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Characterization of retinoid metabolism in the developing chick limb bud

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Summary

Retinoids (vitamin A derivatives) have been shown to have striking effects on developing and regenerating vertebrate limbs. In the developing chick limb, retinoic acid is a candidate morphogen that may coordinate the pattern of cellular differentiation along the anteroposterior limb axis. We describe a series of investigations of the metabolic pathway of retinoids in the chick limb bud system. To study retinoid metabolism in the bud, all-*trans*-[^{3}H]retinol, all-*trans*-[^{3}H]retinal and all-*trans*-[^{3}H]retinoic acid were released into the posterior region of the limb anlage, the area that contains the zone of polarizing activity, a tissue possibly involved in limb pattern formation. We found that the locally applied [^{3}H]retinol is primarily converted to [^{3}H]retinal, [^{3}H]retinoic acid and a yet unidentified metabolite. When [^{3}H]retinal is locally applied, it is either oxidized to [^{3}H]retinoic acid or reduced to [^{3}H]retinol. In contrast, local delivery of retinoic acid to the bud yields neither retinal nor retinol nor the unknown metabolite. This flow of metabolites agrees with the biochemical pathway of retinoids that has previously been elucidated in a number of other animal systems. To find out whether metabolism takes place directly in the treated limb bud, we have compared the amount of [^{3}H]retinoid present in each of the four limb anlagen following local treatment of the right wing bud. The data suggest that retinoid metabolism takes place mostly in the treated limb bud. This local metabolism could provide a simple mechanism to generate in a controlled fashion the biologically active all-*trans*-retinoic acid from its abundant biosynthetic precursor retinol. In addition, local metabolism supports the hypothesis that retinoids are local chemical mediators involved in pattern formation.

Key words: retinoid metabolism, pattern formation, limb development, morphogenesis, chick embryo.

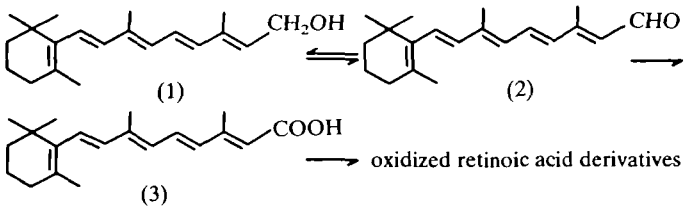
Introduction

It is a long-standing idea that the formation of biological patterns depends in part on diffusible signalling substances known as morphogens that specify the pattern of cellular differentiation in the developing embryo (Crick, 1970; Meinhardt, 1982; Slack, 1987). Recent studies indicate that in the chick limb bud all-*trans*-retinoic acid (see formula 3), a vitamin A derivative, is a candidate morphogen. Briefly, the evidence is as follows. When exogenously applied to the early limb anlage, retinoic acid induces digit pattern duplications in a dose-, stage-, time- and position-dependent fashion (Tickle *et al.* 1982; Summerbell, 1983; Tickle *et al.* 1985; Eichele *et al.* 1985). Instead of a normal **234** digit pattern, a **432234** pattern can arise. Limb anlagen contain endogenous retinoic acid at an average concentration of 25 nM, which is close to the concentration required to induce pattern duplications when retinoic acid is exogenously applied (Thaller & Eichele, 1987). This correspondence indicates that duplications induced by applied retinoic acid reflect a physiological response. Endogenous retinoic acid forms a concentration gradient across the bud with a high point at the posterior limb bud margin (Thaller & Eichele, 1987). This region of the limb bud contains the zone of polarizing activity, a previously identified putative inducer tissue of the limb anlage (Saunders & Gasseling, 1968; Tickle *et al.* 1975). The effects of retinoids on regenerating amphibian limbs can also be rationalized in the framework of a morphogenetic substance that influences positional information (Maden, 1984).

It is sensible to assume that the overall mechanism of action of a morphogen should resemble that of a hormone. If one accepts this working hypothesis, then three kinds of questions must be addressed. The first one concerns the biosynthesis of the compound, the second one is about the way a morphogen is sensed by the cells and the third issue concerns the

nature of the specific responses that the morphogen elicits in the target cells. In this study, we examine the biosynthesis of retinoic acid in the developing chick limb.

Retinoids are vitamins and therefore are not synthesized *de novo*, but derive from precursor substances in the form of carotinoids, retinyl esters, and retinol (1). It has been shown for a number of *in vivo* (reviewed by Frolik, 1984) and *in vitro* systems (e.g Frolik *et al.* 1981; Williams & Napoli, 1985; Gubler & Sherman, 1985; Napoli, 1986) that retinoids are linked by the following metabolic pathway:



Scheme I

Recent studies have shown that early limb buds contain in addition to retinoic acid all-*trans*-retinol (Thaller & Eichele, 1987) and all-*trans*-retinal (2) as well as several other unidentified retinoids (Thaller & Eichele, unpublished observation). The concentration of retinol in the limb is about 600 nM, 25 times that of retinoic acid which is consistent with the possibility that retinol serves as a precursor for retinoic acid. In contrast, limb buds contain little retinal (the tissue concentration is about 15 nM) suggesting in agreement with Scheme I that this compound represents an intermediary metabolite.

The aim of this study was to examine whether retinol and retinal are indeed precursors of retinoic acid in the developing chick limb bud. In other words, does the pool of endogenous retinol provide the substrate for retinoic acid biosynthesis? To examine this question, we have provided physiological doses of all-*trans*-[^{3}H]retinol and also of all-*trans*-[^{3}H]retinal locally to the wing buds of stage-20 embryos and have analysed several of the principal metabolites formed, using a combination of high performance liquid chromatography (HPLC) and chemical derivatization reactions. The main results of this investigation are that applied retinol and retinal are metabolized to retinoic acid. A related problem that we have examined is whether oxidative metabolism as outlined in Scheme I takes place either directly in the limb rudiment or whether retinoic acid is first generated outside the limb and later imported back into the limb bud. We found that the conversion predominantly takes place in the limb bud.

Materials and methods

Chemicals

The retinoids used were all-*trans*-retinol, all-*trans*-retinal (both from Sigma), 11- and 13-*cis*-retinol (a gift from Dr W. C. Law, Harvard Medical School), all-*trans*-retinoic acid, 13-*cis*-retinoic acid (both from Hoffmann-LaRoche), and 4-oxo-all-*trans*-retinoic acid (given to us by Dr C. Smith, National Cancer Institute, Bethesda). 11- and 13-*cis*-retinal were prepared according to established procedures (Bridges & Alvares, 1982). 4-hydroxy-all-*trans*-retinol was a kind gift of Drs Olson and Barua (Iowa State University). All-*trans*-[10,11-$^3H_2$]-retinal, all-*trans*-[10,11-$^3H_2$]-retinoic acid (both synthesized by SRI International under contract to the National Cancer Institute) had a specific activity of ~3 Ci mmol^{-1}, and all-*trans*-[11,12-$^3H_2$]-retinol (Amersham) had a specific activity of ~50 Ci mmol^{-1}). Whenever possible, work involving retinoids was carried out under yellow and/or red light and in the presence of nitrogen gas.

Local treatment with radioactive retinoids

'Utility' grade fertile chicken eggs were purchased from Spafas (Norwich, CT) and incubated at 37·5 °C for 3·5 days to develop to stage 20 (Hamburger & Hamilton, 1951). About 20 AG1-X2 ion-exchange beads (BioRad, Richmond, CA) of 200–250 μm diameter were impregnated in DMSO solutions of all-*trans*-[11,12-$^3H_2$]-retinol (58 μg ml^{-1} for 90–120 min), or all-*trans*-[10,11-$^3H_2$]-retinal (350 μg ml^{-1} for 90 min) or all-*trans*-[10,11-$^3H_2$]-retinoic acid (12·5 μg ml^{-1} for 20 min). In the case of retinol and retinal, the beads were removed from the soaking solution and rinsed for about one minute in an excess of phosphate-buffered saline. Retinoic acid impregnated beads were washed as previously described (e.g. Eichele & Thaller, 1987). After washing, the beads were implanted into the right wing buds of stage-20 embryos at the posterior margin (see Fig. 1) below the apical ectodermal ridge as described (Tickle *et al.* 1985). The operated embryos were incubated for 3 h at 37·5 °C. 20–40 embryos were used per experiment. Since applied retinoids are radioactive, they can be detected in minute amounts.

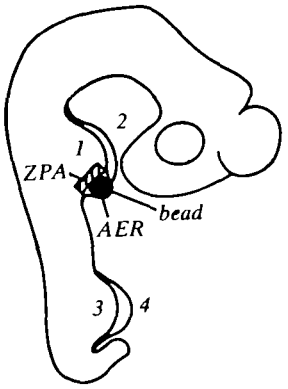


Fig. 1. Schematic drawing of a Hamburger & Hamilton stage-20 chick embryo. Beads releasing retinoids were applied to the posterior margin of the right wing bud (*1*). In order to hold it in place, the implant was pushed below the apical ectodermal ridge (*AER*) next to the zone of polarizing activity (*ZPA*). The numbering of buds is as follows: *1*, treated right wing bud; *2*, left wing bud; *3*, right leg bud; *4*, left leg bud.

Isolation of retinoid metabolites

After incubating the embryos, the beads were removed, the embryos were dissected out of the egg and rinsed in ice-cold stabilizing buffer (Eichele *et al.* 1985). All four limb buds were removed from the embryo, rinsed in 3 ml of stabilizing buffer and separately collected into microfuge vials kept on dry ice. The time span between dissection and storage in dry ice was less than 2 min. The rinse effectively removes the egg plasma that contains traces of [^{3}H]retinoids. To the frozen limb buds were added 200 μl of stabilizing buffer, 100 μl of a saturated Na_2SO_4 solution, 40 μl ethanol, 40 μl of ethylacetate/methylacetate (8:1) containing 2 mg ml^{-1} butylated hydroxytoluene (antioxidant) and 20 μl of a nonradioactive internal standard retinoid cocktail (100 ng of all-*trans*-retinol, all-*trans*-retinal, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 50 ng 4-oxo-all-*trans*-retinoic acid). The sample was sonicated and extracted three times with 800 μl of the ethylacetate/methylacetate mixture. The combined organic phases were evaporated to dryness at room temperature with nitrogen gas and the residue was dissolved in 20 μl methanol for subsequent HPLC analysis. We found that this procedure routinely results in retinoid recoveries >90 %.

Characterization and quantification of the metabolites by high-performance liquid chromatography

Chromatographic equipment

A Waters 6000 A solvent pump combined with a Waters 441 detector (set at 280 nm) and Shimadzu SPD-6A detector (set at 325 or 350 nm) were used. Absorbance signals were recorded and integrated by a Shimadzu integrator. Samples were applied to the column using a Waters UK6 injector and fractions of 1·2 ml (solvents A and C) or 1 ml (solvent D) were collected.

Columns. 4·6×250 mm Microsorb C_{18} and silica 5 μm columns (both from Rainin Instruments, Woburn, MA).

Solvent systems. (A) acetonitrile: methanol: 2 % aqueous acetic acid (6:2:2); (C) methanol: acetonitrile: water: acetic acid (80:10:9:1); (D) *n*-hexane: dioxane (95:5).

Scintillation counting. Aliquots or whole fractions were mixed with 17 ml of Instafluor (Packard) and counted in a Packard Tri-Carb 1500 scintillation counter.

Quantification. Cts min^{-1} data were converted to disints min^{-1} using a quench curve correction method. The quantity Q of retinoid metabolite formed per bud (in disints min^{-1}) was calculated with the following formula: $Q = C_{tot}/n \cdot R$, where C_{tot} equals the total disints min^{-1} per peak, n is the number of buds used and R the recovery of the internal standard (actual peak area of internal standard observed divided by the peak area expected if no loss had occurred).

Chemical derivatization reactions

Since derivatizations require nonaqueous solvents, samples eluted with solvent A were transferred into the appropriate solvent in the following way. 2 ml water was added to the sample fractions, followed by two extractions each with 2 ml *n*-hexane. The organic phase was evaporated to dryness and the residue dissolved in methanol (for methylation) or in acetonitrile (for acetylation). The methyl ester of retinoic acid was prepared by treating the sample with 200 μl of an ether solution of diazomethane. Acetylation of retinol and other putative hydroxylated retinoids was carried out by standard procedures (e.g. Gubler & Sherman, 1985).

Characterization of the slow release system

All-*trans*-retinoic acid when released from Dowex beads into tissue culture medium is partly isomerized to its 13-*cis*-isomer (Eichele *et al.* 1984). This raises the possibility that other retinoids can also undergo tissue-independent, nonenzymic reactions. The main concern for the present study was of course not isomerization but whether the oxidations at the terminal carbon (Scheme I) can occur in the absence of tissue (see Frolik & Olson, 1984 for a discussion of experimental artifacts with retinoids). To test for nonenzymic reactions, beads were first soaked in 5 μl all-*trans*-[^{3}H]retinol (58 μg ml^{-1}) for 40 min, rinsed in phosphate-buffered saline for 1 min, washed twice for 10 min in phosphate-buffered saline and immersed into 200 μl Dulbecco's modified Eagle's medium containing 10 % fetal calf serum (FCS, from Gibco). After 3 h of incubation at 37·5 °C, the released retinoids were extracted from a 30 μl sample and analysed by HPLC as described above for tissue extracts. Fig. 2 shows a reversed-phase chromatogram of the products formed. It is most useful to compare these data with those obtained after releasing retinol into limb buds (Fig. 3A). Figs 2 and 3A are different in two important aspects. First and most obvious, there is no indication for formation of metabolite A_1 *in vitro*. Second, in contrast to the *in vivo* experiment, the *in vitro* data reveal no radioactivity above background at the elution position of retinoic acid. To be sure about this, we have treated the fraction containing retinoic acid standard (arrow 4) with diazomethane and fractionated the products on a C_{18} column. No radioactive peak above background was detected at the elution position of all-*trans*-methylretinoate. Note the small peak at 25·5 min in Fig. 2 which might represent all-*trans*-retinal. However, refractionation of the peak by normal phase HPLC does not confirm this suspicion: a small peak twice background level that was obtained upon rechromatography does not comigrate with all-*trans*-retinal (data not shown). Similar investigations were also conducted with retinal with the result that retinal is not converted to retinoic acid or retinol *in vitro*.

Results

Applied retinol is converted to retinal and retinoic acid

To determine whether retinol can be converted to retinal and retinoic acid, AG1-X2 beads were impregnated with all-*trans*-[^{3}H]retinol and implanted to the posterior margin of the right wing bud of stage-20

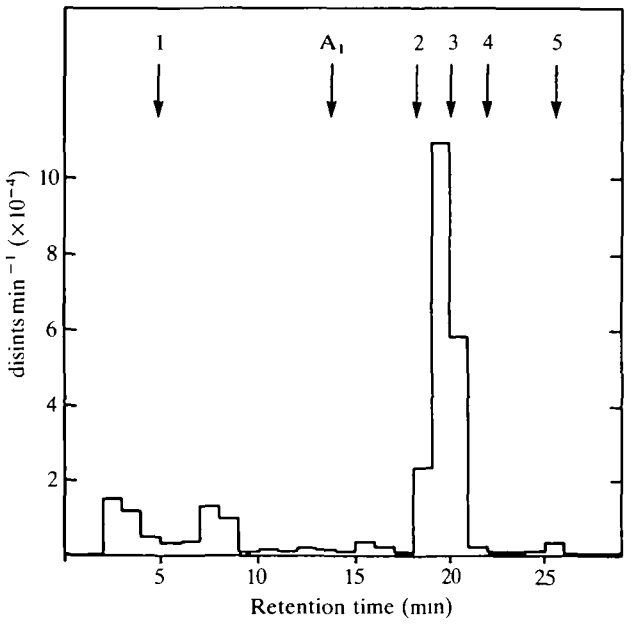
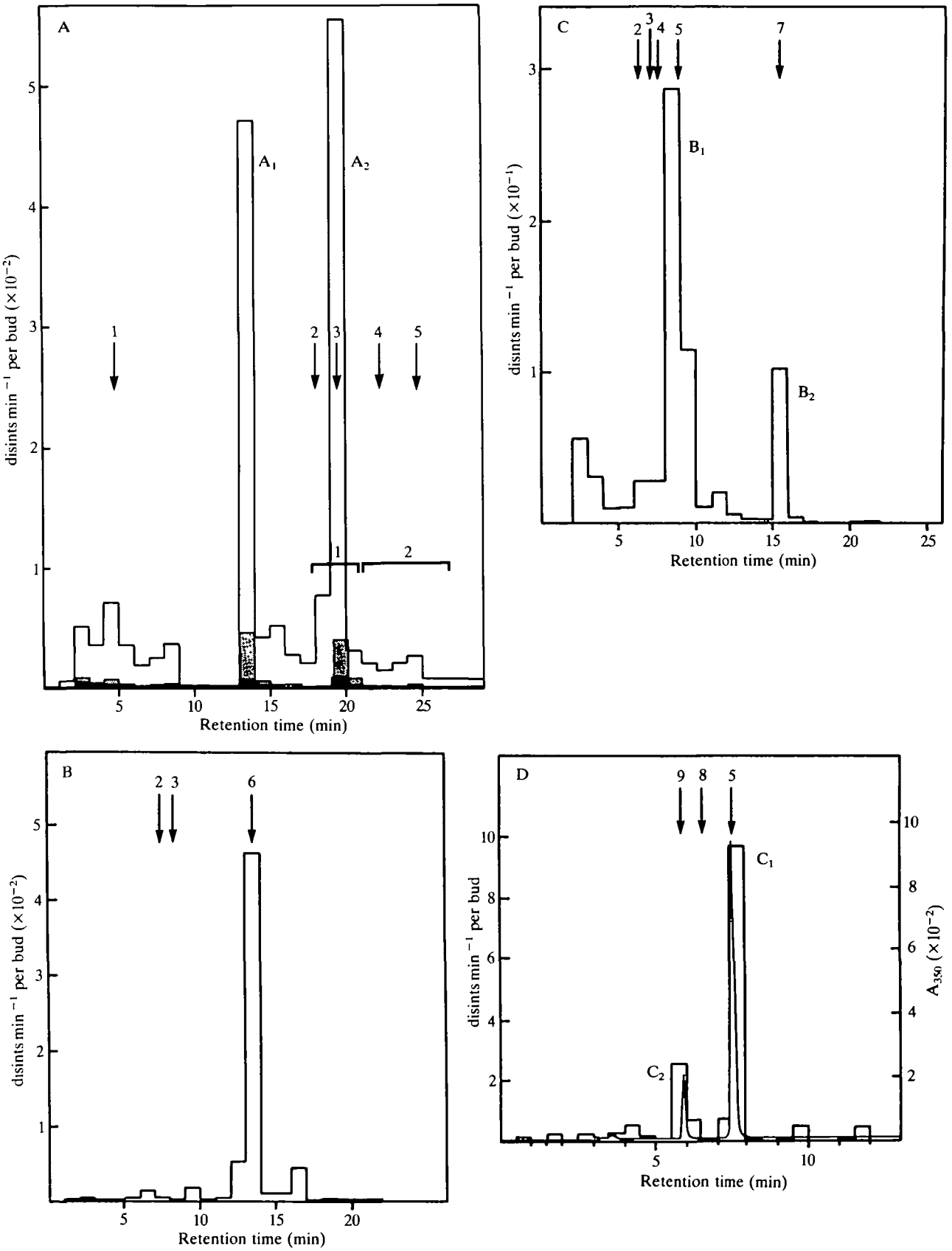


Fig. 2. Chromatogram of products formed over a period of 3 h at 37°C from all-*trans*-retinol that was released into tissue culture medium. In the region where retinoid A_1 and retinoic acid (4) elute, the radioactivity has background levels. The small peak eluting near the internal standard all-*trans*-retinal (5) is not retinal (see Materials and Methods for details). Chromatography: C_{18} column eluted with solvent C at a flow rate of 1·2 ml min^{-1}. *Arrow marks* (in this and subsequent figures): elution position of several internal standards that are always added to the samples prior to extraction; (1) 4-oxo-all-*trans*-retinoic acid, (2) 13-*cis*-retinoic acid, (3) all-*trans*-retinol, (4) all-*trans*-retinoic acid, (5) all-*trans*-retinal, (6) all-*trans*-retinyl acetate, (7) all-*trans*-methylretinoate, (8) 9-*cis*-retinal, (9) 11-*cis*-retinal.

embryos (Fig. 1). The bead was positioned posteriorly in order to target the retinoid into the posteriorly located zone of polarizing activity, the postulated source of limb morphogen (Tickle *et al.* 1975). After 3 h of incubation, the buds were removed and, after adding 20 µl of nonradioactive internal standard mixture, the tissue was extracted (see Methods). Fig. 3A shows separation on reversed phase of the radiolabelled metabolites present in each of the four limb buds. While all four buds display a rather similar pattern of metabolites, the treated bud contains much larger quantities of radioactivity than the other three buds (see also Table 1). In the chromatogram of the treated buds, there are two major peaks (A_1 and A_2) and a series of smaller but often distinct peaks. Because of comigration with authentic retinol, peak A_2 most likely is the residual, not metabolized all-*trans*-retinol. It can be argued that the shoulder preceding the main peak is 13-*cis*-retinoic acid. To clarify this point, the fractions marked by bracket 1 in Fig. 3A were pooled and extracted with *n*-hexane. After acetylation the sample was fractionated on a C_{18} column that was eluted with solvent C. As can readily be seen in Fig. 3B, more than 90 % of the radioactivity coelutes with the authentic retinylacetate internal standard. Also note that no radioactivity can be detected at the position where 13-*cis*-retinoic acid standard elutes. Hence the shoulder in Fig. 3A is not 13-*cis*-retinoic acid and we conclude that peak A_2 represents all-*trans*-retinol. Using the known specific activity of applied retinol (370 disints min^{-1} pg^{-1}) we have calculated the quantity of radiolabelled retinol present in a treated wing bud as ~2 pg. The amount of endogenous retinol per bud is approximately 140 pg (Thaller & Eichele, 1987), and consequently the local retinol treatment does not alter the endogenous level of retinol to any significant extent.

In order to determine whether all-*trans*-retinal and/or all-*trans*-retinoic acid are amongst the products generated from retinol, the fractions that contain the corresponding internal standards were pooled (bracket 2 in Fig. 3A). After transferring the sample into methanol, diazomethane was added, which methylates retinoic acid but will not react with retinal. Typically, fractionation of the reaction products on a reversed phase column leads to two main peaks (Fig. 3C). The larger one (B_1) coelutes with all-

Fig. 3. Sample chromatograms that illustrate the HPLC analysis and identification of *in vivo* metabolites of all-*trans*-[^{3}H]retinol formed in chick limb buds. (A) The chromatogram drawn with open bars represents the primary reversed-phase fractionation of tissue extract of the treated wing bud (1). The other three chromatograms show metabolite fractionations for bud 2 (hatched), bud 3 (shaded) and bud 4 (solid black). (B) Analysis of peak A_2 and its neighbouring fractions (bracket 1 in A) after acetylation results in one major peak of radioactivity that coelutes with authentic all-*trans*-retinyl acetate (6) on a reversed-phase column. (C) Fractions marked by bracket 2 in A were pooled and methylated. Subsequent reversed-phase chromatography resulted in one peak coeluting with retinal (5) and a second peak that comigrates with authentic all-*trans*-methylretinoate (7). (D) Alternative method of identification of retinal. The tube that contains internal standard all-*trans*-retinal in the reversed-phase separation was reanalysed on a normal-phase column. Two radioactivity peaks are seen, one comigrating with authentic all-*trans*-retinal (5) and the other with 11-*cis*-retinal (9). Arrow 8 marks the elution position of 9-*cis*-retinal. The elution profiles of the internal standard (solid line) consists of all-*trans*-retinal and its 11-*cis*-isomer. We believe that 11-*cis*-retinal most likely is an artifact created by extraction and chromatography. HPLC conditions: for A–C we used a reversed-phase C_{18} column that was eluted with solvent A (A) or solvent C (B,C); for D we used a straight-phase column and eluted with solvent D.


A
A1
A2
1
2 3
4
5
1
2
disints min⁻¹ per bud (×10⁻²)
Retention time (min)
C
2 3 4 5
7
B1
B2
disints min⁻¹ per bud (×10⁻¹)
Retention time (min)
B
2 3
6
disints min⁻¹ per bud (×10⁻²)
Retention time (min)
D
9 8 5
C1
C2
disints min⁻¹ per bud
A350 (×10⁻²)
Retention time (min)

trans-retinal standard and the smaller one (B_2) cofractionates with authentic all-*trans*-methyl retinoate. As an alternative identification procedure for retinal, fractions containing the putative retinal recovered in the primary fractionation was repurified on a normal-phase column that is able to resolve all-*trans*-retinal from the 9-, 11-, and 13-*cis*-isomers (e.g. Groenendijk *et al.* 1980). The major peak seen (C_1, Fig. 3D) comigrates with authentic all-*trans*-retinal. Peak C_2 coelutes with authentic 11-*cis*-retinal. We believe that this isomer had formed during the extraction and derivatization procedure and hence almost certainly is not a natural metabolite. This interpretation is consistent with the observation that the internal standard all-*trans*-retinal had also partly isomerized to 11-*cis*-retinal (Fig. 3D).

A rather unexpected finding was that the major metabolite of retinol is neither retinal nor retinoic acid but a species represented by peak A_1 (Fig. 3A). In a typical experiment, radioactive A_1 amounted to 470 disints min^{-1} per bud compared with 660 disints min^{-1} for retinol, ~30 disints min^{-1} for retinal, and ~50 disints min^{-1} for retinoic acid. It is important to be aware of the fact that chromatograms of whole early embryos as well as of limb bud extracts show that A_1 is also an abundant endogenous retinoid (Thaller & Eichele, in preparation). Therefore, A_1 is unlikely to be an experimental artifact of the local application procedure (see also Materials and Methods). Preliminary analyses uncover the following properties of A_1. It is not a carboxylated retinoid since diazomethane treatment does not alter its chromatographic behaviour. A_1 can be acetylated and upon acetylation its retention time on a reversed-phase column eluted with solvent C increases from 6·5 to 10·5 min (data not shown). Thus acetylation alters a polar functional group (e.g. -OH or $-NH_2$) to one which is measurably less polar. A_1 is not a *cis* isomer of retinol, because it does not coelute with either 13-*cis*-, 11-*cis*- or 9-*cis*-retinol. We conclude that in the chick limb bud all-*trans*-retinol is metabolized according to the established pathway of Scheme I. The major metabolite A_1 remains to be identified, but both retinaldehyde and retinoic acid are definitely generated *in vivo* from retinol.

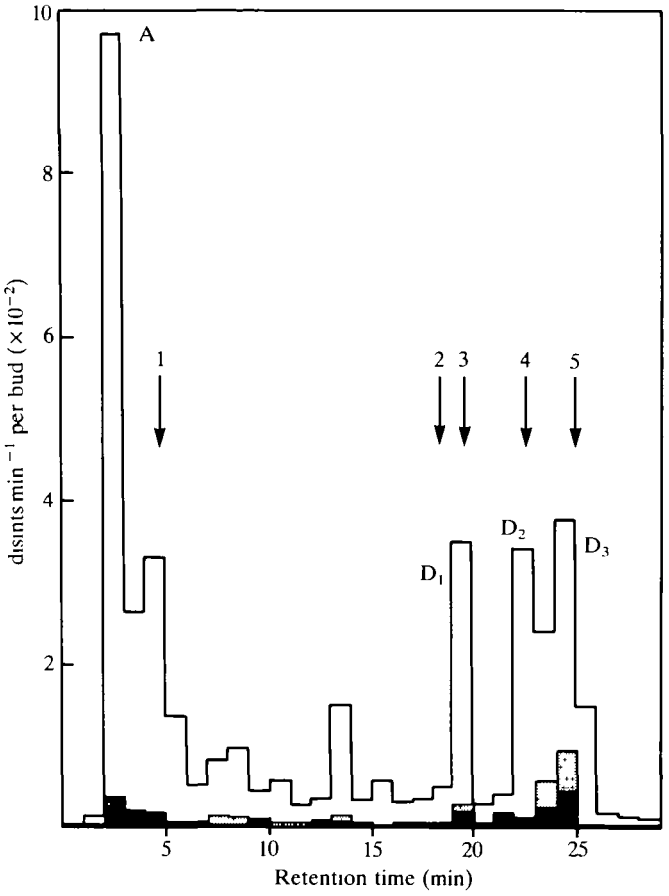
Applied retinal is converted to retinol and retinoic acid

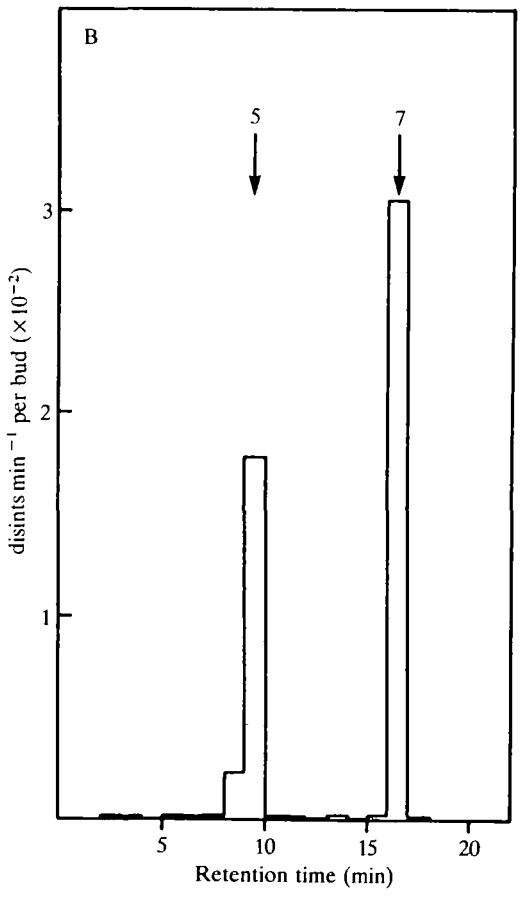
The first set of analyses ascertained that retinol is converted to retinal and to retinoic acid. The question we were asking next is whether in the developing limb bud oxidation from retinol to retinal is reversible or not. Thus beads soaked in all-*trans*-[^{3}H]retinal were implanted into wing buds. After exposing them for 3 h, the buds were dissected, spiked with nonradioactive internal standard, homogenized and extracted. Fig. 4A shows for each of the four buds reversed-phase fractionation of the metabolites resulting from retinal treatment (see also Table 1). Three principal groups of products can be seen. First, there are substantial quantities of polar compounds (retention times less than 7 min). The accumulation of substantial amounts of polar metabolites is not seen after retinol treatment (Fig. 3A). Second, there is a distinct peak coeluting with retinol (D_1). In between the polar metabolites and D_1 there is a small but discernible peak with a retention time of 13·8 min, which most likely represents A_1. Third, the chromatogram displays two nearby, not entirely resolved, peaks coeluting with retinoic acid (D_2) and retinal (D_3), respectively. For unambiguous identification, fractions containing D_1 were acetylated and fractionated on a C_{18} column with solvent C. Under these conditions the only radioactive peak present coeluted with authentic all-*trans*-retinyl acetate, as one expects if D_1 is retinol (data not shown). To identify peaks D_2 and D_3, all fractions between 22 and 27 min were pooled, treated with diazomethane and chromatographed. This resulted in the profile shown in Fig. 4B in which there are only two peaks, one coeluting with authentic retinal (presumably a mixture of all-*trans*-retinal and a small quantity of 11-*cis*-retinal, see Fig. 3D), the other comigrating with methyl retinoate standard. We conclude that local application of all-*trans*-retinal results in formation of all-*trans*-retinol, all-*trans*-retinoic acid, and A_1.

Since the specific activity of applied retinal is 23 disints min^{-1} pg^{-1}, the retinal peak area of Fig. 4B amounts to 8·7 pg per bud. The amount of endogenous retinal is about 5 pg per bud, hence the local treatment results in a concentration that exceeds the physiologic retinal level by threefold. However, we found that the pattern of metabolites does not change if the retinal dose is 15-fold reduced or 1·5-fold increased (data not shown). Therefore we conclude that reduction and oxidation of retinal as illustrated in Fig. 4A and B are physiological reactions.

Applied retinoic acid is not converted to retinal or retinol

Application of all-*trans*-retinoic acid primarily results in a series of very polar metabolites (Fig. 5). Such an accumulation of polar products has been observed previously (Tickle *et al.* 1985). Several of these polar metabolites have been characterized in other systems (reviewed by Frolik, 1984). The important feature to note in Fig. 5 is the absence of either retinal, retinol or A_1. To make this point very clear, we show in Fig. 5 a set of data based on a dose of retinoic acid that results in levels of retinoic acid 10 times above that of endogenous retinoic acid. One can argue that the low level of radioactivity in fractions between 24





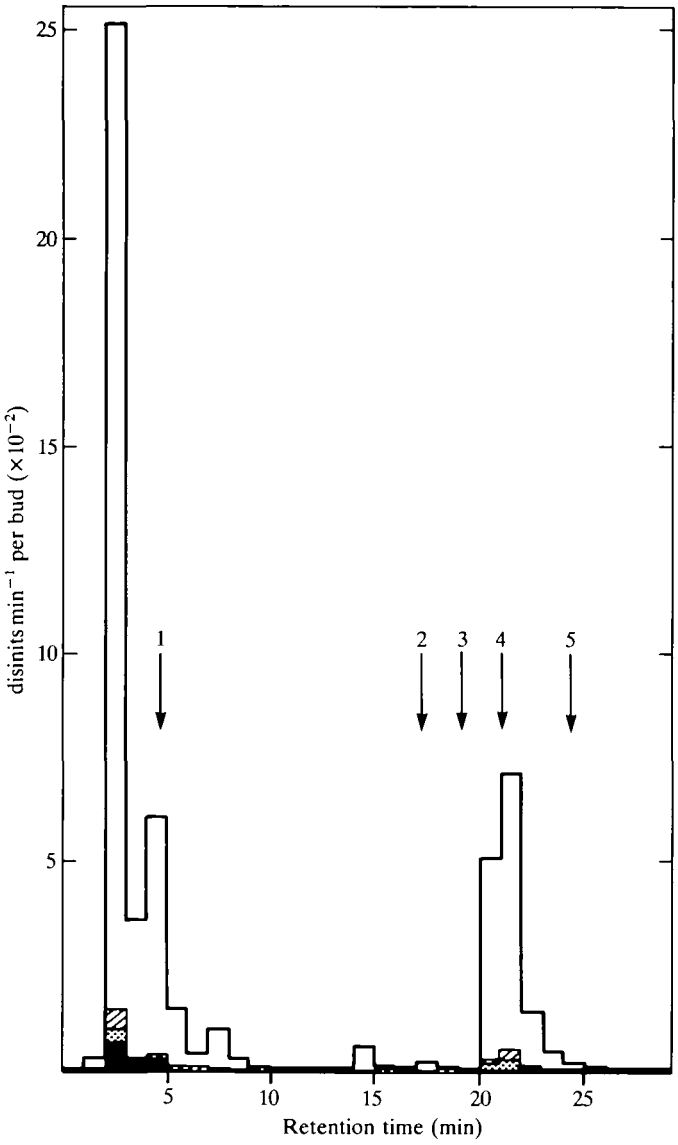


Fig. 5. Sample chromatogram illustrating the analysis of metabolites generated from all-*trans*-[^{3}H]retinoic acid in bud 1 (open bars). Nontreated buds contain little retinoic acid (bud 2, hatched; bud 3, shaded; bud 4, black). HPLC condition: a C_{18} column which was eluted with solvent A.

Fig. 4. Sample chromatograms illustrating the purification and identification of metabolites that arise from all-*trans*-[^{3}H]retinal in the developing chick limb bud. (A) Fractionation of extract from the treated limb bud (bud 1, open bars), and the nontreated buds (bud 2, hatched; bud 3, shaded; bud 4, black) on a C_{18} column eluted with solvent A resulted in a series of peaks. (B) Tubes containing D_2 and D_3 were pooled and treated with diazomethane. This led to the fractionation pattern shown here. Two distinct peaks can be detected, the earlier one represents retinal (5) and the other coelutes with all-*trans*-methylretinoate (7) on a C_{18} column eluted with solvent C.

and 25 min might represent retinal. To find out, the corresponding fractions were reanalysed on a normal phase column. No radioactive peaks above background are detected (data not shown). Using the same approach we have examined the fraction containing nonradioactive retinol standard (arrow 3). Again we found that they are devoid of any radioactive peak, when reanalysed on a normal-phase column (data not shown). We are driven to the conclusion that in limb buds retinoic acid cannot be reduced to its precursors, which is consistent with Scheme I.

Retinoid metabolism is local

The above experiments clearly establish that locally applied retinoids are metabolized. However, there remains the question as to whether metabolism takes place directly in the limb bud or not. In principle one can imagine that locally applied retinoids first diffuse into the body and that metabolism happens there, and that the products are subsequently imported back into the limb bud. One way to examine whether this is so, is to compare the levels of retinoid metabolites in each of the four buds following [^{3}H]retinol or [^{3}H]retinal application to the right wing bud (no. 1 in Fig. 1). If metabolism is local, then the amount of metabolite present in the treated bud will be significantly greater than in any of the nontreated buds. If metabolism takes place at some central site, followed by redistribution into the limbs, then all four limb buds should contain approximately equal amounts of metabolites. Inspection of the chromatograms in Figs 3A, 4A and 5 and the listings in Table 1 will convince that the amounts of metabolites in the treated bud exceed those in any of the other three buds by an order of magnitude or even more. For example, following retinal application, the treated bud contains 280 disints min^{-1} retinoic acid (Table 1), whilst the other buds have between 6 and 13 disints min^{-1}. Hence, at most, a few percent of the tritiated retinoic acid present in bud 1 is recruited from a pool outside the limb. We conclude that retinoid import is quantitatively a minor process and that metabolism is therefore predominantly local.

Discussion

One important type of biological regulation depends on a mechanism in which a ligand binds to a receptor protein, thereby altering the physiological state of the receptor. A classical example is the lac repressor, which upon binding of inducer is released from the operator site, permitting transcription of the downstream genes (Jacob & Monod, 1961; Riggs *et al.* 1970). Similarly, the gene-regulatory activity of steroid hormones is mediated by receptors, whose capacity to bind to regulatory sites on the DNA ultimately depends on ligand binding (reviewed by Ringold, 1985; Yamamoto, 1985). Hence small molecule ligands, bound to receptor proteins, have a central role in the regulation of biological processes. It is likely that morphogens, i.e. diffusible substances of low molecular weight that are thought to control the generation of certain biological patterns, function according to this general scheme. The argument for retinoic acid acting *via* such a mechanism is considerably strengthened by the recent discovery of its receptor (Guiguere *et al.* 1987; Petkovich *et al.* 1987) which belongs to the family of nuclear *trans*-acting enhancer factors that includes the receptors for steroids, vitamin D and thyroid hormone. For pattern formation there seem to be other features necessary in addition to those seen in the above-mentioned regulatory systems. There is need for a mechanism to interpret ligand doses in terms of thresholds (Lewis *et al.* 1977); and, unlike classical hormones, morphogens are thought to act in the form of concentration gradients (Meinhardt, 1982). Hence reactions that generate and affect the concentration profile such as local synthesis and degradation of morphogen, are an integral part of its mechanism of action.

Studies of the effects of retinoids on the developing and regenerating vertebrate limb indicate that retinoids have several characteristics commonly at-

Table 1. *Distribution pattern of tritiated metabolites in limb buds*

Retinoid applied†	Retinol in bud*				Retinal in bud				Retinoic acid in bud				Peak A_1 in bud				Polar metabolites in bud			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Retinol	660	9	47	9	37	n.d.	n.d.	n.d.	51	n.d.	n.d.	n.d.	470	10	54	4	192	9	27	12
Retinal	180	20	25	11	260	31	46	32	280	12	13	6	76	11	14	5	1699	83	73	44
Retinoic acid	0	0	0	0	0	0	0	0	1288	43	61	25	0	0	0	0	3621	135	208	94

* disints min^{-1} per bud, determined after primary HPLC fractionation on a C_{18} column eluted with solvent A.

† Note, the specific activity and the soaking concentrations differ for the three retinoids. Therefore, the figures between lines of the matrix cannot be directly compared.

n.d., below the level of detection of about 2 disints min^{-1} per bud.

tributed to morphogens (see Introduction for a synopsis of the current knowledge of the role of retinoids in the chick limb system). To substantiate such a claim it is necessary to explore, at a biochemical and cellular level, the mechanism of action of retinoids in the early limb anlage. One pivotal question is that of the origin of retinoic acid in the limb anlage. Is retinoic acid continuously generated from its precursors retinol and retinal directly in the limb bud? Is it synthesized primarily outside the limb anlage and becomes sequestered into the limb bud? It is important to recall that all-*trans*-retinol is very abundant in limb bud and embryo and thus would represent an essential unlimited precursor pool for retinoic acid. We found that indeed the biosynthetic precursor retinol is converted to retinoic acid *via* retinal and that this reaction can take place directly in the limb bud.

As an experimental technique to demonstrate local metabolism, we have used slow-release carriers that targeted radiolabelled retinoids into the posterior region of early wing buds. The implicit assumption of this approach is that exogenously provided radioisotope mimics the endogenous compound, e.g. partitions into the proper tissue region and cellular compartments. Local application will primarily expose tissue surrounding the bead within the range of diffusion which amounts to a few hundred μm (Eichele & Thaller, 1987). Undoubtedly among the exposed tissues will be the zone of polarizing activity that extends immediately next to the bead (Honig & Summerbell, 1985; Hinchliffe & Samson, 1985). It is therefore logical to posit that this putative signalling tissue can convert retinol to retinal and retinoic acid. It goes without saying that the present investigation does not test the possibility that non-ZPA tissue is also competent of retinol oxidation. In other words, our data do not mean that retinoid metabolism is restricted to the ZPA.

An HPLC analysis of metabolites generated *in situ* following site-directed treatment clearly demonstrates that locally delivered all-*trans*-retinol is converted to all-*trans*-retinal and all-*trans*-retinoic acid. Similarly, all-*trans*-retinal delivered to wing buds is converted to all-*trans*-retinoic acid and to its precursor all-*trans*-retinol. Hence, the alcohol/aldehyde reaction is reversible (Scheme I). In contrast, local application of all-*trans*-retinoic acid does not yield any detectable levels of retinal or retinol. This agrees well with the classical observation that retinoic acid cannot be converted back to retinaldehyde or retinol (Dowling & Wald, 1960).

An unexpected, yet interesting, finding was that relatively little retinal and retinoic acid are made from retinol in the limb. This is reminiscent of a study of retinol metabolism in LLC-PK_1 kidney cells (Napoli, 1986) and F9 cells (Williams & Napoli, 1985), where retinal and retinoic acid account for a minor percentage of the metabolic products. In the limb system, the initial HPLC fractionation of retinol metabolites on a reversed-phase column usually gave only small peaks for aldehyde or acid (Fig. 3A). However, subsequent rechromatography and/or chemical derivatization have permitted clear-cut identification (Fig. 3). This difficulty arises partly from the dilution of applied [^{3}H]retinol by a substantial pool of nonradioactive endogenous retinol. More important, we have discovered that the major metabolite of retinol is neither retinoic acid nor retinal, but a species represented by peak A_1 (Fig. 3A). We are still in the process of elucidating the chemical structure of A_1. It appears to have a polar functional group (e.g. -OH or -NH_2), and is not a downstream metabolite of retinoic acid since A_1 is not formed after application of retinoic acid (Fig. 5). Consistent with this notion is that A_1 does not have a free carboxyl group because it cannot be methylated. Leo & Lieber (1985) have described in liver tissue a pathway of retinol degradation that bypasses retinoic acid and hence is different from that outlined in Scheme I. One of the major metabolites in this new pathway is 4-hydroxyretinol. However, A_1 does not seem to be 4-hydroxyretinol, because solvent C elutes this polar molecule at 4·5 min, while A_1 elutes at 13·6 min on a reversed-phase column. That A_1 is likely to be of physiological significance is underlined by the fact that it is also a major endogenous metabolite (Thaller & Eichele, unpublished data).

Why can we be confident that the observed retinol conversion in the limb bud is a physiological process? First, the amounts of retinol and retinal that build up in the tissue following local delivery are in a physiological range (see Results). Second, in the absence of tissue, these metabolites are not formed (see Materials & Methods). Third, the metabolites that we have identified are also present as endogenous metabolites. A fourth and evolutionary argument is that the pattern of metabolites in the wing bud agrees with the universal pathway of Scheme I, with the exception of the compound represented by peak A_1 (Fig. 3A) for which a place in the equation still must be sought.

Since apparently little retinal and retinoic acid are generated from retinol, one might question whether local metabolism can account for the known endogenous quantities of these two compounds. The following order-of-magnitude calculation indicates that this is the case. As shown in Table 1, a limb bud with ~2 pg (660 disints min^{-1}) applied [^{3}H]retinol, contains 37 disints min^{-1} [^{3}H]retinal. The radiolabelled retinol is predominantly found in the posterior half of the bud (Eichele & Thaller, 1987), where it is diluted with ~70 pg endogenous retinol (Thaller & Eichele, 1987).

This 36-fold dilution reduces the specific activity from 370 to 10 disints $min^{-1} pg^{-1}$. Therefore 37 disints min^{-1} of tritiated retinal represent 3·7 pg of total retinal. This amount is close to the experimentally determined steady-state value of ~2·5 pg (Thaller & Eichele, unpublished observation). A similar line of thought can be applied successfully to retinoic acid, leading to the conclusion that the local turn over can account for the equilibrium quantities of retinoids in the limb bud.

What is the purpose for a steady synthesis and degradation of retinoic acid in the limb field? It would seem simpler to provide the organism at the onset of development with as much retinoic acid as needed for embryogenesis. However, high concentrations of retinoic acid would certainly be toxic to the embryo (Kochhar, 1977) and hence this mechanism is not practical. Another good reason for a steady-state system is that it can regulate the level of a transient effector substance. In particular if one posits a receptor–ligand complex as the biologically active species, it becomes crucial to continuously fine-tune the amount of ligand available to the receptor. While binding to the abundant cellular retinoic acid-binding protein could be one way to achieve this goal (Maden & Summerbell, 1986), it seems to be more economic to regulate directly the levels of retinoic acid *via* its synthesis and catabolism. A second argument for a steady-state system bears on the generation of the retinoic acid gradient. Because retinoids diffuse rather rapidly in limb bud tissue, a static retinoic acid gradient set up as a one-time event, would decay soon after it has been formed (Eichele & Thaller, 1987). In contrast, if retinoic acid is continuously synthesized, a steady-state gradient can readily be produced. If one accepts this line of reasoning, then the conclusion is that retinoid metabolism in the limb bud is an important facet of the mechanism leading to specification of the pattern of cellular differentiation along the anteroposterior limb axis.

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