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High pH prevents retinoic acid-induced teratogenesis

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ABSTRACT Exogenously applied retinoic acid is known to cause teratogenic effects in a variety of animal systems. We examined whether the formation of teratogenic effects may be influenced by the electrical charge of retinoic acid. The pKa of retinoic acid ranges from 6 to 8, indicating that it is electrically neutral in a pH 5 medium and is negatively charged in a pH 9 medium. With this idea in mind, embryos of the pond snail *Lymnaea stagnalis* were pulse-treated with retinoic acid and cultured in media of different pH. The percentage of embryos with retinoic acid-induced eye defects was 6-fold lower in the pH 9 medium as compared to the pH 5 medium. In contrast, the apical plate defects induced by retinoic acid were not pH-dependent. The observation that high pH prevents eye defects but not apical plate defects can be explained by taking into account an electrophoretic redistribution of retinoic acid resulting from the voltage gradients that are generated by the *Lymnaea* embryo.

KEY WORDS: *embryogenesis, retinoic acid, molluscs, ionic currents, voltage gradients*

Introduction

Retinoids, a family of vitamin A metabolites, are thought to be positional signalling molecules which may be responsible for pattern formation in various animal systems. In vertebrates, retinoids are required for normal embryonic development (Lohnes *et al.*, 1993, 1994; Kastner *et al.*, 1994; Mendelsohn *et al.*, 1994; Sukov *et al.*, 1994) but excess is teratogenic, inducing deformations in the limb (Tickle *et al.*, 1982), the central nervous system (Durston *et al.*, 1989), and the craniofacial region (reviewed by Brockes, 1989; Maden and Holder, 1992; Morriss-Kay, 1993). The claim that retinoids may act as morphogens in development is supported by the presence of endogenous gradients of retinoids (Thaller and Eichele, 1987; Chen *et al.*, 1994; Scadding and Maden, 1994) and high-affinity receptors in the developing organism in patterns that are consistent with the role as a positional signalling molecule (Dollé *et al.*, 1989). In many experiments, excess of retinoids is brought about by incubating whole embryos in various concentrations of the agent. Retinoids are rapidly taken up and are retained in the embryo, as demonstrated in *Xenopus* embryos (Durston *et al.*, 1989). Similar to the endogenous retinoids, the applied retinoids may become localized to specific regions within the embryo. Such localizations of applied retinoids have been shown to occur in the chick limb bud for example (Tamura *et al.*, 1993).

For both endogenous and applied retinoic acid, the mechanisms of localization have remained unclear. Possibly, endogenous retinoic acid gradients may result from the presence of specific sites of retinoic acid synthesis, such as Hensen's node in mouse embryos (Hogan *et al.*, 1992). Another mechanism that has been proposed, is the binding of retinoic acid to cytoplasmic proteins (CRABPs). These receptors are present, for example, in the mouse embryo and display differential expression during early morphogenesis and development of the limbs (Dollé *et al.*, 1989; Ruberte *et al.*, 1991). CRABP-I is known to reduce the intracellular half-life of retinoic acid (Boylan and Gudas, 1992). This change in half-life may function as a sink for retinoic acid resulting in a non-uniform distribution within the embryo. Diffusion of retinoic acid between source and a sink may explain the establishment of a gradient. However, this explanation need not to be the only one. A novel view which we put forward in this study takes into account the electrical properties, both of retinoic acid and of the embryo.

It is well known that most developing and regenerating organisms generate voltage gradients that can be measured in the surrounding medium using a special electrode called vibrating probe (reviewed by Nuccitelli, 1990). The behavior of electrically-charged molecules both outside and inside the embryo will be affected by these voltage gradients. For example, the endogenous voltage gradient between the insect oocyte and its nurse cells results in electrophoresis of proteins depending on their electrical charge (Woodruff and Telfer, 1980). Intercellular electrophoresis of electrically charged dyes between gap junction-coupled cells can be observed in an applied voltage gradient (Cooper *et al.*, 1989). Furthermore, extracellular voltages can produce asymmetries in cell surface receptors, such as the acetylcholine receptor (Luther and Peng, 1985) and the EGF receptor (Giugni *et al.*, 1987). *In vivo* experiments with retinoic acid are usually performed between pH 7 and pH 8. In this pH range, a considerable proportion of retinoic acid is negatively charged (see Fig. 1). It is to

Abbreviations used in this paper: CRABP, cellular retinoic acid binding protein; EGF, epidermal growth factor.

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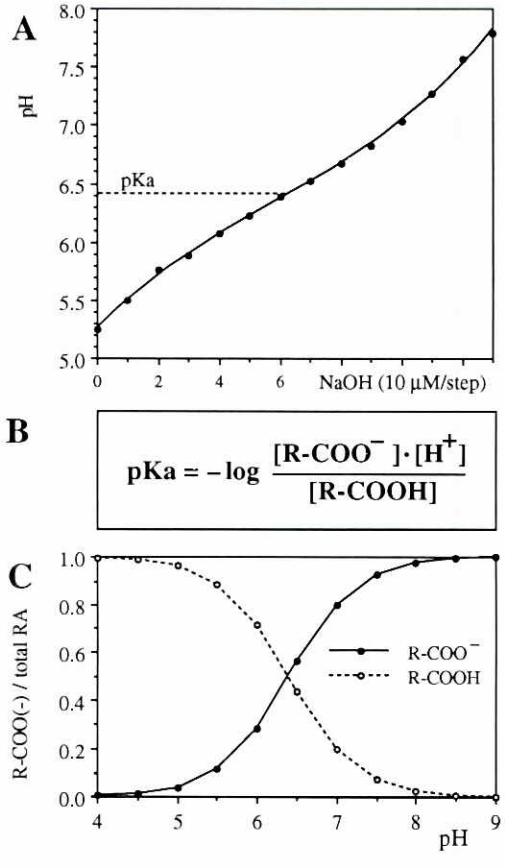

Fig 1. The pKa of all-trans retinoic acid in *Lymnaea* culture medium. **(A)** *Titration of retinoic acid with increasing amounts of NaOH. The midpoint of titration (pH 6.4) indicates the pKa of retinoic acid.* **(B)** *Formula describing the equilibrium between negatively charged (R-COO⁻) and electrically neutral (R-COOH) retinoic acid.* **(C)** *The proportion of negatively charged retinoic acid increases with increasing pH.*

be expected that these charged molecules will be driven away from the negative pole of a voltage gradient within the embryo, whereas they will be drawn actively towards the positive pole. This electrophoretic process may give rise to a non-uniform distribution of retinoic acid within the embryo, thereby influencing both normal pattern formation and teratogenesis induced by this agent.

The aim of the present study was to investigate whether the teratogenic effects of retinoic acid may be influenced by electrical properties, both of retinoic acid and of the embryo. The embryo of the gastropod snail *Lymnaea stagnalis* is a suitable model system for such studies, since both retinoic acid-induced developmental defects and voltage gradients are known to occur in this organism (Créton *et al.*, 1993a,b, 1994).

Results

Retinoic acid is a weak acid that becomes negatively charged at high pH. In order to calculate which proportion of the retinoic acid is negatively charged, one needs to know the pKa (-log dissociation constant) of retinoic acid. It has been shown previously that the pKa of retinoic acid varies between 6 and 8, partly depending on the solvent used (Noy, 1992a). To determine the pKa of retinoic acid in the *Lymnaea* culture medium, we performed a titration with NaOH (Fig. 1A). The midpoint of titration was determined to be at a pH of 6.4, indicating that the pKa of retinoic acid is 6.4 in this particular culture medium. Using the pKa value of 6.4, we calculated the proportion of negatively charged retinoic acid at different pH. This was done with the formula that describes the equilibrium of a dissociation reaction (Fig. 1B). At pH 5, nearly all the retinoic acid is electrically neutral. The proportion of negatively charged retinoic acid becomes progressively larger at increasing pH (Fig. 1C). At pH 9, nearly all the retinoic acid is negatively charged.

Knowing that the electrical charge of retinoic acid is pH-dependent, we examined retinoic acid-induced teratogenesis in embryos cultured at pH 5 and pH 9. Embryos cultured for 5 days

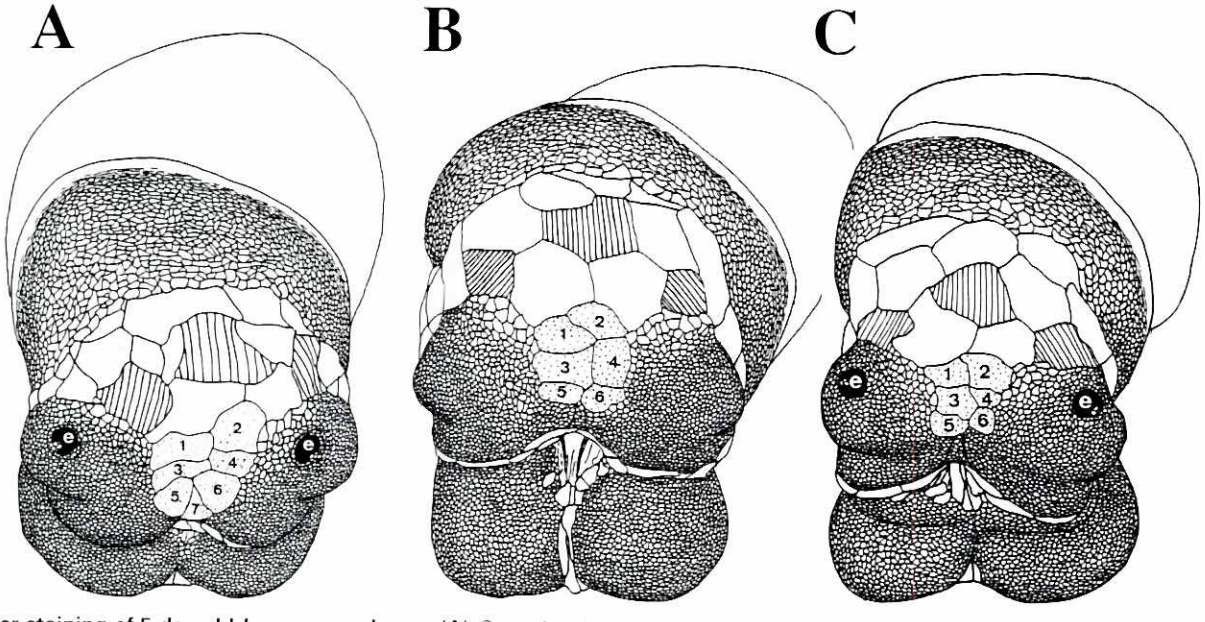

Fig 2. Silver staining of 5-day old *Lymnaea* embryos. **(A)** *Control embryo, showing eyes (e) and 7 apical plate cells (1-7).* **(B)** *Retinoic acid-treated embryos cultured at pH 5 show eye defects as well as apical plate defects.* **(C)** *Retinoic acid-treated embryos cultured at pH 9 show only apical plate defects.*

at different pH were silver stained and examined for developmental defects (Fig. 2). Retinoic acid-treated embryos cultured in the pH 5 medium showed eye defects as well as apical plate defects (Fig. 2B). Retinoic acid- treated embryos cultured in the pH 9 medium did show apical plate defects, but nearly all of the embryos had normal eyes (Fig. 2C). To quantitate the rescue of eye defects with the pH 9 medium, eye defects were scored in a large number of embryos (n= 1224) over 4 successive experiments (Fig. 3A). Control embryos (not treated with retinoic acid) did not show any eye defects when cultured in the pH 5 or pH 9 media. The percentage of eye defects in the retinoic acid-treated embryos was 6-fold lower in the pH 9 medium as compared to the pH 5 medium. In a second set of experiments, the apical plate defects were scored (Fig. 3B). We examined a total of 161 embryos over three experiments. The apical plate was considered to be defective when the number of apical plate cells deviated from the standard number of 7 cells. Control embryos (not treated with retinoic acid) showed a low percentage (8%) of apical plate defects both in the pH 5 and the pH 9 medium. The percentage of apical plate defects increased 6-fold in retinoic acid-treated embryos. No significant difference in apical plate defects was found between the retinoic acid-treated embryos cultured in the pH 5 and the pH 9 medium. Thus, the retinoic acid-induced eye defects are pH-dependent whereas the retinoic acid-induced apical plate defects are not. We subsequently examined whether these different teratogenic effects could be explained by electrophoretic forces that act upon retinoic acid.

To study these electrophoretic forces, we examined the voltage gradient around the *Lymnaea* embryo. This was done by measuring the ionic currents generated by 33 h and 50 h old embryos in both the pH 5 medium and the pH 9 medium (Fig. 4). Several regions were measured and for each region 6 measurements were made in different embryos. The 33 h old embryos showed a distinct antero-posterior pattern of ionic currents. The anterior region of the embryo generates an outward current that loops back through the culture medium to the posterior region of the embryo. The anterior region of the embryo was examined in more detail in a 50 h old embryo in which more structures can be distinguished. We measured the ionic currents at 5 positions within the head of the embryo; 1) the prototroch at the left side of the embryo, 2) the cephalic plate at the left side of the embryo, 3) the apical plate, 4) the cephalic plate at the right side of the embryo and 5) the prototroch at the right side of the embryo. The head region of the 2-day old embryo generates an overall outward current except for the cephalic plates, which generate inward currents. The ionic currents loop through the culture medium from the apical plate to the cephalic plates and will form a voltage gradient in the culture medium along the surface of the embryo. The embryonic battery generating the ionic currents has its negative pole at the cephalic plates (inward current) and its positive pole at the apical plate (outward current). Although the magnitude of the currents varies, the direction of the voltage gradient is the same in the pH 5 and pH 9 medium. Consequently, the direction of the electrophoretic force exerted on the charged molecules will be the same in both media. Nevertheless, there is a difference in teratogenic effect. This difference can be explained by assuming that the efficacy of electrophoretic transport differs between the two media. This efficacy clearly depends on the relative amount of negatively charged retinoic acid molecules and that is exactly the parameter that changes considerably between pH 5 and pH 9.

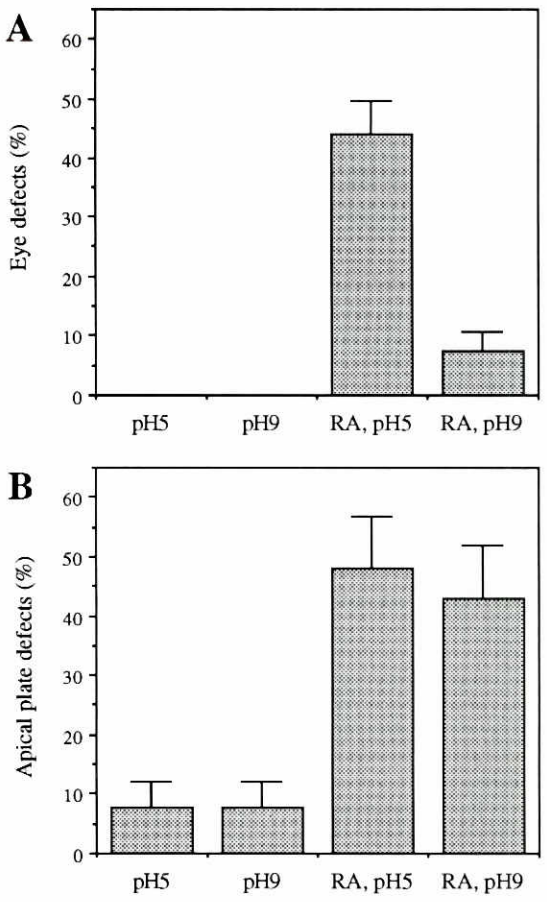

Fig 3. Rescue of retinoic acid-treated embryos. *From left to right: control embryos cultured in pH 5 medium (pH 5), control embryos cultured in pH 9 medium (pH 9), retinoic acid-treated embryos cultured in pH 5 medium (RA, pH 5) and retinoic acid-treated embryos cultured in pH 9 medium (RA, pH 9).* **(A)** *Percentage of eye defects; the difference between RA-pH 5 and RA-pH 9 is significant at a 99% confidence limit (Student's t test).* **(B)** *Percentage of apical plate defects.*

Discussion

The aim of the present study was to find out whether teratogenic effects of retinoic acid may be influenced by electrical properties both of retinoic acid and of the embryo. To this end we examined: 1) the electrical charge of retinoic acid, 2) the embryonic defects caused by negatively charged and electrically neutral retinoic acid, and 3) the voltage gradients generated by *Lymnaea* embryos.

The electrical charge of retinoic acid depends on the pH of the medium. It is a weak acid that dissociates at high pH. The pKa of this dissociation reaction was determined in the *Lymnaea* culture medium. The pKa was shown to be 6.4, which is in close agreement with the pKa of 1 μM retinoic acid in distilled water (pKa= 6.5) or in 0.15 M NaCl (pKa= 6.7), as measured by Noy (1992a). However, the formation of retinoic acid-micelles or the incorporation of retinoic acid into lipid bilayers is known to increase the pKa up to values between 7 and 8 (Noy, 1992a,b). The pKa values of retinoic acid in solution or incorporated in lipid bilayers are all within the physiological range. Therefore, *in vivo* experiments can be carried out to test the effects of altering the charge of retinoic acid by a

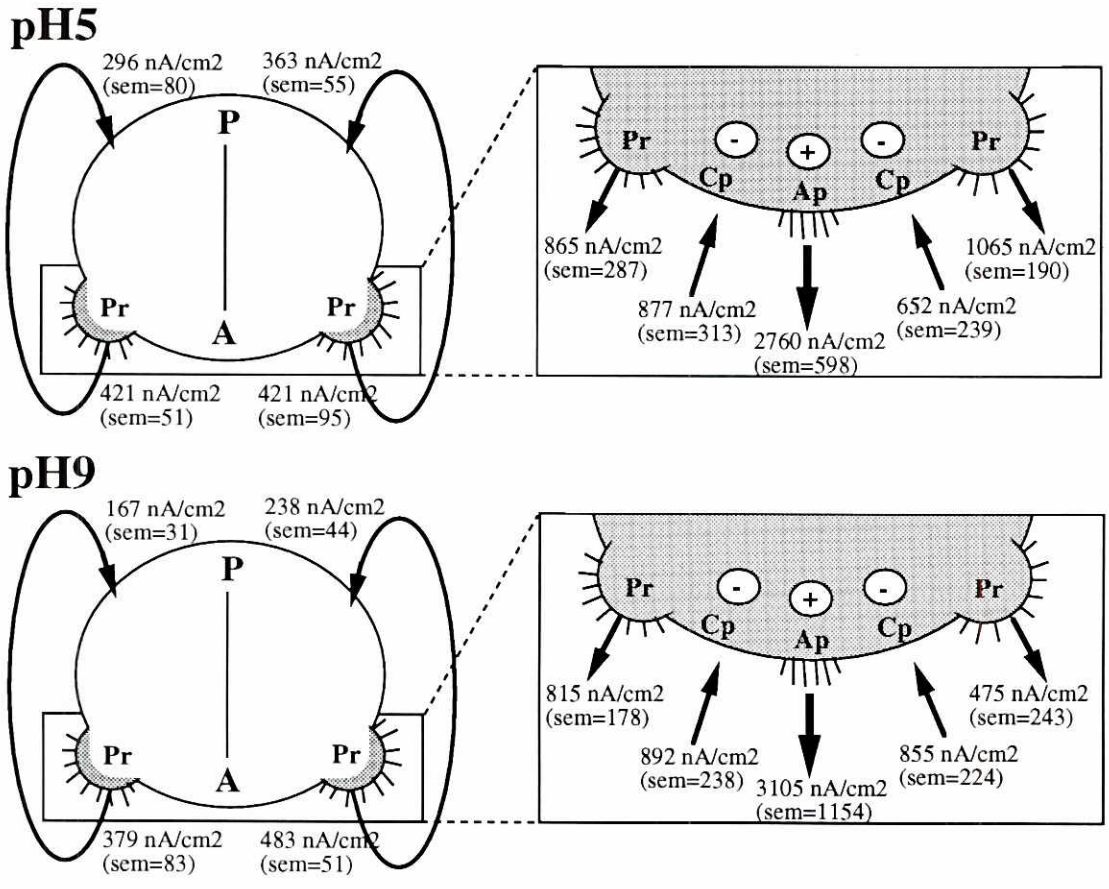

Fig 4. The pattern of ionic currents around the *Lymnaea* embryo. *Ionic currents are correlated with the antero-posterior axis (A-P) of 33 h old embryos (left). The pattern of ionic currents was measured in more detail in the head of 50 h old embryos (right). An outward current is generated by the prototroch (Pr) and the apical plate (Ap). An inward current is generated by the cephalic plates (Cp). The direction of the voltage gradient, which forms concomitant with the ionic currents, is the same in the pH 5 and the pH 9 medium.*

change in pH of the culture medium. We used this strategy to investigate whether electrically neutral and negatively charged retinoic acid differ in their potency to induce developmental defects in the embryos of *Lymnaea stagnalis*.

In normal development, 2 regions of proliferating cells (the cephalic plates) form the eyes and tentacles of the *Lymnaea* larva. The cephalic plates are separated by 7 cleavage-arrested cells which constitute the apical plate (Verdonk, 1965). Treatment with retinoic acid affects both eye formation (Créton *et al.*, 1993b) and the number of apical plate cells (Créton *et al.*, 1994). In the present study, the embryos were first loaded with retinoic acid during early development and subsequently cultured for 5 days in media of different pH. This strategy ensures an equal uptake of retinoic acid in all embryos and allows enough time for internal redistribution by diffusion and electrophoresis. We would like to emphasise that retinoic acid loaded within the cytoplasm of the embryonic cells will be protected from extracellular changes in pH. However, some of the retinoic acid will be present in the lipid bilayers constituting the surface of the embryo, both because retinoic acid was applied from the outside of the embryo and because of the lipophilic nature of retinoic acid. This pool of retinoic acid will be subject to the change in pH of the extracellular medium, because of its configuration in the plasma membranes. The cyclohexene group of retinoic acid will extend towards the centre of the lipid bilayer, whereas the polar carboxylic group will project into the aqueous phase of the membrane surface (Wassall *et al.*, 1988). It is this polar carboxylic group of retinoic acid that makes the molecule susceptible to electrophoresis. We estimate that there are at least 21 h for electrophoresis to take place, since retinoic acid is most likely to exert its effects during late gastrulation (Créton *et al.*, 1993b). In comparison, only 3 to 4 h are needed to establish a gradient of retinoic acid in the chick limb bud (Eichele and Thaller, 1987). In the present paper it is shown that 44% of the retinoic acid-treated embryos had eye defects when cultured at pH 5. A significantly lower percentage (7.5%) of embryos had eye defects when cultured at pH 9. This pH dependence is shown to be specific for the eye development as the retinoic acid-induced apical plate defects did not show such pH dependence. The observation that high pH prevents eye defects but not apical plate defects can be explained by examining the voltage gradients that surround the embryos.

In *Lymnaea*, the polarity of ionic currents correlates with the animal-vegetal polarity of the uncleaved egg (Zivkovic and Dohmen, 1989) and with the antero-posterior polarity after gastrulation (Créton *et al.*, 1993a). For the present study, the pattern of ionic currents was re-examined in the pH 5 and pH 9 media. The attention was focused on the currents in the presumptive head-region of the mid- and post-gastrula snail and these currents were shown to be similar in magnitude and direction to those previously reported for other culture media (Créton *et al.*, 1993a). An outward current leaves the apical plate and an inward current enters the cephalic plates. As a result, a voltage gradient is established in the head region, the apical plate being positive and the cephalic plates negative. This voltage gradient should exert electrophoretic forces on negatively charged retinoic acid. Figure 5 summarizes the electrophoretic forces on retinoic acid at pH 5 and pH 9 and depicts how the electrophoretic forces and the observed developmental

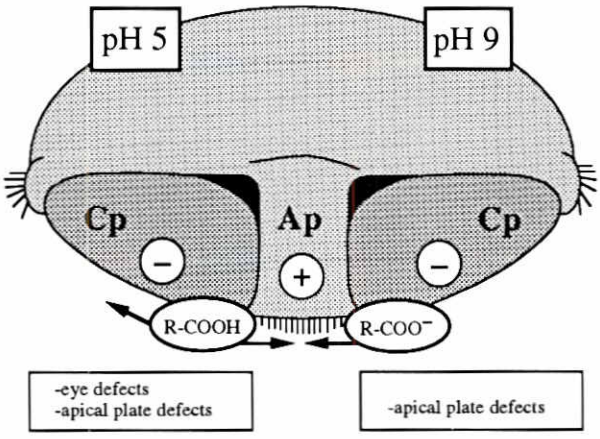

Fig 5. Model showing the electrophoretic forces on retinoic acid in the pH 5 and pH 9 medium, according to the voltage gradient that is generated by the *Lymnaea* embryo. *At pH 5, electrically neutral retinoic acid (R-COOH) will diffuse randomly. At pH 9, retinoic acid is negatively charged (R-COO⁻) and will be subject to electrophoretic forces that remove this negatively charged molecule from the negatively charged cephalic plates.*

defects may be correlated. The main idea is that, at pH 9, negatively charged retinoic acid is driven away from the negatively charged cephalic plates, and is thus incapable of causing defective development of the eyes. At pH 5, retinoic acid is electrically neutral and consequently remains undisturbed in every region of the embryo, including the cephalic plates where its presence induces eye defects. Since we studied electrophoretic forces rather than electrophoretic movements, it remains to be shown to which extent retinoic acid is really removed from the cephalic plates at high pH. The interaction of electrically charged retinoic acid with the voltage gradient that surrounds the *Lymnaea* embryo is, however, unambiguous and provides a simple explanation for the observed pH-dependence of retinoic acid-induced teratogenic effects.

Materials and Methods

The pKa of retinoic acid

The pKa of all-trans retinoic acid (Sigma) was determined by means of titration with NaOH. Retinoic acid was dissolved to a final concentration of 0.1 mM in 100 ml artificial medium, which is used for culturing *Lymnaea* embryos (medium C; 10 mM NaCl, 0.5 mM KCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ in distilled water, pH 6). A fixed amount of NaOH (100 μl, 10 mM) was added in several steps and the pH was subsequently measured with a Beckman electrode. The midpoint of titration indicates the pKa of retinoic acid in this particular culture medium. For measurements of the pKa in other media and in lipid bilayers we refer to the studies of Noy (1992a,b).

Embryos and experimental treatments

The fresh-water snail *Lymnaea stagnalis* is a gastropod mollusc from the subclass Pulmonata. Egg-laying is induced by renewing the aquarium water. The embryos were reared at 25°C in medium C and were kept inside their capsules for the entire experimental procedure. For retinoic acid treatment, the embryos were cultured for 6 h (starting at first cleavage) in 1 μM all-trans retinoic acid in medium C. Following this treatment, the embryos were rinsed twice in medium C and subsequently cultured in pH 5 or pH 9 medium (2 mM Tris-HCl in medium C, setting the final pH at 5.0 and 9.0 respectively).

Morphological analysis

Eye defects were scored in 6 day-old embryos using x20 magnification on a Zeiss dissection microscope. Embryos were scored to have eye defects when either one or both eyes were missing or when one eye was clearly smaller than the other. Apical plate defects were scored after silver staining of 5-day-old embryos. For silver staining, the embryos were decapsulated, stained in a 0.75% $AgNO_3$ solution and exposed to UV light for about 4 min. As soon as the cell boundaries stood out clearly, the embryos were rinsed in distilled water, dehydrated with 70% ethanol (5 min) and dimethoxypropane (10 min), cleared in histoclear (National Diagnostics; Manville, NJ, USA) and mounted in Canada balsam (BHD Chemicals). The embryos were studied in a Zeiss microscope equipped with an auxiliary prism, allowing the exact drawing of the cell boundaries.

Measurement of voltage gradients

Embryos form voltage gradients in their surrounding medium when generating ionic currents. These ionic currents were measured around *Lymnaea* embryos using the extracellular vibrating probe (Jaffe and Nuccitelli, 1974) as described previously (Zivkovic and Dohmen, 1989). We used the one-dimensional vibrating probe system equipped with wire electrodes (Scheffey, 1988). The electrodes had platinum black tips of 30 μm and vibrated with an amplitude of 30 μm. The minimum recording time at one position was 15 sec with a 1 sec time constant of the lock-in amplifier. The probes were calibrated by measuring a known current which was passed through a glass microelectrode filled with 3 M KCl whose the tip was considered a point source of current. The measurements were performed in pH 5 or pH 9 medium, both of which have a specific resistivity of 450 Ωcm. The current densities always exceeded the detection limit of about 20 nA/cm^2. Embryos were measured in plastic petri dishes (Costar, Cambridge, MA, USA). After the embryos had attached to the bottom of the dish, the probe was positioned with the centre of vibration 50 μm away from the surface of the embryo. The plane of vibration was always kept perpendicular to the surface of the embryo. Between 2 measurements, the probe was moved to a reference position 300 μm away from the embryo at which the current density was considered to be 0. The current direction depicted by arrows or indicated as inward or outward refers to the flow of positive charge.

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