as necrosis of single cells scattered between differentiating cells.

**Differentiation processes in infiltrating tumours of the tail and trunk (stage 3 of tumour development)**

Marked differences in the manner of differentiation were seen in infiltrating tumours. Large continuous tumour masses which showed peripheral infiltration differentiated in a similar manner as was seen in the expansively growing tumours described above. An onion-like structure was formed in the centre of
each of these masses and the margin of the tumour became pigmented. An epithelium then appeared between these two tissues. However, the 'onion'-structure did not cornify and developed only a few layers of flat epithelium which soon disintegrated by necrosis. The pigment layer usually was not prominent and mucous glands were seldom observed.

In contrast to the above, the differentiation of infiltrating rod-like tumour projections (as described in Part 1) occurred in a different manner. In these, only a few peripheral cells became pigment cells (Plate 6, fig. 33). The majority of the remaining tumour cells began to elongate in the longitudinal direction of the 'rod' (Plate 6, figs. 32, 33, 34). The cytoplasm of these cells increased and therefore the nuclei moved apart. Furthermore, intense longitudinal fibrillation appeared in this cytoplasm. By continuation of this tendency of differentiation, i.e. the alternation of tumour cells to elongated fibrous cells, the outer layer of the 'rods' took the appearance of fibrous connective tissue (Plate 6, fig. 35), while a centre core of tumour cells remained. Finally, the central core became thinner and even disappeared. This process was continued until only connective tissue-like cells were found at the site of the original tumour 'rod'. These cells initially remained different from the normal adjacent connective tissue, as judged by their more intense staining and increased fibrillation. Finally, they closely approached the appearance of normal connective tissue. Degeneration or necrosis was not seen in these rods.

Therefore two processes may be distinguished, (a) Transformation of the tumour cells into connective-tissue-like cells with rich fibrillation. These cells differ from the surrounding connective tissue, (b) Further transformation of this fibrous connective tissue to cells which closely resemble the surrounding connective tissue. This transformation was also seen in numerous transitional stages.

The transitional stages between tumour cells and fibrous connective-tissue-like cells are as follows. (1) Tumour cell: large spherical nucleus, scanty cytoplasm which stains intensively with haematoxylin (Plate 6, fig. 31). (2) Early transitional stage: oval or long nucleus with increased cytoplasm which stains intensively, beginning of fibrillation (Plate 6, fig. 32). (3) Later transitional stage: nucleus most elongated, cytoplasm intensively staining, with intense fibrillation (Plate 6, fig. 33). (4) Connective-tissue-like cell: most elongated nucleus, cytoplasm further increased and now less staining, but with intense fibrillation (Plate 6, fig. 35). This classification is very artificial, transition between tumour cells and connective-tissue-like cells was quite continuous.

The resulting tissue differed from the last stages of tumour-cell transformation only by its significantly paler tinge and differed from other connective tissues by its large nuclei and its more intense and irregular fibrillation.

In all newts investigated 4 weeks after the appearance of a tumour, only few remnants of the tumour could be found with indefinite transition to the connective tissue of the surrounding areas.
Differentiation of metastases

Spontaneous healing of metastases always occurred simultaneously with the healing of the primary tumour. It took place by differentiation of degenerative mucous cells. When a metastasis had grown to a certain size, single cells in its central part detached from the others and formed loose clusters (Plate 7, fig. 36), which stained with aniline blue. The cytoplasm increased and these cells became mucous cells. Additional cells of the metastasis continuously joined this cell group (Plate 7, fig. 37) and in this way the entire metastasis was transformed into clusters of degenerating mucous cells (Plate 7, fig. 38). These cells were finally extruded via a duct, probably deriving from a destroyed mucous gland. Therefore, spaces resulted which were later assimilated into the normal epithelium by a process of regeneration.

In contrast, the large metastases which were situated in the musculature did not disintegrate to form mucous cells but differentiated exactly in the same way as did the expansively growing tumours. In the centre of these large metastases a cornification process took place (Plate 7, fig. 39) and an onion-like multilayered structure was formed (Plate 7, fig. 40). The peripheral tumour cells differentiated to pigment cells. Between the inner cornified structure and the peripheral envelope of pigment cells an epithelium-like tissue was formed. The formation of mucous glands at the periphery, which was found in the differentiation of expansively growing tumours, was also seen in the differentiation of these large metastases.

The final structure (Plate 7, fig. 40) was identical with the structures formed by the differentiation of expansively growing primary tumours (Plate 4, fig. 24).

DISCUSSION

Part 2

Since differentiation phenomena have been observed in these growths, one might wonder if they are actually tumours. Let us examine the question: is differentiation of a growth a reason to deny its cancerous nature? Certainly the affirmative seems to be a prevalent opinion.

It seems reasonable to us that rather different circumstances prevail for the growth of tumours in animals with great regenerative powers as compared to tumour growth in the non-regenerating mammals. It is well known that newts are capable of differentiating new tissues from undifferentiated blastema cells during regeneration. The existence of this capacity in newts strongly suggested to us that these animals would be also capable of differentiating tumour cells during a process comparable to regeneration.

In fact there have been observations of differentiation phenomena reported even in mammalian tumours. Witten & Zak (1952) reported spontaneous healing with differentiation in a prickle-cell epithelioma. In some tumours of the bladder and the uterus differentiation into cross-striated musculature has been
observed (Hamperl, 1956). Pierce et al. (1960) succeeded in producing differentiations in a malignant carcinoma of the testis in mice. With repeated transplantation of this malignant carcinoma they obtained a terato-carcinoma in which glands, nerves, and even cartilage and muscles were differentiated. Numerous cases of tumour differentiation following X-ray have been compiled by Zöllinger (1960). These observations all show that differentiation of tumour cells can occur even in non-regenerating animals. In those animals such as newts, whose high differentiation capacity typically reveals itself during regeneration, we can even more reasonably expect that differentiation of tumour cells may occur. Therefore the differentiation of tumours in our newts can no longer be a reason to doubt the initial cancerous nature of this tumour which showed numerous malignant characteristics as described in Part 1.

If we assume that tumour differentiation shares certain similarities with a regeneration process, we should attempt to explain how spontaneous tumour differentiation becomes released. A regeneration process is normally released by amputation. As could be shown in triclads by Ortner & Seilern-Aspang (1962), any quantitative disturbance of the biological system, i.e. amputation or an addition of a certain quantity of tissue, released a regeneration process. Thus we would suggest that in the newts the destruction of a certain quantity of normal tissue by the growing tumour is the cause for the onset of the differentiation process.

We observed that the cells of infiltrating rod-like tumour projections differentiated into connective-tissue-like cells. Since the transformation from epithelial cells to mesodermal cells has often been described in newts during regeneration (Rose, 1948; Hay, 1952; Rose et al., 1955), it seems very likely to us that these connective-tissue-like cells had actually differentiated into true connective tissue.

It was striking that only infiltrating tumours differentiated to connective tissue whereas the differentiation of expansively growing tumours, i.e. less-advanced tumour stages, was restricted to the formation of tissues corresponding to the epithelial origin of the tumour (cornified cells, pigment cells, mucus cells). Since we observed increased pluripotential differentiation capacities in advanced tumour stages (differentiation of connective tissue from infiltrative tumours), this suggests that tumour growth not only causes a morphological dedifferentiation of the cells, but also an increase of pluripotency. In consequence of this observation we do not agree with Rose & Wallingford (1948) who suggested that a dedifferentiation of the tumour cells of the newts is still necessary before their differentiation.

The classical histological technique used here to follow the course of tumour-cell differentiation makes our observations probable but not proven. Other techniques which permit marking of individual cells are needed. The use of tritiated thymidine for this purpose is contemplated.

As already mentioned in Part 1 the carcinogen-induced hyperplasias of T.
**SUMMARY**

1. Epithelial tumours have been induced in *T. cristatus* by any of several carcinogens. The tumours arise from the mucous glands of the skin.

2. The tumours showed infiltrative and destructive growth, sometimes penetrating into the peritoneal cavity. Metastasis also occurred. The tumours thus appeared to be malignant.

3. In spite of its malignant characteristics this carcinogen-induced epithelial tumour often regressed. This regression of an apparently malignant tumour occurred spontaneously. During regression of the tumour, differentiation of the tumour cells into normal, non-malignant tissues occurred. Expansively growing tumours differentiated independently of their location into pigment cell layers, cornified layers, mucous glands, and epithelium of the integument. In contrast, infiltrating tumours differentiated in accordance with their surroundings and even connective-tissue-like cells were formed. Tumour metastases differentiated by the formation of degenerative mucous cells or in the same ways as expansively growing tumours. If no differentiation occurred, the tumours were lethal.

4. The capacity of newts to bring their tumour cells under control again by a differentiation process is attributed to the great regeneration power of these animals.

**ZUSAMMENFASSUNG**


2. Diese Tumoren zeigten infiltrierendes und destruierendes Wachstum, gelegentlich drangen sie in die Bauchhöhle ein. Es wurden auch Metastasen beobachtet. Diese Tumoren verhielten sich also wie eine maligne Geschwulst.


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REFERENCES


DIFFERENTIATION OF A NEWT TUMOUR


EXPLANATION OF PLATES

PLATE 1

Fig. 1. Normal integument of *T. cristatus. ct,* subcutaneous connective tissue; *e,* epithelium, with a cornified layer on its outer limit; *g,* mucous gland; *m,* musculature; *p,* pigment cells. × 75.

Fig. 2. Normal mucous gland of the skin. *d,* outlet duct; *g,* germinative layer of the gland pocket. × 300.

Fig. 3. Thickening of the epithelium during an inflammation process caused by injection of lactic acid. × 50.

Fig. 4. Inflammation process. The epithelium migrates from without into two damaged mucous glands. The right gland is already filled with epithelial cells, the left one only in its distal part. *g,* remnants of gland cells. × 100.

Fig. 5. Beginning of proliferation in the germinative layer of a mucous gland following injection of 2 per cent. benzpyrene. × 100

Fig. 6. Further proliferation of the cells of the germinative layer. The gland pocket has become filled in its basal part. Distal parts retain their glandular character. × 300.

Fig. 7. Two mucous glands filled with tumour cells. Outlet ducts are still open and remnants of mucus are being extruded. In the left gland, the growth is still limited by the gland wall, in the right one it has already perforated the gland pocket and coalesced with the epithelium. *m,* mucus. × 150.

PLATE 2

Fig. 8. Numerous mucous gland tumours which have coalesced together and with the epithelium form a large expansively growing tumour on the tail. × 50.

Fig. 9. A tumour of the trunk has penetrated into the musculature. Early infiltration may be seen. × 75.

Fig. 10. An infiltrating tail tumour has already reached the spine. *s,* spine. × 100.

Fig. 11. A region of a trunk tumour which infiltrates the musculature × 150.

Fig. 12. A low magnification of an infiltrating tumour on the trunk. The beginning of rod-like projections are seen. × 75.

Fig. 13. High-power view of infiltrating cells of an infiltrative and destructive tumour. *m,* musculature; *t,* tumour cells. × 400.

PLATE 3

Fig. 14. The tumour penetrates into the peritoneal cavity where it forms an epithelium-like pavement. × 50.

Fig. 15. Growth of a tumour which had penetrated into the peritoneal cavity. × 50.

Fig. 16. Metastases on the dorsal trunk, head, and extremities of *T. cristatus.* Metastases can be seen as white spots within the black skin of the newt. × 1, 5.

Fig. 17. Early metastasis which arises near the vessels of the arteria cutanea. Contact with the vessels not visible in this section. *e,* epithelium, *m,* metastasis, *v,* vessels of the arteria cutanea. × 250.

Fig. 18. Larger metastasis. × 250.

Fig. 19. Metastasis which has coalesced with the epithelium. Connective tissue and mucous glands are destroyed in its region. But it does not penetrate into the musculature. Note that primary tumour occurred only in the tail while the metastases were seen in the trunk. × 250.

PLATE 4

Fig. 20. Beginning of spontaneous healing of an expansively growing tumour. One layer of tumour cells arranges epithelially on the margin of the tumour. *ct,* connective tissue; *t,* tumour. × 250.

Fig. 21. The epithelial layer on the margin of the tumour has become more distinct. It becomes the pigment cell layer (the inner cornified part of the tumour was lost during preparation of the slide). × 200.

Fig. 22. Beginning of the cornification process in the centre of a small tumour. One single spherical cell is seen in the centre around which shell-like layers of cornified cells are arranged. Pigmentation of peripheral cells can be seen. × 400.
FIG. 23. Advanced cornification in the centre of an expansively growing tumour. Cornified layers form an onion-like structure. ×400.

FIG. 24. Large differentiated expansively grown tumour. In its centre note the onion-like structure of numerous cornified cell layers, on its margin an epithelium, a pigment cell layer and several mucous glands all derived from the tumour. ×50.

FIG. 25. Beginning of the formation of a mucous gland from tumour cells. A few peripheral cells have detached from the tumour and agglomerated. Pigment cell layer not yet differentiated. c, cornified cell layer in the centre of the tumour; g, beginning of a mucous gland. ×250.

PLATE 5

FIG. 26. Advanced differentiation of a mucous gland and of the peripheral pigment cell layer from tumour cells. There is no subcutaneous connective tissue between the differentiating tumour and the muscles. ×300.

FIG. 27. Further stage of the differentiation of a gland and of the pigment layer from tumour cells. No subepithelial connective tissue has been formed. ×400.

FIG. 28. Small and deeply penetrated masses of tumour cells which had detached from the tumour, originating following painting with 2 per cent. benzpyrene, have been totally cornified. Neither pigment nor epithelial cells nor mucous glands have been differentiated. ct, cornified tumour; s, spine. ×60.

FIG. 29. A tongue of a tumour which had penetrated into the musculature has been totally melanized. No cornification is present. ×100.

FIG. 30. Irregularly scattered and imperfect differentiation of a tumour into pigment and cornified cells. No tissues were formed. c, cornified cell; p, pigment cell. ×100.

PLATE 6

FIG. 31. Tumour tissue before the start of differentiation processes. ×400.

FIG. 32. Infiltrative tumour at the beginning of the differentiation phase, the cells and nuclei begin to elongate. ×400.

Figs. 33 & 34. Infiltrating rod-like tumour projection during its differentiation. Tumour cells are still more attenuated, pigment cells are differentiated on the periphery. Fig. 33: ×300; Fig. 34: ×200.

FIG. 35. An infiltrating rod-like tumour projection in advanced differentiation. Elongation of the nuclei and formation of fibrous cytoplasm is seen. The former tumour cells, originally deriving from gland cells, now assume a connective-tissue-like appearance. ×300.

PLATE 7

FIG. 36. Metastasis whose degraded cells detach into a newly formed cavity in its centre. ×250.

FIG. 37. One degraded cell can be seen to be advancing into a cavity of the metastasis. There tumour cells become mucous cells. mc, mucous cells; tc, tumour cell which is just entering the cavity. ×400.

FIG. 38. The metastases have been differentiated by progressive detachment of tumour cells into the enlarging cavities and by their transformation to mucous cells. ×150.

FIG. 39. Early stage of the differentiation of a metastasis in the musculature of the trunk (primary tumour on the tail). In the centre of the metastasis a few layers of cornified flattened epithelium already have been formed. cl, cornified layers; m, musculature; met, metastasis. ×150.

FIG. 40. Late stage of differentiation of a large metastasis situated within the musculature of the trunk (primary tumour on the tail). Cornification has set in at two centres and therefore two 'onions' of cornified layers have been formed. Differentiation of pigment cells has occurred at the periphery of the metastasis. Furthermore, three mucous glands have been differentiated. cl, cornified layers; m, musculature; mg, mucous glands; pc, pigment cells. ×75.

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Plate 1

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Plate 5
Plate 7