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A new teratogenic agent applied to amphibian embryos

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It has been demonstrated that the application of the magnetic probe to *Drosophila* pupae has a pronounced influence on the pattern of morphogenesis (Levengood, 1966). In addition to morphological abnormalities, there was a retardation of development which continued to occur without further treatment in succeeding generations. A theoretical basis for these disturbances has been proposed (Levengood, 1967).

The present study was undertaken to find out whether comparable developmental disturbances would be produced in vertebrates. Amphibian eggs and embryos were exposed to magnetic probes at various stages of development. The results demonstrate that a brief treatment of early amphibian embryos produces several types of abnormalities, some of which are not expressed until the climax stage of metamorphosis.

MATERIALS AND METHODS

The magnetic probe devices used in these experiments have been previously described in detail (Levengood, 1966). The probe coils contain a core with a 1 in. section at one end prepared with a conical taper down to a final diameter of 0·125 mm with the tip machined flat. The field characteristics of the probes were mapped with a gauss meter positioned at various distances below the probe tip. The field strength and gradients in the working space were determined by extrapolation from the linear portion of a curve obtained by plotting the gauss reading as a function of the reciprocal of the distance squared. Probes of two different field strengths but with identically shaped conical pole pieces were used in these experiments. In Table 1 the design characteristics are listed along with field strengths and gradients.

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Rearing procedure

Amphibian eggs were collected during the spring breeding season from a woodland pond, in a region approximately 25 miles west of Ann Arbor, Michigan. This pond was in a large wooded area and was free of industrial or artificial contamination. Egg masses were placed in fresh well water, and, for a given test series, 12–15 eggs were allowed to develop at room temperature (22 °C with a ± 1 °C extreme variation) in crystallization dishes approximately 10 cm in diameter and 6 cm deep. During the period of the egg development, the water was maintained at a 5 cm depth; fresh water was added as needed. After hatching,

Table 1. *Design and field characteristics of the magnetic probes*

Characteristic	Probe P1	Probe P3
Diameter of probe tip	0·125 mm	0·125 mm
Field strength 0·5 mm from probe tip (working distance)	$6{\cdot}3 \times 10^3$ G	$17{\cdot}7 \times 10^3$ G
Field gradient in first 2 mm from probe tip	$3{\cdot}9 \times 10^3$ G/mm	$11{\cdot}0 \times 10^3$ G/mm
Applied voltage	22·5 V	90·0 V
Approximate current (d.c.)	12 mA	12 mA

the larvae were placed in plastic tanks (30 cm long, 16 cm wide and 9 cm deep) containing 3 l of well water with not more than 15 specimens in each tank. The larvae were fed on a diet of cooked spinach and there was no evidence of oxalate crystal formations in either treated or control specimens.

Specimen exposure

An egg to be probe tested was placed on a glass microscope slide which in turn was fastened to a vertically adjustable stand (small lab-jack). Sufficient water was left on the slide so that the eggs did not dry up during the exposure intervals. The outer gelatinous envelopes of each egg were removed with dissection tools before the probe treatment (vitelline membrane left intact). Control eggs were treated in the same manner as the test groups; that is, envelopes were removed, they were positioned on the microscope slide and exposed to air for the same length of time as those placed under the magnetic probes. After exposure, the control eggs and treated eggs were placed in their respective crystallization dishes containing well water. The eggs in these exploratory studies were allowed to assume their normal position of rotation and the probe was applied at the animal hemisphere. Approximately a 0·5 mm air gap was provided between the probe and the test specimen.

RESULTS

In the following discussions of the abnormalities produced with magnetic probes, the experiments with the salamanders have been separated from those using frogs. Control specimens were examined and compared anatomically with the probe treated specimens in all of the groups.

Salamander embryos—abnormalities at hatching

Most of the experiments with salamander eggs were carried out with the *Ambystoma maculatum* species. Severe oedema appeared in larvae from eggs treated in the early gastrula stages of development (Harrison stages H7–H10). The oedematous blebs appeared on the flanks and ventral side of the treated specimens (not observed in any of the control specimens). Larvae with oedema died 4–5 days after hatching.

A severe alteration in morphogenesis was observed in a larva from an egg group treated at stage H4 (four cell) of development. Five days after hatching the test larvae died and at this time was less than one-third the size of controls. The treated larva also had a severe growth on the abdomen. This growth region left a white deposit on the bottom of the crystallization dish (possibly yolk material). The head was very incompletely formed with only an indication of eye spots and rudimentary gills.

Table 2. *Probe-induced malformations in hatching* Ambystoma maculatum *larvae*

Exposure	Total eggs treated	Percentage hatched	Percentage abnormal	Type of malformation
Controls	94	88·4	2·4	2 scoliosis
Probe P1	34	88·3	13·3	2 microcephalic, 1 oedema 1 retarded (abnormal) growth
Probe P3	94	73·5	10·2	1 microcephalic, 3 oedema, 3 retarded (abnormal) growth

A somewhat different alteration in the development pattern was observed in a larva from eggs exposed 10 min to P_1 in the gastrula stage (H11–H12) of development. A microcephalic larva was found in the test group and this larva lived for 17 days after hatching with no mouth, eyes, or balancer organs. The larva swam in an exceedingly erratic manner; when disturbed, it would gyrate in a corkscrew motion and then after this very erratic swimming would drop like a dart to the bottom of the tank. It would often be seen resting in an inverted position. The gills were also less developed in the microcephalic larva.

The three examples discussed here are representative of the abnormalities

appeared in a salamander larvae hatched from the probe-treated eggs. Table 2 summarizes the collective data from the induced abnormalities in salamander eggs treated at various stages of development. Even though some treated stages gave no abnormal larvae, the combined data (not all stages were treated) demonstrate a much higher percentage of abnormal larvae from the probe-treated eggs than in the control groups. It is interesting to note in Table 2 that the hatching potential is not seriously affected by the magnetic field treatment; however, the group treated with P3 disclose about a 15% lower hatching than controls. Because of the difficulty in rearing salamander larvae, the studies were discontinued less than 3 weeks after hatching.

Frog embryogenesis

The eggs from the wood frog, *Rana sylvatica*, were collected after natural fertilization and at early stages of development. Groups of 12–15 eggs were exposed 5 min to both of the magnetic probes, and a third group served as the control for each development stage. The abnormalities produced in the hatching frog larvae were on a morphological basis quite different from those appearing at metamorphosis. For this reason, the results with the *R. sylvatica* are discussed separately for these two different stages of early development.

Abnormalities in hatching tadpoles

In the spring of 1966, the probe-treatment programme was initiated and the main emphasis was placed on abnormalities appearing at larval hatching. Because of the large number of eggs treated and the time-consuming procedure of

Table 3. *Probe-induced malformations in hatching* Rana sylvatica

Exposure	Total eggs treated	Percentage hatched	Percentage abnormal	Type of malformation
Controls	132	93·2	2·4	3 scoliosis
Probe P1	122	92·6	4·4	1 scoliosis, 2 oedema, 2 retarded (abnormal) growth
Probe P3	127	93·7	11·8	3 scoliosis, 2 oedema, 8 retarded (abnormal) growth, 1 spina bifida

bringing large numbers of frogs to maturity most of the animals were killed soon after hatching. The induced hatching abnormalities in the 1966 tests are summarized in Table 3, and it is apparent from these data, as in the salamander tests, that the abnormalities are higher in the probe-treated eggs. A number of different developmental stages were exposed to the probes, and the data in Table 3 include those stages where the effect was minimal as well as those showing high percentages of abnormalities. At the early pregastrula stages over 25% of the hatching larvae were deformed.

Delayed teratogenic effects

In the spring of 1967, *R. sylvatica* eggs were again collected and treated with the two magnetic probes, in the same manner as in the 1966 tests. In these studies, the surviving larvae were allowed to develop through metamorphosis. At the onset of premetamorphosis, many of the treated tadpoles developed severe leg malformations as well as a pronounced alteration in the pattern of histogenesis which took the form of subepidermal blistering. In some cases the larvae developed both limb abnormalities and subepidermal blistering.

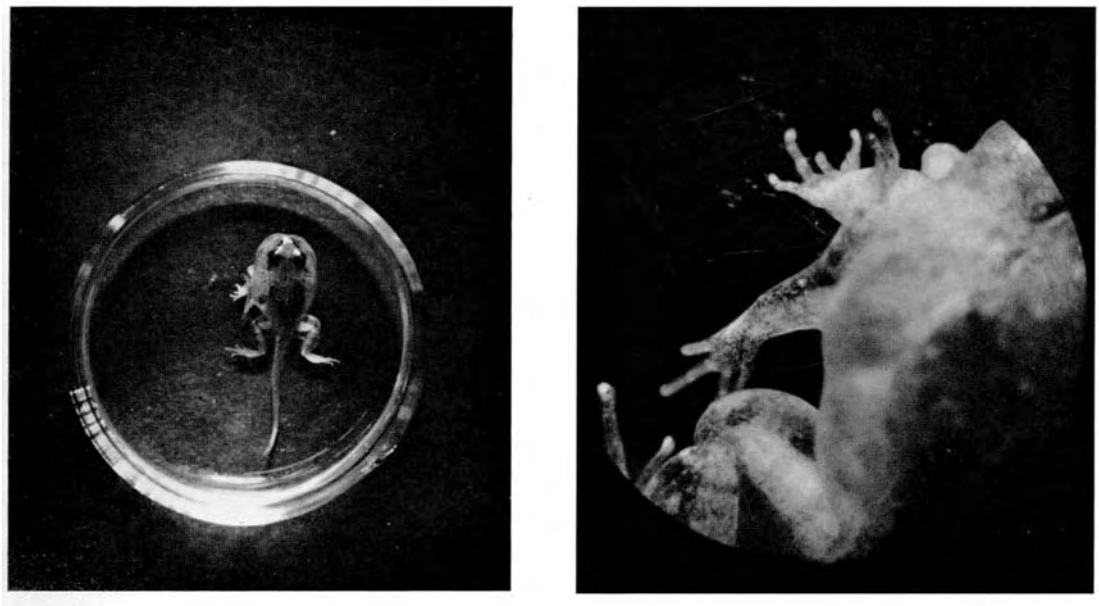

Fig. 1. Supernumerary limb formation in *R. sylvatica* larva exposed 5 min with P3 at stage S8 of development.

Supernumerary limb formation was also observed and an example of this multiple limb proliferation is shown in Fig. 1 with the whole tadpole on the right and a higher magnification view on the left showing the limb development on the left side of this organism. A total of seven legs appeared, six of which were on the left side and one rear leg on the right. This larvae died approximately 48 h after these photos were taken. Post-mortem examination disclosed the presence of an additional leg at the right anterior which had not emerged through the operculum (eight legs total).

Another larva displaying both supernumerary limbs as well as severe subepidermal blisters or oedema is shown in Fig. 2. The rear legs did not function in the usual manner since they were covered with large liquid-containing sacs grouped sequentially along the limbs. These sacs completely covered the pos-

terior limbs. A total of five limbs appeared on this specimen, three on the left side and two on the right. This organism died approximately 24 h after the photos were taken.

In Fig. 3, a histogram is presented to show the appearance of malformations as a function of the development stage (Shumway) exposed to the probes. In this figure the data from both the P1 and P3 exposures have been combined; therefore, each bar represents the results from a group of about 30 eggs. The appearance of subepidermal blistering is indicated in Fig. 3, and, as previously mentioned, a probe-exposed specimen in this period of development may disclose

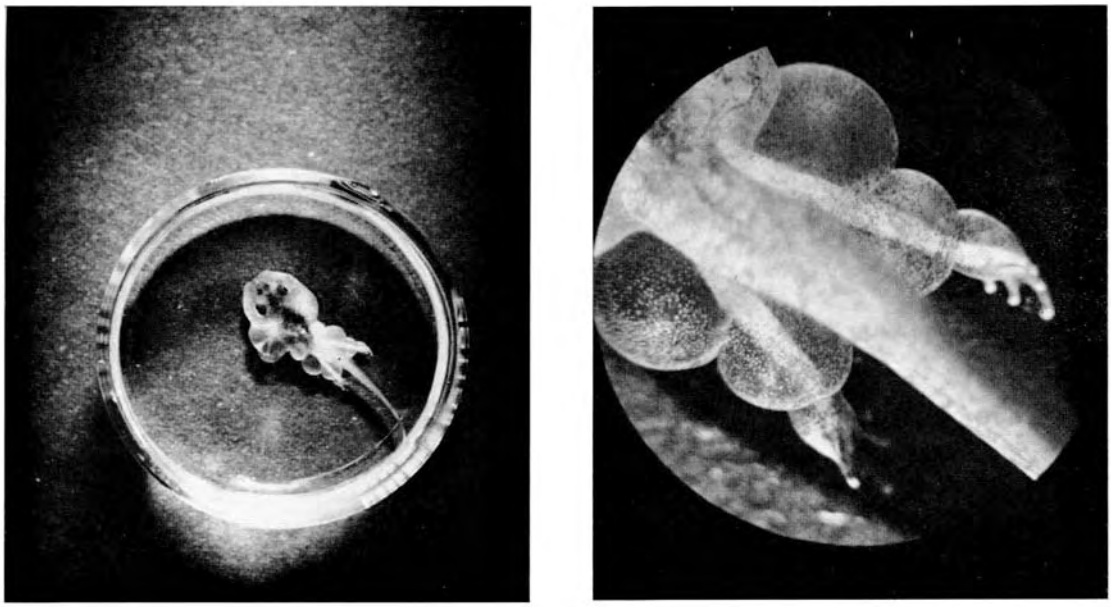

Fig. 2. Subepidermal blisters at the climax stage of an *R. sylvatica* larva exposed 5 min to P3 at stage S8 of development (3 legs on left side and 2 on right).

leg malformations as well as the severe oedema. The bars outside the indicated region of severe oedema represent, primarily, leg malformations. The very short bars at Shumway stages 13 and 14 indicate that eggs were exposed at these stages and no abnormalities were observed. The combined results of all the various exposed stages are listed in Table 4.

Renal malformations in abnormal larvae

Dissection of the larvae disclosed that over 90% of those specimens with leg malformations or severe oedema also have abnormal kidneys. One kidney (in most cases the right) was much larger and disclosed a 'bulging' with the maximum lateral extension located posteriorally. At the maximum bulge, the deformed kidneys were approximately 50% larger in diameter than normal.

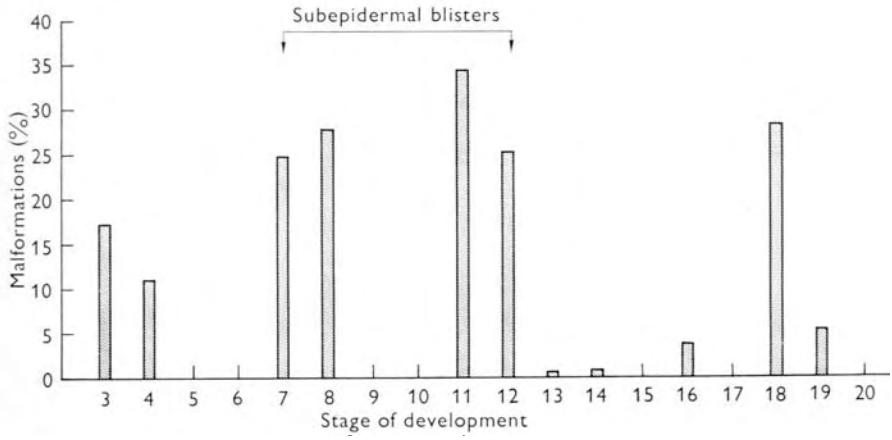

Fig. 3. Histogram showing abnormalities appearing at climax of *R. sylvatica* exposed at different stages of development (Shumway series); stages S13 and S14 were at the zero level of malformation.

Table 4. *Delayed teratogenic effects in* Rana sylvatica *tadpoles from probe-treated eggs*

Exposure	Total eggs treated	Percentage reaching climax	Percentage abnormal at climax	Type of malformation
Controls	165	83·6	1·5	1 leg deformity, 1 retarded growth
Probe P1	160	86·9	16·5	17 leg deformities, 4 subepidermal blisters, 2 oedema
Probe P3	160	76·3	20·4	13 leg deformities, 6 subedpidermal blisters, 2 oedema, 1 single eye, 1 neotenous, 2 super-numerary limbs

DISCUSSION

That subepidermal blistering may be associated with morphologically altered kidneys has been pointed out by Deuchar (1966). She states: 'The pronephric kidney begins to function very shortly after hatching and excretes water to counteract the tendency to endosmosis.' This author points out that water-logging of tissue occurs and subepidermal blisters form whenever abnormality of development has impaired the function of the kidney. The induced oedema in the probe series does not appear until climax and it is presumed that the pronephric kidney functions more or less normally until this period.

The most significant feature of the severely malformed larvae, such as those

shown in Figs. 1 and 2, is the fact that the larvae could not be distinguished morphologically from those in the control groups until they reached the initial stages of climax. Both the supernumerary limb formation and the subepidermal blistering are induced, as indicated in Fig. 3, during the gastrulation stages of development. During this gastrulation period, tissues may be exposed directly to the probe which after being structurally perturbed later invaginate and form the renal and limb bud structures. By carefully mapping the location of the probe (using vital staining techniques) as applied to various types of presumptive tissue on the developing egg, the specific regions perturbed by the magnetic field could be more precisely located. This technique provides a method of altering the embryogenesis without microdissection or gross mechanical damage to the embryo. One important incentive for examining induced alterations in the epigenetic cycle lies in the possibility of influencing genetic diseases through induction or repression of enzyme synthesis.

SUMMARY

The results of an exploratory investigation concerning the influence of a new teratogenic agent applied to vertebrate organisms are described. Exposure of amphibian eggs and embryos to magnetic probe devices produced gross abnormalities in the developing larvae. Two probes of identical configuration but different field strengths were utilized in this study. Both probes were operated in the kilogauss region with high field gradients.

At the hatching stage severe abnormalities were noted in both anuran and urodele larvae from probe treated eggs. These hatching abnormalities took the form of microcephaly, altered development, and oedematous growths.

In probe-treated *Rana sylvatica* a delay was observed in the appearance of a high percentage of the malformations until the climax stage of metamorphosis. Until this point, the larvae were of the same appearance as control specimens. The frog abnormalities at metamorphosis were somewhat different on a morphological basis from those appearing in the hatching tadpoles and consisted primarily of severe subepidermal blistering and leg malformations including the formation of supernerary limbs. Over 90 % of the morphological alterations at climax were found to be associated with deformed kidneys. The characteristics of these teratogenic alterations were examined in relation to the particular developmental stage exposed to the probes and in a very cursory sense the type of tissue subjected to the magnetic force field. In terms of the delayed effects, the gastrula stages of development appeared to be the most sensitive.

RÉSUMÉ

Un nouvel agent tératogène expérimenté sur des embryons de batraciens

Les résultats d'une étude préliminaire de l'influence d'un nouvel agent tératogène sur des vertébrés sont décrits.

L'application de probes magnétiques produit chez les larves des anomalies marquées. Deux probes de géométrie identique mais de sensibilités différentes ont été utilisées pour cette étude, chaque probe étant employé dans la gamme des kilogauss avec gradients de champs élevés.

De graves anomalies ont été observées chez les larves d'urodèles et d'anoures au moment de l'éclosion: microcéphalie, développement anormal, oedème.

Une certaine latence s'observe en ce qui concerne l'apparition des anomalies chez *Rana sylvatica*. Jusqu'au moment critique de la métamorphose, les larves traitées ressemblent aux témoins. Les anomalies constatées chez les jeunes grenouilles après la métamorphose diffèrent de celles observées chez les têtards et comprennent surtout le boursoufflement de l'épiderme et le développement de membres surnuméraires.

Plus de 90 % des modifications morphologiques observées au moment critique s'associent à des malformations rénales. Ces modifications tératologiques ont été examinées par rapport au stade de développement auquel le traitement a débuté, ainsi qu'au type de tissu soumis au champ de force magnétique. Les gastrulas paraissent être les plus sensibles en ce qui concerne les effets tardifs.

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