Determination of anteroposterior polarity in the axolotl forelimb by an interaction between limb and flank rudiments

By J. M. W. SLACK

From the Department of Biology as Applied to Medicine, The Middlesex Hospital Medical School, London

SUMMARY

1. It is shown that the mesoderm in the prospective forelimb-bud of the axolotl embryo is thickened and divided into somatic and splanchnic layers, while that of the flank is thinner and undivided. The first sign of the limb-bud itself appears at stage 38.
2. A whole, a half or a third of a limb rudiment can develop into a normal or reduplicated limb when transplanted to the flank.
3. An anterior half of a limb rudiment fails to develop when transplanted to the head but will do so if accompanied by a small piece of flank tissue.
4. Small pieces of tissue from a wide area of the flank will cause reduplication of the forelimb if grafted to the anterior margin of the rudiment. It is shown that the whole of the reduplication is formed from host tissue and has the morphological character of the host.
5. Reduplications have posterior structures arranged symmetrically on both sides of the midline. Both muscles and cartilages are duplicated.
6. It is suggested that the same interaction between prospective flank and limb is responsible for the capacity for growth on the head, the induction of reduplications and the formation of the anteroposterior pattern of the limb in normal development.
7. A simple rule is proposed which explains the occurrence of reduplications in classical work on the amphibian limb.

INTRODUCTION

The textbook account of the determination and development of the amphibian limb (e.g. Balinsky, 1970) seems rather at variance with what we know of other regulative fields in embryonic development. There seem to be no dominant regions, no competence and no ‘gradients’. This account derives from the work of R. G. Harrison (1918, 1921) and was extended in the interwar years by other workers in America, Germany and Japan (review: Swett, 1937). Harrison showed first that the prospective limb tissue, which he called the ‘limb disc’, lay in the mesoderm between the levels of somites 3 and 6 and ventral to the pronephros. This disc showed great powers of regulation. After almost complete extirpation its peripheral regions could still form a complete limb, although it is the central portion which is fated to form the limb in the course of

1 Author's address: Imperial Cancer Research Fund, Mill Hill Laboratory, Burtonhole Lane, London, NW7 1AD, U.K.
normal development. The disc would develop into a limb when transplanted to other sites of the body and was therefore called by Harrison a 'self differentiating, equipotential system', following the usage of Driesch.

Since the pattern of the limb is asymmetrical in three dimensions, Harrison addressed himself to the problem of the determination of this pattern. By reversals of discs at different stages Harrison (1921), Swett (1927) and Detwiler (1929, 1933) showed that the three geometrical axes, anteroposterior, dorso-ventral and mediolateral, were determined sequentially. The first was established by the stage of the yolk plug gastrula, the second in the late tail-bud stage and the third shortly afterwards at the commencement of limb-bud outgrowth. It was believed that the physiochemical basis for these events was an orientation of molecules within each cell in the manner of a liquid crystal. An X-ray search for the oriented arrays was conducted in 1940 (Harrison, Astbury & Rudall, 1940) but without success.

This theory is very unsatisfactory in the light of modern thinking. If the limb disc is really equipotential and homogeneous, there is no good reason why the polarization of the cells should occur in the correct directions. Also it is difficult to see how an orientation of molecules such as actin or tubulin could give rise to the large number of different states of determination which must precede the appearance of the rather complex pattern of the limb along any one of its three axes.

I have reinvestigated the determination of the anteroposterior axis using the axolotl (Ambystoma mexicanum) which is very similar to the urodele species used by the classical investigators. In this paper I shall present evidence that the ‘limb disc’ is not equipotential but consists of two distinct parts: prospective limb and prospective flank, Throughout this work I shall use the work ‘flank’ to indicate the parts of the body wall lying between the forelimb and the hindlimb. I wish to suggest that the specification of pattern along the anteroposterior axis depends on an interaction between these two tissues. Much of the argument will depend on the production of reduplicated limbs. These were often encountered in the classical experiments but the conditions for their onset and the mechanism of their formation could not be explained within the framework of the thinking current at the time. The model presented in this and the accompanying papers is capable of explaining most of the classical results as well as bringing our ideas about the amphibian limb closer to those on other well known embryonic fields such as the sea urchin (Hörstadius, 1973), the insect egg (Sander, 1976) or the chick limb (Wolpert, Lewis & Summerbell, 1975).

**MATERIALS AND METHODS**

Axolotl eggs were obtained by natural matings of animals kept in the laboratory, and allow to develop in ‘standing tap water’, which is tap water that has been allowed to stand for 24 h or more to remove the chlorine. After a few
days development they were sorted into cohorts of different stages, which were kept in the refrigerator. For each day's operations the appropriate cohort(s) were taken out the previous evening and allowed to resume development at 20 °C overnight. Staging was carried out using the table of Schreckenberg & Jacobson (1975).

All solutions and instruments for the operations were sterilized before use. The embryos were decapsulated, washed in several changes of 1/10 × Holtfreter solution, and operated on in ½ × Holtfreter containing 1/3000 MS222 (Sandoz). Operations were carried out with electrolytically sharpened tungsten needles, and the grafts were held in place for 1–2 h with glass bridges. The embryos were kept in ½ × Holtfreter overnight and then in 1/10 × Holtfreter which was changed every 3 days until the hatching stage. Both these solutions contained 20 mg/l of nystatin (Squibb) and 100 mg/l sulphadiazine (May & Baker) to suppress infection. After the hatching stage the larvae were kept in standing tap water and fed on brine shrimps (California Brine Shrimps Inc.) until the limbs had reached a suitable stage of development. Up to 96 % survival could be obtained with this procedure.

For sectioning, embryos were fixed overnight in 1:1 Bouin-Dioxan, dehydrated in two changes of dioxan (2 h) and embedded in 54 °C wax (Rugh, 1941). 10 μm sections were cut and stained reggressively in Ehrlich's haemat- oxylin. Larval limbs were fixed in Karnovsky's fixative (Karnovsky, 1965), dehydrated, embedded in Araldite, sectioned at 2 μm, and stained in toluidine blue. For the preparation of whole mounts, the limbs were fixed in 5 % trichloroacetic acid for 3 h, bleached overnight in Mayer's bleach (Lee, 1946), stained for 30 min in 0·1 % alcian green in acid alcohol, differentiated overnight in acid alcohol, dehydrated, and cleared in oil of wintergreen.

The magnifications quoted on the figure legends refer to the 35 mm negatives of the published photographs.

RESULTS

Nomenclature

Most of the following results arise from grafting experiments involving parts of limb and flank rudiment. The positions of the cuts are defined in relation to the somites. So 3 indicates a cut made ventral to somite 3, 5/4 indicates a cut ventral to the cleft between somites 5 and 4. Zones of tissue are indicated by parentheses. So (3–4) indicated that tissue lying ventral to somites 3 and 4 and between the levels of their midpoints. (5/4–3/2) indicates all the tissue between levels 5/4 and 3/2. Grafts are shown by arrows and their orientation by a for anterior, p, for posterior, d, for dorsal and v, for ventral. So (6/5–3/2) → (9–7) aadd means that tissue from the first position was grafted to the second position with orientation preserved in both axes. (6) → (3/2) apdv means that a strip of tissue ventral to somite 6 was grafted to the level lying ventral to the junction of somites 3 and 2 and both axes inverted.
A one-somite-wide strip of mesoderm is about 0.2 mm wide and may contain about ten cells along a file.

**Histology of limb rudiment**

The disposition of the lateral mesoderm is well shown by the section of a stage-30 embryo in Fig. 1. The plane of section is tilted from the frontal, so that the right side of the figure is more ventral than the left side. Thus on the left side of the figure may be seen somites 1, 2, 3 and 4 at the anterior end. Outside somites 3 and 4 lies the prospective pronephros. On the right side of the figure in the region (5–3/2) lies a mesodermal thickening which may be shown by transplantation experiments to be, or to contain, the forelimb rudiment. In transverse sections at this stage it may be seen that the mesodermal thickening consists of a somatic (outer) layer, and a splanchnic (inner) layer, the latter lying medial as well as ventral to the pronephric rudiment. The thickening only extends posteriorly as far as the centre of level 5, beyond which the lateral plate mesoderm is a thin layer of 1–2 cells thickness and is not visibly divided into somatic and splanchnic layers.
**Polarity in axolotl forelimb**

Table 1. *Growth of limb rudiment on the flank*

(A) Whole rudiment (6/5–3/2), stage 30

Of 14 oriented grafts: 7 *ap* gave 4 reversed normal, 3 resorbed; 7 *aa* gave 4 normal *aa*, 3 reduplicated.

(B) Anterior halves (4–3/2), stage 30

18 grafts oriented *aa* gave: 4 no growth, 4 bud only, 10 limbs (6 normal *aa*, 3 reversed normal, 1 reduplicate).

(C) One-somite-width strips, stage 34

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At stage 30 and 34 there is no morphological sign of a limb-bud, but only undifferentiated somatopleure whose morphogenetic properties must be demonstrated experimentally. The first sign of a limb-bud is at stage 38 (some 3 days after stage 30) and only by stage 40 (hatching) is a limb-bud readily visible externally. The formation of the limb-bud occurs later in the axolotl than in *Ambystoma punctatum* used by the classical investigators and it should be borne in mind that at the stages of most of the experiments described here, the limb-bud has not yet been formed.

It should also be noted that when the limb-bud does appear it contains only tens of cells and is much smaller in area than the mesodermal disc which gives rise to it. So either the fate map of the limb rudiment contains only a small area destined to form the bud, or there must be a migration of cells to the bud from the surrounding region.

**Growth of the limb rudiment on the flank**

When the region described above was grafted to a distant site on the flank, i.e. the graft St 30 (6/5–3/2) → (9–7), in most cases it developed into a complete and well formed forelimb. Of 26 grafts, 23 gave limb structures. Some of these were reduplicated limbs. For those grafts whose orientation was recorded it was found that the grafts harmonic to the body (*aa*) tended to form reduplicates and those disharmonic to the body (*ap*) gave single limbs (Table 1). These results are in accord with those of Harrison (1921).

These grafts, and others unless stated, include both mesodermal layers, pronephros if present, and the ectoderm.

Not only will the region (6/5–3/2) self-differentiate on the flank, but so will much smaller pieces. In Table 1 are shown the results of grafting anterior halves (4–3/2) and the one-somite-wide strips (2)–(6) inclusive. The anterior halves
Polarity in axolotl forelimb

Table 2

(A) Isolated anterior half on head: host stage 28–30

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(B) Anterior half and flank strip on head: donor and host stage 28–30

<table>
<thead>
<tr>
<th>Cases</th>
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<th>Limb</th>
<th>Character</th>
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<td>4</td>
<td>10</td>
<td>7 normal, 2 extra ventral digits, 1 only 2 fingers</td>
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Gave 10 limb structures out of 18 grafts. The results from the strips show quite clearly that it is the tissue ventral to somites 3 and 4 which is most active. (5) forms a few buds, but (2) and (6) are completely inactive.

Results scored as 'limb structures' in the tables are always well individuated. Some are single and some reduplicated, a few have supernumerary digits, but long formless outgrowths were not included and were in fact extremely rare in these experiments. Limb structures grown from an anterior half rudiment and from the strip (4) are shown in Figs. 2 and 3.

It seems that tissue with the ability to form the forelimb is located within the region (5-3/2), and that fragments from this region are able to regulate and form all levels of the limb.

Growth of limb rudiments on the head

A series of grafts were carried out in which anterior halves of limb discs from embryos of various stages were grafted to the heads of stage 28–30 hosts between the eye and gill rudiments, i.e. (4–3/2) → head aadd. From Table 2 it may be seen that of 27 such grafts, none formed limb structures, although a number formed small buds which subsequently regressed. There appears to be no significant difference between the stages used as donors. So the striking ability for growth and regulation, which anterior halves show on the flank, is not shown on the head.

Figures 2–5

Fig. 2. Axolotl larva showing supernumerary limb grown on the flank from an anterior half limb rudiment (case Q34). Dorsal view × 8.

Fig. 3. Larva with reduplicated supernumerary limb grown on the flank from the strip (4) (case L27). Dorsal view × 8.

Fig. 4. Larva with supernumerary limb grown on the head from a graft of (4–3/2) + (7) (case Q32). Dorsal view × 8.

Fig. 5. Whole mount of limb grown on the head, showing the normal skeletal pattern (case Q36). × 50.
Fig. 6 (A) Whole mount of normal axolotl forelimb; ×32. (B) Whole mount of reduplicated limb (case E17); ×40. (C) Reduplicated limb in situ (case J21); ×16.
A very different result was obtained when a one-somite-wide strip of flank tissue was grafted along with the anterior half-disc: (7) + (4–3/2) → head aadd. Here 10 out of 18 formed well individuated limbs (Figs. 4, 5; Table 2). This is the same frequency of success as obtained with grafts to the flank although it should be noted that none of the limbs grown on the head was reduplicated.

The fact that normal limbs could be formed by the action of a small piece of flank tissue makes it unlikely that the failure to grow isolated limb rudiments on the head is due to unspecific factors such as inadequate vascularization or innervation. A specific interaction between limb and flank primordia required for the normal development of the limb seems more likely.

Effect of flank tissue on the limb rudiment in its normal position

When a flank strip was grafted to the anterior margin of the limb rudiment of tail-bud embryos ((6) → 3/2 aadd or apdd), reduplicated limbs were formed with high frequency (Fig. 6). At stage 30, 8 out of 10 grafts gave a reduplication, and at stage 34, 21 out of 33 did so. A detailed analysis of the character of
reduplications will be presented later, most of them are perfectly symmetrical and appear as two posterior portions arranged around a central axis of symmetry.

Reduplications were not formed when wounds were made in the position 3/2 (six of six cases), not when anterior strips were inserted into such positions: (3) → 3/2 (six of six cases). The phenomenon seems therefore to be a specific consequence of the presence of flank tissue.

When the level of the graft was varied it was found that the effect was only given by grafts in a narrow band of positions (Table 3). In the more posterior positions the graft split the limb rudiment in two, and posterior to level 4/3 it seemed to suppress the growth of the posterior half. Anterior to level 3/2 the graft is being inserted not into limb rudiment but into gill rudiment (Fig. 1) so it is possible that intimate contact between limb and flank primordia is required to give reduplications.

A large number of grafts were performed to establish the extent of the active region. Of 76 grafts, 27 were positive, and 23 of these came from the dorsal half of the region (10/9–5/4) (see Table 3). So it seems that the active region corresponds to a large proportion of the prospective flank. It is not a ‘point source’ or ‘boundary region’.

The grafts taken from the ventral part of the flank contain very little mesoderm and it was thought that this might be the reason for their relative lack of activity. So a series of grafts were carried out using separated ectoderm and mesoderm from the flank position (67). The tissue layers were separated after the fragment had been soaked in 0.25% trypsin for 10 min at room temperature. 7/8 mesoderm grafts gave reduplications but none of the 5 ectoderm grafts did so. So it appears that the flank activity, like the limb rudiment itself, resides in the mesoderm.

Self-differentiation, instruction or evocation?

All the reduplications formed in these experiments are symmetrical and double posterior. The only variable which was found to have a reproducible effect on their character was the position of the grafts in the host: the more anterior the graft the more elements are produced. This will be discussed more fully in another paper. Conversely, the position of origin of the grafts, and their
Fig. 8. (A) Whole mount of normal pleurodele limb; x 32. (B) Reduplication induced by pleurodele-pleurodele graft (case M3); x 32. (C) Reduplication induced by pleurodele-axolotl graft (case N13); x 32.
orientation \((aadd\ or\ apdd)\) had no effect. This rather suggests that the morphological character of the response is determined solely by the host.

Two experiments were carried out to establish whether the graft participated in the outgrowth of the limb-bud. In the first, the donor embryos were stained overnight in \(1/100000\) neutral red. Then seven cases of the graft \(6\to 3/2\) were carried out (donor and host stage 34), of which four gave reduplications. The vital stain remained visible for several days, and Fig. 7 shows a camera lucida drawing of one of the positive cases at stage 40. The region which is stained is clearly not participating in the growth of the limb-bud.

Vital staining, although suggestive, is not wholly satisfactory, because most of the stain is located in the ectoderm and the internal migration of mesodermal cells cannot be excluded. Also, it cannot settle the question of whether the signal is evocative (i.e. evoking normal limb elements in an abnormal position of the limb rudiment), or instructive (i.e. causing the pattern of new elements by its own distributions in space).

To answer this, an interspecific graft was performed between embryos of the Spanish salamander \((Pleurodeles\ waltlii)\) and the axolotl. Note that the published stages of \(Pleurodeles\) (Gallien & Durocher, 1957) are not homologous with those for \(Ambystoma\). Six grafts were carried out from pleurodele to pleurodele at stage 26 (similar to axolotl st. 30) of which five gave reduplications. Thirty grafts were carried out from pleurodele to axolotl with the donors varying from stage 24–30 (similar to axolotl stages 27–37) and the grafts from positions \((5)\), \((56)\), \((6)\), \((7)\) or \((67)\). The grafts were inserted into position 3/2 of stage-34 hosts. Five gave positive results, and all the reduplications were composed of axolotl-type elements alone (Fig. 8). It is possible to say this because the pleurodele limbs have narrower and spindle-shaped phalanges, and a fusion between intermediate and ulnare in the proximal row of carpals. The rather low frequency of positives can probably be ascribed to the variation of donor stages. But in this type of experiment, only one positive result is necessary, and those which were obtained show clearly both the evocative character of the signal and the fact that the same interaction occurs in two amphibian genera.

**Nature of reduplications**

For technical reasons the cartilage elements are the most convenient markers in studies of limb development. Although it was reported by Blount (1936) that the soft tissues are also mirror symmetrical in reduplicated limbs, it was thought wise to confirm this in the present study.

It is very striking that the growth pattern of buds for reduplicated limbs differs from the normal. Normal buds point dorsoposteriorly at an early stage and the tip undergoes a complicated torsion such that the \(ap\) and \(dv\) axes of the hand eventually lie parallel to the \(dv\) and the mediolateral axes of the body respectively. Reduplicating buds grow laterally and undergo no torsion at all. If the
Fig. 9. Diagrams of the three-dimensional reconstructions of the muscle patterns in a normal and reduplicated axolotl lower forelimb (case U8). (A) Normal dorsal muscles. (B) Reduplicated dorsal. (C) Normal ventral muscles. (D) Reduplicated ventral.

Muscle nomenclature from Grim & Carlson (1974). *edc*: m. extensor digitorum communis; *ecr*: m. extensor carpi radialis; *ear*: m. extensor antebrachii radialis; *eacu*: m. extensor antebrachii et carpi ulnaris; *ps*: m. palmaris superficialis; *facr*: m. flexor antebrachii et carpi radialis.
development of overall form can be regarded as a manifestation of the *ap* asymmetry of tissue behaviour then the reduplications are symmetrical in this respect.

To examine the muscles, a specimen with a reduplicated limb was allowed to develop for 117 days before the limb and the control limb were embedded in Araldite. Transverse sections, 2 μm thick, were cut every 200 μm along the lower arm and a three-dimensional reconstruction of the muscles was carried out. The lower arm was selected because of the simplicity of the normal muscle pattern.

If it can be assumed that the reduplicate has the muscles *eacu* fused to *edc* to which it is closely apposed in the control, then the muscles of the reduplicate appear to be an accurate double posterior version of the normal (Fig. 9). Although the pattern of nerves does not appear to be very symmetrical it is striking that there are major nerve bundles in both the intercartilage spaces, which rather suggests that the cues guiding the growth paths of the nerves are present on both sides.

**DISCUSSION**

These results present us with three separate problems of pattern formation: (i) what controls the anteroposterior pattern of the normal limb? (ii) how can flank tissue convert the anterior edge of the limb rudiment into a posterior edge? (iii) how does a piece of flank permit the growth of limb rudiment halves on the head?

I wish to suggest that these three phenomena all arise from the same interaction, namely a signal from flank to limb rudiment which determines the posterior end of the latter. The possible mechanisms of the interaction will be discussed more fully in the next paper (Slack, 1977), at present we shall simply assume that such a signal exists. So the normal polarity is guaranteed by the fact that the flank always lies posterior to the limb rudiment. If the flank is removed, which is equivalent to the graft of anterior half rudiment to the head, there is no signal. So the cells do not have all the cues which determine their programme of division and differentiation, and there is no development of the limb. When flank tissue is grafted to the anterior edge of the limb rudiment, both edges become posterior in character and a reduplication ensues.

According to this view, there are two quite distinct steps in the ‘determination’ of anteroposterior polarity. The first is the segregation of the limb and flank primordia, which presumably occurs when the mesodermal shield is regionalized in the course of gastrulation. The second is the actual passage of the signal and specification of the posterior edge of the limb rudiment. The timing of this event will be discussed in a later paper, but present evidence indicates that it occurs around stage 37–38.

It seems probable that this mechanism exists in all urodeles. The present paper indicates a common signal in *Ambystoma* and *Pleurodeles*. The classical authors used several genera (*Ambystoma, Triturus, Hynobius*), all of which behave in a
similar manner. Anura do not afford such favourable material for the study of limb development but Kleinebeckel (1975) showed that Xenopus forelimb-buds could be caused to reduplicate if implanted next to the hind limb-bud; also Cooper (1964) obtained reduplications 'by accident in the course of immunological experiments on Rana catesbeiana. So it is possible that the determination of limb polarity has a common mechanism in all amphibia.

When we read the classical literature, it is important to note that the 'limb discs' of Harrison extend as far back as the level of the 6th somite. They therefore consist both of limb and flank tissue. Accordingly they grow on the head as well as the flank (Detwiler, 1930), and they appear to have their antero posterior axes determined however early they are rotated (Detwiler, 1929, 1933).

No results baffled the classical workers more than the frequent appearance of reduplications. But with the view explained above it becomes possible to explain the onset of practically all reduplications with one simple rule: a limb rudiment, or portion of a rudiment, will form a reduplication if it has flank tissue in intimate contact on both sides.

Clearly this rule can be satisfied when a flank strip is grafted to the anterior edge of the limb rudiment. The frequency of positives is not 100 % and this is presumably because a number of grafts were slightly too far anterior to be in intimate contact. Reference to Table 3 shows that moving the graft half a somite width from level 3/2 to level 2 reduces the incidence of reduplications from 64 % to zero. The rule explains why no reduplications are found on the head (Detwiler, 1930 and Table 2). It explains why 100 % of (4)s grown on the flank give reduplications, but only 22 % of (3)s (Table 1). This is because both grafts have flank on both sides, but many of the (3)s have a little gill tissue anteriorly.

The classical literature is full of reduplications for which no satisfactory explanation was ever advanced. To give three examples: (i) Harrison (1921) showed that when a limb disc was grafted to its normal site in normal orientation (aadd) it gave a normal limb. When grafted in reversed orientation (apdd) it gave a reduplicated limb. It is now clear that the 'limb disc' contains some flank and on reversal it will have the flank of the host posteriorly and its own flank anteriorly. (ii) Swett (1926) showed that division of the limb rudiment with 'indifferent' tissue caused the anterior half to form a normal limb and the posterior half a reduplication. In fact the 'indifferent' strip of tissue came from the flank and on reversal it will have the flank of the host posteriorly and its own flank anteriorly. (iii) The most mystifying of all cases was first performed by Harrison (1921) and confirmed by Swett (1932) with heteroplastic grafts, by Takaya (1941) and by the present paper (Table 1). A limb disc grafted to the flank in reversed orientation (apdd) usually gives a normal reversed limb. If grafted with normal orientation (aadd) it gives a reduplicated limb in about 50 % of cases. This was particularly worrying because it was the 'harmonic' grafts which gave the reduplications. Presumably the reversed discs have their original anterior faces posterior to the limit of
polarizing activity, around level 10, whereas the normally oriented discs have their original anterior faces in the centre of the active region. The reduplication frequency is around 40% and not 100% because some of the grafts have gill tissue at the anterior edge and cannot receive the signal.

In a recent paper, Bryant & Iten (1976) attempt to account for the occurrence of reduplications in the classical experiments by means of the 'clock face model' of French, Bryant & Bryant (1976). According to this model, the limb rudiment is considered to be a flat disc of cells whose positional values are representable as 'polar coordinates', i.e. one value for the distance to the centre and another value for the angular separation from an arbitrary starting point. The rule is that whenever unequal values are apposed by grafting, extra tissue is regenerated in an intercalary manner with positional values which fill the gap. For the angular coordinate the new values always fill the gap by the shorter of the two possible routes. If the regeneration causes a complete new set of angular values to arise (0–360°) then a supernumerary limb will grow.

The experimental evidence on which this model is based comes not from experiments on embryos but from rotations and transplantations of regeneration blastemas in adults. I find it unsatisfactory when applied to embryos for several reasons. First, the limb is asymmetrical in three dimensions and so no model can account for its formation in terms of less than three independent signals. Secondly, it assumes with the classical authors that the 'limb disc' is homogeneous, whereas evidence has been presented above that it is composed of two parts by both histological and morphogenetic criteria. Thirdly, while this model can explain the occurrence of complete supernumerary limbs it is much more difficult to explain the type of reduplications obtained in experiments on embryos. Quite often a reduplication may have only three fingers (see Slack, 1977) and such 'narrow' structures cannot have arisen from a rudiment having a full set of angular positional values, which is a necessary condition for outgrowth in the clock face model.

There is one final series of classical experiments to which the present work is of some relevance. This is the repeatedly confirmed fact that limb structures, including occasional well individuated limbs, may be induced from the flank by implantation of nasal placode tissue. This occurs in a variety of urodele species with the unfortunate exception of the axolotl (Filatov, 1927; Glick, 1931; Balinsky, 1933; Takaya, 1941). Balinsky's results, which are the most extensive, are usually taken to show that the anteroposterior polarity of induced limbs is normal in the vicinity of the host forelimb and hindlimb but inverted in the intervening flank region. It was this result which first led me to postulate the existence of a signalling region posterior to the limb rudiment and I suggested in a preliminary communication (Slack, 1976) that the inverted polarity of induced limbs could be explained if new competent tissue was created posterior to the active region in the flank. However, it is clear from the results described above that the active region is very extensive and that many induced limbs must
have arisen from implants well within it. According to the arguments presented here this means that they should be reduplicated. This seemed to call for another look at the original results, and my current impression from them is that digit number in induced limbs is extremely variable and that at least some of the cases which are described in detail are definite reduplicates (for example Balinsky's C337 which he counts as an inverted normal). So I believe that limb induction can be brought into the framework of the explanation of polarity which is presented here.

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