

Characterization of Concentration Gradients of a Morphogenetically Active Retinoid in the Chick Limb Bud

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Abstract. It has long been suggested that the generation of biological patterns depends in part on gradients of diffusible substances. In an attempt to bridge the gap between this largely theoretical concept and experimental embryology, we have examined the physiology of diffusion gradients in an actual embryonic field. In particular, we have generated in the chick wing bud concentration gradients of the morphogenetically active retinoid TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid, a synthetic vitamin A compound. Upon local application of TTNPB the normal 234 digit pattern is duplicated in a way that correlates with the geometry of the underlying TTNPB gradient; low doses of TTNPB lead to a shallow gradient and an additional digit 2, whereas higher doses result in a steep, far-reaching gradient and patterns with additional

digits 3 and 4. The experimentally measured TTNPB distribution along the anteroposterior axis, can be modeled by a local source and a dispersed sink. This model correctly predicts the site of specification of digit 2, and provides an empirical estimate of the diffusion coefficient (*D*) of retinoids in embryonic limb tissue. The numerical value of $\sim 10^{-7}$ cm²s⁻¹ for *D* suggests that retinoids are not freely diffusible in the limb rudiment, but interact with the previously identified cellular retinoic acid binding protein. In addition, *D* affords an estimate of the time required to establish a diffusion gradient as 3 to 4 h. This time span is in a range compatible with the time scale of pattern specification in developing vertebrate limbs. Our studies support the view that diffusion of morphogenetic substances is a plausible mechanism of pattern formation in secondary embryonic fields.

GENERATING a biological pattern in a multicellular embryonic field is thought to involve direct cell-cell interaction and long-range cues in the form of gradients of diffusible substances. That the generation of simple or intricate morphogen gradients is feasible from the physical-chemical point of view has been well documented (for a review, see Meinhardt, 1982; Slack, 1983). To test the gradient concept in an actual embryonic field, we have studied gradients of the morphogenetically active retinoic acid analogue TTNPB,¹ or (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid, in the chick limb bud. TTNPB, when applied from a local source (a bead) to the anterior margin of a limb bud, will distribute in the form of a concentration gradient and eventually induce digit pattern duplications (Eichele et al., 1985). This observation has prompted us to examine at a quantitative level how the strength of the local source influences the shape, extent, stability, and biological effect of the resulting gradient. We find that the experimentally generated TTNPB gradients can be modeled in terms of a local source and a dispersed sink. Using this model, we have determined the magnitude of the diffusion constant (*D*) for TTNPB in the chick limb bud.

1. *Abbreviations used in this paper:* CRABP, cellular retinoic acid-binding protein; TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (see Loeliger et al., 1980; Strickland et al., 1983); ZPA, zone of polarizing activity.

The chick limb rudiment is an excellent system to study morphogenetic gradients, because the limb bud is a geometrically simple, autonomous (Hamburger, 1938) and experimentally accessible secondary embryonic field. Furthermore, in the limb system retinoic acid is a candidate morphogen for the following reasons. First, locally applied retinoic acid (or TTNPB) induces mirror-image duplications of the digit pattern (Tickle et al., 1982; Summerbell, 1983; Tickle et al., 1985; Eichele et al., 1985; Eichele, 1986). These patterns are indistinguishable from those generated by grafting tissue from the zone of polarizing activity (ZPA, also known as polarizing region) to a host limb bud (Saunders and Gasseling, 1968; for a review, see Tickle, 1980; Bryant and Muneoka, 1986). Secondly our recent work demonstrates that retinoic acid is present endogenously in the chick limb bud, and that it forms a concentration gradient with a high point in the ZPA-containing posterior limb bud tissue (Thaller and Eichele, 1987).

Materials and Methods

Chemicals

(*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-6,7-³H₂)-1-propenyl] benzoic acid ([³H]TTNPB, 348,500 mol wt and 24.1 Ci/mmol specific activity) was synthesized by SRI International, Menlo Park, CA, under contract to the National Cancer Institute. Nonradioactive TTNPB was a kind gift from Hoffmann-LaRoche, Basel, Switzerland.

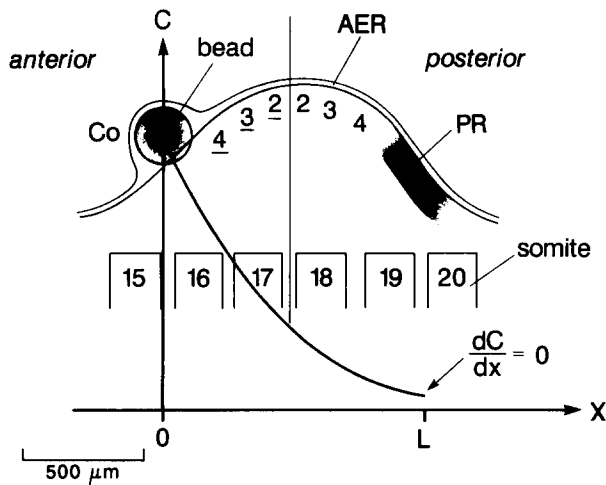


Figure 1. Schematic representation of the experimental system. A small ion-exchange bead impregnated with radioactive TTNPB is placed below the apical ectodermal ridge (AER) at the anterior margin of an early chick wing bud. The bead acts as a local source of TTNPB and will release the retinoid at an approximately constant rate for a period of about 1 d (Eichele et al., 1984). The slow release is balanced by clearance of TTNPB from the limb tissue, resulting in a steady-state level of TTNPB which persists for at least 20 h (Eichele et al., 1985). Superimposed onto the bud is a coordinate system with a position axis (*abscissa*) and a concentration axis (*ordinate*). A schematic concentration gradient of TTNPB is shown with $C = C_0$ at $x = 0$ and $dC/dx = 0$ at $x = L$, where L is the width of the field. The polarizing region (PR) is situated opposite the bead, extending along the posterior margin of the bud. Plain numbers indicate the prospective tissue areas for each of the three native digits (Hincliffe et al., 1981) and underlined numbers represent the prospective areas assigned to the induced digits.

Chick Embryos

Fertile "utility" grade chicken eggs were purchased from Spafas, Norwich, CT. All experiments were carried out with stage 20 embryos (Hamburger and Hamilton, 1951).

Slow-Release Beads and Their Implantation

AG1-X2 ion-exchange beads were loaded with TTNPB essentially as previously described for retinoic acid (Tickle et al., 1985) with the following modifications. 15 formate form beads of 250 μm diam were placed into a 1.5-ml microcentrifuge tube and vigorously shaken for 20 min in 200 μl of solution of TTNPB in dimethylsulfoxide. After removing the soaking solution, the beads were washed three times with 200 μl phosphate-buffered saline (for 1, 10, and again 10 min) and implanted into wing buds of stage 20 embryos (Fig. 1). The embryos were further incubated at 37°C for various amounts of time, depending on the type of experiment.

Analysis of Digit Patterns

After 6 d of incubation the embryos were killed and processed as previously described (Tickle et al., 1985). To construct a dose-response curve the digit pattern duplications are expressed in terms of percentage of respecification values as discussed by Tickle et al. (1985).

Extraction of Released [^3H]TTNPB

TTNPB-treated wing buds were cut into four strips (Fig. 2) and individual fragments were collected as described by Eichele et al. (1985). No radio-label is lost during the dissection process. A control experiment reveals that the amount of radioactivity per bud with and without dissection differs by <1%. To extract [^3H]TTNPB from the tissue, 100 μl of stabilizing buffer (Eichele et al., 1985) were added, followed by 100 μl of a saturated

Na_2SO_4 solution and 1 μg of nonradioactive TTNPB (a carrier and an internal standard). The sample was sonicated, and extracted twice with 800 μl of an 8:1 mixture of ethylacetate-methylacetate (containing 100 $\mu\text{g}/\text{ml}$ butylated hydroxytoluene). The organic phases were combined and evaporated with a stream of nitrogen gas, the residue was dissolved in 20 μl ethylacetate-methylate (8:1) and chromatographed.

High Pressure Liquid Chromatography (HPLC)

Purification of crude extract by HPLC is necessary since TTNPB is broken down to yet unidentified metabolites. HPLC was carried out as reported by Eichele et al. (1985) on a C_{18} Microsorb reversed-phase column (Rainin Instruments Co., Inc., Woburn, MA). The flow-through, monitored at a wavelength of 290 nm, was fractionated and fractions were counted with a scintillation counter. The amount of [^3H]TTNPB present in the extract was computed from the recovery (typically >90%) of the internal standard and the specific activity of the radioisotope.

Determination of DNA Content and Volume of Limb Buds

To determine the amount of DNA in a whole bud or in a slice of a bud, we followed the procedure described by Labarca and Paigen (1980). We find 2,440 ng of DNA per bud (stage 21, equivalent to stage 20 + 7 h). The DNA content of each of the four strips is 533 ± 49 ng (most anterior strip), 631 ± 62 ng, 541 ± 49 ng, and 420 ± 22 ng (most posterior strip). To measure

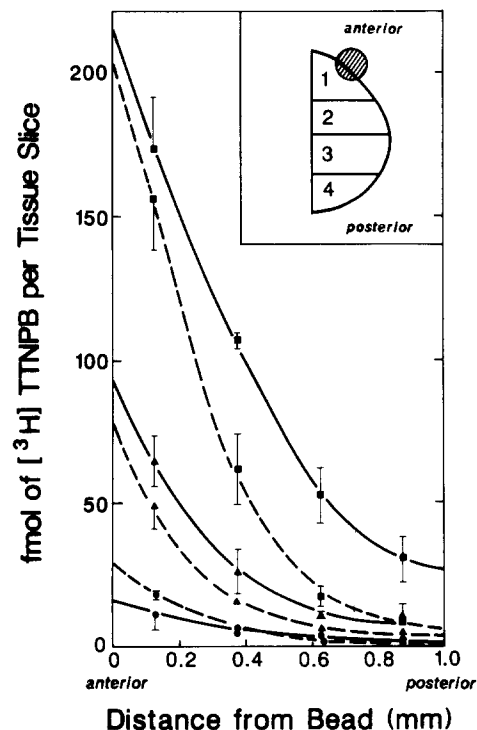


Figure 2. Distribution of [^3H]TTNPB in the developing chick wing bud after treatment with different doses of this compound. The inset indicates how buds were subdivided into strips after local application from the anteriorly implanted bead. Squares, triangles, and circles represent the amount of [^3H]TTNPB per strip. In order to help visualize the distribution of TTNPB in the bud, the data points have been connected by curves. Solid lines represent local application of [^3H]TTNPB for a period of 7 h, and dashed lines represent application for a duration of 15 h. For each point between two (1.5 and 0.1 $\mu\text{g}/\text{ml}$) and six (0.4 $\mu\text{g}/\text{ml}$) sets of measurements were included, each derived from 10–20 tissue strips. Error bars provide an estimate of the agreement between the sets of measurements. Soaking concentrations: (■) 1.5 $\mu\text{g}/\text{ml}$, (▲) 0.4 $\mu\text{g}/\text{ml}$, and (●) 0.1 $\mu\text{g}/\text{ml}$.

the limb bud volume at stage 21, about 50 limb buds were collected into a small tapered test tube, washed with phosphate-buffered saline, and gently centrifuged for 1 min. The volume taken up by the buds, divided by their number, resulted in a bud volume of 0.79 μl (average of two independent measurements given 0.75 and 0.82 μl , respectively). A bud of 0.79 μl in volume and a DNA content of 2,440 ng, yields 3,110 ng of DNA per μl . This number was used to determine the volume of each tissue slice as $0.171 \pm 0.016 \mu\text{l}$ (most anterior slice), $0.203 \pm 0.02 \mu\text{l}$, $0.174 \pm 0.016 \mu\text{l}$, and $0.135 \pm 0.007 \mu\text{l}$ (most posterior slice).

Equation of Diffusion

If a substance (e.g., TTNPB) is released from a constant source, such as an implanted bead and freely diffuses into the surrounding tissue, where it is irreversibly removed in a first-order reaction, then the equation of diffusion in one dimension becomes (see e.g., Carslaw and Jaeger, 1959; Crank, 1975; Slack, 1983):

$$dC/dt = D_a d^2C/dx^2 - kC, \quad (1)$$

where D_a is the apparent diffusion constant, k the rate constant of the first-order clearance reaction, and C the concentration of the released TTNPB. Eichele et al. (1985) and Fig. 5 (below) show that TTNPB is cleared from the bud in a first-order process. Moreover, the distribution of TTNPB in the limb field is in a good approximation in steady state (Fig. 2). Thus, dC/dt is equal to zero:

$$D_a d^2C/dx^2 - kC = 0. \quad (2)$$

To solve Eq. 2, the following boundary conditions were chosen (see also Fig. 1):

$$C = C_0, \text{ at } x = 0; \quad (3a)$$

$$dC/dx = 0, \text{ at } x = L. \quad (3b)$$

L is the width of the bud at the level of the implant. Eq. 3a means that at the site of the source ($x = 0$), the concentration of TTNPB has a certain finite value C_0 that depends on the concentration of TTNPB in which the bead was soaked. Eq. 3b implies that the slope of the gradient is zero at the posterior margin of the bud, a condition that is fulfilled as can be seen in Figs. 2 and 4. The solution of Eq. 2 is given by

$$C(x) = C_0[\exp(-ax) + b \sinh ax], \quad (4)$$

$$\text{where } a = \sqrt{k/D_a}; \quad (5)$$

$$b = \exp(-aL)/\cosh aL. \quad (6)$$

Results

Influence of the Strength of the Retinoid Source on the Shape and Biological Effect of the Resulting Gradient

Fig. 1 shows a scheme of the experimental approach. The major asset of this setup is that one can readily vary the strength of the source by soaking beads in different concentrations of $[^3\text{H}]\text{TTNPB}$. By slicing the bud into small portions, one can then directly visualize the spatial distribution of $[^3\text{H}]\text{TTNPB}$. Beads were soaked in solutions of 1.5, 0.4, or 0.1 $\mu\text{g/ml}$ $[^3\text{H}]\text{TTNPB}$ and implanted below the apical ridge of stage-20 wing buds and left in situ for 7 (stage 21) or 15 h (stage 22/23). Subsequently, the beads were removed and buds were dissected into four strips as shown in the insert of Fig. 2 and the amount of $[^3\text{H}]\text{TTNPB}$ per strip was quantitated by HPLC. The resulting TTNPB profiles (Fig. 2) reveal that for all three doses and both time points, the amount of TTNPB per tissue slice decreases with the distance from the source (semilog plots show that these curves are exponentials). The higher the dose the farther TTNPB penetrates the tissue. Applied dose and amount of TTNPB in the bud as a whole or in each strip are proportional. For example, after a treatment for 7 h at a soaking concentration of 1.5 $\mu\text{g/ml}$, tissue strip 2 will contain 105 fmol of TTNPB,

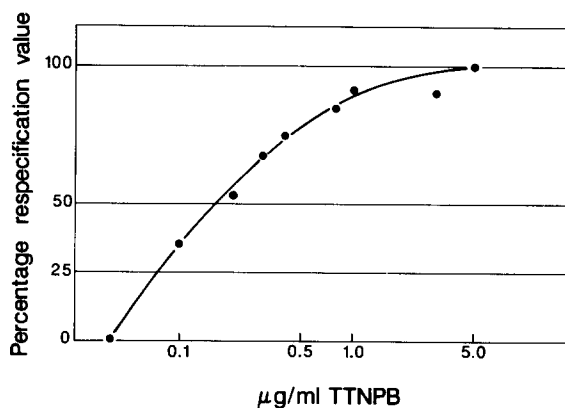


Figure 3. Dose-response curve showing the concentration of TTNPB in which the bead was soaked and the response in the form of percentage of respecification values (see Tickle et al., 1985 for a definition). The three horizontal lines represent a pattern with an average of an additional digit 2 (25%), 3 (50%), and 4 (100%), respectively.

while 0.4- and 0.1- $\mu\text{g/ml}$ treatments will result in 26 and 6 fmol, respectively. The data shown in Fig. 2 make it clear that primarily the strength of the source determines the geometry of the concentration gradient established in the bud. Such a simple correlation is rather surprising, in that limb buds are densely vascularized and exhibit a complex blood flow pattern (Jargiello and Caplan, 1983), a factor that might have led to intricate concentration profiles. In addition, wing buds grow substantially during the period of observation, yet the geometry of the gradient varies little with time.

A second question is whether the extent of each of the gradients shown in Fig. 2 correlates with its biological effect. Fig. 3 shows a dose-response curve covering the range of concentrations used for Fig. 2. At a soaking concentration of 0.1 $\mu\text{g/ml}$, resulting in a shallow gradient, the patterns generated have either an additional digit 2 or 3 (2234 or 32234 patterns, corresponding to a percentage of respecification value of 34%). At 0.4 $\mu\text{g/ml}$, the gradient is much more pronounced and we obtain mostly patterns with an additional digit 3 or 4 (e.g., 43234 or 32234, percentage of respecification value = 75%). Finally, 1.5 $\mu\text{g/ml}$ gives rise to a steep gradient and almost all patterns have an additional digit 4 (e.g., 43234, 43334, percentage of respecification value 93%). This makes it very clear that the spatial distribution of TTNPB directly determines the extent of duplication.

Modeling of the Experimental TTNPB Distribution

A limb bud that receives TTNPB from a bead implant and subsequently can dispose of the substance, can be viewed as a system consisting of a local source and a dispersed sink (e.g., Crank, 1975; Slack, 1983). In our case the sink is a combination of metabolic degradation of TTNPB and TTNPB removal by blood circulation. In one dimension, such a system is described by the following equation (see Materials and Methods):

$$C(x) = C_0[\exp(-ax) + b \sinh ax]. \quad (4)$$

Can Eq. 4 be satisfactorily fitted to the experimentally determined anteroposterior $[^3\text{H}]\text{TTNPB}$ distribution? To explore

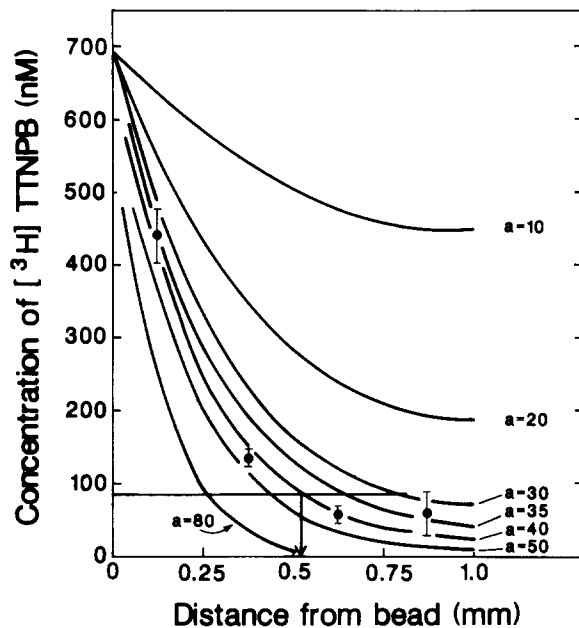


Figure 4. Comparison of the source-sink model with the measured $[^3\text{H}]$ TTNPB distribution. Solid circles denote the experimentally determined concentration of $[^3\text{H}]$ TTNPB in each of the four tissue slices established after a 7-h treatment with a bead presoaked in $0.4 \mu\text{g/ml}$ $[^3\text{H}]$ TTNPB. The concentration in each strip was determined by dividing the quantity of $[^3\text{H}]$ TTNPB per strip (from Fig. 2) by its volume (see Materials and Methods). The curves shown were calculated from Eq. 4 using different values for coefficient a and a C_0 of 690 nM and L of 0.1 cm , respectively. The horizontal line approximately represents the threshold level for digit 2 and the arrow marks the position coordinate of this digit (see text).

this question we have chosen the data derived from the 7-h and $0.4\text{-}\mu\text{g/ml}$ treatment (Fig. 2), a concentration that mostly induces duplications with additional digits 3 and 4. Note that Fig. 2 displays femtomoles of TTNPB per slice. To apply Eq. 4 in a meaningful way, these amounts were converted into concentrations as described in the legend of Fig. 4. Before fitting Eq. 4 to the data, we also need reasonable estimates for C_0 and L . C_0 is the concentration of TTNPB at the source, and cannot be directly measured. To overcome this problem, we have first plotted the concentration data in a semilogarithmic fashion, omitting the most posterior strip and fitted a least squares straight line (not shown). The intersection of this line with the ordinate is a reasonable experimental estimate for C_0 and amounts to 690 nM . For L , the width of the field of diffusion, we used 0.1 cm which is the experimentally measured width of the bud at the level of the bead implant. Using these values several curves $C(x)$, each with a different parameter a , were computed and are superimposed to the experimental data points (Fig. 4). An a of about 40 gives the best fit. It is obvious that small changes in a have little influence on the quality of the fit. An extreme value for a such as 10 or 80, leads to curves clearly far off from the experimental points. We have also varied C_0 and L within reasonable limits ($\pm 20\%$) in order to study how the two parameters affect $C(x)$. The calculated concentration, $C(x)_{\text{calc}}$, was compared with the experimentally observed concentration, $C(x)_{\text{obs}}$ (see Table I). Inspection of the table

leads to the conclusion that, on the whole, changes of L and C_0 in a reasonable range, have a limited effect on the shape of curve. Therefore, the experimentally determined TTNPB distribution agrees quite well with a concentration gradient generated by a local source-dispersed sink setting.

Can $C(x)$ predict the position of digits? Let us consider this question using digit 2 as an example. The dose-response curve (Fig. 3) indicates that an additional digit 2 is formed at a dose of about $0.08 \mu\text{g/ml}$. Although we have not directly measured a TTNPB profile for this dose it should closely resemble that created by a dose of $0.1 \mu\text{g/ml}$ (Fig. 2, circles). The concentration of TTNPB in the most anterior slice of this curve provides therefore an estimate for the threshold for a digit 2 and amounts to 85 nM . One arrives at this figure by dividing the amount of TTNPB in the slice (14.5 fmol , the mean of the 7- and 15-h time point) by its volume ($0.17 \mu\text{l}$, see Materials and Methods). Plotting this threshold level in the form of a horizontal line into Fig. 4 and intersecting it with the $C(x)$ curve of $a = 40$, gives an anteroposterior position coordinate for digit 2 of $\sim 0.05 \text{ cm}$. Thus, the prospective tissue for an additional digit 2 is located about $500 \mu\text{m}$ away from the bead. By analogy, the native 2 would be specified about $500 \mu\text{m}$ away from the polarizing region. These predictions agree with the fate map shown (Hinchliffe et al., 1981; see Fig. 1).

Estimate of the Diffusion Coefficient of TTNPB in Limb Buds

Coefficient a , diffusion constant D_a , and rate constant k of the clearance process are related by $a = \sqrt{k/D_a}$ (Eq. 5). Thus D_a can be estimated if a and k are known. We noticed that the magnitude of k is dependent on the dose of applied TTNPB (unpublished observation). Therefore, we have determined k for the particular dose of $0.4 \mu\text{g/ml}$ as described (Eichele et al., 1985). Briefly, beads were first soaked in $[^3\text{H}]$ TTNPB, implanted into stage-20 wing buds, left in situ for 7 h and then removed. The clearance of TTNPB from the tissue was followed by cutting off buds at various later times and determining the residual TTNPB. As shown in Fig. 5, the clearance reaction is biphasic with a faster initial velocity followed by a slower process. In order for Eq. 4 to be valid, the system must be close to equilibrium. Therefore, we have considered only the initial rate of clearance, represented by the early time points. A fit of an exponential curve reveals that the data up to 120 min follow first-order kinetics with a rate constant k of $1.6 \times 10^{-4} \text{ s}^{-1}$ corresponding to a half-life of 70 min. Using this rate constant and a coefficient a of 40 in Eq. 5 yields a diffusion constant of $10^{-7} \text{ cm}^2\text{s}^{-1}$.

Discussion

Gradients of morphogenetically active substances have been postulated for a long time (Child, 1929, 1941). However, because the underlying compounds are mostly unknown, it has been very difficult to examine the gradient concept in real tissues. Here we describe the geometry of TTNPB gradients spanning the developing chick wing and show that their shape can be modeled by a local source-dispersed sink setting. Moreover, our data demonstrate a quantitative correlation between the experimentally induced TTNPB gradients and the resulting digit pattern duplications.

Table 1. Dependence of $C(x)^*$ from C_0 , a , and L

Parameters	Position x along anteroposterior axis (in cm)				
	0	0.0125	0.0375	0.0625	0.0875
			<i>nM</i>		
$C_0 = 623, L = 0.1$ cm					
$a = 30$	623	429	206	105	66
$a = 40$	623	378	140	54	26
$a = 50$	623	333	95	28	10
$C_0 = 762, L = 0.1$ cm					
$a = 30$	762	525	252	129	81
$a = 40$	762	462	171	65	31
$a = 50$	762	408	117	34	12
$C_0 = 692, L = 0.12$ cm					
$a = 30$	692	476	226	109	57
$a = 40$	692	419	154	57	23
$a = 50$	692	370	106	30	9
$C_0 = 692, L = 0.08$ cm					
$a = 30$	692	479	240	142	127
$a = 40$	692	420	159	71	59
$a = 50$	692	370	107	35	27
$C_0 = 400, L = 0.1$ cm					
$a = 30$	400	275	132	67	42
$a = 40$	400	242	90	35	16
$a = 50$	400	214	61	18	6
Experimental concentrations, $C(x)_{\text{obs}}$	—	440 ± 39	133 ± 11	57 ± 19	57 ± 34

* Calculated using Eq. 4.

Our modeling of TTNPB gradients is based on two foundations; one is Fick's second law of diffusion, the other is a series of experimentally measured concentrations and a rate constant. Given the limited number of data that can be collected in such a complex biological system, we have had to make assumptions: the limb bud was treated in one dimension only, because the data we have collected are one-

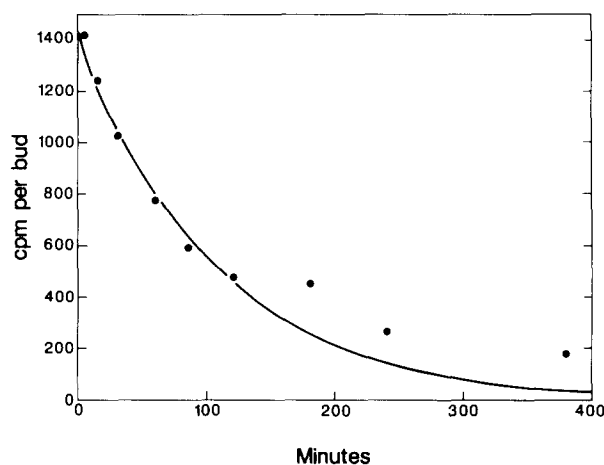


Figure 5. Time course of clearance of $[^3\text{H}]\text{TTNPB}$ from the limb after treating wing buds for a period of 7 h with a bead presoaked in $0.4 \mu\text{g/ml}$ $[^3\text{H}]\text{TTNPB}$. The curve represents a fit of a first-order kinetics including all data points up to a time of 120 min. Each time point represents the average from 5–12 buds.

dimensional (although this does not mean that our conclusions are valid for one dimension only). Furthermore, in that there are no drastic changes in the TTNPB profile (Fig. 2) during the entire period of digit pattern specification of 14 h (Eichele et al., 1985), we have assumed that the system is in steady state. As for other "semiempirical" approaches, it is important to check the appropriateness of our assumptions with model-derived parameters that can be verified by some other means. We have done this by showing that the model correctly predicts the position of digit 2 (see Results). As a second more quantitative test, we can ask whether the diffusion constant for TTNPB is in a physiologically meaningful range. The value of $10^{-7} \text{ cm}^2\text{s}^{-1}$ is certainly small. Diffusion coefficients of low molecular weight substances inside cells are normally of the order of $3 \times 10^{-6} \text{ cm}^2\text{s}^{-1}$ (e.g., Mastro et al., 1984), or 30 times greater than observed in the limb bud. Likewise, diffusion constants in extracellular space would be in the range of $7 \times 10^{-7} \text{ cm}^2\text{s}^{-1}$ (Shaw and Schy, 1981), or an order of magnitude greater than observed. However, retinoids are unlikely to be free floating, neither inside nor outside cells. First, vertebrate limb buds contain a cytoplasmic protein (CRABP, cellular retinoic acid-binding protein; see for example, Chytil and Ong, 1984 for a review) that binds retinoids (Kwarta et al., 1985; Maden and Summerbell, 1986). In the chick limb there are 14–28 pmol CRABP per mg of cytosolic protein, corresponding to $\sim 230\text{--}460$ fmol CRABP per bud (Maden and Summerbell, 1986). The amount of TTNPB per bud after treatment with a dose of $0.4 \mu\text{g/ml}$ is ~ 110 fmol (Fig. 2). Therefore, most of the cellular retinoid will be bound to CRABP. Molecules of the size of

CRABP (16 kD) have diffusion constants inside cells in the range of 10^{-7} cm²s⁻¹ (Paine et al., 1975; Kreis et al., 1982), consistent with our data. Retinoid molecules that traverse the intercellular space will occasionally collide with plasma membrane, and the hydrophobic molecule will quickly partition into the lipid bilayer. This would account for slowing down diffusion in the extracellular environment. We conclude that the observed diffusion constant of TTNPB in the limb falls within the range expected, giving support to the appropriateness of our semiempirical approach. It should be added that estimates by Crick (1970) suggest a diffusion coefficient of small molecules in tissues in a similar range.

How far into the limb bud would a putative morphogen have to diffuse after release by the polarizing region or the bead, and how long would this take? The width of the hand field in the chick wing bud is of the order of 500 μ m (Honig, 1981; Summerbell and Honig, 1982; see also the fate map in Fig. 1). Clearly, retinoids can readily travel such a distance (Fig. 2). Using the diffusion constant of TTNPB, we can estimate the mean time required for a TTNPB molecule to migrate over a distance (x) of for example 500 μ m. With $x \sim \sqrt{2Dt}$ and D of the order of 10^{-7} cm²s⁻¹, the time required amounts to 3.5 h. This agrees with the time it takes retinoids to affect the pattern: recent studies demonstrate that in order to have an effect, retinoids must be present for at least 10–12 h (Eichele et al., 1985). We can take it, then, that diffusion and the time scale for specifying the anteroposterior pattern are in a comparable range.

In our model the slow-release bead acts as a local retinoid source; in the normal limb rudiment, the zone of polarizing activity is believed to function as a morphogen source (Wolpert, 1969; Tickle et al., 1975). Recent results make this view very attractive. We have observed that retinoic acid naturally forms a shallow concentration gradient across the bud, with a high point near the zone of polarizing activity (Thaller and Eichele, 1987). If the source-sink model developed in this study is valid for the endogenous retinoic acid gradient, then two specific predictions can be made: (a) The bud must contain a source for retinoic acid and (b) there must be a dispersed sink. Support for both predictions comes from two kinds of recent experimental data. First, limb buds contain large amounts of retinol, the biosynthetic precursor of RA (Thaller and Eichele, 1987). This pool of endogenous retinol is continuously converted to retinoic acid in the limb (Thaller and Eichele, manuscript in preparation). Whether this conversion takes place preferentially at one site, such as the ZPA, remains to be seen. Second, as reported earlier, retinoic acid is metabolized in the limb bud and also removed by circulating blood (Tickle et al., 1985). At present we do not know whether this clearance reaction is dispersed throughout the entire limb tissue.

The usefulness of a model is reflected partly in the specific predictions it can make. The foregoing discussion has made it clear that the local source-dispersed sink model is useful in this sense. To further refine the model, additional data are required. For example there is an urgent need to biochemically identify the target of retinoids (e.g., a receptor), and localize the target sites at cellular resolution. As more information of this sort becomes available, further refinement of our present concepts will be possible.

We wish to thank Dr. S. Wedden for critical reading of the manuscript.

This work was supported by grant HD 20209 from the National Institutes

of Health to Dr. Eichele with additional support from the Milton Fund (Harvard University) and by Biomedical Research Support grant S07 RR 05381-24 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, NIH.

Received for publication 8 December 1986, and in revised form 27 April 1987.

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