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A Review of the Chemical Induction of Neoplasms in Amphibia

By M. BALLS

Station de Zoologie expérimentale, Université de Genève, Chêne-Bougeries (Suisse)

and L. N. RUBEN

The Reed Institute, Portland (Oregon, USA)

A recent review[1] demonstrated that a wide variety of spontaneous neoplasms has been found in an equally wide variety of amphibian species, and lent further support to the view that these poikilotherms are no less susceptible to neoplastic growth than other vertebrates. Indeed, a number of spontaneous amphibian tumours, most notably the renal adenocarcinoma of *Rana pipiens*, are being used in attempts to deepen our understanding of the neoplastic process[2–5]. The use of amphibia in the study of experimental chemical carcinogenesis has, however, been less satisfactory. LUCKÉ and SCHLUMBERGER[6] considered, in 1949, that there was no conclusive evidence that true neoplasms had been chemically induced in amphibians, and suggested that this was largely due to inadequate experimental conditions. A certain amount of progress has been made since 1949, and the present review represents a summary of what has been achieved in efforts to induce tumours in Amphibia with chemical carcinogens and an attempt to point out those aspects which require further clarification.

Amphibians may be divided into three main groups: the tailed Urodela, which includes the newts and salamanders, the tail-less Anura including the frogs and toads, and the almost legless Apoda. This division is important in any discussion of amphibian carcinogenesis, since the urodeles retain in adult life the ability to regenerate lost extremeties, whereas anurans cannot replace lost parts after the metamorphic change from the tadpole to the adult form without the establishment of special environmental conditions. The Apoda have been largely ignored in this type of experimentation.

A number of authors[7,8] have attempted to summarize previous attempts at chemically inducing tumours in amphibians in the form of tables, but it may be useful to provide additional discussion here regarding some of the features of previous experiments which were not amenable to tabular treatment. Therefore, short abstracts of the reported attempts are given as a basis for discussion.

Abstracts of reported attempts at chemically inducing tumours in amphibians

(The abstracts are placed in chronological order of publication of the original reports. MC = methylcholanthrene, BP = benzpyrene, DBA = dibenzanthracene.)

a. In *Anura:*

STEFKO, 1924[9], painted the skin of *Rana temporaria* and *Bufo viridis* with tar and observed that hyperplasia of the painted epithelium was followed by rupture of the basal membrane and infiltration of the sub-cutaneous tissues. Autopsy showed fatty degeneration of the heart, the liver and, sometimes, the kidneys.

KINOSHITA, 1937[10], treated toads (*Rospo* sp.) with various of the then recently-discovered pure carcinogenic hydrocarbons, but none developed tumours.

DURAN-REYNALS, 1939[11], injected MC, BP and DBA suspended in oil into the thigh muscle of 261 frogs (*Rana pipiens, R. clamitans, R. catesbiana*), but most were dead within one month, all within a few months, and none bore tumours.

BRIGGS, 1940[12], injected MC in choleic acid into 154 *Rana pipiens* tadpoles. Three developed tumours, one of which was a connective tissue tumour of mixed cell type, which spread along the base of the dermis and penetrated the body wall muscle, coelom and lung wall.

BRIGGS and BRIGGS, 1943[13], placed *Rana pipiens* embryos in water-soluble forms of MC and DBA, and found that embryonic development was retarded.

NEUKOMM, 1944[14], injected frog tadpoles (*Rana* sp.) with tar, MC or BP, but none developed tumours.

SKAPIER, 1948–50[15], treated 300 toads (*Rospo* sp.) with tar, MC or BP in oil – by subcutaneous injection or by painting the paws twice. All the toads died within 30 days, but none bore tumours.

SCHLUMBERGER, 1949[16], implanted crystalline MC directly into the kidney of 111 *Rana pipiens*, but more spontaneous renal adenocarcinomas were found in the uninoculated controls than renal tumours in the treated animals.

[1] M. BALLS, Cancer Res. *22*, 1142 (1962).
[2] W. DURYEE, Ann. N.Y. Acad. Sci. *63*, 1280 (1956).
[3] T. J. KING and R. G. MCKINNELL, in *Cell Physiology of Neoplasia* (University of Texas Press, Austin 1960).
[4] L. N. RUBEN, Rev. suisse Zool. *70*, 224 (1963).
[5] K. TWEEDELL, J. Morph. *107*, 1 (1960).
[6] B. LUCKÉ and H. G. SCHLUMBERGER, Physiol. Rev. *29*, 91 (1949).
[7] V. LEONE, Tumori *39*, 420 (1953).
[8] S. NEUKOMM, Oncologia *10*, 107 (1957).
[9] W. STEFKO, Z. Krebsforsch. *21*, 432 (1924).
[10] R. KINOSHITA, Trans. Soc. Path. Japan *27*, 665 (1937).
[11] F. DURAN-REYNALS, Yale J. Biol. Med. *11*, 613 (1939).
[12] R. W. BRIGGS, Nature (London) *146*, 29 (1940).
[13] J. B. BRIGGS and R. W. BRIGGS, Cancer Res. *3*, 1 (1943).
[14] S. NEUKOMM, Mém. Soc. Vaud. Sci. nat. *8*, 137 (1944).
[15] J. SKAPIER, Acta Un. int. Cancr. *6*, 65 (1948–50).
[16] H. G. SCHLUMBERGER, J. Nat. Cancer Inst. *9*, 111 (1948).

PFLUGFELDER, 1949[17], produced papillary adenomas (epidermal hyperplasia – see [18]) in *Rana ridibunda* treated with chloral hydrate, chloroform, *o*-amido-toluol or follicular hormone. PFLUGFELDER also found[19] that, by growing *Xenopus laevis* tadpoles in water containing potassium perchlorate, he could prevent metamorphosis and prolong larval life from six weeks to 18 months. Tumour-like lesions developed in those larval organs which are normally resorbed at metamorphosis and whose life span had been so greatly prolonged.

BALLS, 1962[20] and 1963[21], found that 20 of 42 *Xenopus laevis laevis* given MC crystals alone or in arachis oil developed lymphosarcomas after an average of 260 days. The implantation of BP crystals also gives rise to lymphosarcomas in this species[22]. The induced tumours were similar to those occurring spontaneously in *Xenopus*[1], and, like them, could be transferred to other *Xenopus*[23,24].

STRAUSS and MATEYKO, 1963[25], found that renal adenocarcinomas developed in *Rana pipiens* given intra-renal injections of MC, BP, DBA or other carcinogens suspended in olive oil. We look forward to the publication of a detailed account of these experiments, since LUCKÉ[26] showed that 2.7% of frogs caught in the Lake Champlain area bore spontaneous renal adenocarcinomas, but RAFFERTY[27] has observed much higher incidences (up to 50%) in frogs maintained in a laboratory.

RUBEN and BALLS[28] implanted MC crystals into the non-regenerating or regenerating forelimbs of 63 post-metamorphic, immature *Xenopus laevis laevis* and into the abdominal cavity of 15 animals of the same species. Lymphosarcomas developed in 100% of the cases in some of the groups regardless of the implantation site, but smaller doses of crystals more often led to tumour formation than larger doses, most of which tended to be ejected by the epidermis. Supernumerary normal cartilages as well as lymphoid tumours formed in some limbs. Regenerating limbs in association with MC failed to control the development and spread of the lymphoid tumours, though no neoplastic change occurred in regeneration-competent cells of the imbs themselves.

b. In *Urodela:*

CHAMPY and VASILIU, 1923[29], painted the skin of *Triturus cristatus* with tar but, apart from skin ulceration and cachexia, the results were negative.

HELLMICH, 1928[30], used Sudan III with *Amblystoma tigrinum*, one of which developed a benign tumour, and *Triturus cristatus*, one of which developed a preblastematous process (or epidermal proliferation[18]).

MARTELLA, 1935[31], painted the skin of *Triturus cristatus* with tar in oil, but found that no neoplasms resulted.

DURAN-REYNALS, 1939[11], injected DBA into the thigh muscle of 18 newts (*Triturus pyrrhogaster* and *T. viridescens*), but none developed tumours.

KOCH, SCHREIBER, and SCHREIBER, 1939[32], treated the skin of *Triturus cristatus* and *T. taeniatus* with tar and BP in olive oil. A high percentage of the treated animals developed epithelial neoplasia, which infiltrated and destroyed normal tissues and metastasized to the heart and lungs.

TOKIN, 1940[33], found that a watery tar solution inhibited regeneration in *Amblystoma* and the axolotl (*Siredon mexicanum*).

PEREDELSKY, 1941, see [34], found that 2 of 12 axolotls given DBA and one of 8 given scarlet red developed tumours, though 6 given paraffin oil did not.

SHEVCHENKO, 1941[35], implanted MC crystals into the leg muscles of axolotls, some of which developed spindle cell sarcomas which invaded the leg muscles and bones.

FEDOTOV, 1941, see [36], found epithelial proliferation in the axolotl after treating the skin with DBA.

CIAGGIO and PACCAGNELLA, 1943[37], found that axolotls and *Triturus cristatus* developed granulomas after being treated with BP in oil.

NEUKOMM, 1944[14], gave newts (*Triturus cristatus*) subcutaneous injections of tar, MC or BP in oil. If the injections were into neutral regions (i.e. those unable to regenerate), 'epithelial neoplasia with normal cells' or 'epithelial neoplasia with atypical cells' developed. The tumours developed locally by epidermal hyperplasia, did not metastasize and, after a period of active infiltration, regressed. When carcinogens were injected into regions able to regenerate (limbs and tail), the reaction depended on the regenerative state of the region. Blastemas and regenerates up to 36 days after amputation showed intense epidermal hyperplasia without rupture of the basal lamella or infiltration, but regenerates more than 36 days old and non-amputated limb or tail skin reacted as did that of the neutral regions. NEUKOMM later proposed that the reaction of newt skin to carcinogens was specific and could be used as a rapid and sensitive test of the degree of carcinogenicity of chemical substances, whereby epidermal hyperplasia plus infiltration would indicate a positive result[8,38].

FINKELSTEIN, 1944[36], found non-malignant hyperplasia in four of 200 axolotls one year after their skin was treated with MC.

PRADA, 1947[39], observed effects on regeneration but no carcinogenesis in *Triturus vulgaris* treated with BP.

PFLUGFELDER, 1949[17], induced epidermal hyperplasia in *Triturus cristatus* as in *Rana ridibunda*.

RAUNICH, 1949[40], found epidermal proliferation and infiltration when BP in oil was injected subcutaneously into *Triturus cristatus*.

OVERTON, 1950[41], observed that the implantation of MC crystals under the skin of the dorsal fin of *Amblystoma opaca* and *A. punctatum* larvae stimulated mitosis in the overlying epidermis with the subsequent extrusion of the implanted material.

MORETTI and BELLINI, 1951[42], also found epidermal hyperplasia and infiltration in *Triturus cristatus* skin following BP treatment.

BREEDIS, 1952[43], injected various combinations of olive oil, vaseline, coal tar, MC, BP, acetylaminofluorene, scarlet red and beryllium hydroxide into the limbs of 500 newts (*Triturus viridescens*). Only two males given MC in olive oil developed spindle cell-sarcomas of the limb. The sarcomas were highly invasive and were successfully transferred to other hosts.

STRIGAZZI, 1952[44], injected BP in olive oil subcutaneously into *Triturus cristatus* and subsequently found neoplastic epithelial nuclei.

KARCZMAR and BERG, 1952[45], found that a water soluble form of DBA and MC in oil affected the regeneration of the forelimbs of *Amblystoma punctatum* and *Pseudotriton ruber* larvae, but with no indication of tumorigenesis.

LEONE, 1953[7], injected BP subcutaneously into newts (*Triturus cristatus*) and noted that the resulting epithelial proliferations destroyed the basal membrane of the skin and the chromatophore layer, then invaded the subcutaneous connective tissue and muscles. He called the proliferations 'spindle-cell epitheliomas'.

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[18] E. ARFFMANN and B. C. CHRISTENSEN, Acta path. microbiol. scand. *52*, 330 (1961).
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[20] M. BALLS, Nature (London) *196*, 1327 (1962).
[21] M. BALLS, Rev. suisse Zool. *70*, 595 (1963).
[22] M. BALLS, Exper., *20*, 143 (1964).
[23] M. BALLS, Cancer Res. *24*, 44 (1964).
[24] L. N. RUBEN and M. BALLS, in preparation.
[25] E. STRAUSS and G. M. MATEYKO, Proc. Am. Assoc. Cancer Res. *4*, 66 (1963).
[26] B. LUCKÉ, Ann. N.Y. Acad. Sci. *54*, 1093 (1952).
[27] K. A. RAFFERTY, J. Nat. Cancer Inst. *29*, 253 (1962).
[28] L. N. RUBEN and M. BALLS, in preparation.
[29] C. CHAMPY and J. VASILIU, Bull. Cancer *12*, 111 (1923).
[30] W. HELLMICH, Z. Krebsforsch. *28*, 44 (1928).
[31] N. A. MARTELLA, Riv. Biol. *18*, 197 (1935).
[32] C. KOCH, B. SCHREIBER, and G. SCHREIBER, Bull. Cancer *28*, 852 (1939).
[33] B. TOKIN, Dan. *29*, 518 (1940) (see also FINKELSTEIN[36]).
[34] J. L. HARTWELL, *Survey of Compounds which Have Been Tested for Carcinogenic Activity*, Public Health Service Publication No. 149, 2nd ed. (Bethesda, Maryland 1951).
[35] N. N. SHEVCHENKO, Byull. eksper. Biol. Meed. *11*, 395 (1941).
[36] E. A. FINKELSTEIN, Uspekhi Sovremennoi Biol. *17*, 320 (1944).
[37] C. CIAGGIO and B. PACCAGNELLA, Atti. R. Ist. Veneto Sci. Lett. Arti. *102*, 223 (1943).
[38] S. NEUKOMM, Acta Un. int. Cancr. *15*, 654 (1959).
[39] N. PRADA, Tumori *32*, 151 (1947).
[40] L. RAUNICH, Riv. Biol. *41*, 91 (1949).
[41] J. OVERTON, J. exp. Zool. *115*, 521 (1950).
[42] G. F. MORETTI and L. BELLINI, Boll. Soc. ital. Biol. sper. *27*, 966 (1951).
[43] C. BREEDIS, Cancer Res. *12*, 861 (1952).
[44] I. STRIGAZZI, private communication to LEONE[7] (1952).
[45] A. G. KARCZMAR and G. G. BERG, J. Morph. *91*, 479 (1952).

SCHREIBER and FRANCESCHI, 1954[46], placed cellophane under the skin of *Triturus cristatus* and observed the epithelial reaction we have described above.

CAPUANI, 1957[47], confirmed the results of LEONE[7] using BP and *Triturus cristatus*.

LEONE, 1957[48], placed M crystals under the skin of the flank of 47 newts (*Triturus cristatus*), 22 of which developed lymphosarcomas, which metastasized to the visceral organs, particularly to the liver and spleen. These tumours were readily transplantable.

STOLK, 1961[49], found intestinal carcinomas 40 days after the first of eight daily injections of 0.1 ml of a 5% solution of herring sperm DNA into *Triturus alpestris*.

ARFFMANN and CHRISTENSEN, 1961[18], repeated NEUKOMM's[14] newt test experiments with *Triturus cristatus* and concluded, with reservations, that the test was useful. ARFFMANN has also studied the effects of the solvents which were used to carry the chemicals tested[50]. No positive results were obtained with the solvents.

SEILERN-ASPANG and KRATOCHWIL, 1962[51], treated newts (*Triturus cristatus*) with combinations of MC, BP and DBA by subcutaneous injection of carcinogen suspended in oil or by painting the skin with carcinogen dissolved in benzene. Epithelial tumours derived from the mucous glands of the skin showed infiltrative and destructive growth, and metastasis to lungs, musculature and kidneys. Although apparently malignant, the tumours often regressed and differentiated into normal, non-malignant tissues; expansively-growing tumours differentiated into pigment cell layers, cornified layers, mucous glands and integument epithelium, while infiltrating tumours differentiated into connective tissue cells. If no differentiation occurred, the tumours were lethal.

Discussion

1. *The amphibian species used.* The different amphibian species used in the reported attempts at the chemical induction of tumours are summarized in Table I. The choice of species has often been determined by local availability; thus, for example, *Triturus cristatus* has been widely used by European authors. Other species have been chosen for special reasons; SCHLUMBERGER[16] and STRAUSS and MATEYKO[25] implanted carcinogens into the kidney of Vermont *Rana pipiens* since renal adenocarcinomas occur spontaneously in these animals[26]. The present authors used *Xenopus laevis*[20,21,22,28] since spontaneous lymphosarcomas and other tumours have been found in this species[1] and newly-metamorphosed *Xenopus* still possess considerable regenerative ability[24].

2. *Classification of positive results.* The most frequently observed amphibian reaction to the presence of carcinogens was the proliferation and downgrowth of the skin epithelium, classed, for the moment, in Table II as 'epidermal hyperplasia'. In view of its complicated and controversial nature, this cutaneous reaction will be discussed at length later in this review.

Only nine of the 41 known reports discussed experimental procedures resulting in clearly malignant, neoplastic growths (Table III). LEONE[48] found that 40% of 49 newts developed lymphosarcomas after being treated with methylcholanthrene crystals alone, but BRIGGS[12] induced only 3 tumours in 154 *Rana pipiens* tadpoles given injections of methylcholanthrene in choleic acid, and BREEDIS[43] found only two sarcomas in 500 newts treated with various carcinogens in oil. Moreover, in experiments with *Xenopus*, one of us (M.B.) found that, of 14 animals given methylchol-

Table I. Species used in tumour induction attempts

Species	Total number of reports	Number of reports	
		Positive[a]	Negative[a]
Anura			
Bufo viridis	1	1	
Rana sp.	1		1
Rana catesbiana	1		1
Rana clamitans	1		1
Rana pipiens	5	2	3
Rana ridibunda	1	1	
Rana temporaria	1	1	
Rospo sp.	2		2
Xenopus laevis laevis	4	4	
Urodela			
Amblystoma sp.	1		1
Amblystoma opaca	1	1	
Amblystoma punctatum	2	1	1
Amblystoma tigrinum	1	1	
Pseudotriton ruber	1		1
Siredon mexicanum	6	4	2
Triturus alpestris	2	2	
Triturus cristatus	16	13	3
Triturus pyrrhogaster	1		1
Triturus taeniatus	1	1	
Triturus viridescens	2	1	1
Triturus vulgaris	1		1

[a] Epithelial hyperplasia in adults is, for the present, counted as a positive result, interference with regeneration or embryonic development or the production of granulomas as negative.

Table II. Classification of positive reports

	Number of reports		
	Anura	Urodela	Total
Total number of reports	14	27	41
Positive reports	8	21	29
(1) *Epithelial reactions:*			
Epidermal hyperplasia	2	15	17
Carcinoma		1	1
Adenocarcinoma	1		1
(2) *Mesenchymal reactions:*			
Sarcoma	1		1
Lymphosarcoma	3	1	4
Spindle-cell sarcoma		2	2
(3) *Miscellaneous:*			
Benign tumour		1	1
Tumours		1	1
Larval-organ tumours	1		1

[46] B. SCHREIBER and P. FRANCESCHI, Boll. Zool. *21*, 273 (1954).
[47] M. CAPUANI, Riv. Biol. *49*, 151 (1957).
[48] V. LEONE, Ist. Lomb. Sci. Lett. Sci. (B) *92*, 220 (1957).
[49] A. STOLK, Exper. *17*, 254 (1961).
[50] E. ARFFMANN, Acta path. microbiol. scand. *55*, 281 (1962).
[51] F. SEILERN-ASPANG and K. KRATOCHWIL, J. Embryol. exp. Morph. *10*, 337 (1962).

Table III. Summary of malignant neoplasms chemically induced in amphibia[a]

Authors (date)	Species used	Chemicals used[b]	Resultant tumour	Sites affected	Transplantable	Similar spontaneous tumours known in same species
BRIGGS (1940)[12]	*Rana pipiens* (tadpole)	MC in choleic acid	Sarcoma	dermis, muscle and lung	–	no
SHEVCHENKO (1941)[35]	*Siredon mexicanum*	MC crystals	Spindle-cell sarcoma	leg muscles and bones	–	no
BREEDIS (1952)[48]	*Triturus viridescens*	MC in olive oil	Spindle-cell sarcoma	limb muscle	yes	no
LEONE (1957)[48]	*Triturus cristatus*	MC crystals	Lympho-sarcoma	limb muscle	yes	no
STOLK (1961)[49]	*Triturus alpestris*	Herring sperm DNA solution	Carcinoma	intestine	–	no
BALLS (1962–63)[20,21]	*Xenopus laevis*	MC in arachis oil or as crystals alone	Lympho-sarcoma	liver, kidneys, spleen and others	yes	yes
BALLS (1964)[22]	*Xenopus laevis*	BP crystals	Lympho-sarcoma	liver, kidneys, spleen and others	yes	yes
RUBEN and BALLS[28]	*Xenopus laevis*	MC crystals	Lympho-sarcoma	liver, kidneys, spleen and others	yes	yes
STRAUSS and MATEYKO (1963)[25]	*Rana pipiens*	MC, BP, DBA, etc. in olive oil	Adeno-carcinoma	kidneys	–	yes

[a] Not including 'epidermal hyperplasia', or the 'miscellaneous' results from Table II. [b] MC = methylcholanthrene, BP = benzpyrene, DBA = dibenzanthracene.

Table IV. The reaction of amphibian skin to carcinogens

Authors (date)	Species used[a]	Carcinogen used[b]	Diagnosis	Invasion	Metastasis
STEFKO (1924)[9]	*Rana temporaria* and *Bufo viridis*	Tar	Epidermal proliferation	+	
HELLMICH (1928)[30]	*Triturus cristatus*	Sudan III	Preblastematous processes		
KOCH, SCHREIBER, and SCHREIBER (1939)[32]	*T. cristatus* and *T. taeniatus*	Tar, BP	Epithelial neoplasia	+	+
FEDOTOV (1941)[36]	*Siredon mexicanum*	DBA	Epithelial proliferation		
NEUKOMM (1944)[14]	*T. cristatus* and *T. alpestris*	Tar, BP, MC	Epithelial neoplasia with typical and atypical cells	+	
FINKELSTEIN (1944)[36]	*Siredon mexicanum*	MC	Non-malignant hyperplasia		
PFLUGFELDER (1949)[17]	*T. cristatus* and *Rana ridibunda*	Chloral hydrate etc.	Papillary adenoma		
RAUNICH (1949)[40]	*T. cristatus*	BP	Epithelial proliferation	+	
OVERTON (1950)[41]	*Amblystoma opaca* and *A. punctatum*	MC	Epithelial proliferation		
MORETTI and BELLINI (1951)[42]	*T. cristatus*	BP	Epidermal hyperplasia	+	
STRIGAZZI (1952)[44]	*T. cristatus*	BP	Neoplastic epithelial nuclei		
LEONE (1953)[7]	*T. cristatus*	BP	Spindle-cell epithelioma	+	
SCHREIBER and FRANCESCHI (1954)[46]	*T. cristatus*	Cellophane	Epithelial reaction		
CAPUANI (1957)[47]	*T. cristatus*	BP	Epithelial reaction	+	
ARFFMANN and CHRISTENSEN (1961)[18]	*T. cristatus*	MC, BP, DBA	Epithelial proliferation	+	
SEILERN-ASPANG and KRATOCHWIL (1962)[51]	*T. cristatus*	MC, BP, DBA	Epithelial tumours	+	+

[a] *T. cristatus* = *Triturus cristatus*. [b] BP = benzpyrene. MC = methylcholanthrene. DBA = dibenzanthracene.

anthrene in arachis oil, 5 (36%) developed lymphosarcomas, whereas 49 of 66 (74%) of those given methylcholanthrene or benzpyrene crystals alone subsequently bore similar tumours[21,22]. Some of the *Xenopus* in the former experiments died as a result of a lipogranulomatous reaction of the peritoneum, presumably as a result of the presence of the oil. It would thus seem that the implantation of carcinogen crystals alone is more likely to lead to malignant tumour formation than the injection of carcinogens suspended in oils or fats.

A common factor in the experiments producing lymphoid tumours in *Triturus* or in *Xenopus* was the use of a 5% solution of urethan (ethyl carbamate) in water for the anaesthetization of the hosts during carcinogen implantation. Moreover, in some of our experiments[28] urethan was used as anaesthetic at weekly intervals during the experiment for the immobilization of the hosts to allow macroscopic observation of the progress of the interaction of regenerate and carcinogen. In view of its co-carcinogenic or tumouraugmenting properties[52], the possibility that the urethan played a part in the induction of the tumours must be considered. Tests of the role of urethan in experiments involving *Xenopus* are now in progress.

Although one of us (M.B.) had previously noted[21] that there was a much longer latent period between the implantation of methylcholanthrene crystals into *Xenopus* and the appearance of lymphoid tumours than was observed by LEONE in crested newts (*Triturus cristatus*)[48], we found in a later series of experiments[28] that 14 of 15 immature *Xenopus* developed widespread and highly lethal lymphosarcomas only 39 days after methylcholanthrene treatment. It appears that there is a wide variation according to parentage in the ability of *Xenopus* from different matings to form tumours at all and in the latent period involved.

The plea of TANNENBAUM[52] that investigators should differentiate between the augmentation of normally expected neoplasms and the *de novo* induction of tumours must also be borne in mind. On the basis of the known occurrence of spontaneous neoplasms in amphibian species[1], the production of lymphosarcomas in *Xenopus* and of renal adenocarcinoma in *Rana pipiens* would be an augmentation of the incidence of normally expected tumours, whereas, within the limits of this present knowledge, the other experiments summarized in Table III seem to involve *de novo* induction.

Attempts to transfer the sarcomas induced in *Triturus viridescens*, *Triturus cristatus*, and *Xenopus laevis* to other hosts were successful, which speaks for their true, neoplastic nature.

We have not sufficient information concerning the benign tumour produced in *Amblystoma* by HELLMICH[30] or the tumours induced in axolotls by PEREDELSKY (see [34]) to permit their inclusion in Table III.

3. *Reaction of amphibian skin to carcinogens.* A number of authors treated the skin of amphibians with carcinogens, usually tar, MC, BP or DBA in oil, and observed epithelial proliferation with or without invasion of the subcutaneous layers and distant metastasis (Table IV). Most of the experiments involved the European crested newt, *Triturus cristatus*, though similar cutaneous reactions have been observed in other urodele species and in anurans. The names used to describe the reaction vary from non-malignant epithelial hyperplasia through epithelial neoplasia and papillary adenoma to spindle-cell epithelioma.

Since this cutaneous reaction so dominates the question of experimental chemical carcinogenesis in amphibia, three aspects will be discussed in an attempt to clarify the significance of the reaction and the associated questions needing further clarification:

(a) *The essential similarity of the observed reactions.* There seems little doubt that, on the whole, whatever the species or carcinogen used, and whether the regeneration-competent or neutral areas of the animal are treated, the amphibian skin shows a characteristic reaction when carcinogens suspended in oil are injected into it. The normal amphibian skin consists of an outer, cornified layer of flattened cells which is limited by a basement lamella beneath which is a layer of pigment cells, blood vessels and nerves. Also in the dermis are large mucous and other glands surrounded by connective tissue. The dermis is bounded by a thin muscle layer. In urodeles subcutaneous connective tissue binds the skin to the muscle layers beneath, but anurans have lymph sacs beneath the skin.

A few days after carcinogen in oil is injected into the skin, there is a thickening of the epidermis and epithelial downgrowth into the dermis by rupture of the basal lamella and infiltration of epithelial cells into the pigmented layer. The downgrowth continues to include the dermal glands and connective tissue and subcutaneous muscle.

Various degrees of this reaction were reported by numerous authors both before and after the extensive investigation of NEUKOMM[14]. Only SEILERN-ASPANG and KRATOCHWIL[51] seem to have observed a very different reaction. These authors found that if carcinogens were injected into the mucous glands of the skin, the epithelial hyperplasia occurred in the glands rather than in the epidermis. Whether this is different from the general reaction in kind rather than in degree is not known.

(b) *The specificity of the cutaneous reaction.* NEUKOMM[14] found that tar, MC and BP were 100% effective in producing the cutaneous reaction in newts, and later[8] suggested that this reaction could be used as a rapid, highly specific test for the carcinogenicity of chemical substances. It was found, however, that the

[52] A. TANNENBAUM, Acta Un. int. Cancr. *17*, 72 (1961).

test was less positive for nitrogenous hydrocarbons and azo-dyes known to be carcinogenic in mammals, so the proposed applicability of the test was limited to polycyclic hydrocarbons. In a subsequent report[38] a positive result was defined as epidermal hyperplasia plus downgrowth. NEUKOMM and LUDER-HUGUENIN[53] then suggested a quantitative method of expressing the neoplastic power of chemical compounds on the basis of the strength of response of the newt skin and the time of appearance of the response.

ARFFMANN and CHRISTENSEN[18] repeated and confirmed NEUKOMM's experiments with BP, MC and DBA, but criticized the quantitative estimations of neoplastic power in view of the subjective nature of the grading of the histological response. OVERTON[41] found that methylcholanthrene crystals, paraffin and glass beads were extruded a few days after they were implanted under the skin of larval *Amblystoma*. Histological sections showed the early breakdown of the basement lamella, the inward migration of cords of epidermal cells, then the walling off and extrusion of the implanted material. Such extrusion probably occurred when BRIGGS[12] injected methylcholanthrene into 154 *Rana pipiens* tadpoles and only 12 retained the carcinogen, and crystal extrusion was certainly observed when methylcholanthrene was placed in the forelimbs of *Xenopus*[28].

LUCKÉ and SCHLUMBERGER[6] considered the cutaneous reaction to be unspecific and the result of prolonged irritation, and other authors[24,54,55] have provided evidence of the role of massive epithelial movements in removing foreign bodies or cellular implants placed under the amphibian skin. Thus, epidermal hyperplasia and downgrowth is not a specific response to the presence of carcinogens alone.

(c) *The neoplastic nature and malignancy of the cutaneous response.* Although no definition of a neoplasm is completely satisfactory, that of WILLIS[56] could well be mentioned here: 'A tumour is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change'. The response of amphibian skin to the presence of carcinogens has been called neoplastic and malignant because of the observed epithelial downgrowth and infiltration of the dermis and muscle layers. Yet, this does not completely satisfy WILLIS's definition, for it seems likely that the downgrowth may occur in response to the presence of foreign material and that the depth of infiltrative penetration is related to the depth of influence of the foreign material rather than to an autonomous, increasing, cell-destroying, neoplastic progression. Indeed, the reactions observed by NEUKOMM[14], ARFFMANN and CHRISTENSEN[18] and SEILERN-ASPANG and KRATOCHWIL[51] *healed*, which seems to point to a cessation of excessive growth after cessation of the stimulus.

Malignant tumours tend to be distinguishable from benign ones on the basis of their infiltrative, expansive, rapid and unceasing growth and metastasis. Therefore, in view of the normal massive movements of amphibian epidermis, findings of distant metastases take on increased importance in this discussion. KOCH, SCHREIBER, and SCHREIBER[32] reported metastases of the epithelial lesions in the heart and lungs, and SEILERN-ASPANG and KRATOCHWIL[51] found metastases in the lungs, musculature and kidneys, though it is not clear whether this was due to the spread of the carcinogen or of tumour cells.

Thus, with the possible exception of the two reports of apparent metastasis, it seems that none of the reports of epithelial reactions summarized in Table IV may be said with any certainty to have involved malignant neoplasia. Indeed, it is our opinion that most of the observed epithelial downgrowths represent attempts at removing the irritating agents from the body rather than specific cancerous reactions to the presence of carcinogens.

4. *Carcinogenesis, regeneration and development.* In spite of the importance of the hypothesis developed by WADDINGTON[57] and later restated by NEEDHAM[58] that cancer formation represents a morphological escape from developmental controls, there have been few attempts to use the post-embryonic morphogenetic fields of amphibia, that is the regeneration-competent appendages, for the study of carcinogenesis. No cases of spontaneous primary cancer formation in amphibian limb tissues have been reported[1]. Furthermore, although some authors[14,25,45] report a retardation of regeneration in the presence of carcinogens, in only two reports have tumours of non-cutaneous, regeneration-competent limb tissues been induced with carcinogens, spindle-cell sarcomas in both cases[43,35]. The experiments of BREEDIS[43] are especially interesting since they demonstrate the ability of urodele limbs to form supernumerary normal tissues *or* tumours in response to carcinogenic substances. In fact, while some 60 implanted sites developed supernumerary limbs, only two sarcomas resulted.

Our own experiments have recently led us to restate our view of the significance of the morphological escape hypothesis. In one group of experiments[24], the highly lethal, metastatic lymphosarcoma of *Xenopus laevis laevis* was placed within non-regenerating and regenerating forelimbs of post-metamorphic, immature *Xenopus*. New lymphoid tumours subsequently de-

[53] S. NEUKOMM and M. LUDER-HUGUENIN, Oncologia *13*, 294 (1960).
[54] L. N. RUBEN and J. STEVENS, J. Morph. *112*, 279 (1962).
[55] M. SINGER and M. M. SALPETER, in *Growth in Living Systems* (Basic Books Inc., New York 1962).
[56] R. A. WILLIS, *The Pathology of Tumours*, 3rd ed. (Butterworths, London 1960).
[57] C. H. WADDINGTON, Nature (London) *135*, 606 (1935).
[58] J. NEEDHAM, *Biochemistry and Morphogenesis* (Cambridge University Press, London 1942).

veloped at the limb site as the donor material was broken down by the homograft rejection mechanism, and metastases spread into unimplanted, contralateral, regenerating limbs as well as to the visceral organs. It was then clear that the individuation field could not effectively control the growth or metastatic behaviour of an already formed cancer associated with it. In a second group of experiments[28] methylcholanthrene crystals were brought into contact with non-regenerating and regenerating *Xenopus* limb tissues. Lymphosarcomas subsequently developed in almost 100% of some of the groups of treated animals regardless of the regeneration state of the implantation site, which clearly demonstrates that the regeneration field failed to control the development of cancer within it, though the cells of the tumour were not regeneration-competent cells of the limb. Accessory limb structures were formed in some limbs, a further demonstration of the dual response of amphibian appendages to the presence of carcinogens. We have concluded on the basis of our recent results and those obtained previously[59–62], that although an adult field capable of post-embryonic morphogenesis is not able to control cancer within it if the cancer is derived from cells not originally comprising the field, the difficulty of inducing cancer from regeneration-competent limb tissues and the absence of spontaneous primary tumours of these tissues suggests that control is exerted over cells which are members of a system of this kind.

The lack of exploitation of the properties peculiar to amphibia, which was noted with regard to regeneration-competent tissues, applies even more strongly to the question of carcinogenesis and embryonic development. In spite of the detailed, analytic knowledge concerning the embryonic differentiation, morphogenesis and metamorphosis of amphibia, there have been few attempts to use these processes of normal development in the study of the carcinogenic aberration of differentiation.

The most significant experiments seem to be those of BRIGGS[12] and PFLUGFELDER[19]. BRIGGS produced three tumours in 154 *Rana pipiens* tadpoles given methylcholanthrene injections and one of the tumours appeared to be malignant. PFLUGFELDER found that tumour-like lesions developed in the larval organs of *Xenopus* tadpoles whose larval life was prolonged from six weeks to 18 months by rearing them in a potassium perchlorate solution.

A number of authors[63] have studied the effect of the *Lathyrus odoratus* factor on amphibian development, and LEVY and GODMAN[64] found that tumour-like lesions of the notochord developed in *Amblystoma punctatum* larvae reared in a weak aqueous solution of the crystalline factor. Since the 'true' neoplastic nature of these benign growths has not yet been demonstrated, these reports are noted here but not included in Tables I–III.

Conclusions

In conclusion it must be said that, while neoplasms have sometimes been produced in amphibians treated with carcinogenic chemicals, the potential uses of these lower vertebrates in researches to improve our understanding of the neoplastic process have not been widely realized. Further information is needed concerning the defensive hyperplasia of the skin in response to the presence of carcinogens and other foreign material and the role of urethan in some of the reported experiments. It is especially regrettable that while amphibia are being used in many different approaches to the problem of normal differentiation, so few attempts have been made to interfere with embryonic differentiation and regeneration with carcinogenic substances[65,66].

Résumé. Ce rapport analyse et résume les résultats des travaux de différents auteurs sur la possibilité d'induire des tumeurs par des substances chimiques chez les Amphibiens.

Les substances cancérigènes peuvent, dans les cas positifs: (1) induire des tumeurs caractérisées, (2) augmenter l'incidence des tumeurs spontanées, (3) produire des hyperplasies de l'épithélium cutané.

On peut se demander si les hyperplasies observés sont une réaction spécifique aux substances cancérigènes et sont réellement de nature néoplasique.

On peut s'étonner, aussi, que les Amphibiens dont le pouvoir de régénération est élevé et dont le développement embryonnaire, la vie larvaire et la métamorphose sont bien connus, n'aient pas davantage été utilisés dans l'étude du cancer.

[59] C. BREEDIS, Fed. Proc. *13*, 1390 (1954).
[60] S. INOUE and M. SINGER, Cancer Res. *23*, 1679 (1963).
[61] L. N. RUBEN, J. exp. Zool. *128*, 29 (1955).
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[65] M.B. is supported by the Fonds national suisse pour la recherche scientifique (No. 2551), L.N.R. by Grant No. CA-02913-C8 of the National Cancer Institute, N.I.H., Bethesda (Maryland USA).
[66] *Note added in proof.* Three important articles have come to our notice since this review was written. ARFFMANN[67] has continued his studies of the newt test for carcinogenicity using weak-carcinogenic and non-carcinogenic hydrocarbons. The negative reaction to 1,2,4-trimethylphenanthrene and the positive response to 3,6-dimethylbenz(a)anthracene were in disagreement with the reviewed reactions in mammals. ARFFMANN[68] has also found that quantitative experiments support the conclusion that the degree of response parallels the concentration of the test solution. SEILERN-ASPANG and KRATOCHWIL[69] have greatly extended their studies of the epithelial reactions of the skin of *Triturus cristatus*. These articles, however, do not remove our reservations expressed in this review concerning the true neoplastic nature of the cutaneous activity so frequently observed in the newt.
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[68] E. ARFFMANN, Acta path. microbiol. scand. *60*, 13 (1964).
[69] F. SEILERN-ASPANG and K. KRATOCHWIL, Arch. Geschwulstforsch. *21*, 113 (1963).