Although EW-IIA and -IIC were still contaminated with minor proteins, EW-IIB was shown to be pure on a gel slab of 7.5% w/v polyacrylamide with Tris-glycine pH 8.3 as electrode buffer. Amino acid analysis of the purified EW-IIB was carried out along with assays of total sugar[9] and metal contents. The amino acid composition listed in the table indicated a high half-cystine content and no aromatic amino acids in the cadmium-binding protein, EW-IIB. The absence of sugars and the cysteine-to-metal ratio of 2.4 also coincided with the accepted metallothionein characteristics. However, there are several characteristics which distinguish the earthworm cadmium-binding protein (EW-IIB) from the established metallothionein concept for mammalian metallothioneins; namely, a) low cysteine residue percent (15.4% compared to 33%), b) lack of methionine, c) low serine residue percent (6.1% compared to 14%), d) high level of acidic amino acid residues, and e) longer polypeptide chain. The longer polypeptide chain of EW-II was already suggested by co-chromatography with rat liver metallothionein on a Sephadex G-75 column[6]. The characteristic properties of EW-IIB are rather similar to the cadmium-binding proteins isolated from mussels[10] and blue green algae[11].

The absence of aromatic amino acids in EW-IIB accounts for the UV-absorption spectra in figure 3. The decrease of the shoulder around 250 nm with the decrease of pH value agrees with the dissociation of cadmium from the protein by the cleavage of mercaptide bonds.

The present communication revealed the amino acid composition and some characteristic properties of only 1 of the isoproteins among the 3 different mol. wt cadmium-binding proteins. The isoprotein characterized has properties consistent with the metallothionein concept.

The unique induction of 3 different mol. wt cadmium-binding proteins in the earthworm facilitates further studies not only on biochemical and biological functions of the inducible cadmium-binding proteins but also on evolutionary aspects.

1 M.Y. and K.T.S. thank Dr K. Kubota for encouragement.
2 Sewage Works Division, Yokosuka Municipal Office, Miharu-cho, Yokosuka, Kanagawa 238, Japan.
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## Growth inhibition and occurrence of cleft palates due to hypervitaminosis A[1]

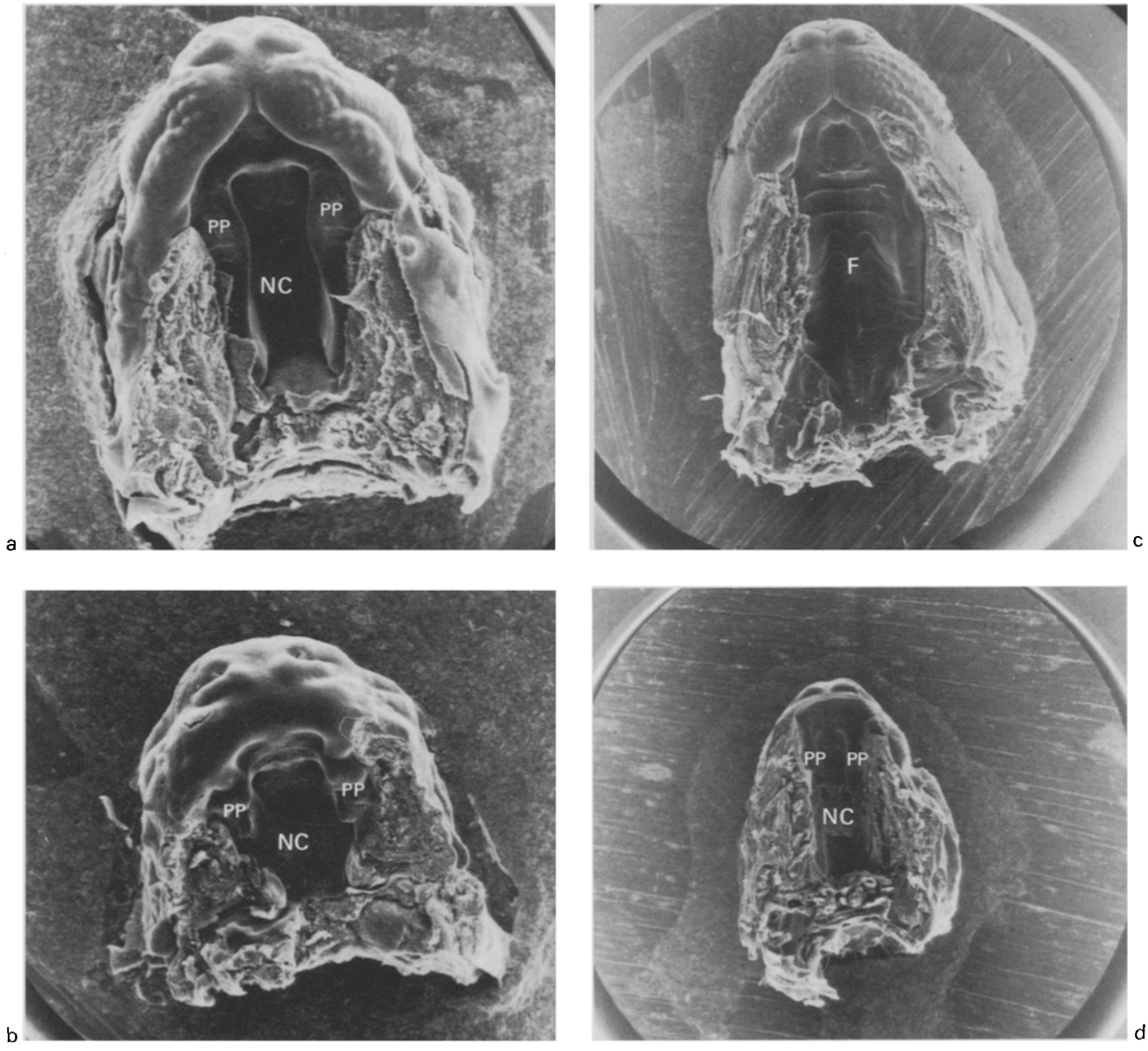
J.B. Pick and C.A. Evans

*Harvard School of Dental Medicine, Boston (Mass. 02115, USA), 14 August 1980*

*Summary.* DNA synthesis in palatal processes obtained from fetal rats was 36% lower in the presence of excess vitamin A, but vitamin A-exposed maxillary explants showed only a modest decrease. Scanning electron micrographs of fetuses exposed to hypervitaminosis A in utero demonstrated both decreased head size and stunted palatal processes. The results suggest that cleft palates occur in this model system because the palatal processes are more sensitive to the actions of vitamin A than are the surrounding maxillary tissues.

The production of cleft palates by giving excess vitamin A to pregnant laboratory animals has been well documented. Nanda reported an 86% occurrence of cleft palate in Wistar albino rats when 40,000 IU/day of retinyl palmitate was given on days 9–13 of gestation[2] and a 74% rate following 60,000 IU/day on days 10–13[3]. Myers et al.[4], Yarington and Shrivers[5], and Nanda[2,3] all reported that palatal tissue from fetuses of vitamin A-treated mothers and palatal tissue cultured in vitamin A-containing medium appeared stunted in comparison to palatal tissue of controls. That palatal shelves might not come into contact at the proper time due to reduced size was offered as an explanation for palatal clefting. This explanation was supported by the work of Lorente and Miller[6] who showed that vitamin A-exposed palatal processes cultured in contact are able to fuse, implying that vitamin A affects events occurring prior to fusion. Sauer and Evans[7] compared DNA synthesis in control and vitamin A-treated maxillary explants of Wistar albino fetal rats and showed that maxillary explants were also sensitive to the growth-inhibiting effects of vitamin A. The present study examines differential responses of tissues to hypervitaminosis A. The aims of the study were: 1. to compare the effects of vitamin A in vitro on rates of DNA synthesis in palatal processes and in maxillary explants having the palatal processes removed; 2. to evaluate the palatal morphology of 16-day and 19-day rat fetuses treated with excess vitamin A in utero using the scanning electron microscope.

*Materials and methods.* A) In vitro. Palatal processes and maxillary explants with palatal processes removed were dissected from Wistar albino rat fetuses (Charles River Laboratories, Wilmington, Mass.) and cultured in NCTC-135 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 50 µg/ml sodium ascorbate using techniques reported by Nanda[2]. Gestational day 16 was chosen because the palatal shelves on day 16 are horizontal above the tongue but not yet fused in this strain of rat. In each of 8 experiments, 20 tissue samples were cultured (table 1): 5 control explants with palatal processes removed, 5 vitamin A-treated explants with palatal processes removed, 5 control pairs of palatal processes and 5 vitamin A-treated pairs of palatal processes. After a 24-h stabilization period of culture in either control medium or medium containing 30 IU vitamin A palmitate (Grand Island Biological Co.) per ml, $^3$H-thymidine (New England Nuclear Corp., Boston, Mass., 15 Ci/mmole) was added to the medium to give a concentration of 1 µCi/ml and the




Scanning electron micrographs of the palates of 16-day and 19-day control and vitamin A-treated fetuses. PP, palatal processes; F, fused palate; NC, nasal cavity. *a* Control 16-day fetus (× 20). Palatal shelves are horizontal and approaching the midline. *b* Treated 16-day fetus (× 20). The palatal processes appear as small distorted buds. *c* Control 19-day fetus (× 10). Palatal fusion is complete. *d* Treated 19-day fetus (× 10). The palatal processes have formed very small shelves extending medially from the maxillary ridges.

incubation was continued for 1 additional h. The samples were then frozen until analyzed. Within each experiment, the tissues and culture media for each group of like samples were pooled and the pooled samples were then sonicated on ice. Protein was precipitated in each sample by adding 100% TCA to make a 5% solution. The solution was then centrifuged at 12,000 × g for 15 min and the resulting supernatant discarded. The precipitated pellet was washed in 5% TCA, recentrifuged, and again the supernatant was discarded. The pellet was dissolved in 0.2 N NaOH and aliquots were added to scintillation fluid and counted in the scintillation counter. The Burton technique[8] was used to determine the total DNA in each pellet and the rates of DNA synthesis were expressed as cpm/μg DNA. The significance of differences between mean values for various experimental groups was estimated by Student's t-test.

B) In vivo. On days 9–12 of gestation, doses of vitamin A palmitate (40,000 IU/day in 0.5 ml deionized water) were administered to 6 timed-pregnant Wistar albino rats via a gastric tube. 6 control rats received 0.5 ml deionized water via a gastric tube. On days 16 and 19 of gestation, 3 rats from each group were sacrificed. For each pregnant rat, the number of resorbed implantation sites and the number of developing embryos were recorded. Also, sample fetuses from each mother were removed and weighed individually. For scanning electron microscopy, embryos were removed from the uterus, placed in a paraffin receptacle and submerged in 0.2 M phosphate buffer, pH 7.4, containing 3% glutaraldehyde. Under a dissecting microscope the mouth was opened to allow entrance of the fixative, and the tongue was removed. Following fixation for 10–12 h, the embryos were decapitated and the mandible removed. The

embryos were then processed and mounted for scanning electron microscopy using techniques reported by Waterman and Meller[9].

*Results.* The rates of DNA synthesis were determined for tissues cultured in vitro. Comparison of mean values (table 1) suggests a possible modest reduction in DNA synthesis in the vitamin A exposed maxillary explants as compared to the control explants. Counts per min in the vitamin A group were 15% lower than in the control group, a difference that did not attain statistical significance in this sample ($p < 0.10$). In contrast, the rate of DNA synthesis in vitamin A-exposed palatal processes was 36% lower than that in control processes, a reduction that was highly significant ($p < 0.01$). The mean rate of DNA synthesis in untreated palatal processes was approximately 4 times that of untreated maxillary explants ($p < 0.01$).

Scanning electron micrographs of day-16 and day-19 experimental fetuses treated in utero show heads reduced in size and stunted palatal processes (fig.). In the day-16 experimental fetuses, the palatal processes appeared as small distorted buds. In the day-19 treated fetuses, the palatal processes formed very small shelves extending medially from the maxillary ridges but leaving the nasal cavities widely exposed. In the 19-day controls, the palatal processes were fused and the nasal cavities were not visible.

The rate of fetal resorption in day-16 and day-19 vitamin A-treated fetuses was approximately 70% (table 2). No fetal resorption was noted in control animals. For the day-16 fetuses, the mean weight of the controls was 0.32 g; for the experimental fetuses it was 0.21 g, a 35% reduction in weight. For the day-19 fetuses, the mean weight of the controls was 1.61 g; for the treated fetuses it was 0.99 g, a 39% reduction in weight.

Table 1. Rates of DNA synthesis in control and vitamin A-treated rat maxillary explants and palatal processes in vitro. Values are expressed in cpm/μg DNA and each value represents 5 pooled samples

| | Explants without palatal processes | | Palatal processes | |
|---|---|---|---|---|
| | Control | Vitamin A-treated | Control | Vitamin A-treated |
| | 508 | 426 | 2189 | 1482 |
| | 731 | 592 | 2770 | 1904 |
| | 656 | 590 | 1962 | 853 |
| | 485 | 412 | 1988 | 1684 |
| | 604 | 465 | 1573 | 938 |
| | 478 | 382 | 2050 | 1161 |
| | 515 | 513 | 2379 | 1494 |
| | 688 | 564 | 2731 | 1857 |
| Mean ± SE | 583 ± 35.2 | 493 ± 29.5 | 2205 ± 144 | 1422 ± 142 |
| % reduction | 15 ($p < 0.10$) | | 36 ($p < 0.01$) | |

*Discussion.* This study and past research have demonstrated decreased palatal shelf size in the vitamin A-induced cleft palate experimental model. Although growth in general is also reduced, decreased palatal shelf mass remains a likely cause of vitamin A-induced clefting because of the marked sensitivity of the palatal processes to vitamin A.

Normal palatal fusion requires extremely rapid growth of the palatal processes. Even under the in vitro conditions of this study, the palatal processes were sites of greater DNA synthetic activity than maxillary explants during the time period studied. Rapidly growing tissues, such as palatal processes, are vulnerable targets for growth-inhibiting teratogenic agents. The type and extent of abnormality produced by vitamin A may vary with the timing of the insult and the relative rates of growth of different tissues. A final explanation of the experimental phenomenon need not be limited to growth inhibition, however, since vitamin A has many actions.

The second part of this study showed 35% and 39% reductions in weight for the day-16 and day-19 fetuses exposed in utero to vitamin A on days 9–12 of gestation. This weight reduction correlates well with Nanda's[5] observation of an 18% weight reduction on day 18 after intraamniotic administration of varying concentrations of vitamin A on day 13 only. The fact that 70% of the implantation sites in this study were found resorbed for day-19 embryos differs from Nanda's[6] study in which a 10% resorption rate was found under the same general conditions. The difference could be due to different gastric absorption rates of vitamin A palmitate, varying activity of the vitamin A palmitate and/or varying sensitivity within this strain of rat.

Table 2. Effects of hypervitaminosis A on resorption rates and fetal weight

| | 16-day embryos | | | | | | 19-day embryos | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | | Vitamin A-treated | | | Control | | | Vitamin A-treated | | |
| | i | r | $\bar{w}$ | i | r | $\bar{w}$ | i | r | $\bar{w}$ | i | r | $\bar{w}$ |
| Litter 1 | 9 | 0 | 0.36 | 6 | 4 | 0.19 | 10 | 0 | 1.47 | 7 | 5 | 1.07 |
| Litter 2 | 7 | 0 | 0.33 | 8 | 5 | 0.20 | 9 | 0 | 1.72 | 7 | 4 | 0.95 |
| Litter 3 | 10 | 0 | 0.28 | 9 | 6 | 0.22 | 6 | 0 | 1.68 | 5 | 4 | 0.95 |
| Total | 26 | 0 | 0.32 ± 0.02 SE | 23 | 15 | 0.21 ± 0.01 SE | 25 | 0 | 1.62 ± 0.08 SE | 19 | 13 | 0.99 ± 0.04 SE |
| Reduction in weight | | | | | | 35% ($p < 0.01$) | | | | | | 39% ($p < 0.01$) |

i, total implantation sites (embryos + resorptions); r, resorption sites; $\bar{w}$, mean weight of embryos in g.

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