Chapter 7

Spatial sequences of structures under the control of a morphogen gradient

7.1 Intercalating versus non-intercalating sequences

The concentration pattern formed by autocatalysis and lateral inhibition is assumed to be the signal or prepattern, to initiate a particular structure, for instance, the head of a hydra. Frequently, several structures are formed in a precise spatial relationship. They are determined under a common developmental control. Even in hydra it is not only the hypostome, the cone-shaped opening of the gastric column, which is formed during a regeneration but this structure is also surrounded by a ring of tentacles. More evident examples for sequences of structures are the digits of a vertebrate limb, the segments of an insect or an insect leg or the structures within such segments.

Some of these sequences have the ability to regenerate missing elements by intercalary regeneration while others are unable to do so. Examples for both can be found in the development of insects. In cockroaches, an internal part removed from a particular leg segment will regenerate (Bohn, 1965; French, 1978). However, confrontation of different leg segments does not necessarily lead to the regeneration of the missing segments (Bohn, 1970a,b). Similarly, gaps in the basic body pattern of insects are not repaired. For instance, gaps induced by a temporal ligation of an Euscelis egg remain permanently (Armbruster and Sander, quoted after Sander, 1975b) and asymmetric bicaudal embryos of Drosophila (Fig.8.3) develop such gaps without any experimental interference (Nüsslein-Volhard, 1977).

In looking for differences between systems which show and which do not show intercalary regeneration, it is remarkable that most non-intercalating sequences are determined in a period without much change in the geometry. The segments of insects are determined in the non-growing egg and little cell proliferation takes place during the critical period of blastoderm formation. Similarly, in regen-
erating insect legs, the newly formed sequence of segments is laid down in a very minute scale and proceeds without cell divisions. Only later, after the leg segments are already distinguishable, are these structures enlarged by growth (Bulliere, 1972; see Fig.9.6). In the terminology of Morgan (1901), both these processes are presumably morphallactic processes. Further, non-intercalating sequences have in many cases a clear organizing center. In the chicken wing bud, a small group of cells, the so-called ZPA (see Fig.10.7) is decisive for the formation of the digits. In some insects, a small area at the posterior pole of the egg, the activation center, has to be present for a normal development (see Fig.8.1). Frequently after an experimental interference a sequence of structures up to the most terminal structures are formed, e.g. UV irradiation can induce an additional insect abdomen (see Fig.8.4), or an imperfect wound healing of an insect leg can lead to two new distal leg parts (see Fig.9.7).

In contrast, systems which show intercalary regeneration seem to depend much less on special organizing centers but rather on an interaction between neighbouring cells (or groups of cells) at each level of the sequence, detecting and repairing any discontinuity. While in non-intercalating systems the tendency for a unidirectional proximodistal or anteroposterior transformation exists, in intercalating systems there are mainly the distal elements which regenerate the missing parts in a distal-proximal transformation (see Fig.13.1; Bohn, 1972; French, 1976a; Nübler-Jung, 1977). On the basis of these differences one should expect that the two processes are controlled by different mechanisms. Two possibilities can be envisaged for the generation of sequences. On the one hand, the local concentration of a gradedly distributed substance, the morphogen, determine the particular structure at the particular location. In terms of Wolpert (1969, 1971) it is the positional information and its interpretation which causes the sequence. The second possibility consists of a mutual induction of neighbouring structures. Explicit models of both types will be given and comparison with biological systems will reveal that positional information and its interpretation can account for the determination of segmented structures such as the insect body, the insect legs or the digits of vertebrates. In contrast the pattern formation within insect segments seems to be of the mutual induction type (p.?ff). The differences listed above in the ability to intercalate and in the requirement for an organizing region will find straightforward explanations in these models.

7.2 Sources, sinks and the shape of the gradients

It has long been argued (Boveri, 1901; Child, 1929, 1941) that spatial organization could be accomplished by the graded distribution of substances termed morphogens. Wolpert (1969, 1971) developed this idea further into the concept of positional information. He pointed out that the size of an embryonic system is small when determination occurs, of the order of 1 mm or 100 cells across. Diffusion combined with local production and destruction at opposite ends, can form a gradient of this size within a few hours (Crick, 1970). This order of magnitude seems reasonable. Gradient formation involving diffusion in an area with
7.2. SOURCES, SINKS AND THE SHAPE OF THE GRADIENTS

Figure 7.1: (a-c) Concentration profiles of morphogenetic substances, generated by different source-sink arrangements and their dependence on field size. (a) A linear gradient can be formed by a source (here at the right side) and a sink (left). The concentration around the source will depend very much on the distance between the source and the sink, as demonstrated by the long and the short curve. (b) A uniform breakdown in addition to that at the terminal sink leads to an approximately size-independent concentration at the source and at the sink, since the concentration around the source depends more on the local decay rate. The relative concentration increase per unit length is approximately constant which facilitates the discrimination between different concentrations during the interpretation of positional information by the cells. (c) A simpler system consists only of a terminal source and a uniform breakdown. The advantage of simplicity is counterbalanced by the problem that, at the end opposite to the source, the concentration is size-dependent and shallow. Nevertheless, the central and source-containing portion of the gradient can be used to supply positional information; an asymmetric location of the determination of the structures is then to be expected. Positional information produced in this way seems to be used in the determination of segments in insects or of the digits in chicken limbs (Fig.10.7).

a dimension of the order of 1 cm, on the other hand, would require a full day. It is thus tempting to speculate that the spatial development of an organism or of parts of it is controlled by a graded distribution of a substance during a stage of development where the extension of the region is of the order of 1 mm or less, and that the local concentration determines the further developmental pathway of each cell.

There are several ways to set up a graded distribution. The assumption of a morphogen source and/or sink alone would only shift the problem of morphogenesis to another level as long as no explanation is provided as to how and where they arise in an initially undifferentiated tissue. The mechanism of short-range autocatalysis and long-range inhibition described above can provide local high concentrations which may act as sources or sinks, and, in addition, explains why they usually appear at the boundaries of the system. A linear gradient can be obtained with a source at one side and a sink at the other (Crick, 1970), but such an arrangement has some disadvantages. First, the size regulation is poor; the concentration around the source depends on the distance between the source and the sink (Fig.7.1a) unless there is a homeostatic mechanism by which the source strength is increased for smaller sizes. As a physical analogy, if a house has thinner walls, it requires more heating to maintain the same internal temperature, or the same inside-outside temperature gradient. Secondly, a linear gradient signifies that the relative morphogen increase per unit length is high at low morphogen concentration and low at high morphogen concentration. Therefore, the cell must be able to measure high concentrations much more precisely than low concentrations if it is to achieve the same spatial accuracy throughout the tissue. Both problems are avoided if the morphogen decays not only at the terminal sink
but, to some extent, everywhere (Fig.7.1b). The concentration around the source is then nearly independent of the total size and determined mainly by the local decay rate. The slope is steeper in the area of high morphogen concentration with an approximately constant relative change per unit length, as would be desired for uniform reading accuracy. In addition, the time required to reach the steady-state concentration is much shorter. Therefore, much more convenient for cell specification than a linear gradient produced by a morphogen source and sink is an approximately exponential gradient formed by a terminal source with an overall decay of the morphogen. A local sink at the other boundary would maintain the morphogen at a low level, improving in this way the size-regulation.

In sea urchins, both, the animal and the vegetal pole is of importance. Removal of vegetal cells leads to an “animalization” of the embryo and vice versa. As an explanation, Runnström (1929) has put forward the double gradient hypothesis. However, a source, overall degradation plus local sink system would explain the data as well. Let us assume a source at the vegetal and a sink at the animal pole. Removal of the source region, especially of the micromeres, would lead to a general decrease of the morphogen and therefore yield an animalized embryo. The other way round, removal of the sink region (cells at the animal pole) would lead to a general increase of morphogen and therefore to embryos of the vegetal type (enlargement of the endoderm). A combination of the animal half (sink) and micromeres (source) forms a complete larva, despite the fact that most cells of the ventral half are missing since a source-sink combination can show a good size regulation (Fig.7.1c). Lithium ions have a vegetatizing effect. After culture for some time in a medium containing lithium ions, parts of the animal portion of an embryo can act in a similar way as the most vegetal cells, the micromeres (see Hörstadius, 1973). In terms of the model lithium causes a general increase in the source strength. In contrast, rhodamine ions act as an animalizing agent, presumably by poisoning the source.

The question arises whether a terminal sink is required at all in addition to the homogeneous destruction of the morphogen. For the formation of a local sink a separate activator - inhibitor system would be necessary. A relatively simple pattern-forming system would, therefore, consist of a local source only and a uniformly distributed decay of the morphogen but without a local sink. The price paid for this simplicity is that the gradient at the end which does not contain the source is shallow (assuming the boundaries are impermeable), and the absolute concentration here is size-dependent (Fig.7.1c). An appropriate positional information can be supplied only in the central region and that portion of the tissue containing the source of the gradient. In other words, only a certain concentration range of the gradient can be used. An advantage of using only a fraction of the gradient is that the mechanism then becomes insensitive to a size variation of the tissue over a certain range, since the fraction of the gradient used will be present both in a larger and a smaller field (Fig.7.1c). Indeed, if only one organizing center is involved, the area opposite to the organizing center seems, in most cases, not to be used for the specification of structures. Two examples are the determination process in early insect development and the determination
of the digits in chicken limb buds (Tickle et al., 1975) which will be discussed in detail below. The unused cells in the portion of tissue where the gradient is beyond the limit utilized for the relevant development may become necrotic and the constituent material recycled into the growing tissue. Or - vice versa - the utilization of the full region between the terminal boundaries of a diffusible gradient is a first indication that both ends contain organizing centers.
Chapter 8

A gradient model for the early insect development

Early insect development is a very instructive system for studying the determination of several structures within one process (for review, see Sander, 1976; Counce, 1973). After fertilization, the dividing nuclei in the egg spread out into the cytoplasm (cleavage stage) and migrate finally to the egg periphery, coming to rest in a well-defined layer (syncytic blastoderm stage). Only then are cell walls formed between the nuclei, leading to the cellular blastoderm. The embryo proper - the germ band - is formed out of a fraction of this blastoderm. The segments of the larva are linearly arranged and become individually distinguishable during germ band formation. The final pattern can be experimentally disturbed by centrifugation, ligation, thermocauterization, puncture, or UV irradiation. Since the egg is well supplied with nutritional substances, a development into recognizable structures is possible even after severe experimental disturbances. A large amount of experimental data have been accumulated for many different species, providing a challenging testing ground for any model.

There are pronounced differences between species, nevertheless, as a working hypothesis we will assume that the basic developmental control is similar in all species. Keeping this in mind, the results can be generalized in the following way: (i) The basic body pattern is controlled from the posterior egg pole. (ii) An instability exists at the anterior pole to form an abdomen instead of a head. (iii) Gaps can be formed in the sequence of segments which are not repaired by intercalation. (iv) The cells respond to the developmental signals in a stepwise manner. (v) Segmentation and giving individual segments a particular “name” are separate but interdependent processes.

For the positional information it would not matter whether the gradient runs anteroposteriorly or vice versa. Is it possible to distinguish between both possibilities without having yet biochemically identified substances carrying positional information? Experimental interference at both egg poles can have unexpected consequences. For instance, puncture or irradiation of the anterior pole of a Smit-tia egg can lead to the formation of an abdomen instead of a head (see Fig. 8.4) whereas in Euscelis the shift of posterior pole material can lead to up to three
abdominal structures (see Fig. 8.2). Insect development has been assumed therefore to be controlled by anterior and posterior determinants (Kalthoff, 1976). It has been shown, however, that many irradiation, plasma shift and ligation experiments are explicable under the assumption of a gradient arising from the posterior pole alone (Meinhardt, 1977). The sensitivity of the anterior pole reflects more an instability against the formation of a second source. Since it is believed that the control of insect development is a paradigm for the control of development in general, this model should be described in some detail and compared with the experimental observations.

8.1 The “activation center” - an organizer region at the posterior pole

Seidel (1929) found evidence in *Platycnemis* for an “activation center”, a small area at the posterior pole which is necessary for the organized development of the embryo. The fate-map indicates that this activation center does not participate in the formation of the embryo proper; instead its duty is to organize the embryo. An exclusion of the posterior eighth of the egg by a ligation suppresses embryonic development (Fig. 8.1). However, the exclusion of only a slightly smaller posterior fragment leads to a normal development. If the operation is made early in the development, the result is either a completely normal development or no development at all; no intermediate forms are observed. No such center can be detected at the anterior pole since a similar constriction there leads always to normal development.

If one assumes that the anteroposterior organization of the egg is accomplished by a morphogen gradient which is generated by an activator-inhibitor system, these experiments tell much about the orientation and shape of the distributions. Evidently, the autocatalytic center must be localized at the posterior pole. The fact that no pattern regenerates after an early elimination of the posterior pole indicates that a small basic inhibitor production (activator-independent, $\rho_1$ in Eq. 3.2) can suppress the autocatalysis at very low activator concentrations. The smallness of the activation center indicates that the activator maximum is very narrow, otherwise a regeneration of the pattern and therefore normal development would be expected even after removal of a much larger fragment. On the other hand, if the activator maximum is very sharp, the activator concentration is very low in almost the whole egg space and is, therefore, incapable of supplying positional information. However, any substance with a more shallow distribution, produced by the very localized activator maximum could act as morphogen. Since the inhibitor production is activator-controlled and since the inhibitor has, due to its higher diffusion rate, a graded distribution throughout the total area, the inhibitor is a reasonable candidate for the morphogen. Our assumption will be, therefore, that a high activator concentration is formed at the posterior pole and that the cells or nuclei and their immediate plasma environment are instructed by the local inhibitor concentration which segment they must form. In this scheme,
Figure 8.1: The importance of the posterior pole in insect development. (a,b) Normal development of an embryo of a dragonfly is possible only if less than ca. 1/8 of the posterior egg is excluded by an early ligation (Seidel, 1929), otherwise the blastoderm cells do not differentiate (b). A similar ligation at the anterior pole is without effect. (c,d) Simulation: In this and the following simulation, it is assumed that the positional information is generated by an activator (top)-inhibitor (bottom) system and that the distributions have attained a steady state (as shown in Fig.4.1) during oogenesis. To show the reaction of the system to the experimental interference, both concentrations are plotted as function of the anteroposterior position and time. (c) After removal of the activator maximum, regeneration can take place if sufficient activator remains in the egg to initiate the autocatalysis, restoring the gradient. (d) After complete removal of the activator maximum, its reformation depends on the small constitutive activator and inhibitor production ($\rho_0$ and $\rho_1$ in Eq.3.2). To maintain a monotonically graded distribution, secondary maxima have to be suppressed and this requires a low $\rho_0$ and/or high $\rho_1$. This can suppress the reformation of the removed maxima and no positional information would be supplied.

The inhibitor plays a dual role: It activates particular control genes and suppresses the formation of other activated areas.

The all-or-nothing effect after removal of the posterior fractions of the egg is easily explained on the basis of the model: either sufficient activator remains to overcome the basic inhibitor level and to reform the distributions via autocatalysis or all concentrations drop to a very low level (Fig.8.1d).

In further experiments, Seidel (1935) removed large parts of the activation center by burning with a hot needle. He was surprised by the result that even more than half of the posterior pole can be burnt and still yield normal development. With a gradual elimination of a constitutive source one would expect a gradual decrease in the morphogen concentration. But an autocatalytically activated source will restore the pattern as long as sufficient activator is available to initiate the autocatalysis.
Figure 8.2: Evidence of autocatalysis and long range inhibition in insect embryogenesis. (a-e) Experiments of Sander (1961a, 1962) with eggs of the leaf hopper *Euscelis*. Normal (a) and altered germ band pattern after shift of the posterior pole material (●) and ligation: either a symmetric (b) or a reverted sequence of abdominal segments (c) results. (d,e) If some time elapses between the shift (d) and ligation (e), a complete embryo is formed in the anterior fragment. Up to three abdomina can be formed within one egg. (f-h) Model calculation: Shift of the symbionts is assumed to cause some redistribution of the activator. A break in the distributions indicates the time of an experimental interference. Despite the fact that the redistribution can be only vaguely controlled, only two new distributions are possible. Either (f) both maxima coexist keeping maximum distance from each other and a symmetric pattern emerges, corresponding to the result sketched in (b) or (g) the new maximum dominates over the old one via the long-ranging inhibitor, the resulting pattern has a reversed polarity. (h) If some time elapses between the redistribution of activator and the ligation, the anterior part is "infected" with sufficient activator that, due to the autocatalysis of the activator, complete gradients are formed. This corresponds to the result (e). To have a convenient perspective, the distributions in (h) are 90° rotated (after Meinhardt, 1977).

8.2 Evidence for autocatalysis and lateral inhibition - Pattern formation in leaf-hopper embryo *Euscelis*

More support for the positional information concept and for the organization from the posterior pole can be deduced from experiments with the eggs of the leaf-hopper *Euscelis* (Sander, 1959, 1960, 1961a,b). There, a ball of symbionts is located at the posterior pole of the egg. These symbionts, bacteria necessary for the normal development of the embryo, are implanted in the egg by the mother. A dislocation of this posterior pole material in an anterior direction has a dramatic effect on the further development. After shift and ligation, up to three abdominal structures can be formed within one egg, some with reversed polarity. The main results are sketched in Fig.8.2 and can be summarized as follows:

(1.) After a shift of the symbionts and a ligation of the egg, partial embryos are formed in the posterior part. They have either a symmetric or a reversed
(2.) The anterior half of the blastoderm does not participate in the formation of the embryo proper. However, after an anterior shift of the symbionts and a delayed ligation, a complete embryo can be formed in the anterior fragment even if the symbionts are not included in that fragment. Sander (1960) has concluded that the important factor is not the symbionts themselves but some “posterior pole material” spreading out from them.

Similarly as in Seidel’s experiment one has to conclude that a small area controls the whole pattern formation in the anteroposterior dimension. The symbionts provide a handle to manipulate this area. The experiments are explicable by the theory assuming that some activated plasma is shifted with the symbionts (Meinhardt, 1977). Traces of this activated plasma can develop a fully activated source, preferentially at the physical boundaries of the (ligated) egg. In contrast, a stable morphogen source subdivided into two or three parts would lead a much reduced maximum morphogen concentration and no abdominal structure would be expected - in contradiction to the experiment. The newly formed maximum can either be dominant over the old one, leading to a reversed morphogen distribution or both maxima can coexist, leading to the symmetric pattern (Fig.8.2). Obviously, there is some ambiguity between polar and symmetric patterns after an experimental interference. This is also a property of the theory and therefore an explanation is given of why minor and uncontrollable differences can lead to the two strikingly different, but well defined, alternative patterns. If sufficient time elapses between shift and ligation, the newly formed maximum can spread out, the anterior portion becomes “infected” and a complete pattern can be formed there (Fig.8.2h). This observation also supports the autocatalytic aspect of the theory.

Vogel (1978) has separated three fragments of Euscelis eggs by two ligations. He observed that the central fragment has to have a relatively large size if a single pattern element is to occur while some little additional space is sufficient to add further segments. This fits nicely into the model where a minimum extension (range of the activator) is required to form a pattern. Around this critical size, the concentration range depends sensitively upon the size of the field. The maximum concentration and the concentration range of the gradient may be reduced (see Fig.4.1a). The concentration would not be high enough to form the complete abdomen. The most posterior structures are thoracic structures, in agreement with Vogel’s observation.

### 8.3 Formation of posterior structures at the anterior pole

After certain experimental interferences, many species form abdominal structures instead of head structures in their anterior portion. Frequently, a completely symmetric development is observed. The experimental treatments evoking such “double abdomen” (DA) malformation are quite diverse: UV irradiation (Yajima, 1964; Kalthoff and Sander, 1968) or puncturing (Schmidt et al., 1975) of
the posterior pole, temporary ligation (van der Meer, 1978) and centrifugation (Yajima, 1960). Double abdomen formation has also been found in a maternal effect mutant of *Drosophila* (Bull, 1966; Nüsslein-Volhard, 1977).

In the model, the abdominal structures are formed where the inhibitor concentration is high. The formation of additional posterior structures at unusual locations would indicate the triggering of a second activator maximum, establishing a second morphogen source. An especially favorable location for the formation of a second activation is the anterior pole, since here the inhibitor has its lowest concentration and any unspecific reduction of the inhibitor concentration may be sufficient to induce a new center of activation. This is in agreement with the unspecific modes of double abdomen (DA) induction already mentioned. The induction of a DA has similarities with the unspecific induction of a second amphibian embryo (Waddington et al., 1936). However, in amphibians, it is not the anteroposterior axis but the dorsoventral axis which becomes duplicated, forming a dorsoventral-dorsal pattern which leads to two parallel aligned embryos. After DA-formation in insects, the two embryos are not separated. Both gradients overlap because the two sources are not sufficiently remote from each other.

Fig. 8.3 shows photographs of a normal and of DA (bicaudal) larvae of *Drosophila*. Important for the gradient model is that the anterior half of the embryo is not merely transformed into the posterior half. In the center of a normal blastoderm, the Metathorax is layed down (Lohs-Schardin et al., 1979) while in DA-embryos, the plane of symmetry is very variable but always located in one of the abdominal segments. Therefore, the fate map of far more than the half of the blastoderm is changed. As discussed below in more detail, this is expected from the overlap of two gradients and provides a crucial support for the assumption of a diffusible signal.
8.3. DOUBLE ABDOMEN

Figure 8.4: Double abdomen (DA) formation in midge Smittia. (a) normal embryo. (b) Irradiation of the anterior quarter of a Smittia egg can lead to a completely symmetric embryo with one abdomen at each pole (Kalthoff and Sander, 1968; drawn after Kalthoff, 1976). (c-f) Results of the experiments of Kalthoff (1971). The dose of the irradiation of the anterior quarter was adjusted to yield about 50% DA; the dose of additional irradiations was somewhat smaller, in order to minimize the number of eggs which fail to develop at all. The frequency of DA-formation is given in percent (g-j) Model: The inhibitor is assumed to be UV-sensitive. Shown is the response of the activator (top) - inhibitor (bottom) system as function of position and time after irradiation. A reduction of the inhibitor concentration (black bar) at the anterior pole (g) allows an increase of the activator concentration which can, via autocatalysis, develop into a full second maximum. The inhibitor distribution (positional information) at the anterior pole becomes a mirror image of that of the posterior pole. Experiment: While an irradiation of the second anterior quarter is without effect (d), applied together with an irradiation of the first quarter, it considerably increases the probability of DA induction (e). Model: The removed inhibitor in a central area is rapidly replenished by the nearby source (h) and, therefore, without effect. But such a removal delays the restoration of the inhibitor concentration after an irradiation of the anterior quarter. Therefore, the activator increase after an irradiation of the anterior half is much more rapid (i) and the probability of reaching the critical level for the DA-formation is increased. Experiment: (f) An irradiation at the posterior pole is without serious effect (0% DA), but such an irradiation cures partly the anterior radiation damage. (j) Model: Inhibitor reduction at the activated site leads to an overshoot of the activator and, consequently, also in the inhibitor concentration. This is without serious effect, since all concentrations necessary for the determination of any particular structure remain present. But the overall increased inhibitor concentration reduces the activator increase after irradiation of the anterior end; the activator increase may not be sufficient to reach the threshold for further autocatalysis and may, therefore, disappear.

In the midge Smittia, DA-formation can be induced by UV irradiation (Kalthoff and Sander, 1968) or by a puncture (Schmidt et al., 1975) at the anterior pole. According to the model, the UV irradiation may either destroy the inhibitor or the inhibitor-producing structures. The results of a very instructive set of experiments by Kalthoff (1971) are shown in Figure 8.4, together with their explanation in terms of the theory assuming that the inhibitor is UV-sensitive. Due to the inhibitor reduction, the activator concentration increases. If this activator increase is sufficiently high, a new activator maximum develops via autocatalysis, even if the inhibitor concentration is rapidly restored. If the activator concentration fails to reach the critical level, the activator increase will disappear. In agreement with the experiment, the formation of a second activation is an all-or-nothing event. Substantial support for the postulated interaction between an activator and a long-ranging inhibitor can be derived from the fact that a simul-
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8.4 The long range character of the positional signal

The altered central segment in DA embryos offers a crucial support for the gradient model. From a central ligation of a Smittia egg at the blastoderm stage - at a stage when the segment pattern is fixed - we know that the segment No.5, a thorax segment, is laid down in the center (see Fig.8.7). However, in double-abdomen embryos, the plane of symmetry at the center is, as a rule, formed in segment 8 or 9. According to the model, in DA embryos, the morphogen diffuses...
Figure 8.6: Indication for a “negative” size regulation and the absence of anterior determinants in the leaf hopper *Euscelis* (Sander, 1959). (a, b) Only the posterior part of the blastoderm egg is used for the formation of the germband: a ligation at the blastoderm stage at 45 % EL leads to the formation of a complete embryo (b). (c) An early ligation at the same position leads to an omission of the head. In the smaller area, less structure are formed compared to the same region in the unoperated egg (negative size regulation). (d) A complete embryo is formed, however, in a larger fragment (57 % EL), indicating that the omission of head structures does not result from the absence of anterior determinants. Model: (e) Normal activator (—-) and inhibitor (- - - -) distribution. (f,g) Due to the ligation, the inhibitor (+ + + +) accumulates. The low concentration required for head formation is present only if the fragment is larger. (h-j) Assumed fate maps of normal (h) and calculated fate map of the ligated eggs (i,j).

from both sides into the center and the concentration is thus increased there. This has the consequence that a more posterior structure is formed (Fig.8.5). From the simulation of the early ligation experiments one can estimate the diffusion constants and lifetimes of the activator and inhibitor. Applying this to a simulation of a double abdomen formation leads to segment 9 as the central element, in essential agreement with the model. After an early ligation, also the segment 9 is formed in the posterior part of the egg (Fig.8.7). The fact that the same element is formed in the center after the two very different manipulations - anterior UV irradiation and central ligation - is a straightforward consequence of the gradient model. The ligation renders impossible any flow through the center and the morphogen accumulates. Similarly, after DA induction the flow at the center is, due to the symmetry, also zero. Therefore, the same concentration and thus the same structure is expected after both manipulations. This is exactly what has been observed. This may be the best evidence available that the pattern is controlled by a long-ranging diffusible substance and not, for instance, by a chain of induction.
CHAPTER 8. EARLY INSECT DEVELOPMENT

8.5 Negative size regulation - a phenomenon characteristic for gradient systems generated by a local source

In many developmental systems, the complete set of structures is formed even if a substantial portion of the developmental field is removed. Examples are the dorsoventral axis of amphibians (Fig. 13.8, p. 144) or insects (Fig. 12.4, p. 126). However, the pattern regulation of the anteroposterior axis of insects exhibits the reverse behaviour. In a fragment of an egg, resulting from an early ligation (at the nuclear cleavage stage), fewer segments are formed than in an area of the same size as in an undisturbed egg, so to say, a negative size regulation. We will show that this is a straightforward consequence whenever a pattern is controlled by a morphogen gradient which is generated by a local source and diffusion.

Let us regard first only the posterior, or source-containing fragment of a ligated egg. In terms of the model, a ligation during the cleavage stage introduces a diffusion barrier. This leads to an accumulation of the morphogen. Due to the ligation and morphogen increase, a particular cell will get a more posterior specification: Thus a particular structure will appear in a more anterior position. The fate maps of ligated and non-ligated eggs of Drosophila (Newman and Schubiger, 1980) have provided a direct evidence for the predicted anterior shift. The segments which would have been determined just anteriorly of the ligation are instead omitted. This feature of the model has counterparts in many experimental observations. Ligating an egg during the cleavage stage leads to an omission of segments, for example, in Euscelis (Sander, 1959), Calliphora (Nitschmann, 1959), Bruchidius (Jung, 1966), Protophormia (Herth and Sander, 1973) or Drosophila (Schubiger and Wood, 1977). In Euscelis, for instance, a ligation at 44 % EL (EL = egg length, 0 % = posterior pole) (Fig.8.6) at the blastoderm stage leads to a complete embryo in the posterior portion. The same ligation made earlier leads to an embryo lacking the head lobe. Fig.8.6 shows that this is expected from the accumulation of the morphogen. An early ligation placed more anteriorly, at 57 % EL, leads to a complete embryo. In the enlarged space sufficiently low morphogen concentrations are possible despite the accumulation. The latter observation indicates that the incomplete embryo formed after the early 44 % EL ligation does not results from an elimination of “anterior determinants”, spreading out from the anteriorely egg pole since these influences would be eliminated by a 57 % EL ligation as well. These experiments also rule out the argument that the omission of structures result from a damage of cells. As the late 44 % ligation shows, the blastoderm cells located more anterior are normally not used in the embryo formation. The omission of segments does not result from a damage of cells but from a change in the positional information to which the cells are exposed.

The phenomenon of negative size-regulation also provides a strong argument against another type of model stipulating that the most posterior structure is induced at the posterior pole and that the more anterior segments result from a
8.6 Stepwise Interpretation

8.6 The interpretation of positional information is a stepwise unidirectional and irreversible process

Figure 8.7: The influence of a diffusion barrier and evidence for a stepwise, unidirectional interpretation of positional information. (a-f) Schematic drawing of the ligation experiments with eggs of the insect Smittia (Sander, 1975b). Inset in (g): Schematic drawing of a Smittia embryo. Segments are designated H, 1, 2, ... 16. (a-f) Experimentally observed germ band fragments after the ligation. The first and last segment formed in each germ band fragment is indicated. (a-c) After a ligation during the blastoderm stage very few - if any - segments are omitted: the egg behaves as a mosaic. This allows the drawing-up of an approximate fatemap (h). (d-f) If, however, the ligation is made earlier, during the cleavage stage, many segments are omitted, but the terminal segments always remain present. Model: After a ligation, the inhibitor (positional information, - - - -) accumulates on the source-containing posterior side (g), which leads to a shift of the segments in an anterior direction (i). Some of the segments normally determined posterior to the ligation would no longer be formed; in this example the elements 5 - 8 would be omitted (h,i). In the anterior portion, on the other hand, the inhibitor concentration decreases to a level which normally never occurs (g) and no element would be expected to be formed, which is at variance with the experimental observation (e). This contradiction has forced the assumption that the determination proceeds under the influence of the morphogen stepwise and unidirectional to more posterior structures until the determination corresponds to the local morphogen concentration. The structure formed in the anterior part after a ligation reveal how far the determination has already advanced at the time of the ligation (j). The pattern in the posterior portion depends on the extent of morphogen accumulation and the time available to adapt the determination to this increased morphogen concentration.

This sequential triggering must be assumed to be due to a more or less local process. Therefore pinching off regions which would not be used in the normal embryogenes is is expected to be without effect, in contrast to the experimental observation.
known omission of segments in the posterior portion and the minimum time in which a second activation can be formed, one can estimate the diffusion rate of the inhibitor to be $5 \times 10^{-9}$ cm/s and its lifetime at 1 h.

A description of the missing segments in the anterior portion with these parameters is not in agreement with the experiments. From the estimated short lifetime, one would expect a relative fast decay of the inhibitor (morphogen) such that no segments at all would be formed in the anterior part (Fig.8.7). On the contrary, the head lobe is always formed. As Figures 8.7a and 8.7d show, a ligation at 60 % EL of a *Smittia* egg leads to nearly the same segments being formed in the anterior part independent of whether the ligation is made early (during the cleavage) or late (during the blastoderm stage). In such a ligation experiment, a particular cell or nucleus seems to be determined at the time of the ligation if located anterior to the ligation, but the final pathway can be changed if located posterior to the ligation. Extensive reprogramming seems to be possible so that a more anterior structure can be reprogrammed to form a more posterior structure but not vice versa.

The explanation I have proposed for this stipulates how the cells measure the local morphogen concentration. Originally, all cells are programmed to form the most anterior structure. Under the influence of the morphogen, the cells proceed stepwise to higher (more posterior) determinations from “head” to “thorax” etc., until the determination corresponds to the local morphogen concentration. A step in the determination is - as in other developmental systems - essentially irreversible. If the morphogen disappears before the final determination is achieved, the stepping through of the different determinations will be interrupted. The determination would remain unchanged at the stage already reached. Such a situation exists in the anterior part after a ligation. If the morphogen increases due to experimental interference, for instance by the accumulation of the morphogen in the posterior portion after a ligation or after the induction of a second activation by UV irradiation, the determination can proceed. Structures corresponding to more posterior positions would then be formed. The omission of segments on both sites of a ligation results therefore from different reasons. On the posterior side, it depends on the accumulation of the morphogen; at the anterior site, it reveals how far the interpretation had progressed. This explains the asymmetry of the gap. At an early stage, when only the most anterior segments were already determined, a head lobe is formed in the anterior fragment, fairly independent of the position of the ligation (Fig.8.7d,f). In the posterior fragment, the lowest morphogen concentration and therewith the most anterior structure depends essentially on the position of the ligation.

The type of stepwise and irreversible determination described above appears to be a general process. The proximodistal determination of insect legs (p. ) as well as the anteroposterior determination of vertebrate limbs (Fig.10.7) follows the same rules.

Determination - or commitment - of a group of cells to form, say, a head lobe must consist of switching “on” a particular set of genes. Detailed models for the selection of gene activity under morphogen control will be given below (Fig.11.5
8.7 Alternative Models

Other mechanisms which have been proposed for the control of insect development may appear reasonable as well. However, a look into their consequences reveals features which are not supported by the experiments mentioned above. Some of them are listed below, together with conflicting observations. Maybe, some of these conflicts may be cured by additional assumptions. The discussion should show how stringent the experimental observations really are for any model.

Model A: The prepattern specifies only the terminal element, the abdomen; the missing elements are specified by a chain of induction (such as shown in Fig.13.3). Problem: A zone is expected in which the final determination takes place and which moves in a wave-like manner over the field. A ligation at a particular location should lead to different results depending on whether the wave has passed this position or not. If passed already, the development would be normal in both fragments and indistinguishable from a mosaic development. In contrast, when the ligation is made before the wave has passed this location, the development would be normal in the posterior fragment, but no development would be expected in the anterior fragment. In contrast, experimental observations show that gaps in the sequences of segments become gradually smaller if the ligation is made at a later developmental stage and that specially in the posterior fragment segments are missing also (Sander, 1976).

Model B: Two gradients, for instance a and p, with opposite orientations are formed by local sources on each end of the egg (anterior or posterior determinants). The ratio of both concentrations (a/p or p/a) is used as positional information (Sander, 1961a). Problems: After ligation, the concentration of each component drops to very low values in the fragments not containing its source. The values are lower than would be found anywhere in the normal embryo. Therefore the ratio would attain very high values on one side of the ligation and very low values on the other values which are out of the range used to specify structures in the undisturbed organism and this is clearly at variance with the observed gap behavior.

Model C: Head and abdominal structures are determined by anterior or posterior determinants and missing structures are filled in by some sort of intercalation from both sides at the discontinuity. Problems: In a bicaudal embryo (see Fig.8.3) or in a UV-induced double abdomen it has to be assumed that both ends bear posterior determinants. No discontinuity would be present to initiate the intercalation and therefore no pattern formation at all would be expected.

Model D: Similar as model C, but in the center where neither anterior nor posterior determinants are present, thoracic structures are determined (Vogel, 1978) and again missing structures are made by intercalation. Problems: Ligation through the center should contain at each side thoracic structures and normal development is expected. Double abdomina should show thoracic structures in the center. Neither of these expectations is in agreement with the experimental
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Model E: Specification of the field is achieved by a sequence of binary subdivisions (Kauffman et al., 1978). A gradient-like prepattern bisects a field initially into two parts, anterior and posterior (0 and 1). By shrinkage of the diffusion range the prepattern changes into a bell-shaped distribution, subdividing the two halves into four quarters (00, 01, 11, 10), and so on. Problems:

(1) If part of the egg were removed, the pattern would be restored after corresponding shrinkage of the chemical wavelength. In a ligation experiment, such a mechanism would not lead to a gap but would produce a normal set of body parts in both halves of the egg. The only defect could be the absence of some fine structure if the shrinkage of the diffusion range remained insufficient. In contrast, the experiments show that each half produces even fewer structures than would be expected from mosaic development.

(2) At the blastoderm stage, the thoracic segments becomes subdivided into anterior and posterior compartments (Garcia-Bellido et al., 1973, 1976). At that time wings and legs are not yet separated. On the basis of chemical wavelength one would expect an organization of the large dorsoventral dimension first and only then a finer subdivision of the narrow segments into the even narrower anterior and posterior compartments.

(3) A cell which has seen a zero-concentration twice must be in a different state (state zero-zero) compared with a cell which has been exposed to “nothing, but only once” (state zero). This would require, for example, an additional counting or clock mechanism synchronized with the pattern-forming process. The relatively long-time interval in which a double abdomen can be induced in an insect (Ripley and Kalthoff, 1981) argues against a clock mechanism and against early irreversible binary decisions.

8.8 Open questions

The gradient model, despite of providing a unified explanation for the many experimental observations, is not free of problems. Some observations which are difficult to integrate should be mentioned.

1. After temporal ligation of eggs of the beetle *Callosobruchus*, van der Meer (1978) found a double abdomen formation in the right or the left half only while the other half was normal. Possibly, the cells in the non-affected half are unable to respond to the altered morphogen distribution.

2. Centrifugation of eggs of *Smittia* (Rau and Kalthoff, 1980) and of *Chironomus* (Yajima, 1960) can lead to the formation of symmetrical double heads (or double cephalon) embryos which lack thoracic and abdominal structures. A similar pattern has been observed in a mutant of *Drosophila* (Lohs-Schardin and Sander, 1976). A central activator maximum is expected as one possible pattern after an experimental interference (see Fig.4.1) but in such a case, two abdominal structures pointing with the posterior ends towards each other are expected.
Such patterns do occur in an *Euscelis* egg after a shift of the posterior pole material (Fig. 8.2e) but only if the egg is also ligated. It seems that the dorsal and the ventral side of the egg have to be brought into contact to enable a high point for the anteroposterior organization. This would guarantee that the dorsoventral and the anteroposterior pattern are oriented perpendicular to each other. An analogous observation has been made in planarians (see p. ). Therefore, it may be difficult to induce a full height peak in the center of an egg without a ligation. The symmetrical head-like structure mentioned above indicates a much reduced morphogen concentration.

3. *Drosophila* embryos, despite using a large fraction of the blastoderm, show a remarkable insensitivity of the segment pattern against a variation of genetically altered egg size (Nüsslein-Volhard, 1979). The size-regulation seems to be connected with the formation of anterior structures because double abdomen embryos form indeed fewer segments if the egg is smaller. This size-regulation could be achieved by a somewhat stronger sink property of the anterior egg pole, keeping the morphogen concentration low (see Fig. 7.1).
Chapter 9

Pattern formation in subfields: formation of new organizing regions by cooperation of compartments

In the preceding section, evidence has been presented that the positional information in an (insect) embryo is generated by autocatalysis and lateral inhibition. Under the influence of such a morphogen gradient, a subdivision into defined groups of differently determined cells is possible. The final spatial structure is, of course, much more complex than what would be achievable by the interpretation of one (or two orthogonal) gradients. Further subdivisions are clearly necessary. A possibility consists in the formation of secondary gradients and their subsequent interpretation. For example, a primary gradient may specify the future limb area and a secondary gradient can then specify the finer details of the limb, such as the digits. Detailed experimental data about pattern formation in developmental subfields are available for the imaginal disks of holometabolous insects and of the limb field in vertebrates. It will be shown that many of these experiments are explicable under the assumption that the boundaries between patches of differently determined cells, determined under the influence of the primary gradient(s) become the organizing regions for the developmental control of subfields (Meinhardt, 1980). Since the boundaries of existing structures give rise to the new structures, the existing and the new structures have necessarily the correct spatial relationship to each other. This allows a very reliable finer subdivision of a developing embryo.

9.1 Imaginal disks, their fate maps and compartment borders

Epithelial structures such as eyes, antennas, wings, halteres or legs are generated from nests of cells, the so-called imaginal disks (see Gehring and Nöthiger, 1973).
Figure 9.1: The wing and its coordinate system. (a) The dorsal and ventral aspect of the wing. Below: the imaginal disk from which the corresponding adult structures are derived (after Bryant, 1975a; Garcia-Bellido et al., 1976). The border between the anterior (A) and posterior (P) compartments does not coincide with any morphologically recognizable structure while the dorsal (D) and ventral compartments form the corresponding wing surfaces as well as thoracic structures. (b-d) Model for the generation of the coordinate system. By cooperation of the A and the P compartment as well as of the D and the V compartment, two ridge-like morphogen profiles are generated (b,c). The symmetrical distributions are centred over the corresponding boundaries. The product of the A-P and D-V pattern has a cone-shaped distribution (d) which is appropriate to organize the proximodistal axis. Only those cells exposed at least to a low threshold concentration become imaginal disk cells. Cells exposed to a relative high concentration form the wing blade (e). The primary event is therefore the formation of the boundaries. The imaginal disk is formed, in a secondary event, from cells surrounding the intersection of the AP and DV boundary.

In *Drosophila*, the cells of the imaginal disks are almost completely determined before pupation begins, at the end of the third larval stage. Fragments transplanted directly into metamorphosing larvae differentiate according to their original position within their disk. This allows a fate map of the disk to be constructed. Fig. 9.1 shows a wing disk and some of the corresponding adult structures. In the leg disk, the leg primordia are arranged in concentric rings (Schubiger, 1968, see Fig.9.2). The outer rings form the more proximal structures such as thorax and coxa, while the inner rings form the more distal structures such as tarsus and claws. The leg attains its final shape by a telescope-like extension of the central (distal) part.

Two features of the spatial determination of imaginal disks appear to be a key element in the understanding of how subpatterns are formed: their progressive compartmentalization (Garcia-Bellido et al., 1973, 1976; Steiner, 1976, Crick and Lawrence, 1975) and the properties of pattern regulation (Schubiger, 1971; French et al., 1976, Bryant, 1978). In this chapter we will develop a model about how compartmentalization and the generation of positional information in a subpattern are linked with one another. How compartments themselves can be formed is discussed elsewhere (p. pagerefpage:compartment and chapter 14).

One of the earliest developmental decisions is the separation into anterior and posterior compartments (Garcia-Bellido et al., 1973, 1976; Steiner, 1976). It occurs during or shortly after blastoderm formation. A group of cells and all their progeny, once determined to form, for instance, the anterior part of the leg or
9.2 Regeneration, duplication and distal transformation

Fragments of imaginal disks cultivated in the abdominal cavity of adult flies are capable of substantial pattern regulation. Two major types have been observed: either the structures remaining in the fragment are duplicated, leading to a mirror-symmetrical pattern; or the missing structures are regenerated. As the rule, when a disk is fragmented into two portions, the two fragments show complementary behavior: one fragment duplicates itself while the other regenerates (Schubiger, 1971; van der Meer and Ouweneel, 1974; Bryant, 1975a,b). It has further proved possible to predict on the basis of its size and geometric position...
within a disk whether a given fragment will duplicate or regenerate. The borderline between the fragments which regenerate and those which duplicate does not coincide with any compartment border. For instance, a fragment containing constituents of only the anterior leg compartment can regenerate all the missing structures of the leg. This pattern regulation has been successfully described by the formal polar coordinate model of French, Bryant and Bryant (1976). In this model it is stipulated that two positional parameters are of relevance for pattern regulation: position along the proximodistal axis and circumferential position. Circumferential positional values (particular determined states) are assigned to structures around the circumference of a disk, arranged like the numbers of a clock face. Two rules are sufficient to describe how the pattern is regulated: (i) The shortest intercalation rule: Whenever a fragment of a disk is removed, wound closure causes those cells at the wound surface to find themselves close to unusual neighbors. Missing structures are regenerated by intercalary regeneration according to the shortest intercalation rule. Only those structures which are necessary to reform a continuum will be regenerated. Whenever more than half of the positional values are removed, shortest intercalation leads to duplication; otherwise regeneration will occur. (ii) The complete circle rule: Distal transformation and outgrowth occurs whenever all the circumferential positional values are present, forming a complete circle. Duplicated structures (in which more than half the positional values are missing) are therefore not expected to show distal transformation.

As mentioned above, the borderline between fragments which regenerate and fragments which duplicate does not coincide with any compartment border. Compartments are therefore not an element of the polar coordinate model. Compartments are, however, the primary developmental subdivision of a disk. It will be shown how the two concepts can be linked.

### 9.3 Pattern formation by cooperation of compartments

In principle, a subpattern can be generated by autocatalysis and lateral inhibition as described in the preceding chapters. However, after interpretation of a
primary gradient, sharp boundaries exist between the patches of differently determined cells ("compartments"). These boundaries open a new possibility for the generation of positional information. Let us assume two patches with a common boundary which cooperate for the production of a substance which acts as a morphogen. Due to the required cooperation, the synthesis of the morphogen is possible only at the boundary. A symmetrical, ridge-like morphogen distribution centered over the boundary will result. Three compartments - similar as three countries - meet each other only at one point. If three compartments (or two pairs of compartments) have to cooperate, morphogen production is possible only at the point where cells of all compartmental specifications are close to each other. The point of intersection of the compartment borders becomes the source region of the morphogen. By diffusion and decay, a cone-shaped morphogen distribution is formed with the highest concentration at the intersection of the compartment borders (Fig.9.2). The local concentration is a measure of the distance from the intersection and can be used as positional information in the proximodistal dimension. The most distal structures are formed at the intersection of the compartments and the interpretation of the cone-shaped morphogen distribution leads in a straightforward manner to the circular arrangement of structures. The same morphogen distribution can determine which cells form the disk and which form the larval ectoderm. Only those cells exposed to a concentration above a certain threshold would participate in disk formation; no separate positional information system is required. According to this view, an imaginal disk never exists without subdivisions. The formation of the borders necessarily precedes the formation of the disk. Since the boundaries are determined under the influence of the primary organizing gradients, the emerging disks naturally have the correct orientation in respect to the body axis. The handedness of each disk is also determined since three patches, touching each other at one point, are sufficient to determine handedness in an unequivocal way.

Molecular mechanisms for such a cooperation are easily constructed. For instance, each compartment may be responsible for a particular step in the synthesis of the morphogen or each compartment may produce a diffusible co-factor which is required for morphogen production. Fig.9.2 and Fig.9.4 have been calculated in this way. The positional information may be generated in two steps. By the cooperation of the A-P and of the D-V compartments two ridge-like distributions are formed which can supply positional information for the anteroposterior and the dorsoventral dimension. The symmetrical distributions can be interpreted differently in the corresponding compartments, leading for instance to the partially symmetrical pattern of the wing. The product of the two ridge-like distributions then assumes the cone-shaped distribution (Fig.9.1), organizing the proximodistal dimension.

As discussed below, several lines of experimental evidence indicate that interpretation of proximodistal positional information in disks proceeds in a stepwise, unidirectional manner, i.e. in the same way as in the early insect embryogenesis (Fig.8.7). A distal determination, once obtained under the influence of the local morphogen concentration, seems to be irreversible. The “complete circle rule”
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for distal transformation of French et al. (1976) which is difficult to interpret in molecular terms, is thus simplified to yield the straightforward mechanism of “cooperation of compartments”. The achievements of the complete circle rule, such as explanation of supernumerary appendages, remain valid, since demanding a complete circle is formally equivalent to requiring that cells of three or four sectors are close to each other.

The model links early compartmentalization and generation of positional information in the proximodistal dimension. Two stipulations are made: cooperation of compartments in the formation of a cone-shaped morphogen distribution, and response of the cells in a stepwise, unidirectional manner. Both assumptions are supported by experimental observations. It should be pointed out that the local morphogen concentration determines only, for instance, which leg segment a group of cell has to form. The fine structure within a segment is assumed to be generated by a different process (chapter 13).

9.4 Evidence for the cooperation of compartments in the generation of positional information

The most distal structures are formed at the intersections of the major compartment borders. The tip of the wing is determined at the location where the A-P and the D-V border cross each other (Fig.9.1). In the leg disk, the precise location of the D-V border at the center is not known. However, the most distal structures, the two claws, are located on both sides of the A-P border and the D-V border points in that direction (Fig.9.2). The most distal structures are not located trivially at the center of the disk, since the posterior compartment is smaller. This is also true in earlier stages; the posterior compartment is made up of about half as many founder cells as is the anterior compartment (Garcia-Bellido et al., 1973).

Distal transformation of leg fragments requires a close juxtaposition of all compartmental specifications. As can be seen from the experiments of Schubiger and Schubiger (1978) and Strub (1977a) the upper lateral quarter of a leg disk fragment (Fig.9.3f) does not regenerate the removed distal primordia (center of the disk). It does not contain the ventral compartment. Similarly, the lower medial quarter (Fig.9.3b) contains the anterior-dorsal compartment only marginally and shows a low frequency of distal transformation. In contrast, a fragment which contains cells of all compartmental specifications shows distal transformation very frequently (Fig.9.3d).

A complete set of circumferential structures is not required for distal transformation. Distal transformation of leg disks and of the wing disk is possible without an initial regeneration of all proximal structures around the circumference. Schubiger and Schubiger (1978), for instance, have found distal transformation in a fragment as shown in Fig.9.4d without a preceding circumferential regeneration of the missing proximal structures. An analogous observation has been made by Karlsson (1980) for the wing disk. Our model is consistent with these ex-
experimentally discovered violations of the complete circle rule, since only a close juxtaposition of all major compartments is required.

The capability of a fragment which originated exclusively from the anterior leg compartment to show distal regeneration seems to contradict the model. However, compartment borders can be reformed during the regeneration of fragments. According to Schubiger and Schubiger (1978), the distal transformation of an anterior fragment is always associated with the regeneration of structures of the posterior compartment. The formation of new positional information for the proximodistal dimension in such a fragment is assumed to be a two-step process. The first step is regeneration of parts of the missing compartment(s) (see Fig.12.6). The second step is formation of a new morphogen distribution, centered over the new intersection of compartment boundaries. (In the polar coordinate model, ability of an anterior leg fragment to regenerate the missing members of the major compartments is accounted for by the assumption of a non-uniform spacing of positional values, see French et al, 1976).

In the wing disk, the data as to what extent cells can change their compartmental specification after experimental interference are less clear. (Garcia-Bellido and Nöthiger, 1976; Szabad et al.,1979) The A-P border seems to be more rigidly fixed than the D-V border. Thus it would be expected that a wing fragment, if it is to undergo distal regeneration, must include the anteroposterior compartment border. The dorsoventral compartment border, since it can be respecified, would be of less importance. This is in agreement with the experimental observations of Karlsson (1980) and Wilcox and Smith (1980).

Small marginal fragments of a wing disk do not usually show distal transformation by themselves (Bryant, 1975a,b) because they contain at most cells of only two compartments. When two marginal wing fragments derived from opposite positions of the disk are joined together, they frequently show regeneration of the missing distal structures. (Haynie and Bryant, 1976) This would be expected since these fragments together, generally contain cells from all major compartments. The same is valid also for an outer-ring fragment of a disk. Strong distal transformation also occurs after dissociation and reaggregation of imaginal disks (Strub, 1977b). This is expected from the model since in this procedure many new compartmental confrontations and hence morphogen sources are created.

The initiation of cell death in cell-autonomous cell-lethal mutants can lead to a partial or complete duplication or triplication of the leg (Postlethwait, 1978, Rssel et al., 1977). According to the model, the primary event is a compartmental respecification. An explanation of how cell death can lead to a compartmental respecification is given below (see Fig.12.7). It is especially common that structures belonging to the posterior compartment are formed in an anterior environment. Respecification can lead to new intersections of compartmental boundaries and therefore to the establishment of additional morphogen maxima (Fig.9.4). An observation by Girton (1981) provides an especially convincing example of the connection between leg duplications and compartments. He found duplication and distally complete and incomplete triplications of legs (Fig.9.4). Drawing the structures of the triplicated legs at the level of bifurcation in the fate map reveals
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Figure 9.4: Heatshock-induced leg duplications and triplications (Girton, 1981; Bryant and Girton, 1980). In addition to the normal leg (N) a single (D) or a pair of legs (T1, T2) is formed. In the latter case, the pair can be distally complete (b) or incomplete (c) and the pair can be partially (b) or completely fused (photographs kindly supplied by J. Girton). Explanation in terms of the model: Due to the heat shock (and cell death), part of the anterior compartment of a normal disc (d) becomes reprogrammed to posterior (see Fig. 12.7). This can lead to a new intersection (e) and consequent formation of an additional leg with opposite handedness (A, P, V clockwise or counter clockwise, see arrows). If the patch of posterior cells arises in a non-marginal position, two new intersections can be formed (f). This would lead to a pair of additional legs, as shown in (b). The closer the two new intersections are, the more distal the separation of the pair of legs will be. If the patch is close to but does not touch the ventral compartment border (g), cooperation is restricted, the maximum morphogen concentration is not reached and a fused pair of distally incomplete legs will be formed (as shown in c). The model provides an explanation of Bateson's rule (Bateson, 1880) according to which the three limbs are formed in a plane (the new intersections are formed along the AD-AV border line) and the central limb has opposite handedness when compared with the two others (see arrows). (h-k) Computer calculations of the positional information created by intersections shown in (d-g).

that distally complete outgrowth occurs only if cells of the ventral compartment are present (Fig.9.5). All duplicated legs contain the A-P boundary. Figs.9.4 d-k show the expected locations of compartmental respecification and the resulting morphogen distributions.

Some of the observed duplications and triplications indicate clearly that no lateral inhibition is involved in this formation of new organizing regions. Two interactions can appear so close to each other that the resulting legs are fused over almost their entire length (Fig.9.4b). No indication can be found for competition or dominance of one leg over the other (in contrast, for instance, to the formation of new heads in hydra, Fig.6.2). Lateral inhibition is the antagonistic reaction necessary to localize autocatalysis and to suppresses the formation of identical structures in the surroundings. If an organizing region is formed by intersection of compartments, lateral inhibition is not required since the intersection is confined.
9.5. **STEPWISE UNIDIRECTIONAL INTERPRETATION**

Figure 9.5: Evidence that presence or absence of the ventral compartment is decisive as to whether distally complete transformations occur. Shown are the fatemaps of distally complete (a) and incomplete (b) tarsal triplications. Each curved line indicates the structures present at the base of a particular triplication (after Bryant and Girton, 1980). Distally complete legs contain cells of the ventral (V) compartment (a) while distally incomplete legs do not (b). In agreement with the model, an anteroposterior boundary is present in both types of legs. 1-8: tarsal bristle rows. The concentric rings 1-5: the five tarsal segments; thick black bars: approximate location of the two major compartment borders according to Girton and Russel (1981).

In the abdomen, which consists of structures lacking a proximodistal dimension, no compartments have yet been found. (Lawrence et al., 1978). According to the proposed view, subdivision into compartments is a pre-condition for the generation of structures with a proximodistal axis, for instance of wings and legs. Therefore it is not required in the abdomen. It could well be, however, that temporary A-P subdivisions occur in the abdominal segments also during the formation of segments (see chapter 14).

### 9.5 Evidence for a morphogen gradient and a stepwise unidirectional determination

Distal fragments of a disk do not regenerate proximal structures (Schubiger, 1971; van der Meer and Ouweneel, 1974). Distal to proximal segmental respecification does not occur even if proximal and distal fragments are confronted (Haynie and Schubiger, 1979; Strub, 1979). This is in agreement with the proposed model since distal determination, once obtained, is assumed to be irreversible. (This is in sharp contrast to the distal-proximal regeneration within, for example, a leg segment (see Fig.13.1) and emphasizes once more that different mechanisms are involved in these two types of pattern formation).

Mutations are to be expected in which positional information is changed, but not the response of cells to it. Such mutations should not be cell autonomous. For instance, a small clone of mutant cells in a wildtype environment is expected to develop like the wild type cells since they are exposed to the normal positional information. Two known mutations are of this type. *Drosophila* flies carrying the mutation *wingless* can duplicate the dorsal thorax but fail to form a wing blade. However, clones of *wingless* cells can and do participate in wing formation (Morata and Lawrence, 1977). According to the model, either compartmental-
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ization or production of the morphogen by the cooperation of compartments may be affected. This mutant further suggests that the system which generates positional information for the wing is different from that of the leg since formation of the leg is not affected.

Jürgens and Gateff (1979) have found duplication of legs in a temperature-sensitive mutant (mad) of Drosophila. The orientation of the additional legs indicates that in this mutant a second dorsal compartment is formed at the ventral side of the disk while the anteroposterior axis is not affected. Mosaic studies have revealed that both mutant and wild type cells participate in the formation of the duplicated leg. This implies that the positional information and not the response to an unaltered gradient is what is changed. Distally complete duplications can be induced by a pulse of high temperature, applied between 48h and 76h after egg deposition. As the rule, the later the pulse, the more proximal the point of bifurcation. In terms of the model, the disk is larger (or subdivided into more cells) at a later stage and the intersections can, therefore, have a greater distance from each other. This leads to less overlap between the two systems of positional information and therefore to a more complete separation of the two legs.

Distally incomplete structures can occur in triplication of legs (mentioned above) as well as in cockroach legs after an injury (see Fig.9.9). Since only the local concentration of the morphogen is interpreted, distally incomplete structures are expected if the normal maximum concentration is not reached. This can be caused by restricted collaboration of the compartments, e.g. if too few cells of a particular compartmental specification are available or if they are not in close enough proximity. The missing ventral compartment in distally incomplete leg triplication (Fig.9.4, 9.5) supports directly this view.

9.6 Expected mutations

The model predicts mutations in which proximodistal pattern formation is affected. The resulting pattern may be distally incomplete. In extreme cases, the whole disk may be missing. As mentioned, a mutation in which the generation of the signal (morphogen synthesis) but not the local response of the cells is affected would not be cell autonomous. That means, a clone of mutated cells in a wildtype environment will participate in normal pattern formation. However, if such a clone arises close to the intersection of compartments, the prediction is that pattern formation in the whole disk is altered in that only distally incomplete structures are formed, even by the wildtype cells. Further, the model predicts that several mutations exist with the same phenotype but that each mutation is specific for a particular compartment since the mutation effects a compartment-specific function in the cooperation. For instance, a clone has to arise in the posterior compartment and close to the intersection if a pattern alteration is to occur. Such genetic studies combined with DNA sequencing methods may allow one to trace the regulatory pathway of the morphogen responsible for proximodistal determination. Distal transformation of posterior leg fragments
9.7 Strategy for isolation of the morphogen

In a normal disk, the morphogen is produced presumably only in minute amounts rendering a biochemical characterization difficult. However, according to the model, a disaggregation and reaggregation of whole disks should lead to an tremendously increased morphogen production since then cells of all compartmental specifications become close to each other at many locations, not only at the natural intersection as in the intact disk. Comparison of electrophoretic patterns of normal and of reaggregated disk may reveal spots of changed intensity, pointing towards the morphogen. Some experimental evidence is already available that this strategy may be successful. Reaggregates of dissociated leg disks show distal transformation extreme frequently (Strub, 1977b).

9.8 Application to pattern regulation in insect legs

In hemimetabolous insects, the adult appendage emerges not in a unique metamorphosis from a disk but through a sequence of several moults. Between the moults, substantial pattern regulation is possible. The leg of cockroaches is a well-studied developmental system of these insects. Nothing is known about compartments in the cockroach leg, but as a working hypothesis we will assume an analogous compartmentalization as in Drosophila. The compartments would have a stripe-like shape along the tube-shaped ectoderm of the leg. Many features of pattern regulation can then be made understandable by the proposed cooperation of compartments.

The cockroach leg, if removed, is capable of complete regeneration. During closure of the wound, cells of all three compartments come into close contact and positional information is regenerated. All those cells which are exposed to a higher morphogen concentration than that corresponding to their own specifica-
Figure 9.7: Occurrence and failure of intercalary regeneration in systems controlled by positional information. (a) An amputated cockroach leg regenerates all missing parts. (b) However, grafting a mid-tibia (TI) onto a mid-femur (FE) stump does not lead to intercalary regeneration. The parts between the dashed lines remain missing (Bohn, 1970a). Thus, regrafting distal structures suppresses the formation of intervening parts. (c) Incomplete wound healing after cutting and regrafting can lead to distal transformation on both sites of the wound. Then, two additional tarsi (TA) are formed (French, 1976a) analogous to the additional posterior structures in an *Euscelis* egg (Fig. 8.2e). (d-k) Explanation in terms of the model: It is assumed that the morphogen gradient (positional information) causes the determination of the structures 1 - 5. (e) During normal growth, the local morphogen concentration decreases in most of the cells; the gradient is assumed to be unaffected by growth. Since interpretation proceeds unidirectionally, the cells remain stable in their respective states of differentiation. (f) After removal of distal parts and reformation of the positional information, most cells are exposed to a higher morphogen concentration and all structures (g) are formed. (h) If an intermediate section of leg is removed, the positional information that could lead to respecification of the structures at the wound is present only in the very terminal structures of the leg. Therefore, in most of the cells the positional information is lower than the level they were exposed to when the pattern was first established. Whether missing parts are replaced therefore depends on the extent of growth and the range of the morphogen. In this example (i), part of structure 2 becomes respecified to form 3 while structure 4 remains missing since the cells exposed to the appropriate morphogen concentration are already determined to form structure 5. (j) Analogously, no repair of a gap introduced by grafting surplus structures occurs since the morphogen concentration remains too low at the location of the gap. (k) If, however, such an operation triggers a new system of positional information, as would happen if, for instance, cells of all major compartments have come close together, two new distal structures will be determined by respecification. This corresponds to the experimental observation shown in (c).

Figure 9.8: formation of supernumerary limbs after contralateral grafting of a cockroach leg (Bohn, 1965). (a) the operation (b) resulting pattern after several molts. (c) explanation in terms of the model. Two new areas of confrontation of all three major compartments are created at the graft-host junction (squares), leading to two new limb fields.
Figure 9.9: Cutting a notch into the inner ventral (V) site of a cockroach leg (a) leads to outgrowth of additional leg-like structures. This result is very surprising if one assumes that juxtaposition of non-adjacent structures leads to an intercalation of missing structures only. (b) The same operation at the outer dorsal site (D) heals without much additional outgrowth (Bohn, 1965). (c) Explanation based on “cooperation of compartments”. A subdivision similar to that of the leg of *Drosophila* into the anterior (A), posterior (P) and into the much smaller ventral (V) compartment is assumed. After removal of tissue at the ventral side, cells of anterior, posterior and ventral specifications become quite close to each other (d). Cooperation is possible and outgrowth of symmetrical distal structures is expected on the basis of the proposed model. Since anterior and posterior cells could be separated by some ventral cells, the cooperation may be restricted and distally incomplete structures can be formed. After a similar incision at the dorsal site, ventral cells remain far away and cooperation of compartments is impossible.

One strange result of these experiments is that, although a stump regenerates a complete leg, regrafting a leg fragment onto a cut stump can prevent regeneration of structures present neither in the stump nor in the leg fragment. If, for instance, a leg fragment consisting of a leg from mid tibia on is grafted onto a stump cut off in mid femur, the distal femur and proximal tibia will not be regenerated (Fig.9.7). As explained in detail in Fig.9.7, as growth occurs, positional information in most of the cells becomes lower than it was at the time of cell determination. After removal of an intermediate section of the leg, reprogramming is, as a rule, impossible. This failure of gap repair is typical for systems controlled by unidirectional interpretation of positional information, since the cells change their determination only when exposed to a higher morphogen concentration, not when confronted with an unnatural neighbour (see also Fig.8.3). If, however, in such a regraft operation, wound healing is not perfect, two additional distal structures are generated (Fig.9.7c). In terms of the model, as the ectodermal tube closes, cells of all compartments come into close contact and new centers of morphogen production are formed at both wound surfaces. This will lead to the reprogramming of the leg both proximal and distal to the wound such that symmetrical distal structures will be generated at the wound site (Fig.9.7k).

After amputating a limb and reimplanting it either in a rotated position or onto a contralateral stump, supernumerary legs are formed (Bart, 1971a,b; Bohn, 1972; French, 1976b). On the basis of his experiments, Bart (1971a)
has already proposed that new morphogenetic centers arise whenever different sides (anterior-posterior, dorsal-ventral) meet. Such an operation creates new intersections between compartments (Fig.9.8). Their numbers and the handedness of the additional limbs will be discussed in detail for the amphibian limb system (see Fig.10.6). Cutting a V-shaped notch into the ventral (internal) side of a leg leads preferentially to outgrowth of a symmetrical leg which is distally more or less complete (Fig.9.9). This outgrowth is very striking if one expects only an intercalation between mismatching neighbors on the shortest possible route. A similar injury at the dorsal side heals with little, if any, outgrowth. Similarly, artificially produced double ventral legs regenerate to a large extent while double dorsal legs do not (Bohn, 1965). The model predicts this asymmetric behavior. The explanation is given in Fig.9.9.

In conclusion, very different and seemingly unrelated observations can be explained under the assumption that the borders between compartments are used to create new coordinate systems for the finer subdivisions of the developing organism. A still hypothetical morphogenetic substance produced by the cooperation of compartments, provides positional information about the distance of the cells from the border(s). Changes of the geometrical arrangement of the compartments, caused either by surgical interference or by a cell-internal switch in the compartmental specification can lead to new intersections of borders and therefore to the formation of additional structures.
Chapter 10

Boundaries between differently determined cells control pattern formation in the limb of vertebrates

10.1 Polarising and competent zones in the amphibian limbs

Cooperation of compartments has been suggested above as a straightforward mechanism to organized subfields in insects. In vertebrates, one of the best investigated systems of pattern formation in a developmental subfield is the limb (for review see Hinchliffe and Johnson, 1980). Grafting experiments reveal that cooperation of differently determined tissues is also involved in limb organization. In amphibians, two zones are important for limb development: the competent zone and the more posteriorly located polarizing zone (Harrison, 1921; Slack, 1976; 1977a,b). The future limb is formed almost exclusively from the competent zone. However, these competent cells can only form a limb when juxtaposed with cells of the polarizing zone. Grafting polarizing tissue anterior to the future limb area leads to the outgrowth of symmetrical limbs in which the posterior digits are duplicated (P-A-P-pattern, Fig.10.1). In some cases, two almost complete hands are formed while in others, some anterior digits are missing. From these experiments, Slack concluded that an interaction between the two zones is required to generate the positional information that controls anterior-posterior determination of the future limb. By implanting polarizing tissue from a salamander into an axolotl Slack (1976) has provided direct evidence that only the competent tissue responds: The reduplicated leg consists entirely of axolotl type structures. After grafting tissue of the prospective limb area into a more posterior region of the flank, an additional limb with reversed polarity can result (Fig.10.2). In terms of Slack’s model, after such grafting, competent tissue is - in contrast to its normal position - located posterior to the polarizing zone leading to a reversed
CHAPTER 10. VERTEBRATE LIMBS

Figure 10.1: Juxtaposition of two zones is necessary for the anteroposterior (A-P) organization of a limb. (a-c) Graft experiment by Slack (1976) with axolotls. (a) Donor embryo and the location of the polarizing (xx) and competent zone (==) as determined by this and by the experiment described in Fig. 10.2. (b) Host embryo after implantation of the polarizing tissue anterior to the competent zone. (c) The resulting symmetrical (right) limb. The posterior digits are always present and duplicated while some anterior digits may be missing. (d,e) Bones of symmetrical limbs and of a normal limb (f) (after Slack, 1977a,b). (g,h) Model: In the normal situation, confrontation of polarizing and competent tissue leads to a symmetrical morphogen distribution, centered over the common boundary. Only the competent cells can respond and this leads to a monotonic gradient (solid line). After a graft as shown in (a,b) the competent zone is confronted with polarizing tissue both at its anterior and posterior margins, leading to a symmetrical morphogen distribution (h) and therefore to a symmetrical arrangement of skeletal elements. Depending on the overlap of the two gradients, low concentrations and therefore anterior digits could be absent.

gradient and therefore to a reversed limb. It is easy to see how the two zones - the prerequisites for the limb formation - might be formed during development. Interpretation of a primary anteroposterior gradient in the embryo could lead to several belt-shaped patches of differently determined tissues. Two of these could be the polarizing and the competent zones.

Independent of the question of how a limb field is formed, these experiments provide an important indication concerning the origin of polarity in tissues of higher organisms. Harrison (1921) believed that overall polarity results from the superposition of many small polar structures e.g. polar cells. In an experiments such as shown in Fig.10.2 the graft is only transposed, not rotated; the A-P orientation of the graft remains unchanged. Thus, the experimentally observed change in the polarity of the outgrowing limb was regarded as very striking. In fact, the experiment provides strong evidence that polarity does not result from many small polar substructures but from the slope of graded distributions of morphogenetic substances.
10.2 Generation of polar structures by cooperative interaction between two differently determined patches of cells

The need for cooperation between the two zones is reminiscent of the mechanism proposed above for pattern formation in imaginal disks. However, a major difference remains. The vertebrate limb is a structure with clear anteroposterior polarity while the fatemap of the leg disk of Drosophila shows that the elements are circularly arranged (see Fig. 9.2). As has been shown above, cooperation of two zones leads to a symmetrical morphogen distribution, centered over the common boundary. Therefore each zone contains one of the two slopes of the morphogen ridge. If only one of the two zones is able to respond, the cells of this zone are exposed to an exponential gradient. The end result is a polar instead of a symmetric structure (Fig. 10.1).

10.3 Two intersecting boundaries are required to determine a limb field

The common border of the polarizing and competent zone would have a long extension in the dorsoventral direction. The morphogen distribution in the competent cells resulting from the cooperative interaction is high along the whole posterior boundary of the competent zone and low at its anterior side. This morphogen distribution is therefore only able to organize the anteroposterior axis of the limb. The dorsoventral position of the outgrowing limb remains to be deter-
CHAPTER 10. VERTEBRATE LIMBS

Figure 10.3: Steps in the formation of the limb field. (a) A primary anteroposterior gradient can serve to subdivide an embryo into bands of distinct determinations (see Fig 11.5 or 14.5). Among these are the polarizing (posterior, P, crossed) and the competent (anterior, A, blanc) zones. (b) To locate the position of the outgrowing limb a global dorsoventral subdivision of the embryo is required. This leads to the D (==) and V (blanc) stripes. The area around the intersection (o) of the A-P and D-V border (framed in a and b; c) defines the position of the limb field. Cooperation of the A and P and the D and V tissues leads to a symmetrical AP and DV-morphogen distribution. Since only the competent cells respond, the anteroposterior pattern is polar (see Fig. 10.1). The positional information generated in this way (indicated by the triangle) is a measure for the distance of a cell from the borders. The dotted line marks the expected position of the apical ectodermal ridge (AER) on the D-V border in A (competent) tissue. (d) Geometry of the A-P and D-V-stripes in an outgrowing right limb bud, viewed from a posterior-dorsal position.

Regeneration and formation of new limb fields after experimental manipulations

It is not yet possible to test such a model at the biochemical level. Support is provided by the demonstration that the model correctly predicts the altered pattern resulting from certain experimental interferences.

Some amphibians regenerate amputated distal parts of a limb or form super-
10.5. REGENERATION OF SYMMETRICAL LIMBS

Figure 10.4: Regeneration of double posterior (PP) and failure of regeneration of double anterior forelimbs. (a,b) Surgical production of symmetrical PP and AA limbs by reciprocal exchange of anterior and posterior halves of the forelimbs of Axolotl with subsequent removal of the distal parts (b). (c) Result: Symmetrical limb regenerated by a PP stump. The most anterior digit 1 is missing, 2-4 are duplicated (after Holder et al., 1980). An AA stump shows little or no regeneration. (d-f) Wound closure in a normal, in a PP- or in an AA-leg leads to one (d), two (e) or no (f) intersections (circles) of the two borders. Either a normal, a PP leg (as in c) or no leg is expected to regenerate. The triangles indicate schematically the A-P and D-V morphogen profiles, the dots indicate the position of the AER.

numerary limbs after other experimental interferences. According to the model, a new limb field is formed if experimental interference leads to a new intersection of the two boundaries. Under this assumption, regeneration of a limb indicates that the limb does not consist entirely of competent tissue but that at least a small stripe of polarizing tissue is carried along with the outgrowing limb. After truncation of a limb and closure of the wound, a new intersection can be formed, which enables reformation of the limb's removed parts.

10.5 Presence and absence of regeneration of experimentally produced symmetrical limbs

A critical test of any model for limb development is whether it can account for the very striking differences in the regeneration capability of different types of symmetrical limbs. A symmetrical limb consisting of two posterior halves (PP-limb) regenerates a PP leg (Slack and Savage, 1978a,b). In contrast, a limb consisting of two anterior halves (AA) shows little, if any, regeneration (Stocum, 1978). These results are easy to understand in light of the proposed mechanism. Clearly, a PP-limb has two strips of polarizing tissue, one on each site (Fig.10.1; 10.4). This leads to two intersections of all boundaries and hence to a symmetrical P-A-P pattern with some of the most anterior structures missing. After removal of distal parts of such a P-P-leg, two intersections of the two boundaries are re-
formed and therefore the original symmetrical P-A-P pattern is re-established. The formation of the second posterior side is, if the two intersections are sufficiently separated, connected with the formation of a second parallel aligned proximodistal axis. This shows the interdependence of the two axes.

The failure of experimentally produced double anterior (AA) half limbs to regenerate is also described correctly by the cooperation model. An AA limb contains no P strip on either side (Fig.10.4). Only a D-V border is present which is neither sufficient to trigger a proximodistal outgrowth nor an A-P organisation. The different behavior of AA and PP limbs results from the asymmetry of the leg, which contains mainly competent (A) and only a small stripe of polarizing (P) tissue although both tissue types have to be present if distal outgrowth is to occur. Other observations on the regeneration of AA or PP legs are less well understood. The frequency of regeneration depends critically on the time between the surgical production of a symmetrical limb and the removal of its distal part (which eventually induces regeneration). The shorter this time, the higher the frequency of regeneration (Stocum, 1978; Tank and Holder, 1978; Bryant and Baca, 1978). In contrast to upper arm AA-stumps, lower arm or leg AA-stumps regenerate almost as well as PP-stumps (Stocum, 1978; Krasner and Bryant, 1980).

10.6 Formation of supernumerary limbs after rotation or contralateral grafting experiments

It has been known for many years (Bryant and Iten, 1976) that cutting an amphibian limb and regrafting it after 180° rotation can lead to outgrowth of supernumerary limbs. The same happens after grafting a limb tip onto a contralateral
Figure 10.6: Explanation of supernumerary limb formation. (a) After 180° rotation, A and P as well as D and V tissue become juxtaposed at the host-graft junction. (b,c) To visualize the arrangement of tissue with A (blank), P (xxxx), D (—) and V (blank) specificity, the leg cylinder (see Fig 10.3d) has been unrolled. Cells at the right and left margin of the figure are, in reality, neighbors. Condition for a limb field is a DV border in an A-area (dashed/blank border), flanked by posterior (crossed) tissue. As in Fig 10.3, a limb field is indicated by circles, triangles and dots. (b) In a non-operated limb, the A, P, V and D specificities are arranged in stripes, oriented proximodistally. No intersections occur. (c) Arrangement after 180° rotation of the graft (top): 6 intersections occur, two can lead to normal limbs (triangles). The other 4 can lead to symmetrical double posterior (PP) legs, indicated by the M-shaped morphogen distribution. (d-f) Contralateral grafts leads either to a DV (d, e) or an AP-inversion (f). After both types of contralateral transplantation only two normal intersections are formed (no PP-legs) and the supernumerary legs have the handedness of the stump (arrows).

In Fig.10.5, examples of the observed results are shown. It is difficult to imagine offhand which and how many intersections are formed by a particular operation. In Fig.10.6, the leg cylinder has been unrolled and the location of the borders at the graft-host junctions are shown. It can be seen that in all these operations, at least two new intersections of the two borders are created, enabling formation of two supernumerary limbs. Fig.10.6 shows further that the model predicts a very striking difference between a 180° rotation and a contralateral graft of a limb tip. After 180° rotations, very complex intersections occur which depend on the precise alignment between host and graft. Especially, only after 180° rotation formation of symmetrical (PP) limbs is possible. Such complex supernumeraries have been observed by Maden (1980, 1981a,b). Fig.10.5a shows an example. The symmetrical PP-limb can be recognized easily by its bifurcated central digit. The situation is very different after transplantation of a limb blastema to the contralateral side. Depending on the graft, either the A-P or the D-V axis is inverted. According to the model, this leads to two normal but never to PP-type intersections (Fig.10.6). These predictions are fully supported by Maden’s experimental observation. The model leads to an even more precise
prediction: An A-P confrontation should lead to one supernumerary limb derived from the host, the other from the graft. In contrast, after a D-V confrontation, host and stump tissue should contribute to each supernumerary limb. Either the dorsal side is derived from the host and the ventral site from the graft or vice versa (Fig. 10.6d,c). Whether this prediction is true has to wait for further experimental investigations.

The experiments of Maden (1981a) show further a substantial variability in the frequency of supernumerary outgrowth between different amphibian species. Even if the same operation is made repetitively on the same species, the resulting pattern cannot be predicted with certainty. Only a probability for the formation of one or two supernumeraries can be given. The intersection of the boundaries appears therefore to be a prerequisite for a distal outgrowth but other factors such as blood supply or innervations must be involved in the decision whether it occurs or not.

10.7 Pattern formation in the chicken limb bud

Many experimental data are available concerning the developmental control of the chicken limb bud, revealing both differences and similarities to that of the amphibian limb. The most obvious difference is that after truncation, an amphibian limb regenerates while a chicken wing bud does not, unless an apical ectodermal ridge (AER) is transplanted onto the stump. The AER is a thickening of the ectoderm on the wing bud oriented parallel to the anteroposterior axis of the embryo.

A second area important for the pattern formation in the bud is the “zone of polarizing activity” (ZPA), discovered by Gasseling and Saunders (1964). The ZPA is a nest of mesodermal cells located at the posterior margin of the bud. It does not contribute to the limb proper but instead it develops as the ”posterior necrotic zone”. Upon transplantation into a more anterior position, the ZPA can induce a symmetrical anteroposterior pattern and a second proximodistal axis. The grafted ZPA establishes the posterior side of the additional limb structures analogously to the situation observed after transplantation of polarizing tissue in amphibians (Fig.10.1; 10.2). On the basis of its location and orientation, it is tempting to identify the AER with the D-V boundary as discussed above. This view is supported by the observation that in dissociation-reaggregation experiments, the ectodermal hull determines the dorsoventral axis of the leg (MacCabe et al., 1973, 1974). However, transplantation of early limb bud mesoderm under an ectopic ectoderm can induce an AER in the ectoderm (Kieny, 1960), showing that the primary DV organization also takes place in the mesoderm. The ZPA, controlling the anteroposterior axis, is, as mentioned, of mesodermal origin. The ZPA is only effective when transplanted close to an AER (Wolpert et al., 1975). Therefore, similarly as the situation in amphibians, an intersection of an AP border (the ZPA in the mesoderm) and a DV border (the AER in the ectoderm) seems to be required for generation of a limb field. AER and ZPA appear to be specialized tissues which can be formed only during an early em-
10.7. CHICKEN LIMB BUD

Figure 10.7: Determination of the anteroposterior pattern in the chicken wing. (a) The chicken embryo with the wing bud between somite 16 and 19. (b) A wing bud at stage 23 (drawn after Hinchcliffe and Johnson, 1980) showing the Apical Ectodermal Ridge (AER). (c) Normal pattern of a wing. (d-h) ZPA-graft experiments (based on Wolpert and Hornbruch, 1981; Tickle et al., 1975; Summerbell, 1974a): the operations (top), the result (center) and the explanation on the basis of a gradient model. (d) Graft of a ZPA to the anterior side leads to symmetrical development. The ZPA is assumed to be the source of the morphogen. (e) Implantation of the ZPA into the center can lead to a complete limb pattern in the anterior part and an incomplete symmetrical pattern in the posterior part. (f) Two ZPA’s grafted into the center and into an anterior position can lead to the digit pattern 434 in the anterior part. This result argues strongly against an intercalation of structures since the anterior digit 2 is not formed. (g) For the action of the ZPA, close contact with the AER is required. After removal of the anterior part of the AER, implantation of a ZPA does not change the pattern. (h) Graft of a ZPA outside the proper limb field (opposite somite 15). Most of the cells exposed to the high morphogen concentration are incompetent, only digit 2 is duplicated, indicating that pattern formation depends on a long range signal and not on a chain of inductions between neighboring cells.

bryonic stage. The signal for their formation at the appropriate location results presumably from such D-V and P-A juxtaposition. If a limb bud is truncated at a later stage, D and V tissue again comes into contact during wound closure but at that time, an AER can no longer be induced and therefore, the truncated bud fails to regenerate.

Wolpert and coworkers (Tickle et al., 1975; Wolpert et al., 1975; Summerbell, 1979) have shown that the pattern formed after ZPA transplantation can be explained under the assumption that the implanted ZPA acts as a local morphogen source (Fig.10.7). Implantation of a ZPA for a limited period of time indicates that the response of the cell is completely analogous to that deduced from the insect experiments (see Fig.8.7). The cells are “promoted” stepwise and unidirectionally towards a more posterior determination until the actual determination corresponds to the local morphogen concentration. After removal of the morphogen source (ZPA) the cells remain stable in state of determination they have achieved. This mode of interpretation resolves a long lasting controversy (Saunders, 1977) about whether the ZPA is involved at all in normal pattern formation. It has been found experimentally that the ZPA can be removed relatively early without preventing normal development (Fallon and Crosby, 1975).
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The ZPA appears to be superfluous. But at the same time, the ZPA can induce additional structures as mentioned above. In terms of the model, the unidirectional interpretation of the anteroposterior gradient is completed relatively early. After removal of the source, the cells remain stable in the once achieved state of determination. Nevertheless, after implantation into a more anterior position, the increased morphogen concentration is able to reprogram the cells from their more anterior to a more posterior determination state. After very early excision of the ZPA, it is expected that the new juxtaposition of P and A tissues leads to regeneration of the ZPA and therefore also to normal development. Therefore, normal development can follow after ZPA removal in any case.

Tickle (1981) determined how many ZPA cells are required to induce additional digits. About 35 cells are sufficient to induce an additional digit 2 (the most anterior one, demanding the lowest morphogen concentration) and 100 cells can induce the complete sequence. These numbers are surprisingly small. However, if cooperation is involved, it is expected that only the marginal cells which are in contact with the AER contribute to morphogen production. If the number of cells is small, almost every cell has contact and contributes. Another important piece of information can be deduced from this result. Induction of the anteroposterior axis in the vertebrate limb is not a self-amplifying process infecting surrounding cells. It is not characterized by a clear threshold and an all-or-nothing result as observed in determination of the anteroposterior axis of an insect embryo (see Fig. 8.1 and 8.4) or in the induction of new heads in hydra (Fig. 6.2). For generation of the primary embryonic gradient, we have had to assume autocatalytic mechanisms. In contrast, if the morphogen gradient were generated by cooperation of cells a graded (not a switch-like) quantitative relationship between number of cooperating cells and morphogenetic level would be expected. This suggests a strategy for isolation of the morphogen. Creating a close contact between many AER and ZPA cells, for instance, by disaggregation and common tissue culture, should increase the morphogen production dramatically.

10.8 Relation to the polar coordinate model

The proposed mechanism provides a molecular basis for the complete circle rule proposed by French, Bryant and Bryant (1976, see p. ). They stipulated that distal outgrowth occurs whenever a complete set of 12 circumferential positional values are close to each other. Their choice of 12 values was somewhat arbitrary. According to a revised version (Bryant, French and Bryant, 1981) distalization occurs locally whenever the positional values are complete in a particular area of the circumference. If three or four values had been stipulated instead, the two models would have similarities, since demanding a complete set of four quadrants is equivalent to requiring an intersection of two borders. Both models therefore make the same predictions about the handedness of supernumerary limbs since handedness is independent of whether 3, 4 or 12 positional values are assumed. And both models predict that, after a contralateral graft, both supernumeraries should be of the stump handedness. Despite these similarities, the models make
different predictions. According to the model presented above, the morphogen gradients are set up and distal outgrowth occurs whenever an intersection between an A-P and D-V border is present. In contrast to the assumption of the complete circle rule, distal outgrowth is expected to be independent of the limb’s fine structure which results from the interpretation of these gradients. The experiment mentioned above in which polarizing tissue is grafted into a more anterior position (Fig.10.1) should illustrate the different predictions. According to the proposed cooperation model, two intersections of all borders appear close to each other. This leads to two parallel outgrowing proximodistal axes and a symmetrical P-A-P pattern. In agreement with the experimental observation, outgrowth is expected despite the fact that some of the most anterior structures are missing due to overlap of the two resulting morphogen distributions. In contrast, the complete circle rule would predict that missing anterior structures would lead to an incomplete proximodistal outgrowth at best. For ipsilateral 180° rotation the proposed model predicts the observed complex supernumeraries, which was unexpected on the basis of the polar coordinate model. Further, the model presented links the regulatory properties of developing appendages with primary pattern formation while, on the polar coordinate model, it remains an open question as to how the circumferential positional values are generated in the first place, how the handedness of a limb is determined and how a limb obtains its correct position and orientation with respect to the body axes. Nevertheless, the model of French, Bryant and Bryant served to stimulate focussed experimentation which contributed substantially to our present knowledge. It has narrowed the range of possible molecular mechanisms since most of the model’s predictions have proved to be correct and it helped in developing the model discussed above.

10.9 Boundaries in other types of embryonal induction

The creation of a new coordinate system by “cooperation of compartments” is presumably not restricted to appendages. For instance, the diencephalon, the central structure of the forebrain, organizes the adjacent optic tectum (Chung and Cooke, 1975). Normally, the diencephalon is located anteriorly to the tectum. Operations in which diencephalic tissue becomes located at a posterior position lead to a polarity reversal of the tectum as visualized by a reversal of the retinotectal connections. This result is completely analogous to the polarity reversal of limbs after grafting of competent and polarizing tissue (Fig.10.1, 10.2).

Recent rotation experiments with the eye primordium of *Xenopus* have revealed that the eye has a stable anteroposterior polarity even at the earliest state in which the rudiment can be detected (Gaze et al., 1979). The eye results from an inductive interaction between an outgrowing part of the forebrain and the ectoderm. Extending the model developed above, the position of the outgrowth is presumably determined by a hidden boundary (as is the case with insect legs). The determination of “anterior” and “posterior” precedes that of the eye itself. Therefore, a non-polarized eye cannot exist just like an imaginal disk cannot be formed without a preceding subdivision into compartments.
The same mechanism - cooperation of “compartments” - is used in so distantly related organisms such as insects and vertebrates to organize the substructures. It is, therefore, a very general mechanism. It assures that the newly formed structures have the correct position and orientation in relation to the parts already existent in the developing embryo.