Development 99, 127–136 (1987)
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Ventral duplication of the autopod: chemical induction by methoxyacetic acid in rat embryos

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Summary

Administration of 2-methoxyethanol to pregnant rats on day 12 of gestation induced ventral duplication of the autopod, presumably *via* its oxidative metabolite, methoxyacetic acid. Morphological observations indicate that the limb bud periderm is severely damaged by methoxyacetic acid so that large patches of this structure are actually missing during an extended period of limb bud development. A high concentration of methoxyacetic acid (10 mM) was found in the extraembryonic fluid and we postulate that the damage to the periderm was initiated from this extraembryonic exposure. The ventral duplication of the autopod is thought to arise through an attempt by the embryo to repair the periderm lesion.

Key words: ventral polydactyly, periderm, methoxyethanol, methoxyacetic acid, rat embryo.

Introduction

Duplication of limb elements, especially of the autopod (hand or foot anlage), has been repeatedly accomplished by surgical or chemical means. Of the three limb axes, proximodistal, anterior–posterior, and dorsoventral, nearly all duplications have been in the anterior–posterior axis. Duplication in the dorsoventral plane is limited to a single situation, the *Eudiplopodia* chick mutant (Goetinck, 1964), in which duplications of the terminal elements of the wing and leg buds arise from the dorsal surface of the developing limb buds.

Recently we reported that administration of 2-methoxyethanol (ME) to rats on day 12 of pregnancy induced ventral polydactyly of the hindlimbs in a few of the offspring (Ritter, Scott, Randall & Ritter, 1985). Since duplication of the dorsoventral axis occurs so rarely we decided to investigate the pathogenesis of ventral polydactyly induced by ME. In addition we have examined the pharmacokinetic disposition of methoxyacetic acid (MAA) which has been shown to be the proximate teratogen of ME-induced malformations (Brown, Holt & Webb, 1984; Ritter *et al.* 1985; Yonemoto, Brown & Webb, 1984).

Materials and methods

Teratology

Female Wistar rats were kept on a normal day/night cycle in temperature-controlled rooms and received standard food (Altromin®) and water *ad libitum*. The day when vaginal plugs were detected after a 2 h mating period (6–8 a.m.) was designated as day 0 of gestation. At 10 a.m. on day 12 of gestation, 2-methoxyethanol (Fluka AG) was administered by intraperitoneal injection (5 mM kg^{-1}). The rats were returned to their cages until day 20 when they were killed by ether overdosage. The fetuses were removed from the uterus, weighed and fixed in formalin, preparatory to double staining of cartilage and bone with alcian blue and alizarin red. Subsequently the fetal preparations were observed under a dissecting microscope and the limb malformations recorded.

Microscopy

The embryos were removed 1, 2, 4, 6, 12, 24, 48 and 72 h after application of 2-ME and fixed *in toto* either in a 2 % glutaraldehyde solution (phosphate buffer, pH 7·2; 0·1 M) with the addition of 0·5 % tannic acid for electron microscopic inspection or in Bouin's solution for light microscopic inspection. Glutaraldehyde fixation was followed by fixation in 1 % OsO_4 solution (cacodylate buffer, pH 7·2; 0·1 M). Embedding was performed in Mikropal or paraffin.

Reichert microtomes (Ultracut) served for ultrathin sectioning. The sections were contrasted with uranyl acetate/lead citrate and evaluated using a Siemens Elmiskop 101 or a Zeiss EM10. Thicker sections (approx. 1 μm) of the same material were stained for some minutes with Giemsa solution at 60°C; the paraffin-embedded sections were stained with haematoxylin–eosin. The light microscopic pictures were taken using a Zeiss-Ultraphot.

For scanning electron microscopy, the sections were fixed in 3 % glutaraldehyde and 3 % paraformaldehyde in cacodylate buffer (pH 7·2; 0·1 M); postfixation was done in buffered 1 % OsO_4. Dehydration of the preparations was performed in the alcohol series and by critical-point drying. Surface coating with gold–palladium was carried out using an SEM coating unit 5100 (Polaron). Pictures were taken on an ISI-100 scanning electron microscope.

Pharmacokinetics

Pregnant rats were administered 5 mm kg^{-1} (400 mg kg^{-1}) of ME intraperitoneally at 10 a.m. of day 12. 2, 8 or 24 h later the dam was anaesthetized with ether and the uterine horns exposed through a ventral abdominal incision. The uterine wall and decidua were incised and then a sample of extraembryonic fluid was taken with a hypodermic syringe. Next the extraembryonic membranes were removed and the embryo recovered on a stainless steel spatula, any excess fluid being removed with filter paper wedges. When a sufficient number of embryos and extraembryonic fluid were collected (four to five implantation sites) a sample of maternal blood was taken from the abdominal aorta and the mother killed by ether overdosage. The embryos and extraembryonic fluids from a single litter were pooled and frozen as was the serum after centrifugation of maternal blood.

Samples (20–200 μl, depending on the concentrations present) were pipetted into 15 ml disposable microtubes, then 50 μl of 1 N NaH_2PO_4 buffer (adjusted to pH 5·0) and 1 ml of ethyl acetate containing the internal standard (ethyl–methyl–acetic acid) were added. The tubes were shaken for 20 min and then centrifuged for 2 min in a 5012 Eppendorf centrifuge. An 800 μl portion of the organic phase was transferred to a 1 ml disposable glass reaction vial and preconcentrated (N_2-stream) to about 100–300 μl following the addition of 100 μl acetonitrile. The extraction was repeated with 1 ml ethylacetate without the internal standard. The combined extracts were concentrated (N_2-stream) to a final volume of 10–20 μl and trimethylsilylated by adding 30 μl pyridine and 30 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Pierce Chem. Co.) at room temperature. 1 μl samples were injected into the gas chromatograph–mass spectrometer–computer system (Perkin–Elmer F-22 gas chromatograph) and Varian MAT (CH-7A mass spectrometer operated by an SS-100 Varian data system). A 2 m column packed with 3 % OV-17 on 100–120 mesh Gas Chrom Q was used (Suppelco). The initial temperature of 80 °C was programmed to 140 °C with 12·5° min^{-1} rate of increase.

The following ions were selected: ion m/e 89 (MAA) and ion 95 (internal standard). Peak height ratios and calibration curves were used for quantification.

Results

Table 1 indicates that 10 fetuses from a total of 87 exposed to 5 mM kg^{-1} of ME had ventral duplication of hindlimb digits. It should be pointed out that 86 of the 87 fetuses had other fore- or hindlimb malformations, usually ectrodactyly, syndactyly or radioulnar shortening, and thus ventral duplication of hindlimb digits occurs infrequently and only when high doses of ME are administered to the mother. In nine of the ten cases a single digit was duplicated, usually digit **3** (6/9) and in four of these six there was an additional ventral duplication of digit **3** so that the limb possessed three digit 3s (Fig. 1). The duplicated digit is often larger than the normal digits due perhaps to fusion of two or more originally induced ventral digits (e.g. see Fig. 3). The duplicated digits have a normal

Table 1. *Limb malformations in day 20 rat fetuses after 2-ME, 5 mM kg^{-1}, given* i.p. *on day 12 of gestation*

Mother no.	No. of implantation sites	No. of resorption	Mean fetal wt (g)	No. with limb malformation	No. with D/V duplication of hindlimbs
1	12	1	2·86	11	3
2	11	2	3·49	9	0
3	12	1	3·28	11	1
4	10	2	3·42	8	0
5	14	3	2·64	11	1
6	10	2	3·79	8	0
7	12	3	2·66	9	2
8	9	5	2·77	4	2*
9	13	8	2·42	5	
10	9	1	3·04	7	1
11	9	6	2·28	3	0
	121	34 (28 %)	2·97	86/87†	10/87† (11 %)

* The two litters were cleared together and those with dorsoventral duplication cannot be exactly placed with the proper mother.
† No. of fetuses examined.

dorsoventral orientation, i.e. their dorsal surface is apposed to the ventral surface of the normal digit.

Stereomicroscopic inspection revealed changes in the hindlimbs of treated embryos 24 h after drug application. These changes comprise bulge-like thickenings in the proximal region on the ventral aspect of the limb (Fig. 2). After 48 h, these thickenings are even more pronounced. Often two apices are seen on

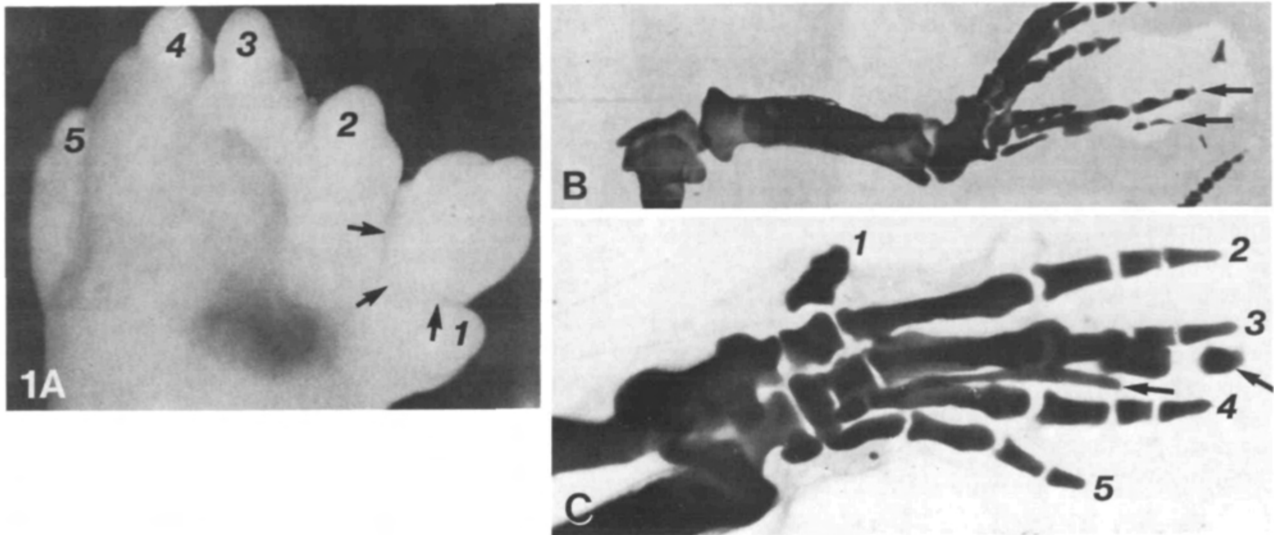

Fig. 1. Treated embryos, day 20 of pregnancy. (A) Dorsal view of a hindlimb with ventral polydactyly (arrows). (Taken from Ritter *et al.* 1985.) (B) Lateral view of a hindlimb with two additional rays (arrows). (C) Dorsal–ventral view of a hindlimb with five normal rays (**1–5**) and two additional rays (arrows).

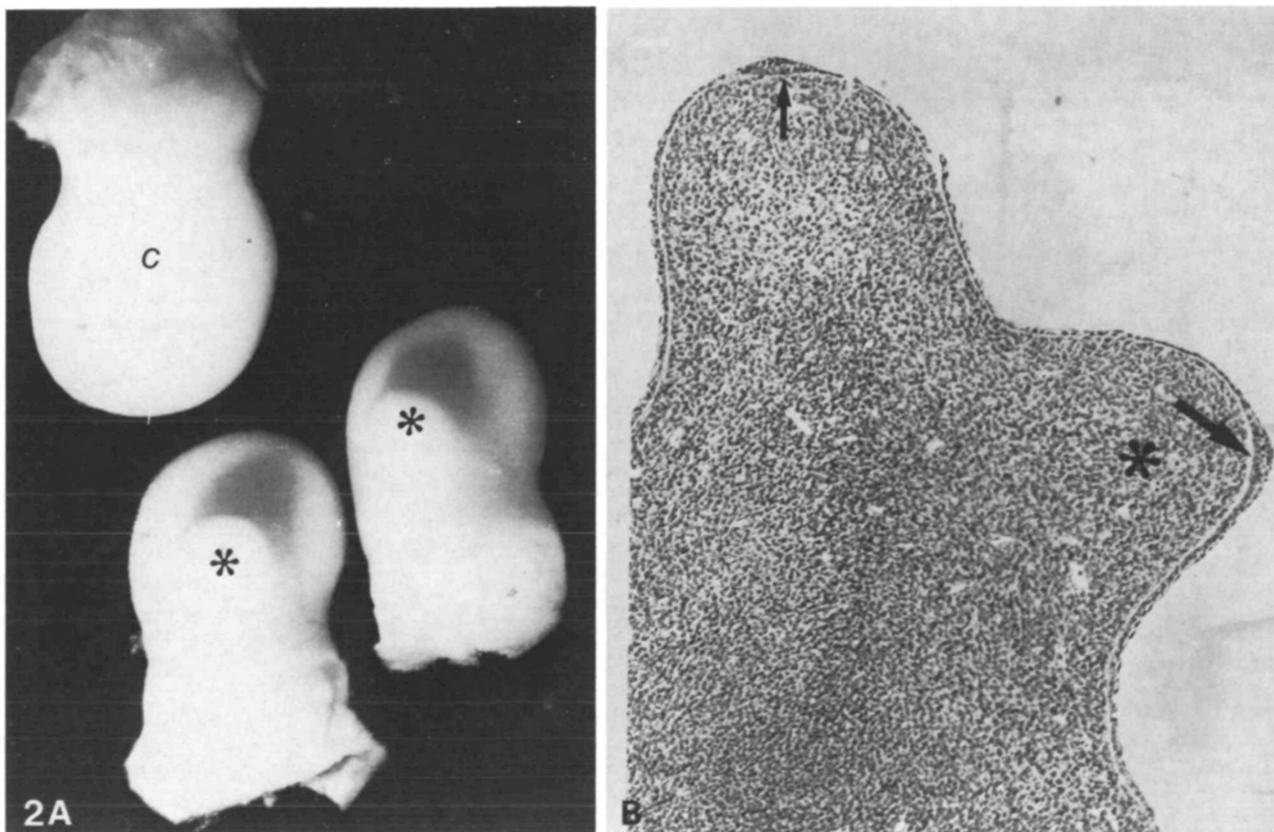

Fig. 2. Embryos 24 h after treatment. (A) Stereomicroscopic picture showing two hindlimbs with ventral bulges (•) and one control hindlimb (*c*). (B) Histological section through a hindlimb with a ventral bulge (•), apical ectodermal ridges at the normal position (small arrow) and at the tip of the ventral bulge (large arrow). 1:27.

the surface of these bulges. Scanning electron microscopy (Fig. 3A,B) was especially suited to demonstrate these changes. Light microscopic inspection revealed apical ectodermal ridges in normal areas as well as at the apex/apices of the ventral bulges (Fig. 2B). These ridges are long or form round plaques.

On days 12 and 14, the surfaces of the hindlimbs (including the apical ectodermal ridge, AER) of the untreated controls consist of flat cells showing a diameter of up to 20 μm. The nucleus and a thin rim at the contact areas stand out against the otherwise smooth surface. The number of microvilli is small. 1 or 2 h after drug application changes are not yet perceptible. Only after 4 h do first changes occur. In certain areas, the periphery of the flat cytoplasm loses contact with neighbouring cells. The periderm cells detach and the apices of the basal cells form hump-like protrusions. After 6 h, large areas have lost their periderm layer (Fig. 4). The apices of numerous basal cells are forming microvilli to an increasing extent. The superficial periderm layer is now missing over long distances of the AER. 12 and 24 h after drug application, large areas of the hindlimbs and the AER have lost their periderm layer. The surface resembles a street paved with cobblestones (Fig. 6). After 48 h, the surface becomes smooth and periderm cells occur to an increasing extent.

The epithelium of the hindlimbs of the untreated controls consists, at the period of investigation (day 12+4 until day 14+4), of two layers. The surface layer is formed by flat, endothelium-like cells, the so-called periderm (Fig. 5A). The contour of the cell membrane is relatively smooth. Only a few short microvilli are observed at the surface. Contacts with the basal cells are accomplished mainly *via* flat protrusions or plump processes. Gap junctions can be demonstrated in the contact areas between periderm cells and between periderm cells and basal cells. The cytoplasm contains, apart from the oval nucleus with a loosely packed karyoplasm and small nucleolus, only a few organelles, e.g. short cavities of the rough ER, but numerous free ribosomes. The basal cells rest on a continuous basal lamina. Their shape varies considerably. Processes of varying length and width radiate from a mostly cuboid cell body and enter into contact with corresponding processes from neighbouring cells. An intercellular space of 20 nm width remains in the contact areas. Only discrete gap junctions are perceptible. Wide intercellular spaces are observed between the cells. Squamous processes that come into contact with one another and form a continuous layer on the basal lamina, derive from the base of the cell bodies. However, the number of gap junctions is small. The nuclei are large, round and show a loosely packed karyoplasm with large nucleoli. The cytoplasm contains numerous free ribosomes, single cavities of the rough ER, oval mitochondria of the crista type, a small Golgi apparatus and a few vesicles and electron-dense granules.

The picture of the epithelium 1 h after drug application resembles the control (Fig. 5A). The only change 2 h after drug application is a permeability of the periderm cells for tannic acid, in other words the cells become tannic-acid-positive and electron-dense. 12 h after treatment the loss of periderm can be confirmed (Fig. 5B). In addition the basal ectodermal cells have changed shape from cuboidal to columnar, acquired microvilli on their apical surface and show special contact zones with neighbouring basal cells.

Duplicative malformations of the forelimb were different in character from those of the hindlimb. Although a few fetuses had ventral polydactyly usually the duplication involved only the metacarpal (Fig. 7). 27 fetuses were affected with this malformation and in almost all cases the second metacarpal was duplicated.

Table 2 contains information regarding the measurement of methoxyacetic acid concentration in maternal serum, embryo and extraembryonic fluid. The concentration of methoxyacetic acid in the embryo was about twice that of maternal serum at the three time points examined and was closely matched by the concentration of this weak acid in the extraembryonic fluid. Methoxyacetic acid was eliminated very slowly from mother and embryo as evidenced by the concentration of chemical still detectable 24 h after administration. In preliminary studies we have been able to detect methoxyacetic acid in maternal blood and embryos 69 h after a single dose of 400 mg kg^{-1} of ME on day 12, giving further evidence that embryonic exposure after a single dose is quite prolonged.

Discussion

Duplication of limb structures in the dorsoventral plane is limited, so far as we are aware, to a single situation, the *Eudiplopodia* chick mutant (Goetinck, 1964). Homozygous embryos have duplication of terminal limb parts that arise from the dorsal limb surface and are more thoroughly developed in the hindlimb. Proximal limb structures are not involved.

In comparison, ME administration to pregnant rats results in dorsoventral limb duplication that invariably arises from the ventral surface of the limb. Again, only terminal limb parts (metatarsals and phalanges) are involved and the hindlimbs are most seriously affected.

The formation of digits is preceded in both cases by the appearance of an aberrant AER which is not a mere extension of the normal AER. In fact the mutant effects of the *Eudiplopodia* gene are expressed

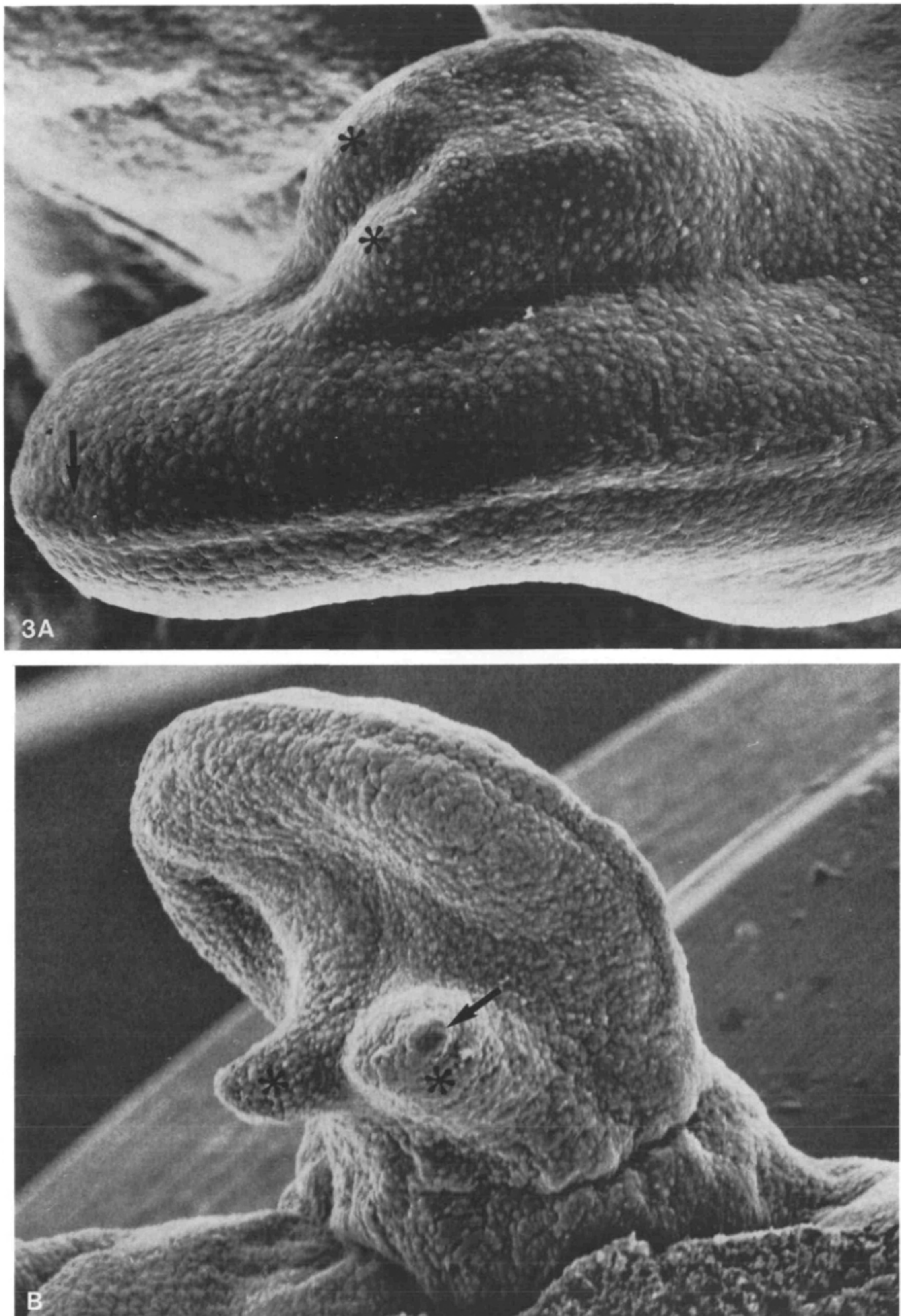

Fig. 3. SEM pictures. (A) Hindlimb of an embryo, 24 h after treatment, with two ventral bulges (*). Arrows mark apical ectodermal ridge. ×42. (B) Hindlimb of an embryo, 48 h after treatment, with two truncated bulges at the ventral side (*). One of the bulges is carrying a plaque-like epithelial thickening (arrow). ×24.

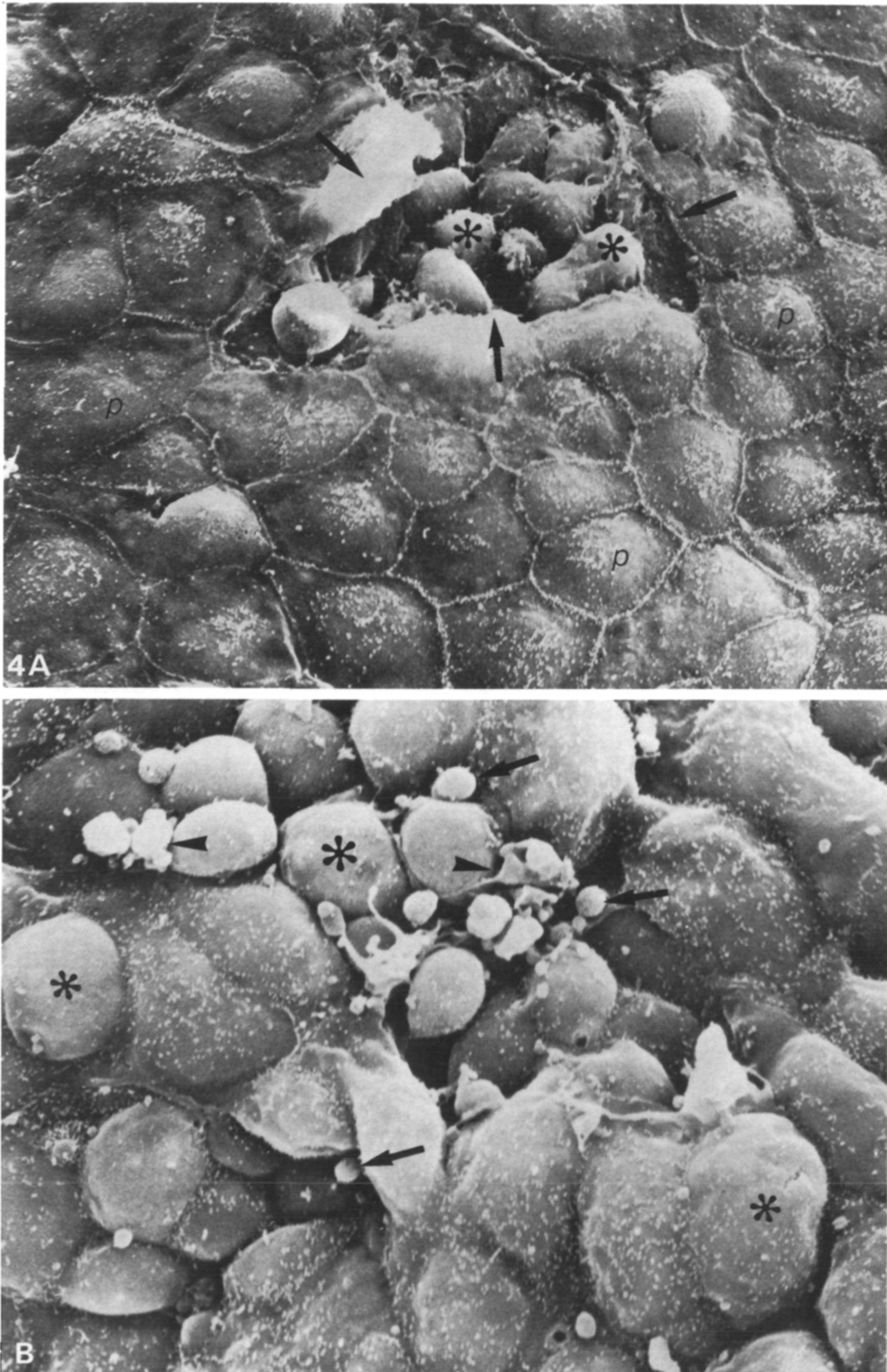

Fig. 4. (A) Ventral surface of a hindlimb 6 h after treatment showing focal shedding of the peridermal cells (arrow). In this area the bulging apices of the basal cells are visible (•), together with normal peridermal cells (*p*) and a smooth surface. ×1870. (B) Ventral surface of a hindlimb 12 h after treatment. Peridermal layer is completely missing in this area. An irregular surface has developed with the apical bulges of the basal epithelial cell layer (•), blebs (arrows) and debris (arrowheads). ×4620.

in the ectoderm as determined from transplantation experiments (Goetinck, 1964; Fraser & Abbot, 1971). ME administration results in changes within the limb ectoderm, especially to the periderm. The damage to this structure by methoxyacetic acid, the proximate teratogen after ME administration, is unique in our experience. We suggest here that the damage to periderm cells may be initiated by methoxyacetic acid

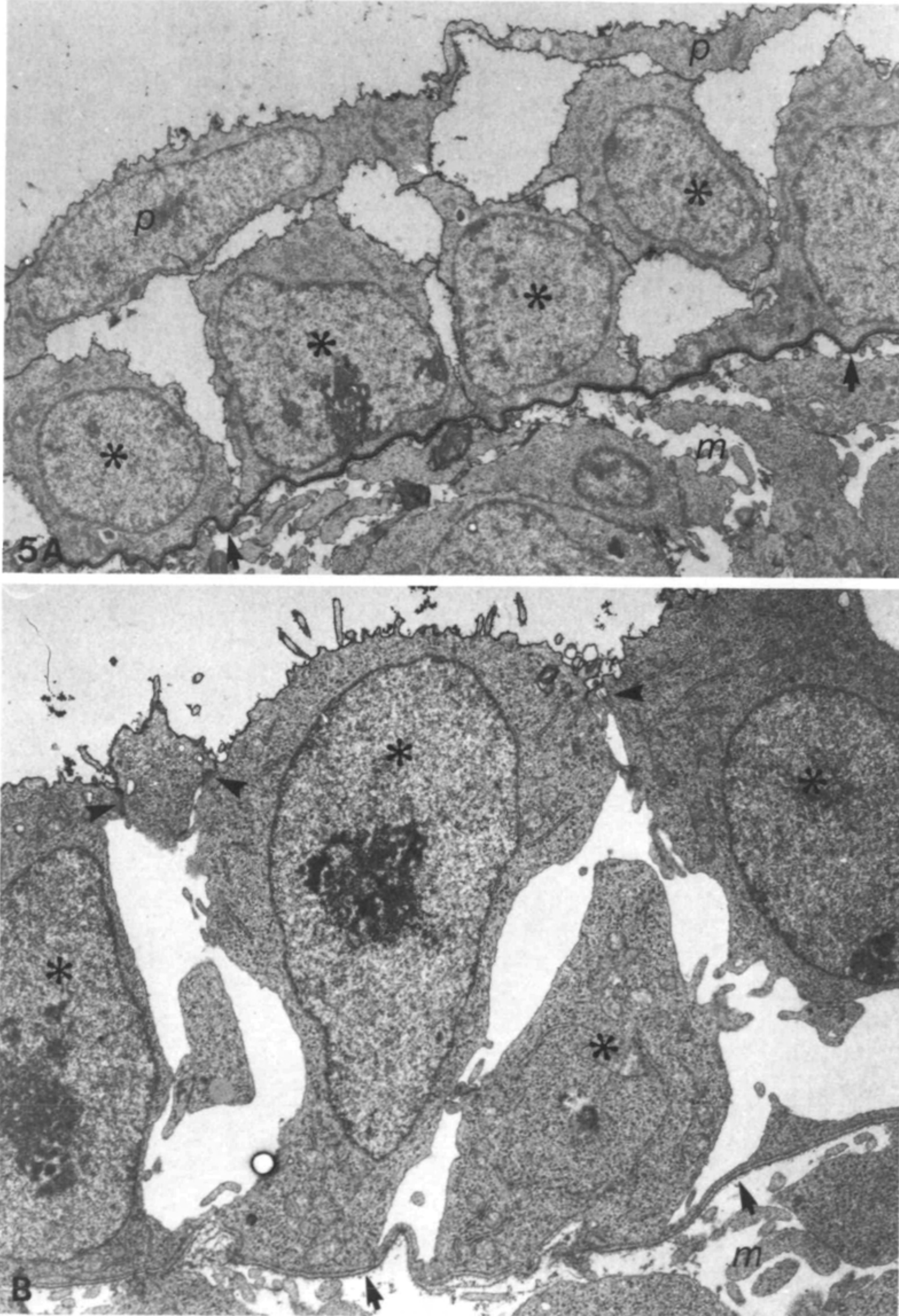

Fig. 5. (A) Untreated embryo, day 12 of pregnancy showing epithelium from the ventral surface of a hindlimb with cuboidal basal cells (•) and flat peridermal cells at the surface (*p*). A basal lamina (arrows) and the mesenchyme (*m*) are seen below the two-layered epithelium. 1:4000. (B) Embryo 12 h after treatment. The peridermal layer is missing. The basal cells are now columnar (•) and show microvilli on the apex and special contact zones (arrowheads). Basal lamina (arrows) and mesenchyme (*m*) are marked. 1:6000.

contained in the amniotic fluid. We have measured the concentration of this weak acid in the exocoelomic fluid and expect that the concentration would not be greatly different in the amniotic fluid that actually bathes the peridermal limb cells. Thus these cells are exposed to greater than 10 mM concentration of a weak acid which intuitively seems a plausible mechanism for the peridermal destruction.

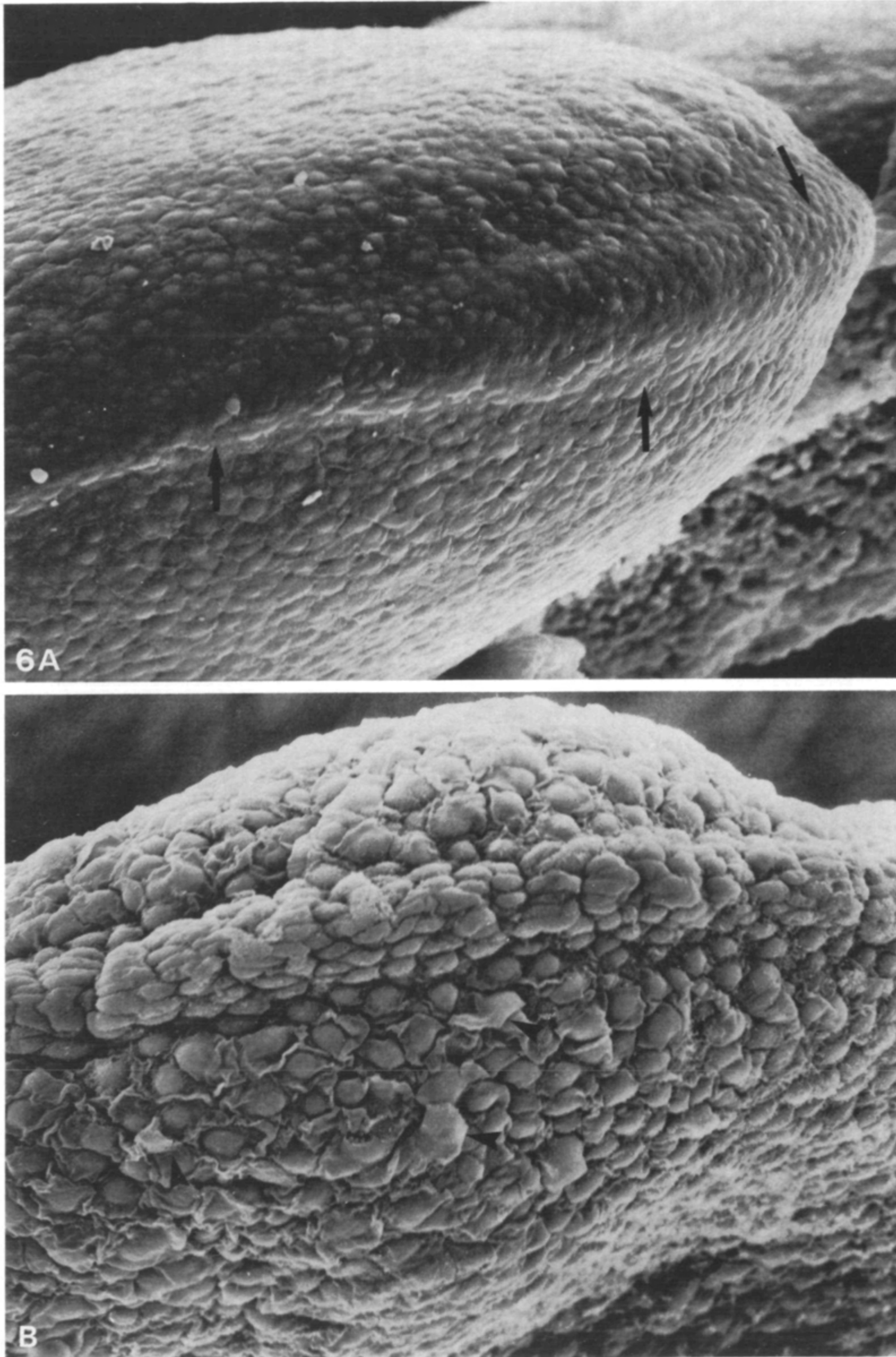

Fig. 6. (A) Rat embryo, late day 12, untreated, showing a hindlimb with smooth peridermal surface and apical ridge (arrows). ×55. (B) Embryo 12 h after treatment. Peridermal layer is completely missing. Some peridermal cells are in the process of shedding (arrowheads). The apices of the basal cells bulge and the smoothness of the surface is lost. ×75.

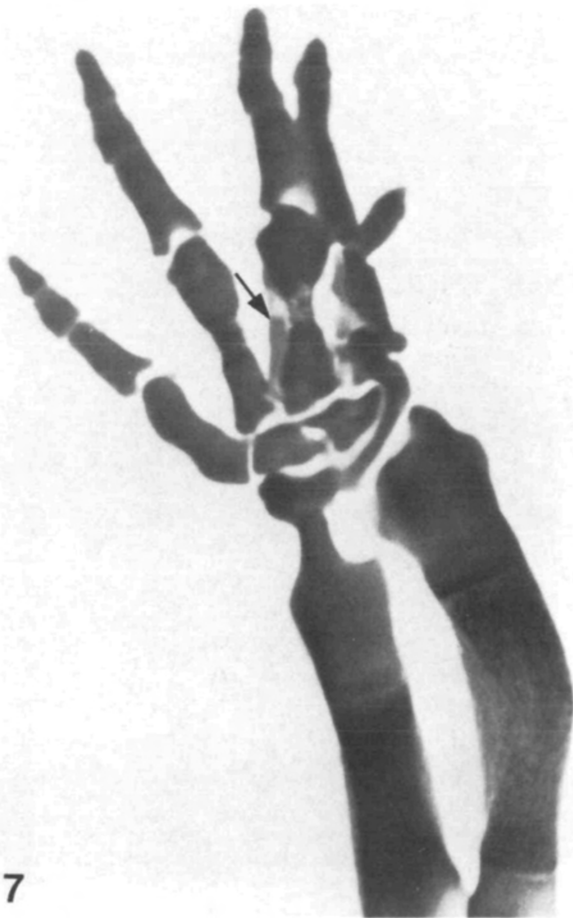

Fig. 7. Forelimb from treated day 20 rat fetus. Arrow indicates ventral metacarpal but the phalanges of digit **2** are not duplicated.

Table 2. *Methoxyacetic acid concentration after administration of 2-ME, 5 mM kg^{-1}* i.p. *on day 12 of rat gestation*

Rat no.	Maternal serum (μg ml^{-1})	Embryo (μg g^{-1})	Extraembryonic fluid (μg ml^{-1})*
2 h			
1	487	1141	1262
2	623	807	1307
3	535	1086	952
4	531	929	840
5	384	1203	1019
	512 ± 86·9	1033 ± 162	1076 ± 201
8 h			
6	273	611	425
7	238	447	374
8	285	554	510
9	158	473	416
10	267	506	471
	244 ± 51·2	518 ± 65·7	439 ± 52·5
24 h			
11	180	458	380
12	140	223	242
13	141	325	252
14	98	245	146
15	82	136	134
	128 ± 38·8	277 ± 121	230 ± 99·2

* Most likely exocoelomic fluid.

† Binding of MAA to plasma or embryos was examined by ultrafiltration through Amicon YMT membranes. No evidence of binding was found in either sample.

A more important question is whether the peridermal changes are associated with the subsequent ventral duplication of the digits. We believe that the two are causally associated and that the ventral duplication represents a regenerative effort by the damaged limb to restore normality. This effort is seen as going awry on the ventral surface perhaps because of a sufficiently high concentration of apical ectodermal maintenance factor. In line with a recent study by Carrington & Fallon (1986) in which a phenocopy of *Eudiplopodia* was induced by grafting normal flank ectoderm from quail and chick donors to denuded wing and leg bud dorsal mesoderm, our results indicate that ridge-inducing ability of limb bud mesoderm is not restricted to apical mesoderm even though under normal developmental conditions the AER appears only at the apex. This property seems to reside in the ventral mesoderm of the rat embryo compared to the dorsal mesoderm of the chick embryo for reasons that are not at all clear. Finally, the present study suggests that the periderm layer of the ectoderm is of some importance for normal limb development. This single cell layer has rarely been the object of attention in studies of limb morphogenesis although Nakamura & Yasuda (1979) suggested that its specific features over the AER can be utilized in detecting abnormalities of this structure.

The authors are grateful for the technical assistance of Helga Sturje and Heidi Wohlfeil. Jan Hagedorn provided secretarial help. This work was supported by grants from the Deutsche Forschungsgemeinschaft to the Sonderforschungsbereiche 29 and 174.

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(*Accepted 4 September 1986*)