Bull. Environm. Contam. Toxicol. 22,297-304 (1979)

Teratogenic Effects of Manganese Ethylenebisdithiocarbamate (MANEB) on Forelimb Regeneration in the Adult Newt, *Triturus cristatus carnifex*

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The wide use of ethylenebisdithiocarbamates (EBDC) in agriculture, as fungicides, poses the problem of potential harm to public health, since it is known that dithiocarbamates and their degradation products contaminate plants, soil and water to various degrees.

Evidence has been presented that these compounds have teratogenic activity in developing mammalian fetuses (PETROVA-VERGIEVA and IVANOVA-TCHEMISHANSKA 1973, LARSSON et al. 1976) in chick embryos (van STEENIS and van LOGTEN 1971) and in amphibian embryos (BANCROFT and PRAHLAD 1973).

The ability of amphibians, especially the urodeles, to regenerate lost appendages is well established and various investigators have stressed the similarity between developmental processes which govern limb regeneration and ontogeny.

Therefore, it was chosen to use the regenerating limb of the crested newt as an experimental model for investigation of the possible teratogenic effects of manganese ethylenebisdithiocarbamate (maneb).

In the present study attention was focused on early regeneration stages, in order to evaluate the effects of maneb on blastema development, and on the subsequent redifferentiation and limb morphogenesis. This report deals with the gross morphology and with the observations of the skeletal elements. A later report will deal with histology.

Maneb treatment resulted in delayed growth, reduced melanogenesis and malformations of regenerating limbs.

MATERIAL AND METHODS

Male and female adult newts, *Triturus cristatus carnifex*, were collected near Perugia and maintained prior to experimentation in running water.

A total of 72 animals were used. They were divided in two groups (a control and an experimental group) of 36 newts of about equivalent sizes, 18 males and 18 females each.

0007-4861/79/0022-0297 $01.60

Animals in the control and experimental group were anesthetized in a 1:2000 dilution of MS 222 (tricaine methanesulfonate, SANDOZ) in boiled water. Their forelimbs were amputated bilaterally through the distal third of the humerus. After the soft tissues retracted, protruding bone was trimmed off to produce a relatively flat amputation surface. Then, prior to receiving treatment, the animals were kept for two days at room temperature in 30-L glass aquaria with repeatedly changed boiled water. No mortality was observed during the experimental period: only one male newt died within 24 h post-amputation.

Experimental animals were exposed percutaneously to maneb of technical grade (Maneb 80, 80% active component, supplied by SIPCAM Milano) at a concentration level of 5 ppm. This level was chosen after preliminary toxicity studies (PACCES ZAFFARONI et al. 1978, in press). Control newts were kept in tap water. A temperature of 24° $\pm$ 1°C was maintained for the duration of the experiment and no attempt was made at regulation of a specific diurnal cycle. The experiment was begun in September.

The following schedule was adopted: on Monday and Wednesday fresh solutions were made, on Friday all animals were photographed and evaluated for the progress of regeneration, on Saturday they were fed a diet of chopped beef liver. The newts were exposed to the toxicant for four days a week and fed in fresh water to avoid ingestion of the test substance.

Individuals animals were identified by the abdominal spot patterns and the developmental stage was recorded weekly, according to the criteria described by ITEN and BRYANT (1973). Animals were killed starting on the 15th day and thereafter at weekly intervals until the 65th day post-amputation. The last twelve newts were killed on the 85th day.

To examine the formation of cartilagineous skeleton, the regenerated limbs were fixed in Bouin's fluid and stained *in toto* with methylene blue. The tissues were dehydrated and cleared in methyl salicylate. Some limbs were processed for histological examination, whose results will be reported later.

RESULTS AND DISCUSSION

Gross morphology

Delayed growth of the regenerate was observed in maneb treated newts. This difference in the rate of regeneration existed until the fifth week post-amputation in the females and until the sixth week in the males. In order to quantify the different growth rates of experimental and control animals, an arbitrary number was assigned to each morphological stage of ITEN and BRYANT (1973). Figure 1 shows graphically the time after amputation at which the developmental stages of regeneration were attained in the two groups of animals. In the females, the difference between the two groups was significant after 3 weeks ($P < 0.05$, _t_ test) and highly significant after 4 and 5 weeks ($P < 0.001$). In the males, the delay of growth was clearly evident at the 5th week ($P < 0.001$) and the 6th week ($P < 0.01$). After six weeks, the regenerates of the 18 control animals were at the stage of late digits (LD), except in two cases, whereas in the experimental group the regenerate had not reached this stage in 8 of 19 animals. Even after seven weeks, digits were not completely developed in 5 of 17 treated newts.

Delayed growth has also been observed in amphibian embryos (BANCROFT and PRAHLAD 1973), in chicken embryos (van STEENIS and van LOGTEN 1971) and in mammalian fetuses (PETROVA-VERGIEVA and IVANOVA-TCHEMISHANSKA 1973, LARSSON et al 1976) following treatment with maneb and other dithiocarbamates. Since antithyroid compounds are formed by decomposition and metabolic processes from EBCD (FISHBEIN 1976), our results are also consistent with those reported by FOX and TURNER (1967), who observed an inhibition of growth in _Xenopus laevis_ and in _Rana temporaria_ larvae after treatment with thiourea and phenylthiourea. However, SCHMIDT (1958) observed a faster rate of regeneration of forelimbs in thyroidectomized adult newts. Therefore, it is doubtful than an inhibition of thyroid function alone could account for the growth retardation observed in our experiment. Because of the trophic role of innervation in limb regeneration (SINGER 1952) the neurotoxic effects of dithiocarbamates must also be considered (EDINGTON and HOWELL 1966). Possible degenerative changes in the peripheral nerves could well result in slower growth and inharmonious development of the limb. In this regard, it seems of interest to note that in our experiments maneb treatment was begun two days after limb amputation, when regenerating nerve fibers already pervade the wound and regenerating tissues (SINGER 1974).

In approximately half of the animals treated with maneb, the regenerates appeared to be less pigmented than those of the control group, especially at the fifth to the seventh week. Subsequently, there was a trend toward normal pigmentation. In almost all the experimental newts, the stump after one week was markedly hyperemic and easily bleeding. In the subsequent stages of regeneration, various degrees of vascular dilatation and extensive hemorrhages were frequently seen through the undamaged skin. Digits also often showed hemorrhagic foci at the tip.

Distal deformities were the most commonly occurring developmental disturbances after exposure to maneb. At the fifth week post-amputation, arthrogryposis was observed in all the experi-

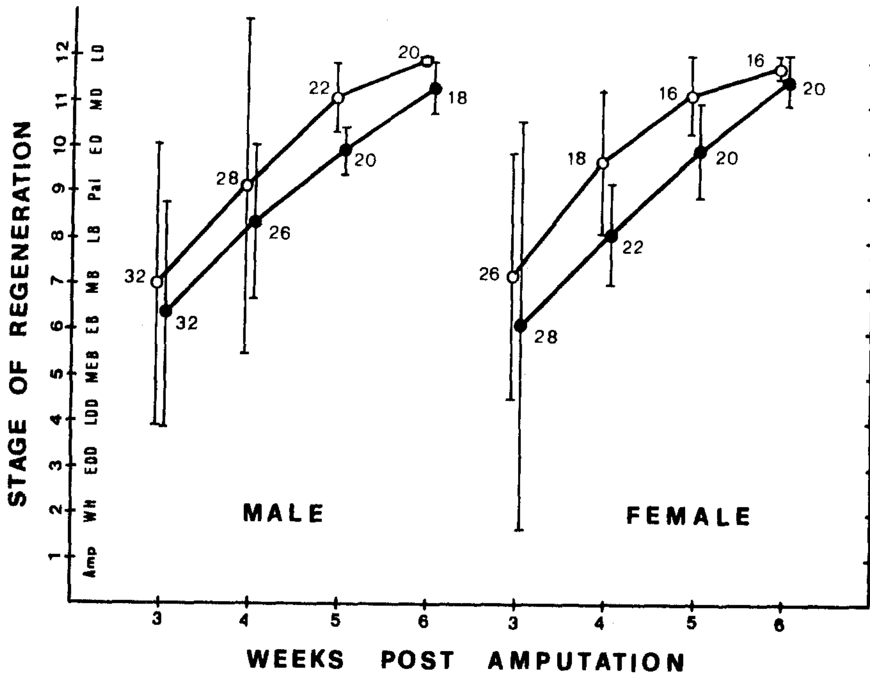

Fig. 1 - Graph comparing the developmental stages of regenerates after amputation in control (open circles) and treated animals (closed circles). The abbreviations for each stage are: Amp, amputation; WH, wound healing; EDD, early dedifferentiation; LDD, late dedifferentiation; MEB, moderate early bud; EB, early bud; MB, medium bud; LB, late bud; Pal, palette; ED, early digits; MD, medium digits; LD, late digits (ITEN and BRYANT 1973). Bars represent standard errors of the mean. The number of limbs is indicated for each point.

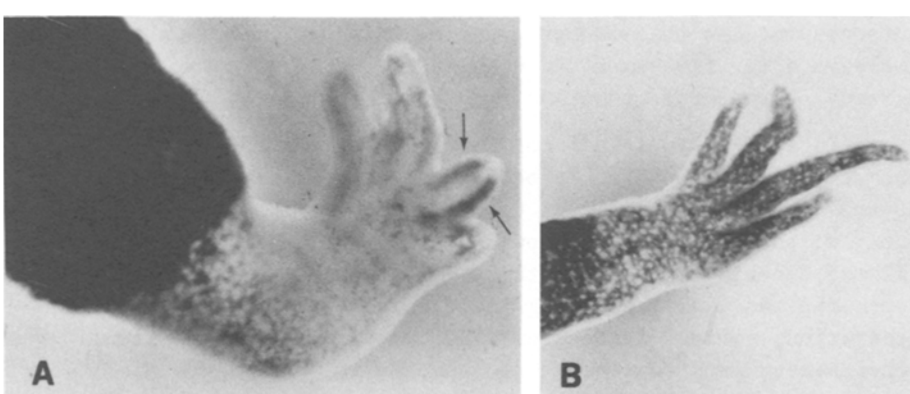

Fig.2 - Forelimbs of maneb-treated newts. A. Day 35 regenerate of a male: arthrogryposis, hyperemia (arrows), and slightly pigmented skin. B. Day 63 regenerate of a female: digit deformities.

mental newts, the autopodium being always twisted dorsally (fig. 2 A). At the late digit stage, arthrogryposis was still present only in some of the animals. Arthrogryposis was also observed by van STEENIS and van LOGTEN (1971) in the hindlimb of chick embryos treated with tecoram. According to them this anomaly could be due to contracture of the musculature. It is of interest that abnormal development of the muscular tissue has also been observed in amphibian embryos treated with nabam and diquat (ANDERSON and PRAHLAD 1976). By six weeks and even more through the following weeks, the majority of digits was variously deformed (fig. 2 B). Regeneration defects appeared to be related to both the severity and the localization of the vascular disturbances observed in the earlier stages. Very often those digits which had shown apical hemorrhages subsequently appeared deformed.

In all the control animals, regenerated limbs were normal except for three males which developed only two or three digits and one female with a supernumerary forelimb.

Skeletal examination

The forelimb of *Triturus cristatus carnifex* has four digits. The phalangeal formula is 2-2-3-2. There are four metacarpals, seven carpal elements (os intermedium and os ulnare always fused), radius, ulna and humerus. The occurrence of carpal fusion and occasional deviations in the number of phalangeal elements (8-9) was considered within normal limits (fig. 3 A depicts what is considered to be a normal regenerated limb).

Nine female and nine male treated newts were examined for skeletal morphology (fig. 3 B,C,D). In the animals exposed to maneb, deformed phalanges were observed in most of the digits. Most often deformities involved distal phalanges, which were hook-shaped and sometimes kinky. This malformation seems to be the most characteristic change induced by maneb. More rarely, deformed metacarpals were found. Other skeletal anomalies were also observed, still involving the autopodium, such as fused or missing phalanges, or fused metacarpals. Occasionally proximal deformities such as shortened radius and ulna were found. Skeletal abnormalities were also observed in limbs with apparently normal gross morphology.

In the females, the incidence of malformed limbs was 100% in the experimental group, which was statistically significant when compared to control newts. Conversely, in male newts the incidence of malformed limbs was not statistically significant when compared to the control group (Table 1).

The skeletal morphology of one or both limbs of seven female and ten male control newts was examined. With one excep-

TABLE 1

Frequency of skeletal malformations of the regenerating forelimb in newts exposed to maneb at a concentration level of 5 ppm

Treatment group		total limbs [a]	normal limbs	malformed limbs No.	malformed limbs %
female	control	10	9	1 [b]	10
	maneb	11	0	11	100 [c]
male	control	10	4	6	60
	maneb	9	0	9	100 [d]

[a] Not all limbs were selected for staining of skeletal elements
[b] Supernumerary limb
[c] Significantly different from control group at 0.1% (χ^2_c test)
[d] Not significantly different from control group

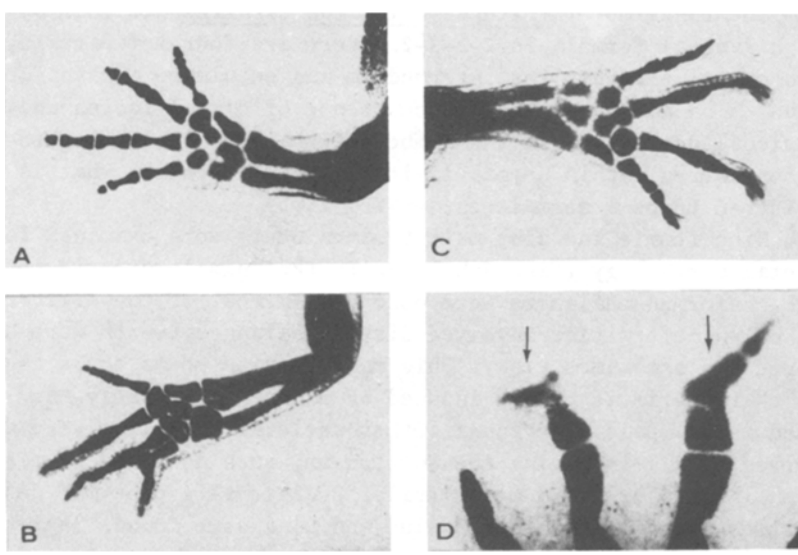

Fig. 3 - Cartilage elements stained with methylene blue; limbs cleared in methyl salicylate. A. Day 57 regenerate of a control female. Normal morphology. B. Day 57 regenerate of a treated female. Incurved phalanges of digits II, III and IV. C. Day 65 regenerate of a treated male. Deformity of middle phalange of digit III and distal fusion of metacarpals III and IV. Incomplete remodeling of carpals. D. Day 58 regenerate of a treated female. Severe abnormalities affecting phalanges of digits II and III (arrows).

tion (where there was a supernumerary limb with some irregular phalanges), skeletal elements of the regenerates were normal in all the female newts. In two male newts, there was an irregularity of the distal phalanges of digits I and/or II, quite similar to but less severe than those observed in the maneb group. In another four males, the following distal deformities were observed: oligodactyly (two limbs), partial fusion of the proximal phalanges of digits III and IV with metacarpal IV (one limb), underdevelopment of metacarpal II, non-articulated with basal carpals (one limb). Four limbs had normal skeletal pattern.

It is tempting to assume that the observed anomalies might be ascribed to toxic compounds with teratogenic activity present in the city tap water. In fact, during the course of the experiment no care was taken to purify the tap water. Since in the control female newts the regenerates were normal, males would appear to be more susceptible than females to pollutants which might be present in the environment.

With regard to the mechanism of the teratogenic effects of maneb, it is well known that the dithiocarbamates and their decomposition products can interfere with many enzyme systems (DU BOIS et al 1961). Accordingly, LARSSON et al. (1976) hypothesized that the teratogenic effects of maneb in rat fetuses may be related to embryonal zinc deficiency, due to the chelating effects of the dithiocarbamates. Severe vascular disturbance may also contribute to altered development through a defect in nutritional supply and impairment of gas exchange. As already mentioned possible peripheral nerve degeneration might also account for the abnormal development of the regenerate.

The conclusion may be drawn that maneb treatment results in teratogenic effects in the regenerating newt forelimb as well as in amphibian embryos and homeothermic vertebrate embryos. Thus, the present data indicate the feasibility of using the newt regenerating forelimb as an experimental model for assessing the teratogenic risks of pesticides.

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