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The effect of local application of retinoic acid to the anterior margin of the developing chick limb

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SUMMARY

Local application of retinoic acid to the chick limb bud produces effects that are dose and/or stage dependent. Low doses and/or old stages tend to give normal limbs or perhaps one or two supernumerary digits of a more anterior character. Medium doses and/or intermediate stages tend to give full mirror-image supernumeraries with two or even three extra digits including particularly digits of a posterior character. High doses and/or early stages give limbs in which supernumerary digits fail to form or are lost, and in which even host skeletal elements are missing or reduced. The effects are graded over the full dose and/or stage range.

Various explanations are discussed in the context of the current hypotheses of limb development. We conclude that one should not necessarily interpret the results as evidence that retinoids normally play a part in the control of development or regeneration.

INTRODUCTION

Vitamin A has been found to exert a dramatic effect on the proximodistal axis of the regenerating axolotl limb (Maden, 1982; 1983*a,b*). Depending on concentration, time window of exposure and analogue used, a variety of additional skeletal elements are produced from an extra row of carpals to the repetition of structures as proximal as the girdle. I have been unable to produce similar effects on the proximodistal axis of the developing chick wing, but instead have found that Vitamin A can influence the pattern of structures across the anteroposterior axis (Summerbell & Harvey, 1983). Similar results have been discovered independently and coincidentally by Tickle, Alberts, Wolpert & Lee (1982); Tickle (1983).

Experiments into this aspect of morphogenesis originated from work describing disturbances in the regeneration of limb and tail regeneration in *Bufo* following treatment with retinol palmitate (Niazi & Saxena, 1968; Saxena & Niazi, 1977).

The teratogenic effects of vitamin A have been widely studied using a variety of analogues and methods of administration to a number of different species. Systemic application has resulted in, for example, defects in limb formation (Jelinek & Kistler, 1981; Kochhar, 1973; Kwasigroch & Kochhar, 1980; Kwasigroch &

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Skalko, 1983; Neubert & Dillman, 1980; Summerbell & Harvey, 1983) central nervous (Langman & Welch, 1966) including anancephaly and malformations of the eye (Marin-Padilla & Ferm, 1965), palate or break formation (Dhouailly & Hardy, 1978; Kochhar, 1967; Morriss, 1975). At the cellular level, vitamin A induces shrinkage of the cytoplasm and increase in the size of intercellular spaces in areas of the mesoderm, also somite necrosis, resulting in somites with fewer than normal cells (Marin-Padilla, 1966).

In vitro experiments have demonstrated that attenuating effect of high doses of vitamin A (1000–03000 i.u. retinol acetate/100 ml medium) on cartilage growth (Fell & Mellanby, 1950), and the lytic action of vitamin A analogues on erythrocyte and mitochondrial membranes (among others) has also been shown (Dingle & Lucy, 1962; Lucy & Dingle, 1964). Vitamin A has also been shown to effect cell proliferation (Harrison, 1965; Kochhar, 1967), movement (Kwasigroch & Kochhar, 1975; Morriss, 1975), survival (Fell & Mellanby, 1950; Marin-Padilla, 1966; Marin-Padilla & Ferm, 1965), and differentiation (Fell, 1957; Fell & Mellanby, 1953; McLoughlin, 1961; New, 1965; Zimmerman, 1980). A feature of many of these studies is the apparent inconsistency of effects, with different doses giving different (sometimes converse) results.

Of particular interest are the studies demonstrating a very specific effect of vitamin A on the control of differentiation. A single systemic dose of retinoic acid acts directly on chick epidermis causing the dermis to initiate feathers rather than scales (Dhouailly & Hardy, 1978; Dhouailly, Hardy & Sengel, 1980; Dhouailly, 1982). The scale-inducing properties of the epidermis, which had been determined for several days, becomes transformed into feather-inducing properties or repressed into a 'neutral' state allowing autonomous expression of the feather phenotype by the dermis.

This paper presents detailed results from experiments in which a local source of retinoic acid is placed in the anterior margin of the developing chick limb bud. I show a dose-dependent response to a range covering two and a half orders of magnitude and a stage-dependent response over a range from stage 17–22 (Hamburger & Hamilton, 1951). The response includes the suppression of digits and /or the induction of supernumerary digits. I chose retinoic acid, the most potent of the vitamin A analogues so far tested (Maden, 1982; Summerbell & Harvey, 1983), on a paper carrier (originally suggested to us by Lewis Wolpert, and subsequently published in Tickle *et al.* (1982)).

METHODS

Fertilized local flock (Needle Farm) eggs were incubated at 38 °C and windowed on the 3rd or 4th day of incubation. The embryos were staged, and a few drops of Hank's BSS added to the egg before sealing with Sellotape and returning to the incubator. Operations were carried out on 740 embryos from stage 17 (the earliest stage at which we found it feasible to apply retinoic acid

using our technique) through 22 (the stage beyond which no effect was observed). In the main experiment (n = 471) a slit was made in the anterior margin of the right wing bud perpendicular to the base of the limb opposite intersomite 16/17, and a piece (500 μm × 500 μm) of newsprint (Richmond & Twickenham

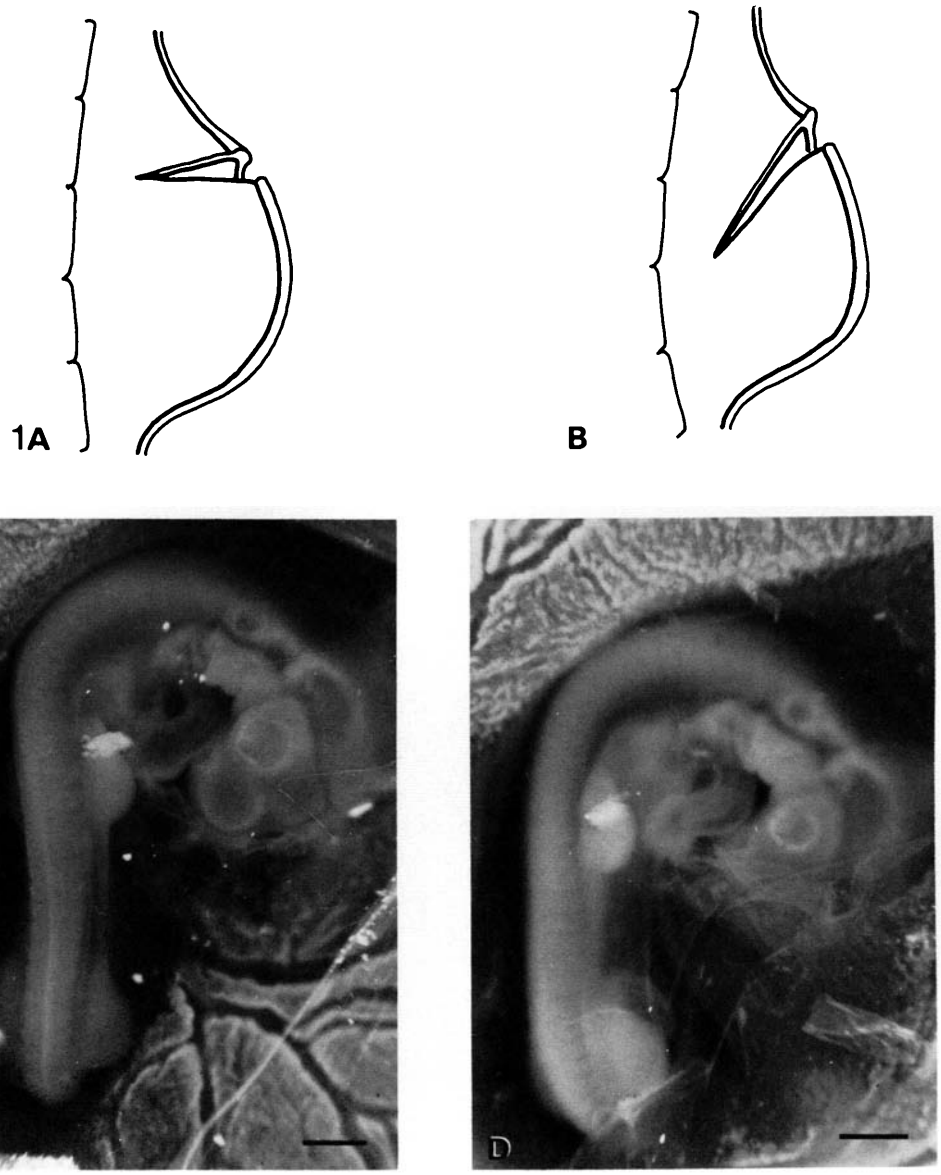

Fig. 1. The two types of operation. (A) Position of slit perpendicular to base. (B) Position of radial slit. (C) Photograph of embryo after insertion of implant into perpendicular slit. (D) Photograph of embryo after insertion of implant into radial slit. Bar = 1 mm.

Times, 112 Chiswick High Rd., London W4) soaked in a solution of retinoic acid (Type XX: all trans, Sigma) in dimethyl sulphoxide (DMSO, BDH Chemicals) was inserted (see Fig. 1A, 1C). At later stages (21–22) the slit was as near as possible perpendicular to the base of the limb, opposite intersomite 16/17 and passing through the apical ectodermal ridge at its most anterior position. In a further experiment (n = 269), carried out by a second operator, the slit was radial from the centre of the base line of the bud to the anterior margin opposite somite 16/17 (ee Fig. 1A, 1D). The concentrations of retinoic acid (mg/ml DMSO), were as follows: 0·25, 0·5, 1, 2, 4, 8, 16, 32, 64 (molarity ranging from 0·8 mM–200 mM; absolute quantity on the carrier ranging from 12·5 ng–3·2 g). In the control operations the paper was soaked in DMSO without retinoic acid. The eggs were then resealed (after adding Hank's BSS) and incubated until 10 days of age when the wings were removed and fixed in 5 % trichloracetic acid, stained

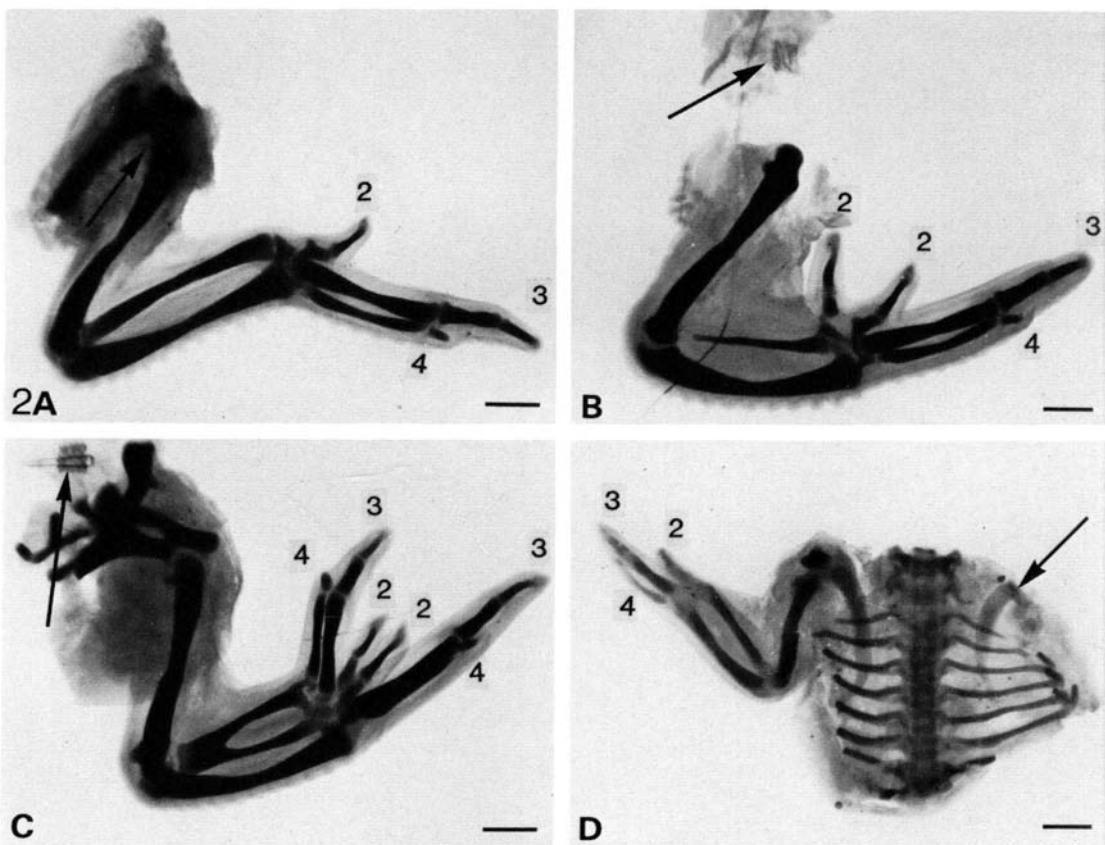

Fig. 2. Examples of results. (A) Normal limb (**HRU 234**). (B) Limb with one supernumerary digit and slightly reduced radius (**HrU 2234**). (C) Limb with full mirror-image hand and one extra zeugopodal element (**HZRU 432234**). (D) Severely reduced limb (−). Arrow indicates implant, bar = 1 mm, for skeletal formula see text, for dose/stage categories see Appendix.

with Alcian green, and mounted in Strand glassfibre resin (Summerbell, 1981). On examination the limbs were arbitrarily classified into three principle groups: normals (see Fig. 2A); reduplications (those with supernumerary cartilage elements or mirror image symmetry, see Figs 2B, 2C); and reductions (those with missing cartilage elements other than that caused by symmetrization, see Fig. 2D). The reduplication category therefore held precedence over the reduction category. Using these criteria I was unambiguously able to classify the data with the exception of two cases with very abnormal hands which have been excluded from our analysis. Analysis of the zeugopod and stylopod was carried out separately from that of the autopod. A record was kept of the number of embryos which died in each category.

Position of source

To compare results of radial versus perpendicular slits, paired t-tests were used for those dose and/or stage categories for which EACH operation type produced sufficient data. Where the test involved a comparison of the proportion of survivors, I used a limit of four operated embryos each. When comparing the proportion of normal, reduplicated, or reduced results, or the strength of activity indices, I used a limit of four surviving embryos (i.e. always a minimum of four in the denominator). Where the test involved proportions I used the transformation $\sin^{-1}$ (P/100) where P was the crude percentage (Armitage, 1971). For the strength of activity index I used the log transformation of the mean index and variance since the standard deviation increased with the mean.

RESULTS

The raw data is included in an appendix at the end of this paper.

Survival

Inspection of the results suggested no obvious difference in survival rate for the two types of operation (overall values 70 % and 68 %). A paired t-test confirmed that the difference was NOT significant ($t = 0{\cdot}88$, d.f. $= 23$, $P = 0{\cdot}19$). I have therefore combined the data into a single table (Table 1).

The overall survival rate of retinoic-acid-treated embryos (n = 648) was 69 % and was similar to that of control operated (DMSO only) embryos (n = 92) at 68 %. The survival rate of the 64 mg/ml embryos was markedly lower (33 %), than that of the other concentrations groups. Apart from this there was no trend to the survival rates for different stages (range 64–75 %), or doses (range 64–81 %). For comparison, the survival rate for embryos in one study involving ZPA grafts (n = 125) was 80 % (Summerbell, 1981).

The two unclassified cases were discarded and the remaining 510 survivors were classified as described above.

Table 1. *Survival (joint)*

Stage	Control	Concentration of retinoic acid in DMSO (mg/ml) 0·25	0·5	1·0	2·0	4·0	8·0	16·0	32·0	64·0	Total
a) *Total number of operations*											
17	13	9	8	9	11	11	18	10	12		101
18	16	6	8	11	11	11	16	15	9		103
19	14	8	12	13	18	16	14	13	11		119
20	18	2	14	8	13	10	19	13	17	11	125
21	18	1	16	15	18	10	18	15	18	30	159
22	13			18	17	15	18	21	18	13	133
Total	92	26	58	74	88	73	103	87	85	54	740
b) *Number of survivors*											
17	11	6	7	9	8	7	11	7	6		72
18	8	6	7	6	9	8	11	10	7		72
19	13	6	9	8	12	9	13*	9	10*		89**
20	9	2	10	6	10	10	15	9	12	4	87
21	12	1	12	13	12	9	12	10	12	8	101
22	10			16	10	12	13	11	13	6	91
Total	63	21	45	58	61	55	75*	56	60*	18	512**
c) *Percentage survival*											
17	85	67	88	100	73	67	61	70	50		71
18	50	100	88	55	82	73	69	67	78		70
19	93	75	75	62	67	56	93	69	91		75
20	50	100	71	75	77	100	79	69	71	36	70
21	67	100	75	87	67	90	67	67	67	27	64
22	77			89	59	80	72	52	72	46	68
Total	68	81	78	78	69	75	73	64	71	33	69

* Includes one case discarded from remainder of analysis, see text.

The proximal skeleton

There were no cases in which a clear supernumerary humerus, or mirror-image symmetrical humerus formed. Occasionally there were minor bumps or perturbations of shape but on the whole if a humerus was present (95 % of all cases) it was approximately normal. At high doses (8 mg/ml) I found that the limb was either truncated or absent (see Fig. 2D), so that the humerus too was truncated or missing. This was more common at early stages (see Appendix).

The zeugopod (overall 62 % normal) was affected more frequently than the stylopod, and at later stages for a given dose (see Appendix). Inspection of the results suggested a difference between radial perpendicular slits and this was confirmed by comparing the proportion of limbs with a reduced zeugopod for the two types of operation (overall values 26 % for perpendicular and 36 % for radial). A paired t-test confirmed that the difference WAS significant ($t = 2{\cdot}73$,

Table 2. *The effect on proximal skeleton (perpendicular slit)*

		Concentration of retinoic acid in DMSO (mg/ml)									
Stage	Control	0·25	0·5	1·0	2·0	4·0	8·0	16·0	32·0	64·0	Total
a) *Number of cases with approximately normal skeleton*											
17	5	4	3	1	3	1	–	–	–		17
18	6	6	4	2	5	1	2	–	2		28
19	5	6	8	4	4	4	4	2	5		42
20	6		6	6	6	3	2	6	3		38
21	5		5	8	5	5	5	3	4	2	42
22	6			5	5	6	7	6	7	3	45
Total	33	16	26	26	28	20	20	17	21	5	212
b) *Number of cases with supernumerary or mirror-image reduplicate elements*											
17	–	2	1	3	–	1	2	–	–		9
18	–	–	–	2	1	3	3	2	1		12
19	–	–	–	–	2	1	1	3	1		8
20	–		–	–	–	1	–	–	–		1
21	–		–	–	1	–	–	–	–	–	1
22	–			–	–	–	–	–	–	–	–
Total	–	2	1	5	4	6	6	5	2	–	31
c) *Number of cases with reduced skeletal elements*											
17	1	–	2	2	3	4	4	6	6		28
18	–	–	3	2	–	3	1	7	4		20
19	1	–	–	2	–	1	1	2	1		8
20	–		–	–	–	2	4	–	3		9
21	1		1	–	–	1	1	3	2	4	13
22	–			1	1	–	–	–	–	3	5
Total	3	–	6	7	4	11	11	18	16	7	83
Total	36	18	33	38	36	37	37	40	39	12	326

– Indicates that no result of this type was found within the dose and/or stage category. I use – rather than 0 because of the better visual impression.

d.f. = 24, $P = 0{\cdot}006$). The results are therefore summarized separately (Tables 2, 3). The most frequent results were relatively normal zeugopods but at intermediate doses there was a tendency to produce reduplications while at high doses there were reductions. There was also a stage effect with a normal zeugopod more common at later stages for a given dose.

The abnormal zeugopods most often comprised mirror-image symmetric ulnas with no radius. Often the anterior element (as judged by the morphology of the epiphyses, see Fig. 2C) was a normal radius proximally but an ulna distally (**Z** or zeugopodal in the Appendix). In other cases, in the main experiment, there were three zeugopodal elements. At low doses an extra radius (**RRU**), at higher doses

a mirror symmetric arrangement of ulna, radius, ulna (**URU**). The radial slits never gave an extra zeugopodal element.

Neither humerus (overall 5 % affected) nor ulna (overall 8 % affected) were ever missing or reduced unless the radius (overall 29 % affected) was similarly affected. An increase proportion of reduced radii was the only effect of radial slits identified. The effect is not surprising as a perpendicular slit does not impinge on the presumptive fate map for the skeleton, while a radial slit does. This is confirmed by comparing the control operations: radial slits caused defects of the radius in 25 % of cases, perpendicular slits caused defects in only 8 % of cases. Similar differences in effect can be shown by replacing tantalum foil barriers at different positions in the bud (Summerbell, 1974). However systemic

Table 3. *The effect on proximal skeleton (radial slit)*

		Concentration of retinoic acid in DMSO (mg/ml)									
Stage	Control	0·25	0·5	1·0	2·0	4·0	8·0	16·0	32·0	64·0	Total
a) *Number of cases with approximately normal skeleton*											
17	3		–	1	1	1	–	1			7
18	2				–	–	1	–			3
19	6		–	1	–	1	2	–	–		10
20	2	1	2		4	1	2	3	–	–	15
21	3	–	4	4	6	3	6	1	3	1	31
22	4			10	4	6	6	4	4		38
Total	20	1	6	16	15	12	17	9	7	1	104
b) *Number of cases with supernumerary or mirror-image reduplicate elements*											
17	–		–	–	–	–	–	–			–
18	–				–	1	1	–			2
19	–		–	–	3	–	–	2	1		6
20	–	–	–		–	1	–	–	3	–	4
21	–	–	–	–	–	–	–	–	2	–	2
22	–			–	–	–	–	–	–		–
Total	–	–	–	–	3	2	1	2	6	–	14
c) *Number of cases with reduced proximal skeletal elements*											
17	2		1	2	1	–	5	–			11
18	–				3	–	3	1			7
19	1		1	1	3	2	4	–	1		13
20	1	1	2		–	2	7	–	3	4	20
21	3	1	2	1	–	–	–	3	1	1	12
22	–			–	–	–	–	1	2		3
Total	7	2	6	4	7	4	19	5	7	5	66
Total	27	3	12	20	25	18	37	16	20	6	184

– Indicates that no result of this type was found within the dose and/or stage category. I use – rather than 0 because of the better visual impression.

application of drugs can also frequently result in preferential loss or malformation of the radius (O'Rahilly, 1951).

The hand

The hand was affected more frequently than the zeugopod and at later stages for a given dose (see Appendix, Tables 2, 3, & 4). Inspection of the results suggested no difference between radial and perpendicular slits and this was confirmed by comparing the proportions of survivors producing each type of result. Paired t-test confirmed that the differences were NOT significant (e.g. proportion of normals, t = 0·26, d.f. = 24, $P = 0{\cdot}60$). The results have therefore been summarized jointly in Table 4. The proportion of normal hands declines with both increasing dose and with earlier stage. At intermediate doses and

Table 4. *The effect on the hand skeleton (joint)*

		Concentration of retinoic acid in DMSO (mg/ml)									
Stage	Control	0·25	0·5	1·0	2·0	4·0	8·0	16·0	32·0	64·0	Total
a) *Number of cases with approximately normal skeleton*											
17	9	4	3	4	4	1	–	–	–		25
18	8	6	5	2	2	2	1	–	2		28
19	12	6	6	3	4	4	1	1	1		38
20	8	2	8	5	3	6	2	1	–	–	35
21	12	1	12	10	9	5	8	2	5	–	64
22	10			16	10	12	10	8	7	3	76
Total	59	19	34	40	32	30	22	12	15	3	266
b) *Number of cases with supernumerary or mirror image reduplicate elements*											
17	2	2	4	5	4	6	5	1	–		29
18	–	–	2	4	7	6	10	4	1		34
19	1	–	3	5	8	5	8	7	4		41
20	1	–	2	1	7	4	11	7	9	–	42
21	–	–	–	2	3	4	4	6	5	3	27
22	–			–	–	–	2	2	3	–	7
Total	4	2	11	17	29	25	40	27	22	3	180
c) *Number of cases with reduced hand skeleton*											
17	–	–	–	–	–	–	6	6	6		18
18	–	–	–	–	–	–	–	6	4		10
19	–	–	–	–	–	–	3	1	4		8
20	–	–	–	–	–	–	2	1	3	4	10
21	–	–	–	1	–	–	–	2	2	5	10
22	–	–	–	–	–	–	1	1	3	3	8
Total	–	–	–	1	–	–	12	17	22	12	64
Total	63	21	45	58	61	55	74	56	59	18	510

– Indicates that no result of this type was found within the dose and/or stage category. I use – rather than 0 because of the better visual impression.

stages there is a strong tendency to produce reduplicated hands. At the strongest doses and earliest stages the proportion of reduced hands is at its highest.

The hand is analysed in more detail using the strength of activity index.

Strength of activity

Current hypotheses for development stress a quantitative variation as one moves from position to position across a field. Our main markers for this variable are by convention the skeleton, and the presence of a particular identifiable digit is assumed to represent a particular local set of positional values. The character or identity of the digit next to the graft therefore tells us something about the potency of the graft to change the positional value of adjacent cells. This has been formalized (for the ZPA hypothesis) in the strength of activity index by Honig, Smith, Hornbruch & Wolpert (1981). A normal limb (Fig. 2A) with no extra digits scores 0; a limb with an extra digit **2** (Fig. 2B) scores 1; an extra digit **3** scores 2; an extra digit **4** (Fig. 2C) scores 3. The index is the % of the maximum possible score (all limbs have supernumerary digit **4**). One of the limitations of this scale is that limbs with the same scores may have different configurations of digits (for example, a score of 2 is given to **32234**, **3234** and **334**). However, it does give intelligible dose or treatment-dependent results in experimental situations (Honig *et al.* 1981; Honig & Hornbruch, 1982). In order to make some attempt of quantifying our results, I initially tried to extend the strength of activity index to include the various reductions we observed. In practise it seems that the activity of the retinoic acid is not necessarily homologous to that of a grafted positional signalling region.

At first inspection it seems that reduced limbs, with fewer digits than normal, were present at higher doses and earlier stages (see Table 4), indicating in some way a more potent effect. There was also a hint of a stage-and/or dose-related series consisting of **S234**, **S2̸34**, **S2̸3̸4**, where **S** represents the source of retinoic acid and / indicates a missing digit). I therefore examined the possibility of extending the Strength of Activity Index to include the various reductions

Table 5. *Strength of activity index % for the hand (joint)*

		Concentration of retinoic acid in DMSO (mg/ml)								
Stage	Control	0·25	0·5	1·0	2·0	4·0	8·0	16·0	32·0	64·0
17	6	8	33	48	29	57	127	181	200	
18	–	–	14	61	70	67	77	143	119	
19	5	–	22	23	54	48	100	93	119	
20	4	–	10	17	33	23	71	89	114	192
21	–	–	–	8	11	22	19	83	63	154
22	–			–	–	–	15	17	46	100

– Indicates that no result of this type was found within the dose and/or stage category. I use – rather than 0 because of the better visual impression.

observed. Having classified the data using several different methods, we finally selected the following series: **S234** = 0, **S2234** = 1, **S3[22]34** = 2, **S4[322]34** = 3, **S234** = 4, **S234** = 5, **S234** = 6 (where digits within square brackets were of variable incidence, and those crossed out were missing). One can make a theoretical justification for numerous other series but we have chosen to use this one on purely pragmatic grounds. This index was simple to apply, it produced no ambiguous cases, it left few anomalous results within a dose/stage category, and it gave good graded dose/stage response curves. None of the other methods

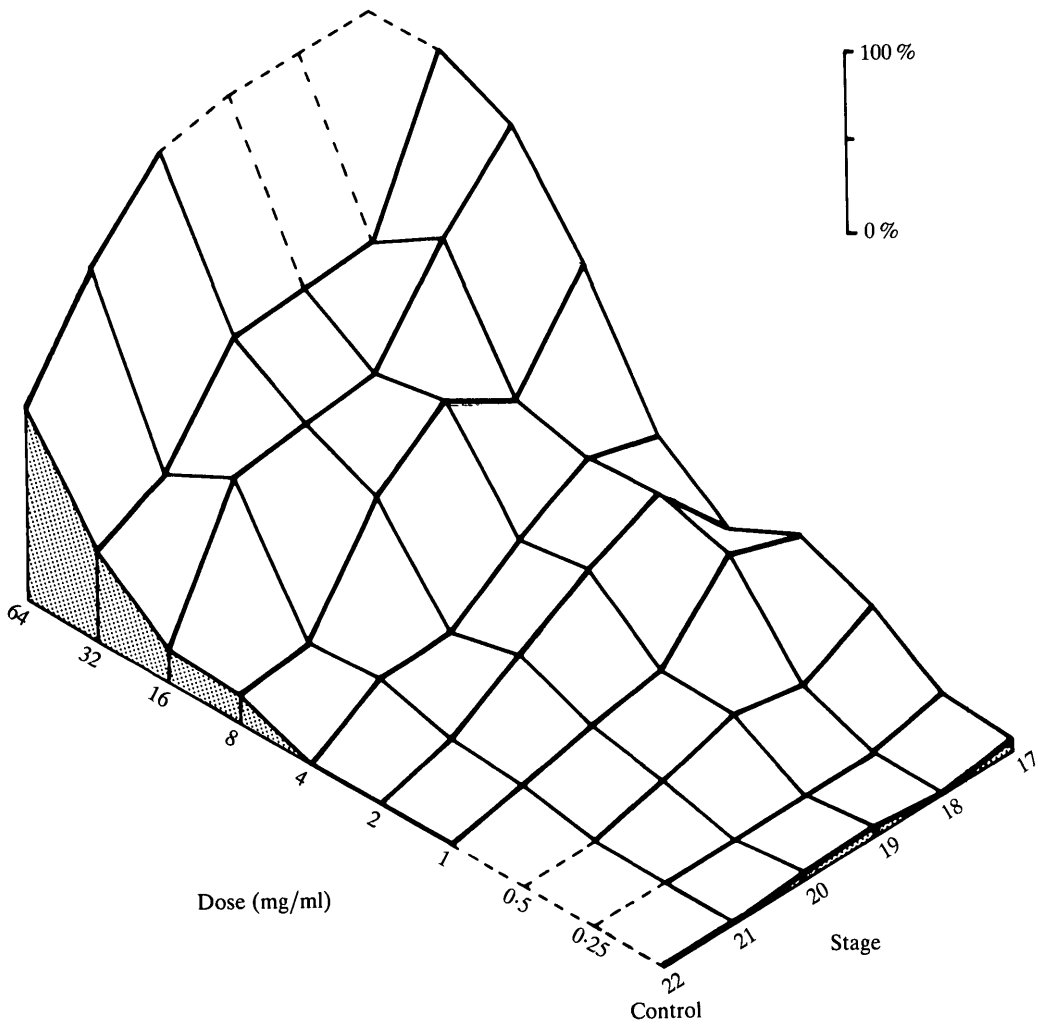

Fig. 3. A 3-dimensional graph showing the modified strength of activity index for the hand. The dose and stage are laid out as a flat 2-D matrix looked at from above. The percentage strength of activity is shown as the vertical height above the appropriate intersection on the matrix. The minimum value (e.g. front corner) is 0 %. The maximum value (e.g. back corner) is 200 %. Missing values on the matrix have been extrapolated to their percentage strength of activity with dashed lines.

of classification that we have tried fulfill these criteria so well. In particular, more complicated series tend to produce problems of identification. A mild supernumerary **S2234** looks the same as a reduced full supernumerary **S432234**. An example of one of these rejected series is included in the discussion.

Inspection of data from the strength of activity index suggested no difference between radial and perpendicular slits and this was confirmed by comparing the indices from each category for the two types of operation. Paired t-tests confirmed that the difference was NOT significant (mean: $t = 1{\cdot}212$, d.f. $= 24$, $P = 0{\cdot}88$; variance: $t = 0{\cdot}614$, d.f. $= 24$, $P = 0{\cdot}73$). The results have therefore been presented jointly in Table 5 and in Fig. 3. Preliminary results were originally presented in Summerbell & Harvey (1983) but subsequent reexamination of the data base has revealed some errors and thse have now been corrected. There are strong dose and stage effects.

Other abnormalities

We also observed an increased frequency of beak defects (reduced or missing beak, especially the upper beak) and incomplete closure of the peritoneal cavity, but we did not keep complete records of these data.

DISCUSSION

Vitamin A as a mimic of polarizing activity?

Superficially the supernumerary wing parts are the most dramatic outcome of this experiment. The additional digits, and in some cases forearm elements are in mirror-image symmetry to the host elements and closely resemble the result of grafting a position signalling region (ZPA) from the posterior border of a donor to the anterior margin of a host wing. If I choose a restricted dose and/or stage range (2–8 mg/ml at stages 18–20) then most embryos (69 %) give this type of result. At lower doses I instead tend to get limbs that are more or less normal. Again this resembles analagous experiments involving a positional signal from the ZPA (c.f. experiments on attenuation: Honig *et al.* 1980; Honig & Hornbruch, 1982; Smith, Tickle & Wolpert, 1978; ticle, 1981). There is also an obvious proximodistal effect similar to that reported for the positional signalling region (Summerbell, 1974). For a given dose the earlier the stage the more likely one is to obtain reduplication at a proximal level.

However in the case of retinoic acid, higher doses applied to earlier stage hosts give a very different result. Most limbs in the range 16–32 mg/ml, and stage, 17–18, had lost elements rather than gained supernumerary ones (mean = 73 %, range 57–100 %). It is not obvious how one could incorporate these results into current hypotheses without significantly modifying or extending the latter.

Reductions

Experiments involving the attenuation of the signal from the ZPA (Honig *et*

al. 1980) first led to the concept of the strength of activity index (described in Results). The formation of digit **2** next to the graft indicates weak activity, digit **3** is stronger, while digit **4** shows a full strength or normal ZPA. The results at low and medium concentrations showed similarly graded activity, but at high concentration the sequence continues as a graded loss of digits. This made it difficult to apply the strength of activity index in its original form. If one assumed that retinoic acid simply mimicked the putative signal from the ZPA then the obvious series for including levels above that normally found in the ZPA would be **S234**, **S2234**, **S32234**, **S432234**, **Sx43234**, **Sxx4334**, **Sxxx434**, etc. (where '**x**' represents a nonsense positional value more posterior than digit 4.) Inspection of the data in the appendix shows that many of the cases fit this series and that there are dose/stage trends. However the range of predicted results do not include the phenotype **S34** or **S334** which were observed at quite high frequencies at higher doses. Even if I arbitrarily insert these cases into the main series as variants of the **S[223]4** phenotype then the results look anomalous for their dose or stage, and the resulting matrix for dose/stage response (comparable to Fig. 4) shows a much weaker trend. However the concept of **enhancement**, the raising of the positional signal above normal levels is an attractive one (see Summerbell & Harvey, 1983), as it complements so well the characteristic **summation**, **attenuation**, **depletion**, and **accumulation**, that are so typical of the ZPA type model (Summerbell & Honig, 1982).

In contrast the index that we set out in the results accounts very well for the reductions. It is not particularly satisfactory for the phenotypes **S4334**, **S434**, **S44**, but they are ascribed unambigously an index of 3, there are few resulting anomalous cases, and the dose/response matrix has good trend lines. Operationally it is the most satisfactory of the many schemes of classification that I have considered.

An alternative (but boring) explanation is that the vitamin A has two coincidental and antagonistic effects. One alters the pattern (Summerbell & Harvey, 1983; Tickle *et al.* 1982) while the other adversely effects cell survival and differentiation, particularly of cartilage (see for example, Fell & Mellanby, 1950). This dual effect at different doses is a common feature of treatment of biological systems with vitamin A analogues. As we have described in the results we found it difficult to produce satisfactory dose/response curves based on this explanation.

Models for a single mode of action of retinoic acid

Throughout this paper we have presented our results within the framework of the ZPA hypothesis of positional information, trying to show that it is in some ways tempting to think of retinoic acid as being a morphogen similar to that putatively produced by the ZPA. In this section on models we discuss our data in a slightly different context. The reaction–diffusion model (Gierer & Meinhardt, 1972) may have the advantage of providing explanations in principle of

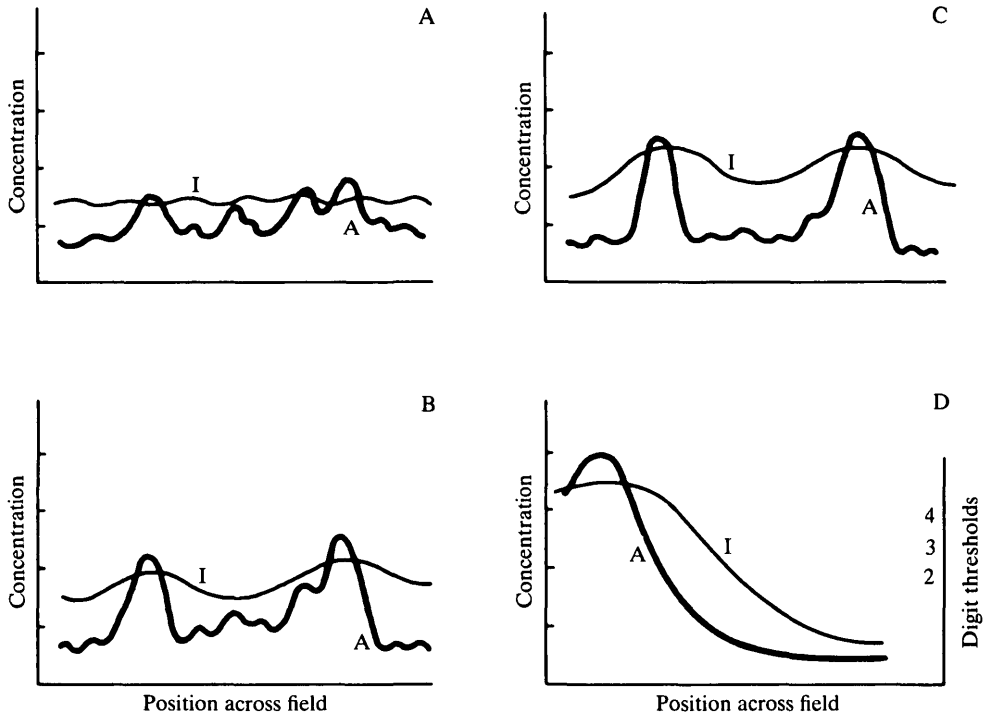

Fig. 4. Application of reaction-diffusion model to an aperiodic field (chick limb bud). See text for detail. Heavy line (A) = activator; light line (I) = inhibitor.

both our results (supernumeraries and reductions) and also of the results of experiments on developing and regenerating amphibian limbs (Maden, 1982; 1983).

Gierer and Meinhardt used two variables to provide positional information across a field. The 'activator' is an autocatalytic molecule enhancing its own production and the production of a second molecule, the 'inhibitor'. The inhibitor inhibits production of the activator but is self neutral. The activator diffuses slowly away from the site of production, the inhibitor diffuses quickly. In an infinite field with near uniform levels of inhibitor and activator chance local fluctuations (Fig. 4A) allow the activator to escape from inhibition forming peaks. Single peaks are stable because the inhibitor diffuses quickly away from the site of maximal production while the activator remains, locally reinforcing itself. Strong peaks suppress formation of adjacent peaks because they 'export' inhibitor (Fig. 4B). As the model nears the steady state the normal result in an infinite field is a set of relatively stable irregularly spaced peaks, with a characteristic wave length (Fig. 4C). The chick limb is commonly seen as an example of an aperiodic pattern (see Hinchliffe & Johnson for a discussion of periodic versus aperiodic patterns in limb development). It requires a special case of the model in which the width of the field is less than a whole wavelength (Meinhardt,

1982). The peak always lies at the posterior margin because of a pre-existing whole body gradient favouring production of activator at the posterior edge of the wing (Fig. 4D). The limb uses the concentration of activator or inhibitor as a concentration profile specifying positional information. (The reaction–diffusion model is in this case formally similar to the source–diffusion model.)

It is easy to produce supernumerary digits by making the assumption that the

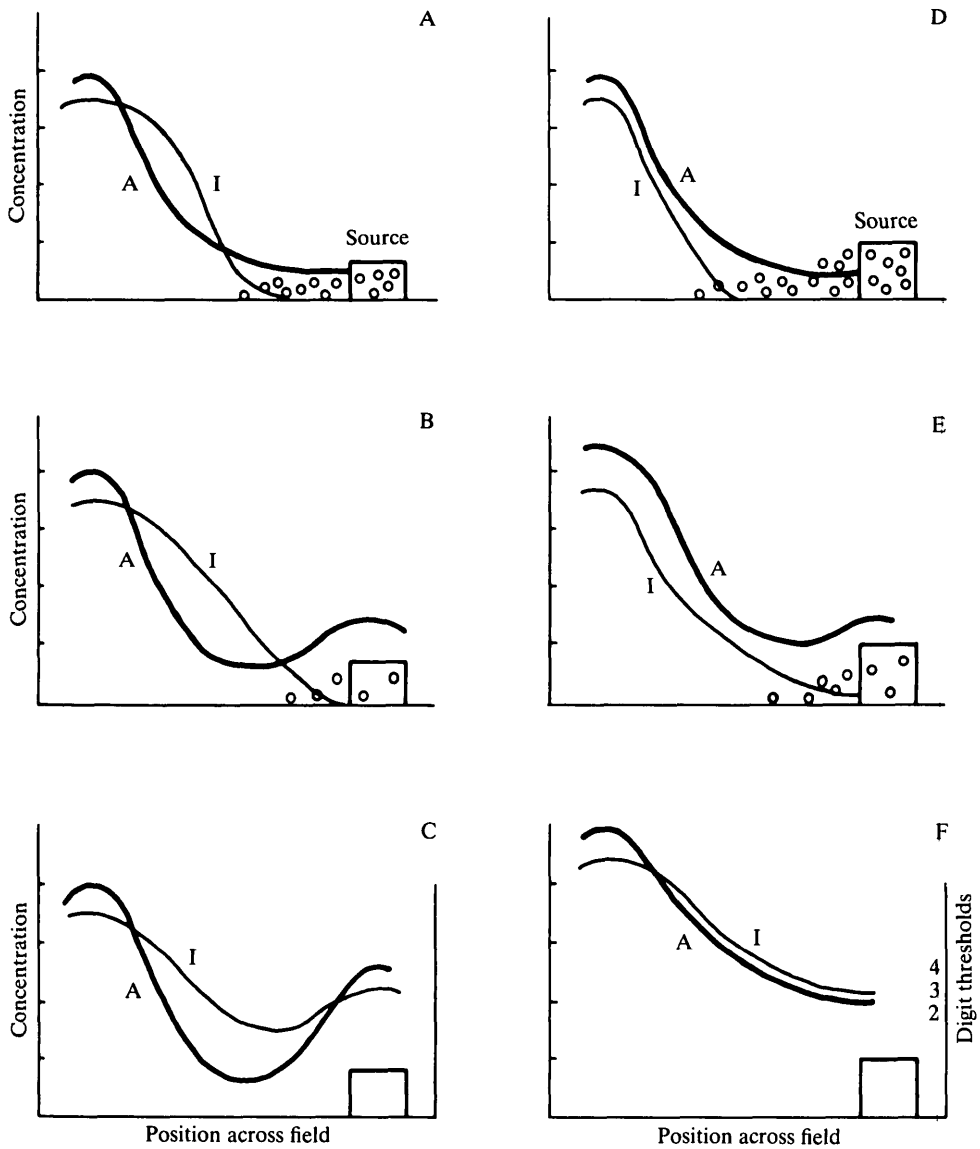

Fig. 5. Series of diagrams showing disruption of reaction-diffusion model by local application of retinoic acid. Heavy line (A) = activator; light line (I) = inhibitor; circles represent retinoic acid molecules. See text for details.

retinoic acid acts as a secondary source of activator, but I have been unable to produce simulations that result in deficiencies. various other assumptions (e.g. allowing the retinoic acid to modify the diffusion rate for activator and/or inhibitor) can result in either reductions or supernumeraries. I have chosen to illustrate one such method in Fig. 5 using a modified version of the program listing in Meinhardt (1982). The additional assumption is that Vitamin A binds to the inhibitor. It therefore lowers the level of free inhibitor below the threshhold at which the activator can escape from inhibition (Fig. 5A). Activators forms a new anterior peak (Fig. 5B) and reaches a concentration at which it can catalyse production of sufficient inhibitor to eventually neutralise the vitamin A. A new steady state is set up with two stable peaks giving supernumerary elements in mirror-image symmetry (Fig. 5C). Excess Vitamin A lowers the inhibitor concentration over more of the limb field (Fig. 5D). The whole system escapes from the negative feedback control and activator concentration rises (Fig. 5E). The net effect is to reset the entire reaction diffusion system but at a higher base concentration. Digits specified by low concentration ranges are therefore progressively lost giving anterior reductions (Fig. 5F). This model has also been discussed in the context of the amphibian limb by Maden (1983). Basically the exlanation for the AP axis is similar, but not that as application to the amphibian limb is systemic, formation of supernumerary digits is dependent on withdrawal of vitamin A so that a new stable concentration profile or inhibitor can form. The effect along the proximodistal axis can be summarized as proximalization of the blastema. Retinoids are again visualized as binding to an inhibitor allowing a proximalizing factor to escape from control until the retinoid is withdrawn. Inhibition then produces a new steady state but the blastema has already proximalized.

A common set of rules can therefore explain much of the phenomenology. This suggess a set of variations on a common molecular theme in which related analogues perform similar roles in different systems, the shared feature is the ability of retinoids to bind the inhibitors.

CONCLUSION

The main purpose of this paper is to set out a detailed data base for the effect of local application of retinoic acid to the anterior margin of the chick limb bud. There are clear dose- and stage-dependent results. Low doses and old stages tend to give normal limbs or perhaps one or two supernumerary digits of an anterior character (**[32]234**). Medium doses and intermediate stage tend to give full mirror image supernumeraries with two or even three extra digits including particularly digits of a posterior character (**4[322]234**). High doses and early stages give limbs in which the supernumerary digits, and even host digits or more proximal skeletal elements are lost (**[234]**). The results over the range of doses/stages are graded.

I then consider possible interpretations starting with the notion that retinoic acid may be *the* morphogen. This would explain supernumerary digits parsimoniously but causes difficulties when one tries to explain some of the reductions. There may of course be a switch from morphogenetic pattern effects at low doses to non-specific growth or differentiation inhibition at high doses. Such behaviour is typical of retinoids in biological systems. Alternatively we have introduced the concept of 'enhancement', the raising of 'ZPA' activity above a level normally encountered. Both of these ideas are worth exploring in more detail in the context of chicken limb development, but neither gives much insight into the amphibian limb regeneration results. Finally we discuss an explanation presented using the terminology of the reaction–diffusion model. This accounts for much more of the experimental data in both developing the regenerating systems.

The principal conclusion is that these experimental results should not necessarily be interpreted as evidence that retinoids normally play a direct role in the control of development or regeneration. Chemicals controlling neural plate induction were described frequently throughout the thirties (review, Needham, 1942). While acclaimed at the time they are not now seen as being of great significance.

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Appendix

We present here the raw data for these experiments. Tables 6 and 7 show in detail the results for the two types of operation: perpendicular slits in Table 6, followed by radial slits in Table 7. Upper case capitals and digits in **BOLD** represent cartilage elements of approximately normal appearance. Lower case and light type signify reduced elements.

H(h) = humerus;
R(r) = radius;
U(u) = ulna;
Z(z) = zeugopodal long bone;
2(2) = digit **2**;
3(3) = digit **3**;
4(4) = digit **4**;
d = unclassified digit;
n = cartilage nodule
– = no limb.
* = limbs illustrated in Figure 2 of the main paper.

The table is laid out with stage down, the concentration of retinoic acid across.

	Control	0.25mg	0.5mg	1.0mg	2.0mg	4.0mg	8.0mg	16.0mg	32.0mg	64.0mg
17	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HrU2234	HRU 234 HRU 234 HRU 234 HRU 234 HRRUd234 HRRU2234	HRU 234 HRU 234 H U 234 HRU 2234 HrU 3234 HUU 43234	HRU 234 H U 234 HZU432234 HUU432234 HUU 434 H u 44	HRU 234 HrU 234 HRU 2234 HRU 32234 H U 32234 H U 334	H U 234 H U 2234 HRU 334 H U 334 H U 4334 HUU 4334	H U 2234 HZU 2234 Huu 434 H U 34 H -	H H - - - -	H -* - - - -	
18	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 H U 2234 HrU 32234	HRU 234 HRU 234 H U 32234 HrU432234 HUU432234 HUU432234	HRU 234 HRU 234 HRU 32234 HRU 32234 HUU 43234 HRU432234	H U 234 H U 234 H U 2234 HRU 43234 HURU 43234 HURU43234 HZU 432234	H U 4334 HZU 4334 HRU 4334 HRU 4334 HURU 43234 HZRU432234*	HZU 334 HZU 334 H U 3234 H U43234 H U 34 H h - -	HRU 234 HRU 234 HUU 434 HrU 34 H U - -	
19	HRU 234 HRU 224 HRU 234 HRU 234 HRU 234 H U3234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 2234 HRU 32234 HRU43234	HRU 234 HRU 234 HRU 234 HrU d234 HRU 2234 HrU 32234	HRU 234 HRU 234 HRU 234 HRU 3234 HrRU32234 HuU432234	HRU 234 HRU 234 HRU 234 HRU 3234 HrU 432234 HUU 432234	HRU 32234 HrU 32234 HRU 4334 HRU 43234 HRU 432234 HURU43234 Discard	H U 234 HUU43234 HRU 4334 HZU 43234 HRU 4334 HUU 434 H U 34	HRU 234 HRU43234 HRU 4334 HRU 43234 Huu 34 HRU - Discard	
20	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU2234		HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 3234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 43234	HRU 234 HRU 234 HRU 2234 HRU 2234 HRU 2234 HRU 43234	HRU 234 HRU 234 HRU 234 H U 234 HZU 2234 HrU 32234	HRU 234 HrU 2234 HrU 2234 HrU 43234 H U 43234 HRU 432234	HRU 2234 HRU4 3234 HRU4 3234 HRU43234 HRU 4334 HRU 4	HRU 2234 HRU 2234 HRU43234 HrU 4334 H h	
21	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 H U 234		HRU 234 HRU 234 HRU 234 HRU 234 H U 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 2234 HRU 2234	HRU 234 HRU 234 HRU 234 HrRU 234 HRU 2234 HRU 3234	HRU 234 HRU 234 HRU 234 HRU 2234 HrU 2234* HRU 2234	HRU 234 HRU 234 HRU 234 HRU 2234 HrU 432234	HRU 234 Hru 234 HrU43234 HRU43234 HRU 434 HrU 434	HRU 234 HRU 234 HRU 234 HRU 234 HrU43234 h u 34	HRU 334 HRU43234 HrU 4 HrU H u H u
22	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234			HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HrU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HrU 234	HRU 234* HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 2234 HRU 2234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 2234	HRU 234 HRU 234 HRU 234 HRU 2234 HRU 3234 HRU 34	HRU 234 HRU 234 HRU 234 HrU H U h u

	Control	0.25mg	0.5mg	1.0mg	2.0mg	4.0mg	8.0mg	16.0mg	32.0mg	64.0mg
17	HRU 234 HRU 234 HRU 234 H U 234 H U$_2$234		H U 2234	HRU 234 HrU 234 H U 234	HRU 234 H U 234	HRU 2234	H U 2234 H U 3$_2$34 - - -	HRU $_3$2234		
18	HRU 234 HRU 234				HrU432234 HrU432234 H U$_{43}$2234	HZU 4334	H U $_2$34 HrU $_2$234 HRUd$_2$234 H U 3$_2$34 Huu 434	-		
19	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HrU 234		H U 234	HRU 2234 H U 2234	HrU 234 HZU d2234 H U 32234 HZU 4334 HZU 43234 H U432234	HRU 234 H U $_3$234 HrU43$_2$234	HRU 234 H U 334 HRU43234 H u 34 H u 4 -	HZU 4334 HZU 4334	Huu 434 H U 34	
20	HRU 234 HRU 234 H U 234	HRU 234 H U 234	HRU 234 HRU 234 H U 234 H U 2234		HRU 234 HRU $_2$234 HRU 2234 HRU $_3$234	H U 234 HrU 234 HRU 2234 HZU 43234	HRU 234 HrU 234 HRU 2234 HrU $_3$234 HrU$_4$3234 H U43234 H U 44 H U 34 H U $_3$4	HRU 234 HRU 43234 HRU 4334	HrU 4334 HZU 4334 HZU 4334 Huu 434 H U 44 -	H u 4 Hru n -
21	HRU 234 HRU 234 HRU 234 H U 234 H U 234 H U 234	H U 234	HRU 234 HRU 234 HRU 234 HRU 234 HrU 234 H U 234	HRU 234 HRU 234 HRU 234 HRU 234 H U 2234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU $_2$234	HRU 234 HRU 234 HRU432234	HRU 234 HRU 234 HRU 234 HRU $_2$234 HRU 3234	HRU 3234 HrU $_{43}$34 HrU $_3$4 H U 34	HRU 234 HRU d234 HRU 43234 HrU$_{432}$234 HZU$_{432}$234 Huu $_3$4	HRU434 H
	HRU 234 HRU 234 HRU 234 HRU 234			HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 234	HRU 234 HRU 234 HRU 234 HRU 234 HRU 234 HRU 34	HRU 234 HRU 234 HrU 234 HRU d234 HRU 34	HRU 234 HRU 234 HRU 234 HRU 234 H U 34 H U 4	