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Journal

Journal of Cell Biology, 101(5)

ISSN

0021-9525

Authors

Eichele, G
Tickle, C
Alberts, BM

Publication Date

1985-11-01

DOI

10.1083/jcb.101.5.1913

Peer reviewed

Studies on the Mechanism of Retinoid-induced Pattern Duplications in the Early Chick Limb Bud: Temporal and Spatial Aspects

GREGOR EICHELE,* CHERYLL TICKLE,‡ and BRUCE M. ALBERTS*

*Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, and ‡Department of Anatomy and Biology as Applied to Medicine, The Middlesex Hospital Medical School, London W1P/6BD, England. Dr. Eichele's present address is Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT All-*trans*-retinoic acid causes striking digit pattern changes when it is continuously released from a bead implanted in the anterior margin of an early chick wing bud. In addition to the normal set of digits (*234*), extra digits form in a mirror-symmetrical arrangement, creating digit patterns such as a *432234*. These retinoic acid–induced pattern duplications closely mimic those found after grafts of polarizing region cells to the same positions with regard to dose–response, timing, and positional effects. To elucidate the mechanism by which retinoic acid induces these pattern duplications, we have studied the temporal and spatial distribution of all-*trans*-retinoic acid and its potent analogue TTNPB in these limb buds. We find that the induction process is biphasic: there is an 8-h lag phase followed by a 6-h duplication phase, during which additional digits are irreversibly specified in the sequence digit *2*, digit *3*, digit *4*. On average, formation of each digit seems to require between 1 and 2 h. The tissue concentrations, metabolic pattern, and spatial distribution of all-*trans*-retinoic acid and TTNPB in the limb rapidly reach a steady state, in which the continuous release of the retinoid is balanced by loss from metabolism and blood circulation. Pulse-chase experiments reveal that the half-time of clearance from the bud is 20 min for all-*trans*-retinoic acid and 80 min for TTNPB. Manipulations that change the experimentally induced steep concentration gradient of TTNPB suggest that a graded distribution of retinoid concentrations across the limb is required during the duplication phase to induce changes in the digit pattern. The extensive similarities between results obtained with retinoids and with polarizing region grafts raise the possibility that retinoic acid serves as a natural "morphogen" in the limb.

The vertebrate limb is an organ in which cell patterning has been studied in great detail (for a review see Wolpert, 1978; Javois, 1984). Vertebrate limbs arise as small buds protruding from the flank region of the early embryo. Saunders and Gasseling (1968) discovered that, when tissue from the posterior part of one bud is grafted to a second bud at the anterior junction with the body wall, extra limb structures (e.g., extra digits) develop which are arranged in mirror-image symmetry to the normal ones. Thus, instead of the normal wing digit pattern *234*, the pattern *432234* can be formed. To obtain duplications, the graft needs to contain only a small number of cells from the posterior tissue of the bud (Tickle, 1981). The cells with this special activity are known as polarizing region cells.

Several experiments suggest that the polarizing region cells release a morphogen, whose concentration specifies the position of the other cells in the bud along the anteroposterior limb axis (Tickle et al., 1975). According to this view, the cells in the limb bud first determine their relative position by measuring the concentration of the morphogen in their surrounding and later differentiate accordingly (Wolpert, 1969 and 1971). However, there are alternative explanations. For example, it has been proposed that, as the limb bud grows, interactions between cells with different positional values

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cause the intercalary growth of cells with the appropriate new positional character (Iten and Murphy, 1980; reviewed by Javois, 1984). Unfortunately, neither the postulated morphogen nor the postulated cell surface molecule involved in the cell–cell interaction model has thus far been chemically identified in the limb.

The controlled release of all-*trans*-retinoic acid (RA)[1] from microcarrier beads implanted at the anterior margin of the developing bud causes the formation of duplicate patterns that are strikingly similar to those obtained after grafting polarizing region cells to the same position (Tickle et al., 1985; see also, Tickle et al., 1982; Summerbell, 1983). These duplications are dependent on the dose of the applied RA: increasing amounts cause first the formation of an extra digit *2*, then a digit *3*, and then a digit *4*; thus the complete duplication pattern *432234* can be formed. The tissue concentration of RA required to cause such duplications is in the physiologically significant nanomolar range, and the local release of RA results in an exponentially shaped concentration gradient of retinoids along the anteroposterior axis (Tickle et al., 1985). As we have discussed previously, these observations suggest that RA could be a natural morphogenetic signalling substance in the limb (Tickle et al., 1985; see also Summerbell, 1983).

To further explore the mechanism by which RA exerts its effect, we report here a study of the time course over which digit duplications are induced by local RA release, showing that the time course is comparable to that observed for grafts of polarizing region cells. We have also exploited the fact that the local release of TTNPB, a synthetic analogue of RA that is much more metabolically stable, causes pattern duplications. Measurements of TTNPB distributions in the limb after different schedules of release suggest that a concentration gradient of retinoid across the limb, rather than just the presence of the retinoid itself, may be required for the biological effects observed.

MATERIALS AND METHODS

Retinoids: The all-*trans*-[11-^{3}H]RA (300-mol-wt) (Fig. 1 *C*) had a specific activity of 2.4 Ci/mmol. The *p*-([E]-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl-6,7-$^3H_2$-propenyl)benzoic acid (TTNPB) (348-mol-wt) (Fig. 1 *C*) had a specific activity of either 24.1 or 3 Ci/mmol. Both the [^{3}H]RA and the high specific activity [^{3}H]TTNPB were synthesized by SRI International, Menlo Park, CA, under contract to the National Cancer Institute. The low specific activity [^{3}H]TTNPB and the nonradioactive TTNPB were a gift from Hoffmann-LaRoche, Basel, Switzerland. Nonradioactive RA was purchased from Sigma Chemical Co., St. Louis, MO, or was a gift from Hoffmann-LaRoche.

Beads and Their Implantation: A detailed account of the properties of the controlled release beads and the implantation scheme used has been published (Tickle et al., 1985; Eichele et al., 1984). Briefly, AG1-X2 ion-exchange beads of 250–300 μm dry diameter (formate form, from Bio-rad Laboratories, Richmond, CA) were first soaked for 20 min in a dimethyl sulfoxide solution of RA or TTNPB and then washed in minimum essential medium containing 10% fetal calf serum. Implants were placed opposite somite border 16/17 into a slit made beneath the apical ectodermal ridge of stage 20 wing buds (see Tickle et al., 1985). These beads can be cleanly removed, with very few adherent cells, at any subsequent time (Lee, J., and C. Tickle, unpublished results).

Tissue Extraction: Treated wing buds were cut off with a watchmaker's forceps, washed twice for 30 s at 0°C in a stabilizing buffer for retinoids (5 mg/ml each of ascorbic acid and Na_3 EDTA dissolved in phosphate-buffered saline [PBS], pH readjusted to 7.3 with NaOH) and collected in a plastic vial immersed in liquid nitrogen. For several experiments, the buds were cut into four parallel strips using electrolytically sharpened tungsten needles; in this case the bud was deposited on an agarose gel layer, covered with a drop of the stabilizing buffer, cut, and each strip was collected and frozen. After thawing, more stabilizing buffer was added to a volume of 400 μl, followed by 400 μl of ethanol containing 100 μg/ml butylated hydroxytoluene, and 20 μl of an ethanol solution containing either 500 ng RA or TTNPB (carrier). The mixture was sonicated briefly to disrupt the tissue, heated to 60°C for 2 min, chilled on ice, and extracted three times with 2 ml of *n*-hexane containing 100 μg/ml butylated hydroxytoluene. The hexane extracts were pooled, the hexane was evaporated with nitrogen gas, and the residue was redissolved in 30 μl of dimethyl sulfoxide for subsequent high pressure liquid chromatography analysis. In a few cases, the residual aqueous phase was re-extracted with a mixture of ethylacetate and methylacetate (1:5) and further processed as described for the hexane extract. All work with retinoids, including the extractions, was performed under argon in a room illuminated with yellow light.

High Pressure Liquid Chromatography Analyses: Chromatography was carried out on a reverse phase C_{18} column as described previously (Tickle et al., 1985). The column was eluted isocratically with methanol/acetonitrile/1% aqueous acetic acid (20:60:20) at a flow rate of 1 or 1.2 ml/min. Fractions were collected every 0.2 min and counted after adding 1 ml of scintillation cocktail (Liquiscint, National Diagnostics, Somerville, NJ).

RESULTS

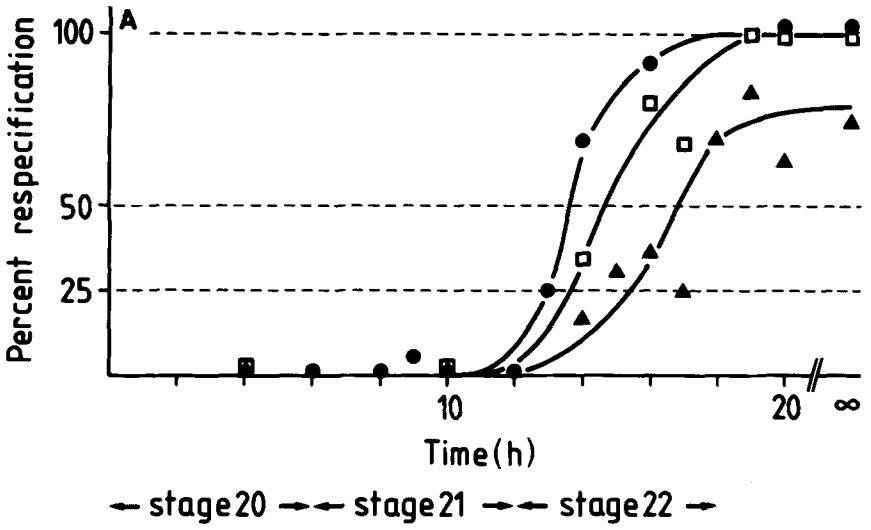
Evidence for Separate "Priming" and "Duplication" Phases in the Induction of Additional Wing Digits

The observations of Summerbell and Harvey (1983) suggested that only a limited exposure to locally released RA is required to change the digit pattern. To define the time requirements for this RA action, we have implanted ion-exchange beads (AG1-X2) that deliver different doses of retinoids into stage 20 wing buds, removed them at varying times thereafter, and analyzed the wing digit pattern that eventually forms. To quantitate these patterns, we have calculated a number called the "percentage-respecification value" (PRV), which represents an average of digit patterns obtained from identically treated embryos. The higher the PRV, the more posterior is the character of the additional digit that forms nearest to the implant (see Fig. 1 *A*). In Fig. 1 *A*, the PRV is plotted as a function of the exposure time of the wing buds to beads presoaked in either 33 μM RA, 330 μM RA, or 14 μM TTNPB. It can be seen that, regardless of the dose of retinoid, if the controlled release bead is removed between the first 10 and 12 h after its implantation at stage 20, a normal *234* digit pattern is invariably seen (PRV = 0%). If the bead is removed at progressively later times, additional digits are formed as a consequence of continued retinoid delivery from the implant. First, we observe an extra digit *2* (digit pattern *2234*; PRV = 25%), then a digit *3* (digit patterns *32234*, *3234*, *3334*, and *334*; PRV = 50%), and finally an extra digit *4* (digit patterns *432234*, *43234*, and *4334*; PRV = 100%). Photographs of wings with two of the above digit patterns are shown in Fig. 1 *B*.

The synthetic retinoid, TTNPB, is much more metabolically stable than RA, and it is therefore active at much lower applied doses (see below). Thus, in Fig. 1, beads presoaked in 14 μM TTNPB (circles) cause a similar extent of duplication as beads presoaked in 330 μM RA (squares), even though about 20 times more total retinoid is released from the latter beads. In addition, the slopes of the curves drawn in Fig. 1 *A* indicate that TTNPB produces additional digits at a somewhat faster rate than RA.

Inspection of the results in Fig. 1 *A* suggest that the first 12 h of retinoic acid release has little effect on the wing bud

[1] *Abbreviations used in this paper:* PRV, percentage respecification value; RA, all-*trans*-retinoic acid; TTNPB, *p*-([E]-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl-6,7-$^3H_2$-propenyl)benzoic acid.



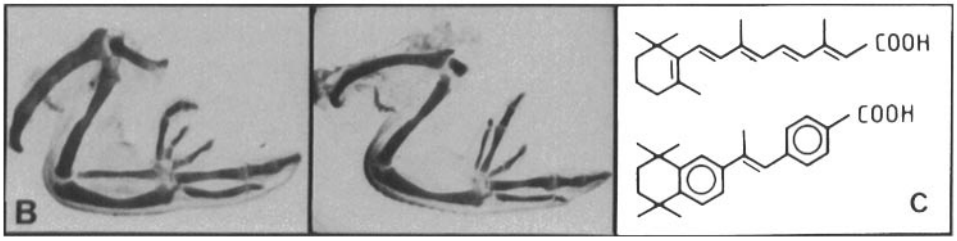


FIGURE 1 Effects on the digit pattern of changing the time of exposure of the wing bud to retinoids. (*A*) PRV plotted as a function of the time of exposure of the wing bud to beads soaked in 33 μM (▲) or 330 μM (□) RA, or in 14 μM TTNPB (●). The infinity sign marks the values for buds in which the bead was never removed, and Hamburger–Hamilton stages of chick development are marked below the time axis. Embryos were incubated for 6 d after treatment, sacrificed, fixed with 5% trichloroacetic acid, and the wings stained with Alcian Green. A score, S_i, was assigned to the most anterior digit found in the treated wing, which is equal to 1, 2 or 4 if this digit is a *2*, *3* or *4*, respectively (S_i is equal to 0 for a normal or completely truncated wing). The PRV plotted is calculated as $\Sigma_i \frac{S_i \cdot 100}{4n}$, where *n* is the number of embryos tested (see Tickle et al., 1985). From the intersection of each curve with the horizontal lines that represent duplicate digits *2*, *3* and *4*, respectively, one can estimate the bead exposure times, on average, required to specify each particular extra digit. For example, for the TTNPB treatment, these times are ~13 h for digit 2, 13.5 h for digit *3*, and 17 h for digit 4. (*B*) Photographs of two wings that developed following the application of RA on AG1-X2 beads to the anterior margin of stage 20 wing buds. (*Left panel*) *32234* digit pattern; a bead soaked in 33 μM RA was removed after 16 h of incubation. (*Right panel*) *43234* digit pattern; a bead soaked in 33 μM RA was removed after 20 h of incubation. (C) Structures of RA (*top*) and of TTNPB (*bottom*).

tissue. If this is true, the same digit pattern should be obtained with an identical bead implantation performed 12 h after stage 20. To maintain a consistent bead position in such "delayed-release experiments," a plain bead (no retinoid) was implanted at stage 20 and later substituted by a fresh bead that had been presoaked in 330 μM RA. Bead substitutions of this type made after 4, 8, and 11 h resulted in the following wing digit patterns: 4 h, *43234* (5 cases); 8 h, *2234* (3 cases) or *234* (12 cases); and 11 h, *234* (4 cases). When the plain bead was left in place throughout limb development, the normal *234* pattern was invariably obtained. From these experiments, we conclude that, although the first 12 h of retinoic acid release has no demonstrable effect on wing pattern, nevertheless this exposure is required to lead to the subsequent pattern changes. Thus, there is an initial "priming phase" in the experiments shown in Fig. 1, which must last for at least 8 h in order to yield a maximum response; a shorter priming period results in much weaker or no duplications.

In part, the inefficiency of the delayed release experiments may result from an increasing distance between the bead and the tissue forming the digits, since the bead remains behind as the bud grows distally and the presumptive digit cells are located near the tip of the bud (for a fate map see Hinchliffe et al., 1981). Thus, if a plain bead is implanted at stage 20 and then replaced at stage 22 with a new bead that releases large amounts of RA, there is no effect on the limb pattern (Eichele, G., unpublished results). In contrast, duplications are obtained if such a bead releasing RA is implanted closer to the tip at stage 22. However, these duplications are much less complete than those obtained from earlier implantations (*2234* in most cases), showing that the potential of the limb tissue to produce complete duplications in response to retinoids is greatly reduced at this time (see also Summerbell, 1983).

A plot of the mean number of digits as a function of the exposure time reveals that for the higher RA concentration used (330 μM), the mean number of digits increases from 3

to 4 at about 12 h in Fig. 1, but that it remains constant thereafter in three-quarters of the embryos (data not shown). The character of the fourth digit, however, changes with time: a *2234* pattern at 12 h becomes a *4334* pattern a few hours later. Thus, the most anterior digit appears to be serially "promoted" from a *2* to a *3* and then to a *4* as the exposure to RA continues. In addition, the tissue that would form a digit *2* in a normal limb appears to be promoted to form a digit *3* instead.

The Concentration and Clearance Rates of Retinoids Released in the Wing Bud

Determination of both the concentration and the half-life of retinoids in the wing bud is required for any understanding of the results of Fig. 1. To obtain this information, beads soaked in 330 μM [^{3}H]RA or 14 μM [^{3}H]TTNPB were implanted into the anterior margin of stage 20 wing buds. The amount of each compound found in the bud at various times after bead implantation is plotted in Fig. 2*A*. As determined by a high pressure liquid chromatography analysis, the RA level dropped steadily during the 20-h period of bead implantation to ~20% of its initial value. In contrast, the level of TTNPB in the bud remained almost constant during this time, the slope of the least squares line being ~4–5 times steeper for RA than for TTNPB.

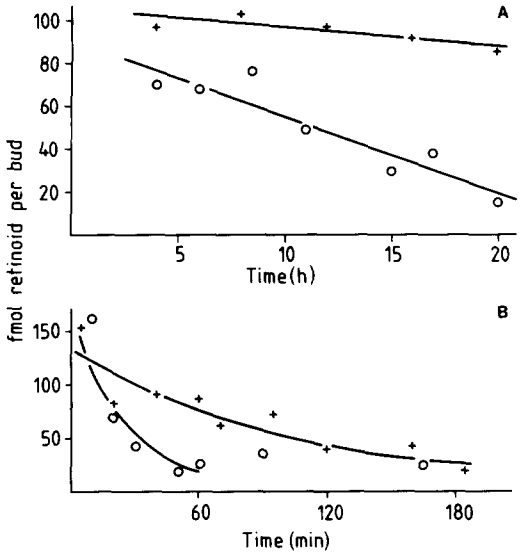


FIGURE 2 Concentration and stability of experimentally applied retinoids in the chick limb bud. (*A*) The amount of [^{3}H]RA (○) or [^{3}H]TTNPB (+) present in a wing bud as a function of the time of exposure to a bead releasing either of the two chemicals. AG1-X2 beads, soaked in 330 μM [^{3}H]RA or 14 μM [^{3}H]TTNPB, were implanted at stage 20 (zero time in the plot) at the anterior margin of the wing bud (Tickle et al., 1985). At the time points indicated, the bead was removed, and the bud was excised and washed. 10 to 15 buds were pooled and extracted. Each extract was analyzed by high pressure liquid chromatography to determine the amount of [^{3}H]RA or [^{3}H]TTNPB present. (*B*) Disappearance of [^{3}H]RA (○) and [^{3}H]TTNPB (+) from the wing bud after removal of a bead implant releasing the radioactive compound. Stage 20 wing buds received AG1-X2 beads, presoaked in nonradioactive 330 μM RA or 14 μM TTNPB. Between 12 and 14 h later these beads were replaced with substitutes pretreated with the same concentrations of the radioactive compound. After 1 h, the radioactive bead was removed and the amount of radioactive parent compound in the limb determined immediately and at various later times by means of a high pressure liquid chromatography analysis. For each data point, 12 to 15 limb buds were pooled. A least squares exponential was fitted to the data to obtain an estimate of the half-time of each compound in the tissue (for RA, only time points up to 60 min were included).

We have directly measured how fast RA and TTNPB disappear from wing buds by setting up pulse-chase experiments. In these experiments, beads releasing non-radioactive compound were first implanted into stage 20 buds and left there for 12–14 h. These implants were then replaced by beads releasing radioactive RA or TTNPB, which were left in place for 1 h. After this period, the second bead was removed, and the total amount of [^{3}H]RA and [^{3}H]TTNPB present in the bud at various later times was determined by high pressure liquid chromatography analysis. The results are plotted in Fig. 2*B*. Between 5 and 10 min after bead removal, both RA and TTNPB were present in about equal amounts in this experiment. However, the concentration of RA dropped much faster than that of TTNPB. An experimental decay curve fitted to the early data points of RA yields a half-life of ~20 min. Although TTNPB also disappeared exponentially, its half-life was longer (80 min). Because RA and TTNPB are structurally related (see Fig. 1*C*) and therefore will have similar diffusion constants, the large difference in their clearance times is likely to be caused by their different metabolic breakdown rates (see below).

The Metabolism of RA and TTNPB in the Wing Bud

Could the priming phase shown in Fig. 1 indicate a need to synthesize some active species from the parent retinoids? To explore this option, we have analyzed the metabolites produced from both RA and TTNPB in wing buds. Fig. 3A shows a sample chromatogram of an *n*-hexane extract obtained from wing buds that had been treated with radioactive RA. The parent molecule (the peak with the longest retention time) steadily disappears with time, compensated by the appearance of a large number of more polar metabolites. In addition, a considerable amount of the total radioactivity (30–50% depending on the time of incubation) is present in very polar breakdown products, which remain in the aqueous phase after the *n*-hexane extraction and can only be recovered by re-extracting with ethylacetate/methylacetate (see Tickle et al., 1985). Despite the obvious complexity, the pattern of metabolites resolved by our analysis remains qualitatively and to a large extent quantitatively the same over the entire 20 h of RA release (data not shown).

An analogous sample chromatogram of TTNPB and its metabolites is shown in Fig. 3*B*. A much less extensive metabolism is seen. The major peak is the active E isomer of TTNPB while the nearby minor peak co-migrates with the inactive Z isomer. The metabolites eluting in tubes 25 to 35 represent unidentified polar molecules (presumably oxidized and hydroxylated derivatives). Again the pattern of metabolites is invariant with time. However, here only 5–10% of the TTNPB-derived radioactivity is insoluble in *n*-hexane, and analysis of an ethylacetate/methylacetate extract of the residual aqueous phase yields a chromatogram devoid of any distinct peaks, with low amounts of radioactivity eluting during the entire analysis (data not shown).

In conclusion, our high pressure liquid chromatography analysis provides no support for the possibility that a special

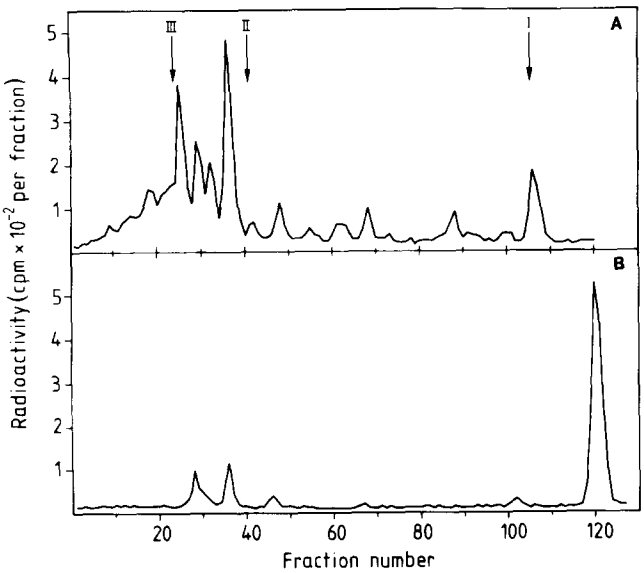


FIGURE 3 High pressure liquid chromatography analysis of radioactive metabolites produced from [^{3}H]RA and from [^{3}H]TTNPB in the chick wing bud. (*A*) Sample chromatogram of RA metabolites present in wing buds. An analysis of hexane-soluble radioactive metabolites produced from locally released [^{3}H]RA after 14 h is shown. The arrows on the chromatogram mark the elution position of RA (*I*), 5,6-epoxy-RA (*II*), and 4-keto-RA (*III*). A total of 10 to 15 wing buds of embryos that were treated as described in Fig. 2 were used. Very similar results were obtained at 6, 8, 12, 17.5, and 20 h (not shown). For details, see Materials and Methods. (*B*) Sample chromatogram of [^{3}H]TTNPB metabolites present in wing buds. An analysis of the radioactive metabolites produced from locally released [^{3}H]TTNPB after 16 h is shown. The major peak is the E isomer of [^{3}H]TTNPB and the minor peak eluting around tube 100 represents the Z-isomer. AG1-X2 beads were presoaked in 14 μM [^{3}H]TTNPB prior to implantation. Extracts were obtained by pooling limbs from 15 treated embryos. Identical peaks were observed at 4, 8, and 20 h (data not shown). For details, see Materials and Methods.

active intermediate is synthesized during the priming phase that becomes abundant only after a prolonged incubation. All-*trans*-retinoic acid is closely homologous in its three-dimensional conformation to the E-isomer of TTNPB (Loeliger et al., 1980; Strickland et al., 1983). From our data, one can make a good argument that these parent retinoids themselves are the active species, as follows. Even though a bead pre-soaked in 14 μM TTNPB will release only ~5% as much retinoid per unit time as a bead soaked in 330 μM RA (Eichele et al., 1984; Eichele, G., unpublished results), the steady-state concentration of the TTNPB is approximately twofold greater than the steady-state concentration of RA when these two beads are used for implant studies (Fig. 2*A*). Thus, if RA and TTNPB are directly responsible for the digit duplications found, the two treatments are expected to be about equally efficient in causing duplications, and this is the result observed (Fig. 1*A*; also, Lee, J., and C. Tickle, unpublished results).

The Spatial Distribution of TTNPB in the Limb

Since it appears from our studies that RA and TTNPB are the active species that cause pattern duplications in the limb, some other explanation besides metabolism is required to account for the long priming phase required. In addition, it is not clear why bead removal at 10 h produces weak or no duplications in the case of treatment with beads presoaked in 14 μM TTNPB, in view of the expected persistence of this relatively stable compound in the limb throughout the duplication phase that follows.

Could the spatial distribution of the released retinoid during the duplication phase be important? Our previous studies have shown that locally released RA and its metabolites form a concentration gradient in the limb at steady state (Tickle et al., 1985). Making use of the much greater chemical and metabolic stability of TTNPB, we have therefore determined both the steady-state spatial distribution of this analog in the limb bud and the stability of its distribution after removal of a controlled release implant. For these experiments, beads presoaked in 14 μM [^{3}H]TTNPB were implanted into the wing buds of stage 20 embryos. After 10 h, the implants were removed. Following further incubation for various times, the distribution of [^{3}H]TTNPB in the buds was determined by high pressure liquid chromatography analysis. In Fig. 4*A*, we present this distribution at 7 min, 2 h, 4 h, and 6.5 h after removing the bead. In the 7-min sample, the distribution is very asymmetrical (open bars), with a maximum concentration near the source (left margin). This asymmetry is quantitatively the same as found previously in an analogous experiment for RA (Tickle et al., 1985). At 4 and 6.5 h after bead removal, the gradient is much less pronounced (stippled and solid bars). For example, the twofold difference in TTNPB concentration between the two anterior strips has disappeared, resulting in an almost even distribution of TTNPB throughout the anterior half of the bud.

It is striking that during the period following the bead removal there are appreciable amounts of TTNPB left in the tissue (Fig. 4*A*). Nevertheless, the digit patterns resulting from this particular treatment are mostly normal (PRV of 13%, Table I and Fig. 1*A*). This is particularly intriguing in view of the fact that a dose of 10-fold less TTNPB produces quite pronounced duplications when the implant is continuously present during the duplication phase (see Table I, beads soaked in 1.4 μM TTNPB).

To investigate this point further, we have compared the above spatial distributions resulting from beads soaked in 14 μM TTNPB with the distributions observed when TTNPB is continuously released from a bead soaked in 1.4 μM [^{3}H]-TTNPB. In Fig. 4*B* (*left panels*), we show the spatial distributions after 12 h (*a*) and 18 h (*b*) of such a continuous low dose release. The data reveal a 3–4-fold difference in TTNPB concentrations between limb sections 1 and 2 during the duplication phase. These two sections comprise the anterior half of the limb, the tissue in which new digits are reproducibly induced by this low dose treatment (Table I). As mentioned above, when the bead releasing 10-fold more TTNPB is removed at 10 h and the tissue analyzed 4 and 6.5 h later (*c* and *d* show these data on the same scale as for *a* and *b*), an equivalent amount of TTNPB remains in this half of the limb throughout the duplication phase. Nevertheless, the biological effect of such a bead implantation is much less (Table I).

The results in Fig. 4*B* and Table I suggest that bead removal at 10 h in Fig. 1 yields a weaker biological response to TTNPB, not because the TTNPB disappears, but because the spatial distribution of TTNPB in the limb becomes too symmetrical during the duplication phase. We conclude that the induction of digit duplications in the limb by retinoids requires some aspect of the very asymmetrical spatial distribution that is established by a continuous local release process.

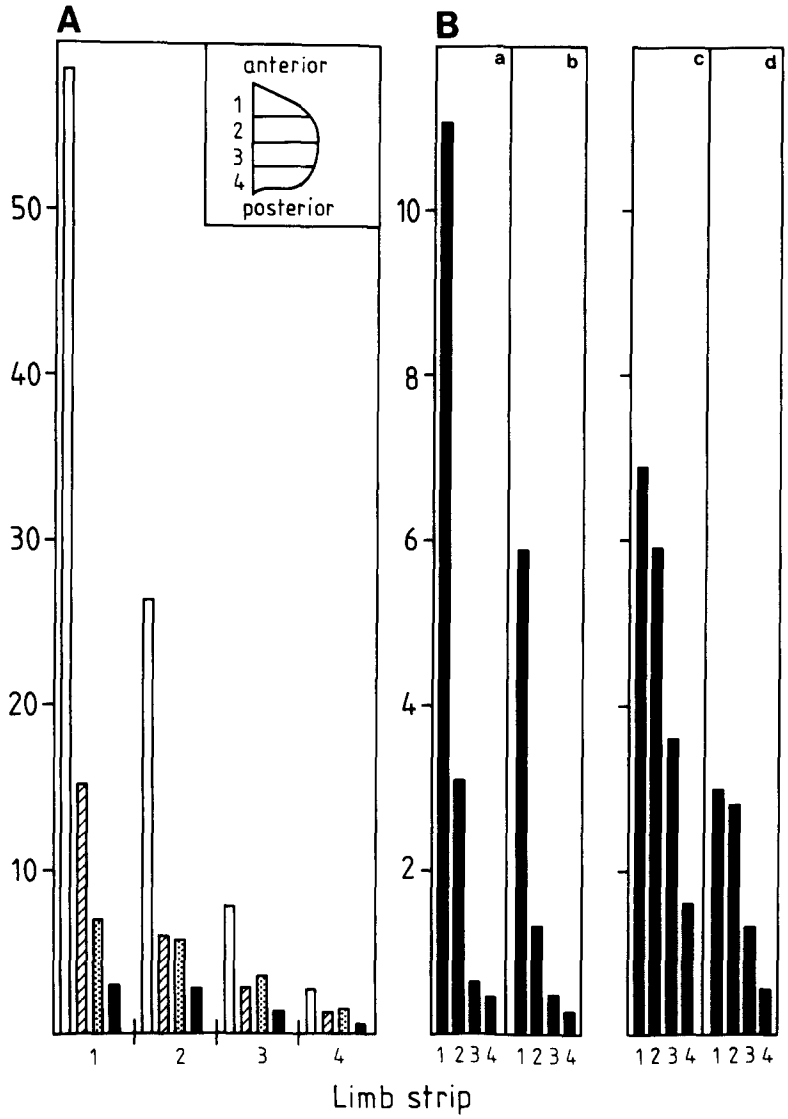


FIGURE 4 Analysis of the gradient of [^{3}H]TTNPB produced in the wing bud by its local release from a bead implanted at the anterior limb bud margin. (*A*) Distribution of [^{3}H]TTNPB in a wing bud that has been sectioned into four strips as shown in the insert. For this experiment, AG1-X2 beads were soaked in 14 μM [^{3}H]TTNPB and implanted into stage 20 wing buds, and 10 h later the bead was removed. Buds were cut off either after 7 min (open bar), 2 h (striped bar), 4 h (stippled bar), or 6.5 h (solid bar) of additional incubation. For analysis of the [^{3}H]TTNPB gradient, each bud was quickly washed at 0°C in stabilizing buffer, and subsequently cut into four strips. The corresponding strips from 12 to 15 buds were pooled, extracted, and analyzed by high pressure liquid chromatography in order to measure the amount of [^{3}H]TTNPB present. The ordinate plots the fmol of [^{3}H]TTNPB detected per tissue slice. (*B*) Comparison of the TTNPB distribution in the limb caused by two treatments that have very different biological effects. *c* and *d* (small biological effect) are a replot of the 14-h and 16.5-h data points from *A*, 4 and 6.5 h after bead removal of a bead releasing [^{3}H]TTNPB, respectively. *a* and *b* (large biological effect) show the gradient of [^{3}H]TTNPB produced in the wing bud by the continuous release of a low amount of [^{3}H]TTNPB. For this experiment, AG1-X2 beads were soaked in 1.4 μM [^{3}H]TTNPB (a concentration 10-fold lower than that used in *A*). The beads were then implanted into stage 20 wing buds and left in place for either 12 h (*a*) or 18 h (*b*). The distribution of [^{3}H]TTNPB in the limb was then determined 7 min after bead removal by the method described in *A*. The ordinate plots the fmol of [^{3}H]TTNPB detected per tissue slice.

DISCUSSION

Local release of all-*trans*-retinoic acid or its structurally related synthetic analogue TTNPB at the anterior margin of an early wing bud induces the anterior tissue to form duplicate digits. This reproducibly results in mirror-image pattern duplications such as a *43234* pattern (Tickle et al., 1982; Summerbell, 1983; Tickle et al., 1985). By removing the bead releasing RA or TTNPB after its implantation at stage 20, we find that the formation of additional structures is a biphasic process consisting of about an 8-h-long priming phase, followed by a duplication phase of 3 to 6 h (Fig. 1). Cell commitment during the duplication phase that will subsequently lead to digit formation appears to be a rapid process: on average, an exposure to RA or TTNPB of ~1–2 h is sufficient to create or promote a digit. It is not clear what changes occur during the priming phase. However, they are not permanent, since removal of the implant during the priming phase produces the normal pattern. In contrast, the processes initiated during the duplication phase result in an irreversible change, which only becomes visible 2 d later (during stage 27) as emerging additional digits. It is interesting to note that a similar two-step process, requiring several cell generations, is observed for the production of irreversible changes in teratocarcinoma cells that have been treated with retinoids (Ogiso et al., 1982; Mummery et al., 1984). The average cell cycle time in the early chick limb is 6 to 8 h (Cairns, 1977).

With later and later times of bead removal, the additional digits formed appear in a defined temporal sequence, with digit *2* being the first and digit *4* being the last to appear. This order is reminiscent of the hierarchy seen in dose-variation experiments, since low doses of RA result in an extra digit *2*, while increasing doses lead first to a digit *3* and then to a digit *4* (Tickle et al., 1985). By increasing the RA dose, the critical time required during the duplication phase to induce each digit can be decreased somewhat. However, a long lag time still remains. By removing polarizing tissue grafts, Smith (1980) has experimentally determined that a total of 15 h is required for such a graft to give rise to duplications. Moreover, he observed a lag period lasting for ~12 h before any duplications are found. Thus, duplications mediated by tissue grafts and by retinoids exhibit very similar requirements.

An Asymmetrical Distribution of Retinoid Across the Limb May be Required for Pattern Duplications

Exposure of the limb bud to a bead soaked in 14 μM

TABLE I. *Biological Effect of Various Types of TTNPB Treatments on the Wing Digit Pattern*

Exposure time to TTNPB before bead removal	Concentration of TTNPB in bead soaking solution	Digit pattern observed (*234* = normal)	PRV
	μM		%
17–18 h	1.4	*43234* (1)*	
		4d234 (1)‡	
		32234 (3)	33
		2234 (3)	
		234 (5)	
Bead not removed	1.4	*4d234* (1)	
		32234 (2)	
		3234 (2)	31
		2234 (4)	
		234 (4)	
9–12 h	14	*32234* (3)	
		2234 (5)	13
		234 (13)	
Bead not removed	14	*43234* (18)	
		4334 (3)	
		4334 (2)§	96
		⊔	
		432234 (2)	
		3234 (1)	
		32234 (1)	
10 h	140	*43234* (1)	
		32234 (2)	30
		2234 (2)	
		234 (1)	
Bead not removed	140	trunc (3)‖	
		44 (3)	
		434 (1)	100
		4334 (1)	
		⊔	
		4334 (2)	

* The number of cases is given in parentheses.
‡ *d* stands for a digit not identified.
§ A bracket indicates that the two digits are fused proximally.
‖ Prolonged exposure to very high concentrations of either TTNPB or RA causes truncation of the limb and loss of all digits (Tickle et al., 1985); truncated wings are ignored in calculating the PRV.

TTNPB that is removed at 10 h has almost no effect on the normal digit pattern (see Fig. 1 and Table I). In contrast, significant pattern duplications are obtained with 10 times less TTNPB provided that the bead is not removed. Yet, the experiment in Fig. 4*B* demonstrates that, during the crucial duplication phase (12 to 18 h after bead implantation), the average concentration of TTNPB in the anterior half of the wing bud is about the same in these two treatments. Moreover, the removal at 10 h of a bead soaked in 140 μM TTNPB causes about the same extent of duplication as the release of 100 times less TTNPB for a more prolonged period (Table I). Our preliminary measurements of the tissue concentration of TTNPB resulting from this high-dose treatment show that at 16 h, 6 h after bead removal, there is ~5 times more TTNPB in the limb than is found with continuous treatment at a dose of 1.4 μM TTNPB. How can such results be explained?

There are two obvious possibilities. First of all, pattern duplications may require a graded concentration of retinoid across the anterior portion of the limb. In this view, the cells in the limb respond to an elevated concentration of retinoid, but they also require that their neighboring cells experience a different retinoid concentration in order to differentiate to produce an ordered tissue. How such an integration across the limb might be performed is unclear, although microsurgery experiments reveal that pattern regulation can smooth out experimentally induced discontinuities in the positional values of neighboring cells (Kieny, 1977; Summerbell, 1977). Thus, some sort of extensive cell–cell communication is obviously occurring in the normal limb.

It seems likely that the concentration of retinoid immediately adjacent to a bead during the duplication phase is higher for the continuous release of TTNPB from a bead soaked in 1.4 μM TTNPB than it was in the same region of tissue for any treatment in Table I in which the bead was removed at 10 h. Thus, the second possible explanation for our results is that an extremely high concentration of retinoid in a special "active zone" very close to the bead is all that is necessary to cause duplications. For example, bead removal at 10 h might be relatively ineffective because the concentration of retinoid drops drastically in the adjacent tissue that immediately surrounds the bead. In this view, it is only the absolute concentration of retinoid in nearby tissue that determines the differences in biological response seen in Table I, and the retinoid could be doing something as simple as turning adjacent tissue into polarizing region cells.

This second explanation implies that only the retinoid most recently released from a bead matters, because only these molecules are in proximity to the active zone, while those released earlier will have diffused away. The substantial difference in the breakdown rates found for RA and TTNPB (Fig. 3) should have essentially no effect on the most recently released molecules. Thus equal doses of TTNPB and RA would be expected have the same biological effect, which is clearly not the case (Fig. 1*A*). For this reason, we interpret our data as favoring the first hypothesis, which assumes that a gradient of retinoid concentration across the limb is important.

In studies with amphibians, it has been shown that the addition of retinoids to the tanks in which the animals are regenerating limbs gives rise to pattern duplications along the proximodistal axis for axolotls (Maden, 1982), and to duplications along both the proximodistal and the anteroposterior axis for *Rana* and the newt (Maden, 1983; Thoms and Stocum, 1984). Although the retinoids are being applied systemically in these cases, they may permeate into the regenerating tissue unevenly, so as to establish a local concentration gradient of retinoid. In agreement with this view, Maden et al. (1985) have recently reported that a local release of retinoic acid from a silastic carrier implanted at a regenerating axolotl blastema frequently changes the anteroposterior pattern of the resulting limb. Some of the patterns observed resemble the duplications caused in chick limbs by a local release of retinoic acid. Moreover, the effect is position specific, with the greatest pattern duplications being found for a dorsal implant. It therefore seems likely that an asymmetric distribution of retinoids is effective in inducing pattern duplications in amphibians, as well as in the chick.

Is Retinoic Acid a Naturally Occurring Morphogen?

Position specificity in gene expression is a crucial aspect of pattern formation (e.g., Fjose et al., 1985; Gehring, 1985).

This raises the intriguing question of how cells know their location. Wolpert and colleagues have proposed a model, in which a gradient of a morphogen released by the polarizing tissue provides the limb cells with knowledge of their position (a positional value: Wolpert, 1969; Wolpert, 1971; Tickle et al., 1975). Each different positional value would subsequently specify a corresponding program of differentiation. We have previously shown that experimentally released retinoic acid and its metabolites form an exponential concentration gradient along the anteroposterior axis very similar to that shown in Fig. 4 for TTNPB (Tickle et al., 1985). In this report, we provide suggestive evidence that some aspect of the retinoid gradient, rather than just the presence of the retinoid, is required to obtain experimental pattern duplications (Fig. 4*B*). Finally, the time pattern of induction of duplications by RA and polarizing region cells is strikingly similar. Could retinoic acid itself be a natural morphogen that provides positional information in the limb?

From a very different point of view, there is now convincing evidence from several tissue culture systems that retinoids influence gene expression (Wang and Gudas, 1983; Eckert and Green, 1984; Thiele et al., 1985); moreover, these compounds seem to be an important class of biologically active molecules in search of a biological role. Thus, in our opinion, the proposal that RA functions as a natural morphogen in the limb (being the signalling molecule normally released by polarizing region cells) is a reasonable although tentative one. The idea clearly needs to be tested directly by determining whether polarizing region cells are enriched in RA. To date, by pooling extracts made from 1300 stage 20/21 whole limb buds, we have been able to detect a peak that co-migrates with RA on both reverse-phase and straight-phase high pressure liquid chromatography chromatograms. This peak represents about 15 fmol of RA per bud (Eichele, G., and C. Thaller, unpublished results), which is in the range that is found to be effective when retinoic acid is exogenously applied from beads (Tickle et al., 1985). How this compound is distributed in the limb is not yet known.

We wish to thank Professor Wolpert for his encouragement and Kathleen Raneses for preparation of the manuscript.

This study was supported by the Medical Research Council United Kingdom (C. Tickle) and by grant GM 23928 from the National Institutes of Health (B. M. Alberts). G. Eichele was supported by fellowships from Ciba-Geigy, Basel, Switzerland, and the Swiss Science Foundation. The radiolabeled retinoic acid used was synthesized by SRI International, Menlo Park, California, under contract to the National Cancer Institute. Several of the retinoids used were gifts from Hoffmann-LaRoche, Basel, Switzerland.

Received for publication 24 June 1985.

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