THE EFFECTS OF 5-BROMODEOXYURIDINE AND 5-FLUORODEOXYURIDINE ON DIFFERENTIATION AND METAMORPHOSIS IN *XENOPUS LAEVIS* TADPOLES

Caroline Anne Christie

A Dissertation Submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg
for the Degree of Master of Science

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I hereby declare that this project is my own work and
that it has not been submitted to any other university

C. A. Christie

ABSTRACT

The effects of 5-FUdR and 5-BUdR on differentiation and metamorphosis in Xenopus laevis tadpoles were studied. In particular, a detailed study was made of the effects of 5-FUdR on cellular patterning and tissue differentiation during hindlimb development. Xenopus laevis tadpoles grown in solutions of 5-FUdR and 5-BUdR demonstrated hindlimb deformities, which were analysed by staining for cartilage visibility. Furthermore a comparison of the uptake of exogenous radioactive thymidine in the presence and absence of 5-FUdR by Xenopus laevis tadpoles showed that 5-FUdR depressed exogenous thymidine uptake.

A buoyant density gradient analysis of 5-BUdR-substituted DNA was undertaken using the analytical ultracentrifuge. The resulting ultraviolet absorption photographs showed bands of heavy and normal DNA.

A model is proposed to explain the patterning of the Xenopus laevis hindlimb in the presence or absence of 5-FUdR.

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1. INTRODUCTION

1.1 Aim

The aim of this project was to study hindlimb development during the metamorphosis of Xenopus laevis tadpoles. The aspects of hindlimb development focussed on were cell differentiation and cell patterning. Cell differentiation is affected by 5-BUdR (5-bromo-deoxyuridine) which replaces thymidine in the DNA molecule. 5-FUdR (5-fluorodeoxyuridine) prevents the formation of thymidine, which thus prevents DNA replication, preventing cell division from taking place. These two drugs were therefore used to gain further insight into cell differentiation and cell patterning of the Xenopus laevis hindlimb during metamorphosis.

1.2 Metamorphosis and its relation to hindlimb development in the frog

The limbs of amphibian tadpoles develop during the process of metamorphosis. This is the process which involves the transition of the tadpole larva to the adult animal. In addition to the development of the limbs, the process of metamorphosis also involves other anatomical and physical changes to suit the frog's adult mode of life. Some of the more obvious external changes are : the loss of the tail, the widening of the mouth, a change of the skin to the adult pattern (Brown 1970). Furthermore, internal gills are replaced with lungs, pronephros with mesonephros, urea cycle enzymes are induced in the liver (Cohen 1970), histological changes are induced in the pancreas,and the oxygen affinity of the haemoglobin is changed (Grisswold and Miller 1977). Xenopus laevis is unusual in that the adult animal remains aquatic. However, this is a secondary adaptation and the animal still undergoes the typical anuran pattern of metamorphosis outlined above.

1.3 Development of the hindlimb of Xenopus laevis

As indicated above, one of the externally visible metamorphic changes which occurs during metamorphosis is the development of the limbs. The hindlimb develops first, beginning at stage 43 in Xenopus laevis, whereas the forelimb only starts to develop from stage 46.

(All stages referred to in the text will be Nieuwkoop and Faber 1967 stages and will subsequently be referred to as NF stage.)

1.3.1. Detailed description of hindlimb development in Xenopus laevis

The first trace of limb development may be found in the lateral plate mesoderm. The somatic layer of the lateral plate becomes thickened just underneath its upper edge. The cells of this thickening soon lose their epithelial connections and are transformed into a mass of mesenchyme without the somatic layer having lost its continuity. The mesenchyme accumulates between the remaining lateral plate epithelium and the epidermis soon becomes attached to the inner surface of the epithelium (see Figure 1.1). The epidermis over the mesenchyme mass becomes slightly thickened and bulges outward (see Figure 1.2). In the regions where the fore and hindlimbs are to develop, the protrusion consisting of a thickened epithelial covering and of an internal mass of densely packed mesenchyme increases in size and becomes the limb bud. Figure 1.1 shows the mesenchyme accumulating under the lateral plate epithelium while Figure 1.2 shows the formation of the protrusion.

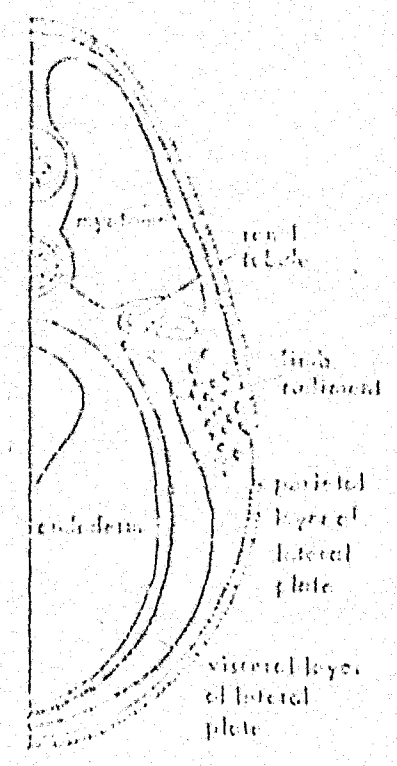


Figure 1.1 *Drawing showing the accumulation of the mesenchyme under the lateral plate epithelium (from Balinsky 1951)*

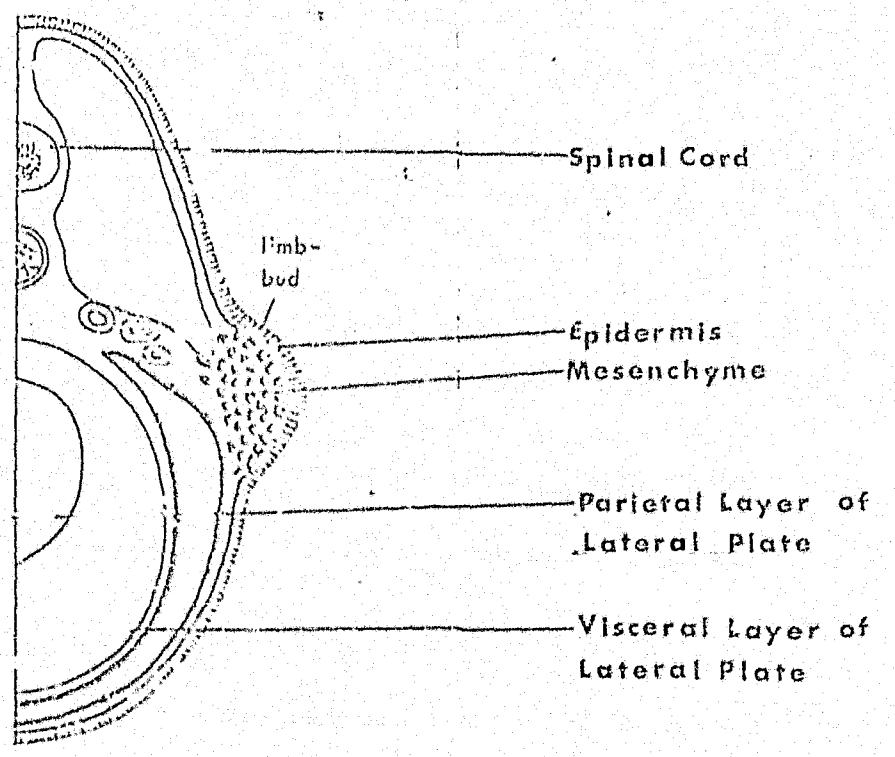


Figure 1.2 *Drawing showing the protrusion of the mesenchyme and the thickening of the overlying epidermis to form a limb bud (from Balinsky 1981)*

For a detailed analysis of the tadpole development during the metamorphosis of Xenopus laevis refer to Appendix A. Below follows a detailed description of the subsequent development of the hindlimb as described in Nieuwkoop and Faber (1967). The mesenchyme cells proceed with the formation of cartilage. The cells first clump together (although not very clearly described by D.R. Reuth in the above book; this seems to be what Reuth refers to as "indicated" cartilage formation). The aggregated mesenchyme cells then secrete "chondromucoproteins" and the aggregates i.e. clumps of cartilage cells, are noted; this stage is the procartilage stage. Meanwhile the extracellular matrix has now been laid down separating the chondroblasts and this association of cells and extracellular matrix is known as cartilage. The process during which it is formed is called chondrification. The cartilage is slowly replaced by bone from the outer radius inwards and this process is called perichondral ossification. This development is summarised in table 1.1 and discussed stage for stage on pages 4 to 9.

NF Stages 43 - 47

At NF stage 43 the hindlimb bud is recognisable for the first time as a slight concentration of mesenchyme cells, dorsal and lateral to the anal tube, under the epidermis which thickens over them. This is the stage shown in detail in Figure 1.2. The mesenchyme then becomes concentrated directly under the epidermis in NF stages 44 and 45. By NF stage 46 the limb rudiments are represented by clearly defined masses of mesenchyme. Up to stage 47 mesenchyme continues to migrate from the lateral plate mesoderm (Fig. 1.1), thereafter it condenses under the epidermis, which thickens over it and becomes a double layer of cells (Fig. 1.2). Subsequent to all this, growth takes place by mitosis of existing mesenchyme cells. The external location of the hindlimb bud in stage 43 is indicated below. (The hindlimb is not seen externally at this stage.) All sketches taken from Nieuwkoop and Faber (1967).

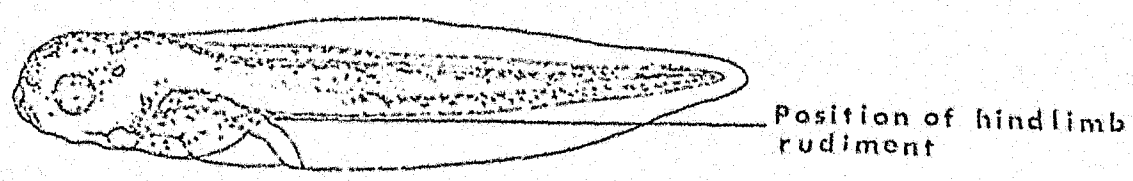


Figure 1.3 *Lateral view of Stage 43 Xenopus laevis tadpole showing the location of the hindlimb bud.*

NF Stage 48

During this stage the hindlimb bud is visible for the first time as shown in Figure 1.4 below.

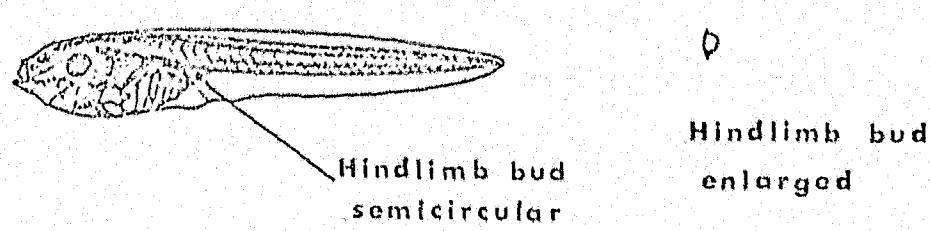


Figure 1.4 *Lateral view of Stage 48 tadpole showing hindlimb bud*

NF Stage 49

During this stage the limb bud increases in size and becomes vascularised.

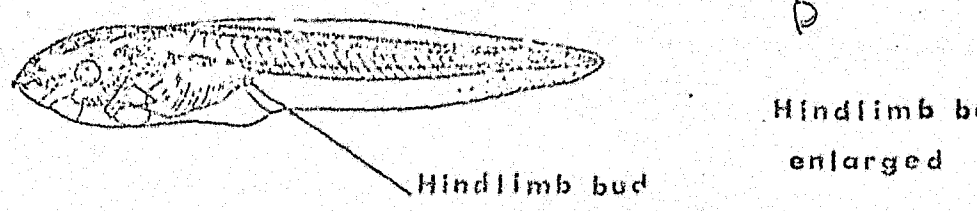


Figure 1.5 *Lateral view of Stage 49 tadpole showing the location of the hindlimb bud*

NF Stage 50

The future pelvic girdle now becomes "indicated" by a mass of mesenchyme at the base of the limb bud. This stage 50 limb bud differs externally from the stage 49 bud by a constriction at the base. The bud begins to elongate distally.

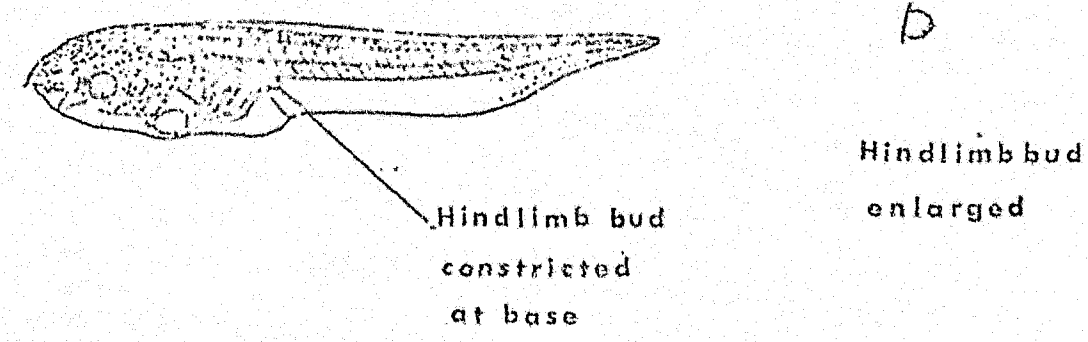


Figure 1.6 *Lateral view of Stage 50 tadpole*

NF Stage 51

At this stage the limb bud becomes innervated. The femur is "indicated". The bud is distally elongated and referred to as the "cone" stage. During this stage the mesenchyme begins to condense preliminary to cartilage formation.

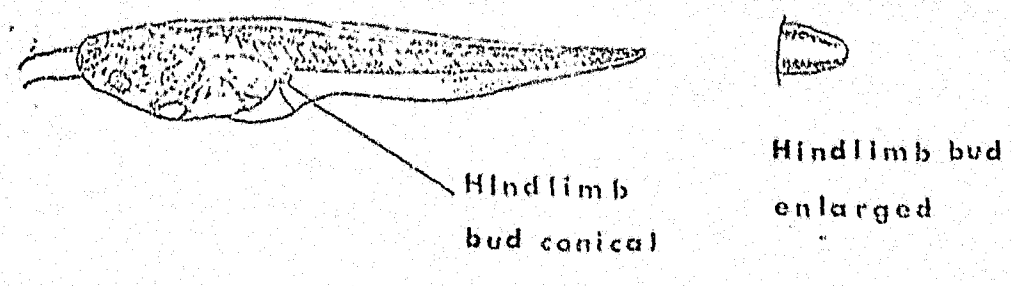


Figure 1.7 *Lateral view of Stage 51 tadpole*

89

NF Stage 52

At this stage the femur is procartilage and the tibio-fibula is "indicated". The bud is externally recognisable by a constriction in the ankle region. We refer to this stage as the "paddle stage".

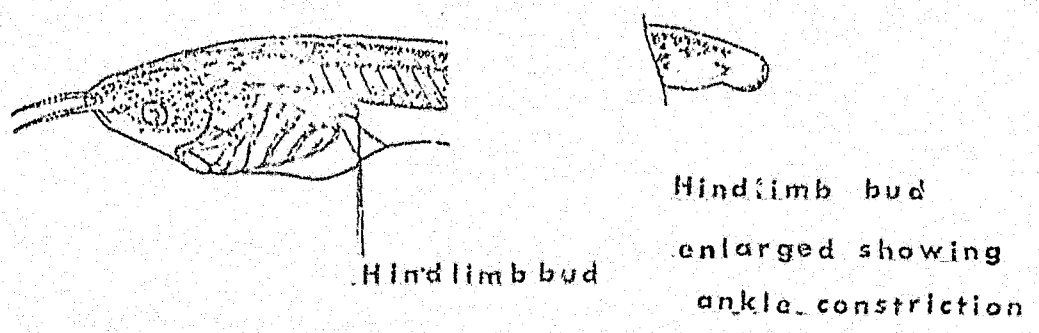


Figure 1.8 *Lateral view of Stage 52 tadpole*

NF Stage 53

At this stage, the masses of mesenchyme begin chondrifying centrally in the pelvic girdle. The femur chondrifies while the tibio-fibula and the tibiale fibulare are procartilage. The beginnings of the digits are noticeable distal to the ankle constriction as a slight protrusion in the paddle region. The forerunners of digits 4 and 5 are seen.

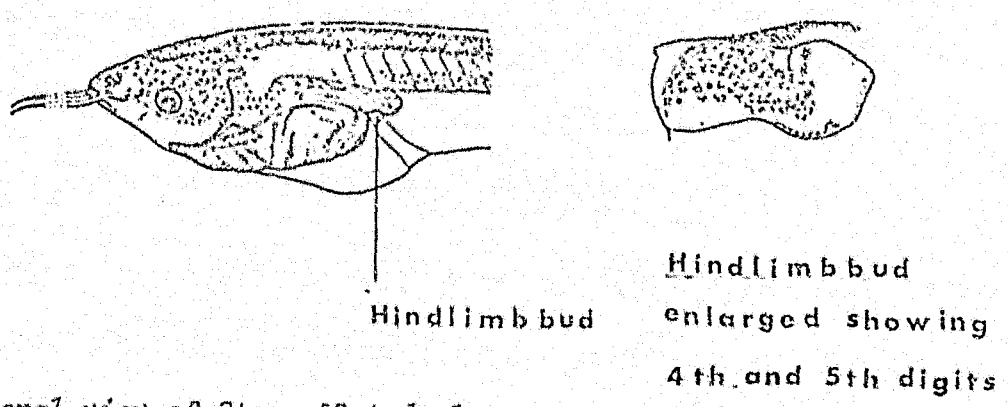


Figure 1.9 *Lateral view of Stage 53 tadpole*

NF Stage 54

At stage 54 the femur and tibio-fibula are completely chondrified, while the tibiale fibulare is chondrifying and the metatarsals are procartilage. The beginnings of the digits are noticeable distal to the ankle constriction as a slight protrusion in the paddle region.

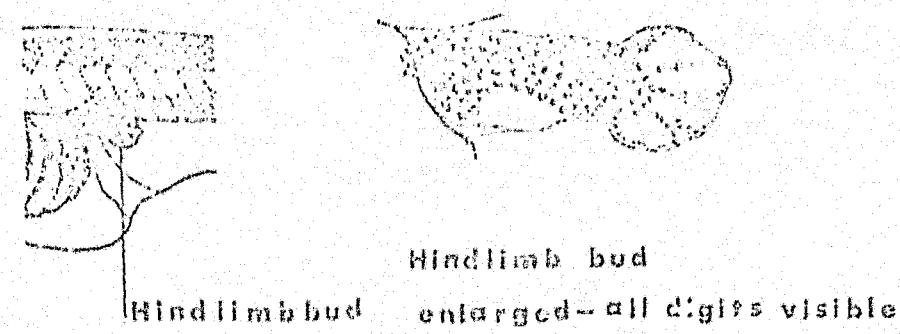


Figure 1.10 *Lateral view of Stage 54 tadpole, abdomen only*

NF Stage 55

During this stage the femur undergoes perichondral ossification and the stage is recognised by the presence of 5 clearly demarcated digits.

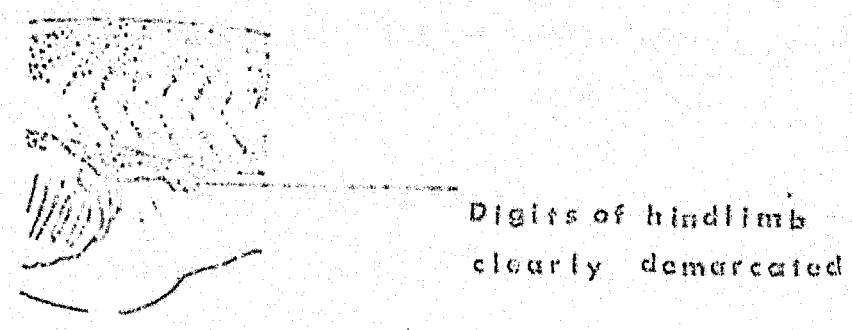


Figure 1.11 *Lateral view of Stage 55 tadpole, abdomen only*

NF Stage 56

During this stage the tibio-fibula undergoes perichondral ossification. The tibiale fibulare ossifies. The metatarsals ossify while the phalanges are cartilaginous. The digits are longer than those in stage 55.

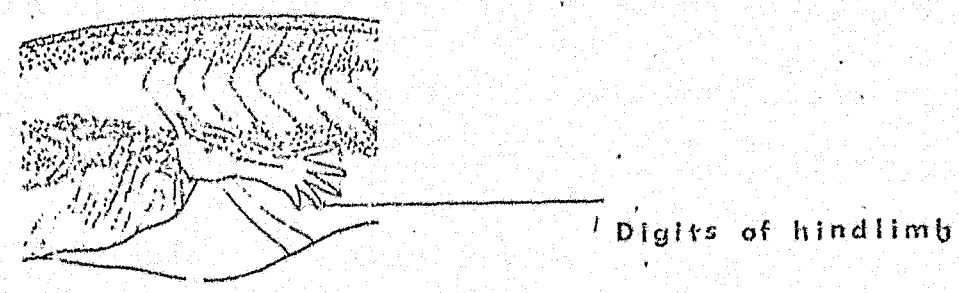


Figure 1.12 *Lateral view of Stage 56 tadpole*

NF Stage 57

During this stage the phalanges ossify and the claws become visible as a result of the cornification of the epidermis at the tips of digits 1, 2 and 3 to form the claws .

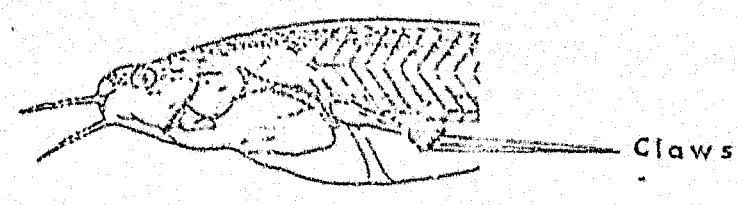


Figure 1.13 *Lateral view of Stage 57 tadpole.*

NF Stage 58

During this stage the tibio-fibula is enclosed in a bony sheath in the middle layer. The forelimb erupts from its pouch, (at this stage) indicating the beginning of the metamorphic climax in which the final rapid changes occur to convert the tadpole into a frog.

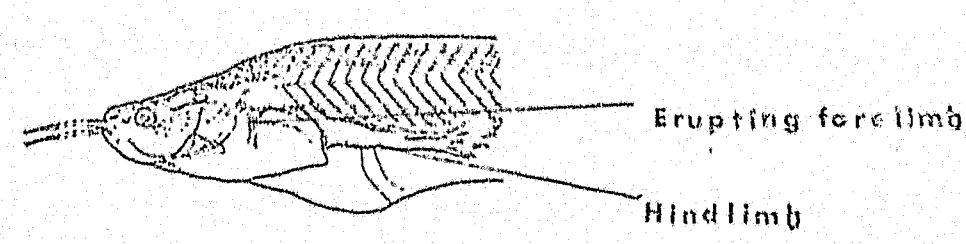
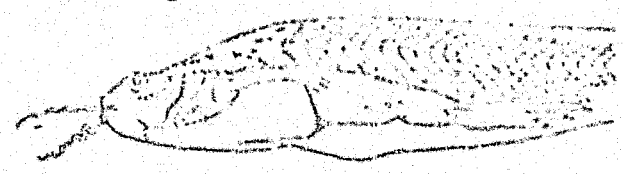


Figure 1.14 *Lateral view of Stage 58 tadpole*

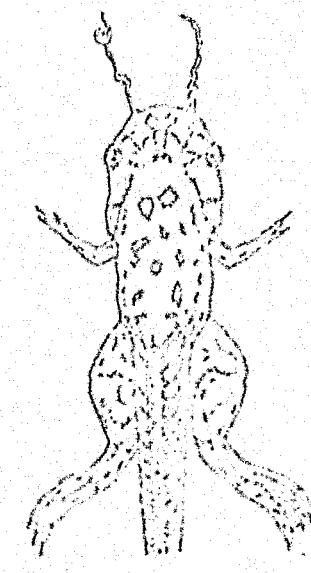
NF Stages 59 to 66

During these final stages of metamorphosis the hindlimb bud continues growing, cartilage continues ossifying and the adult skin forms. Figure 1.15 below shows these remaining stages.

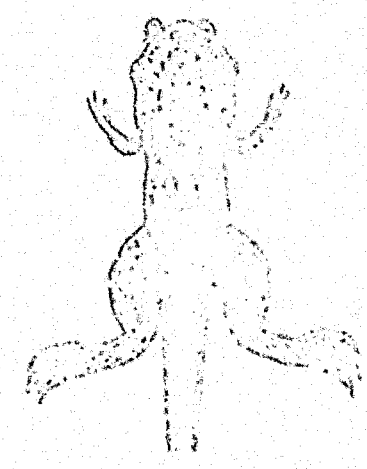
Stage 59



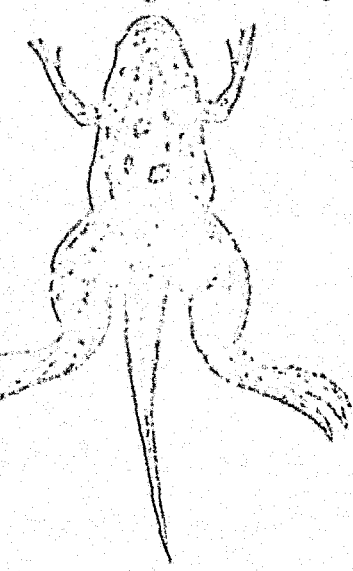
Stage 60 Lateral view



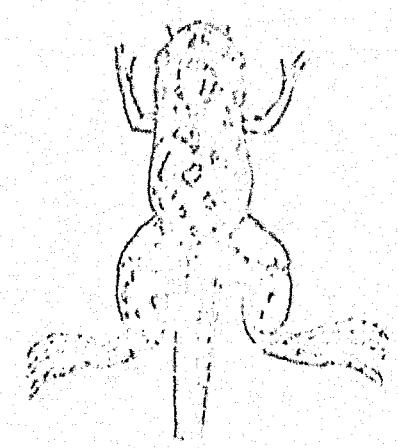
Stage 60 Dorsal view



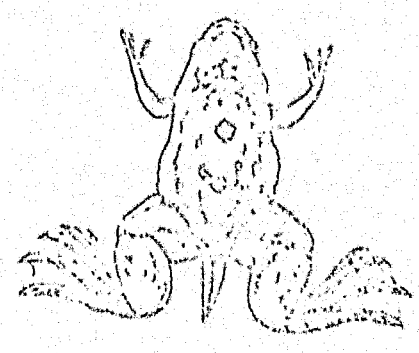
Stage 61



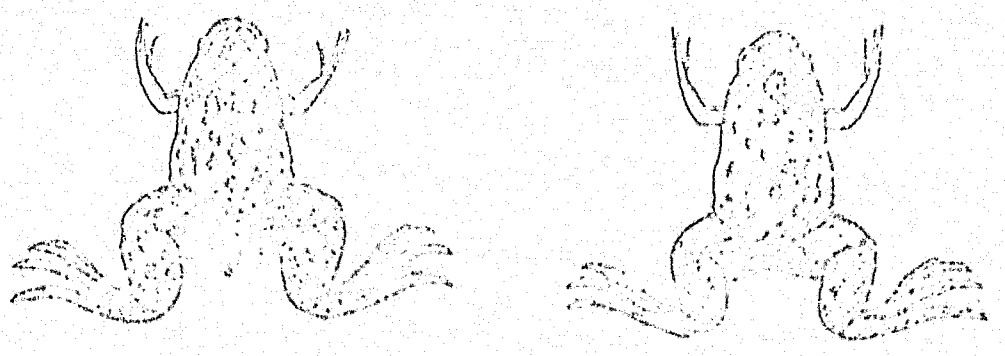
Stage 63



Stage 62



Stage 64



Stage 65 Stage 66

Figure 1.15 *Remaining stages of metamorphosis showing growth of the forelimb, formation of adult skin, shrivelling of the tentacles, regression of the tail and change of shape to adult form*

Table 1.1

As development of the hindlimb of Xenopus laevis proceeds, the future proximal elements develop before the distal elements. For example, the proximal element such as the femur already shows perichondral ossification before the phalanges are "indicated". This sequential development of cartilage along the proximo-distal axis of the limb is shown in Table 1.1 below (after Tschumi 1957).

ST.	FEMUR	TIBIO-FIBULA	TARSALS FILAMENT	METATARSAL	PHALANGES
51	Indicated				
52	Precartilage	Indicated			
53	Chondrification	Procartilage	Procartilage	Precartilage	
54	Chondrification	Chondrification	Chondrification	Procartilage	
55	Perichondral Ossification				
56		Perichondral Ossification	Perichondral Ossification	Perichondral Ossification	Cartilage
57					Perichondral Ossification 1 - 3 centers to form claw
58		fusion			

Table 1.1 *Sequential formation of the elements of the hind*

1.3.2. Structure of the adult frog limb

In this investigation, an analysis of the possible deformities in the developing hindlimb from experimental treatments i.e. the use of 5-BUdR and 5-FUdR, is carried out, by comparison to the normal adult amphibian hindlimb.

Figure 1.16 below shows the arrangement of the skeletal elements of the normal adult amphibian hindlimb (after A. Milnes-Marshall 1947)

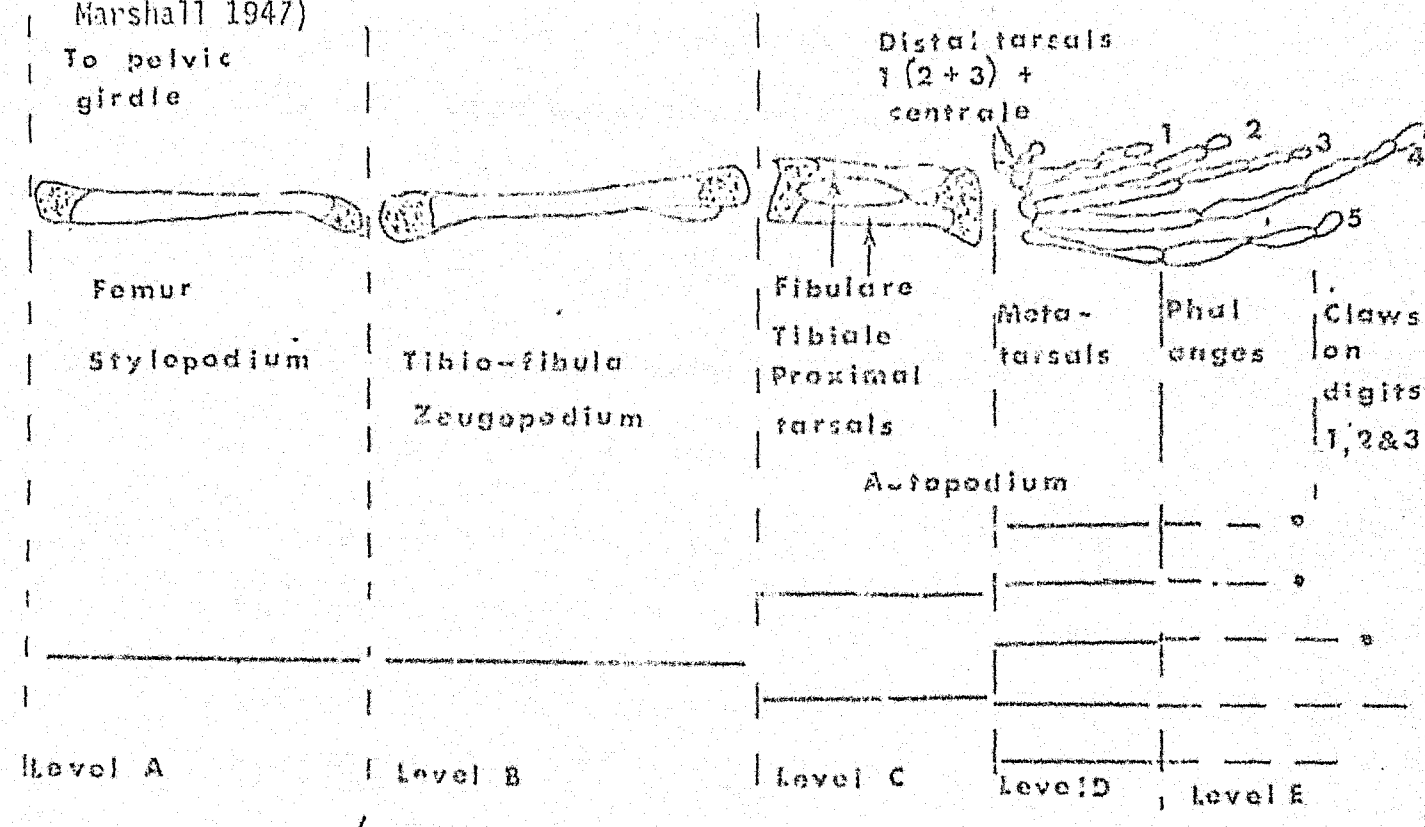


Figure 1.16 *A diagram showing the arrangement of the skeletal elements of the normal adult amphibian hindlimb (after A. Milnes-Marshall 1947)*

For convenience the limb elements have been straightened out. The limb has also been sketched in a more convenient layout. For the present analysis it is useful to arrange the hindlimb elements into five levels, namely A, B, C, D and E.

Level A consists of the femur. This is the stylopodium.

Level B consists of the tibio-fibula which is formed by the fusion of the tibia and the fibula at about stage 58. This is the Zeugopodium.

Level C consists of the proximal and distal tarsals. The proximal tarsals are known as the tibiale and fibulare. The tibiale and fibulare are the first of two rows of tarsals which in the frog have become lengthened to form an extra joint. These are followed by the

second row of tarsals, the distal tarsals, which consist of tarsal number 1, tarsals 2 and 3 fused, the centrale and a prehallux which is a remnant of what may have been an extra element in ancestral amphibians.

Level D consists of the five metatarsals which make up most of the foot.

Level E consists of the phalanges which are present as the five digits. The phalanges are arranged in the digits as follows:

Digit Number	Number of Phalanges
1	2
2	2
3	3
4	4
5	3

This is a total of fourteen phalanges. Digits 1, 2 and 3 end in claws. (Nieuwkoop and Faber 1967.)

Balinsky (1981) uses other terms for various areas of the hindlimb. These are also shown in Fig. 1.16 on page 11.

Stylopodium - femur

Zeugopodium - tibio-fibula

Autopodium - metatarsals, tarsals and phalanges.

According to Balinsky (1981) digits 1 and 2 form first, followed by digits 3, 4 and 5, while Tarin and Sturdee (1971) claim that digits 4 and 5 form first.

1.4 The three axes of the hindlimb, with special reference to Xenopus laevis

There are three main axes in the hindlimb, the proximo-distal axis, the antero-posterior axis and the dorso-ventral axis. As illustrated in Figure 1.17 below, the proximo-distal axis is the axis from the thigh to the toes, where the thigh is proximal and the toes are distal. The antero-posterior axis is that from the first toe to the last toe with the first toe being at the anterior end, pointing towards the head. The dorso-ventral axis distinguishes the top of the foot from the bottom of the foot i.e. dorsal is the uppermost part.

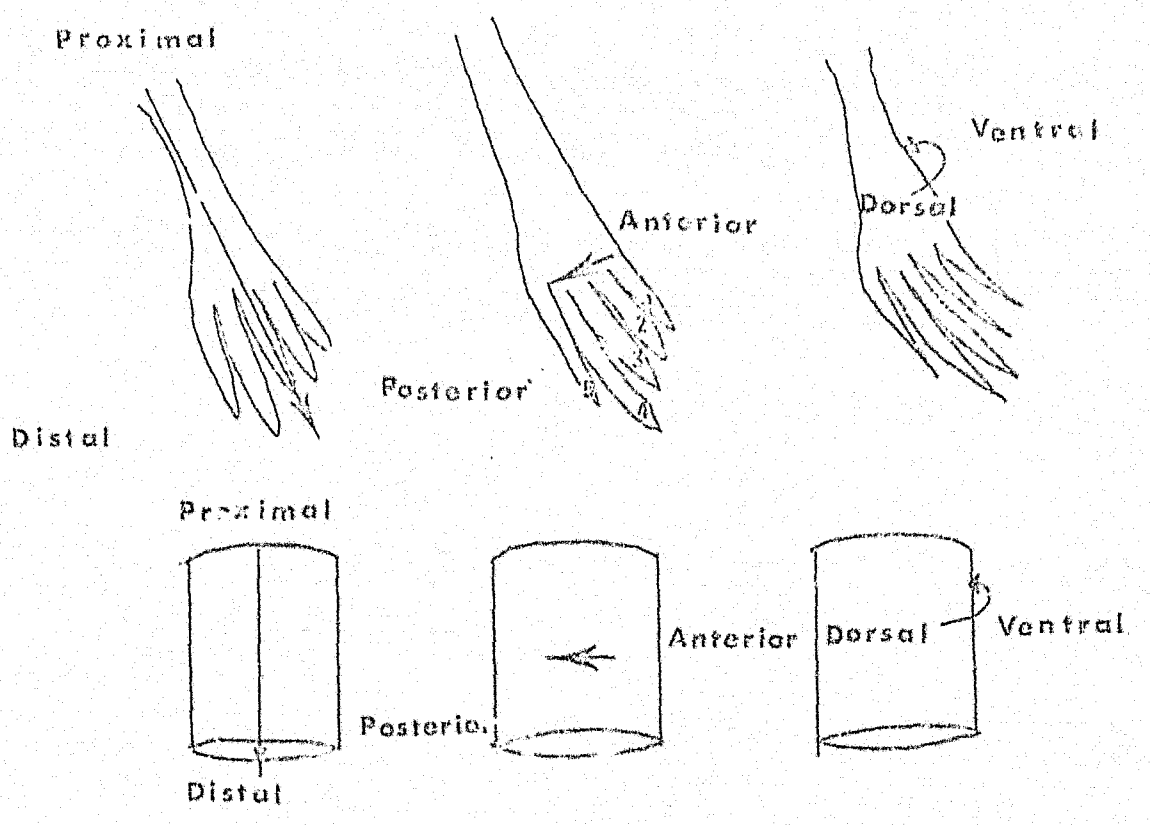


Figure 1.17 *Sketch to show the orientation of the three axes.*

1.4.1. The proximo-distal axis

1.4.1.1. The experimental evidence for this axis

Saunders (1948) found that the wing bud of the chick embryo grows mainly at its distal end. This comes from a study in which carbon particles were inserted into all parts of an early wing bud where it was found that the distal particles spread more widely than the proximal particles. At the extreme distal end no carbon particles were found, which led him to the conclusion that the distal elements are laid down successively during limb development i.e. they are not present from the earliest stage. These experiments of Saunders were repeated and extended by Tschumi (1957) on Xenopus laevis. He inserted the carbon or carmine particles into the mesenchyme using the tip of a very fine steel needle. After a period of limb growth, the larvae were fixed and stained with Methyl Green and cleared in Benzyl Benzoate to show up the cartilage skeleton. After two to three days, the marks which were at first compact and well-delimited, spread into several aggregates of variable size as shown in Figure 1.18 on p.14.

Extensive spreading of the marks was noted in certain areas i.e. distal areas, while clumping of the marks or little growth occurred in the proximal areas This confirmed Saunders' findings of distal growth being greater than proximal growth.

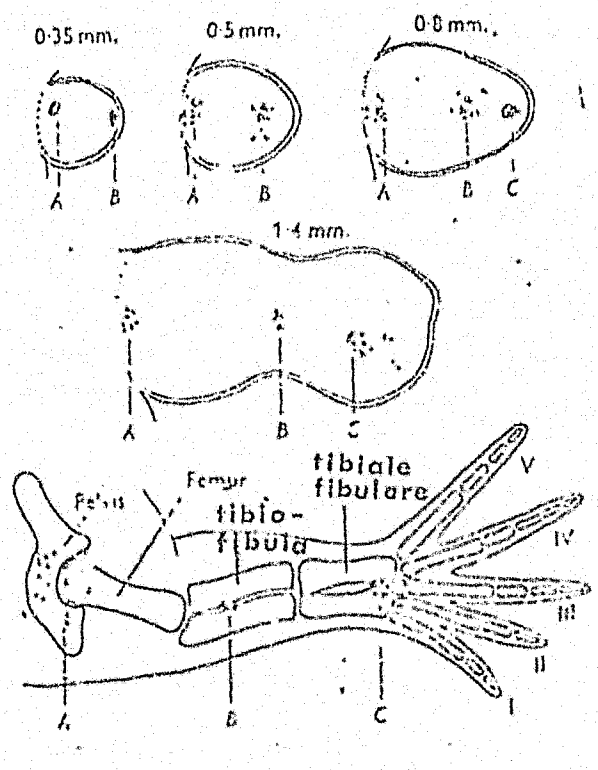
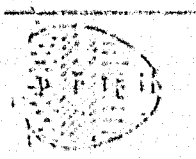
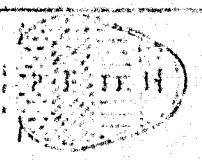
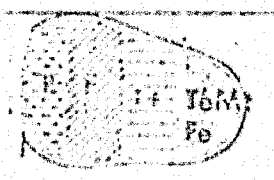
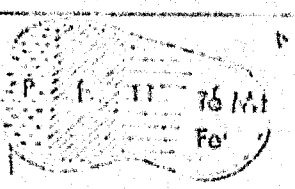
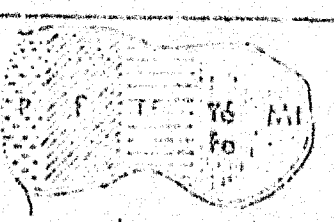
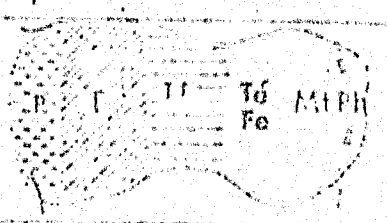
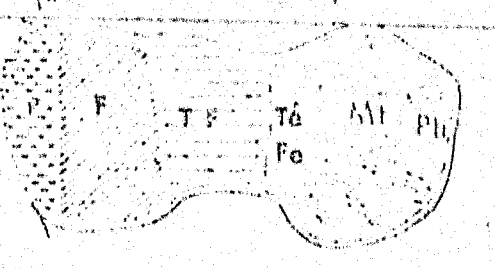


Figure 1.18 *Results of a carbon-marking experiment showing the proximal clumping and distal spreading of the carbon particles.*

As the limb developed, Tschumi traced back the parts that developed to their presumptive areas in the original buds and from this constructed a series of fate maps. The results shown in Figure 1.19 on page 15 show that, for example, in NF stage 48 only presumptive pelvis is present while at NF stage 50 presumptive pelvis, femur and tibio-fibula are present.

LENGTH mm.		NF STAGE
0,2		48
0,3		50
0,4		50
0,5		51
0,6		51
0,8		51
1,0		52
1,2		52
1,4		53
1,6		54
2,0		54

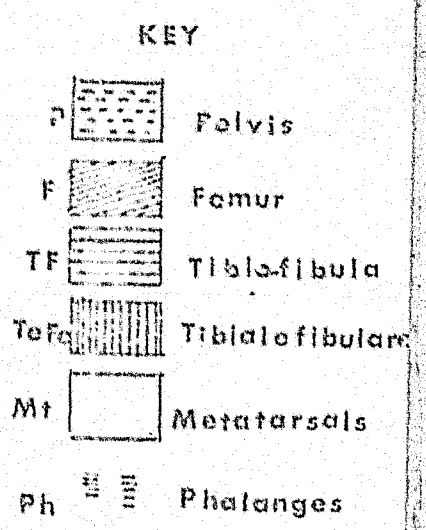


Figure 1.19 *Tochami's sketches of presumptive limb tissues. The various stages have been analysed into NF stage by the present author.*

From Tschumi's fate maps Table 1.2 below has been constructed. It correlates well with the "indicated" stage of the cartilage in Table 1.1. on page 10.

Stage	Pelvis	Femur	Tibio-Fibula	Tibiale Fibulare	Metatarsals	Phalanges
48	X					
50	X	X	X			
52	X	X	X	X	X	
53	X	X	X	X	X	
54	X	X	X	X	X	X

Table 1.2 *Summary of Tschumi's results*

Stark and Searls (1973) conducted more sophisticated experiments on this aspect of limb development by means of autoradiography. They made maps of embryonic chick wing by implanting blocks of ^{3}H-thymidine-labelled cells into chick limbs. The final location of the cells was established by autoradiography of histological sections. Contrary to the findings of Tschumi (1957) the authors located prospective hand cells at the earliest stages. They argue that these cells would have been left unmarked by the cruder techniques of Tschumi (1957). They conclude that all the prospective cells are present from the earliest stages and although the distal and proximal cells grow at the same rate, the distal cells grow for a longer period of time.

Dent (1962) did studies on the regeneration of the Xenopus laevis hindlimb bud, by cutting it off at different proximo-distal levels. The same type of experiment was attempted chemically in the present dissertation using 5-BUdR and 5-FUdR. For a detailed discussion of the effects of 5-BUdR and 5-FUdR see Section 1.8. Dent cut off the developing hindlimb at various stages from 51 to 60 (NF stages) and the resulting regenerates are shown in Figure 1.20.

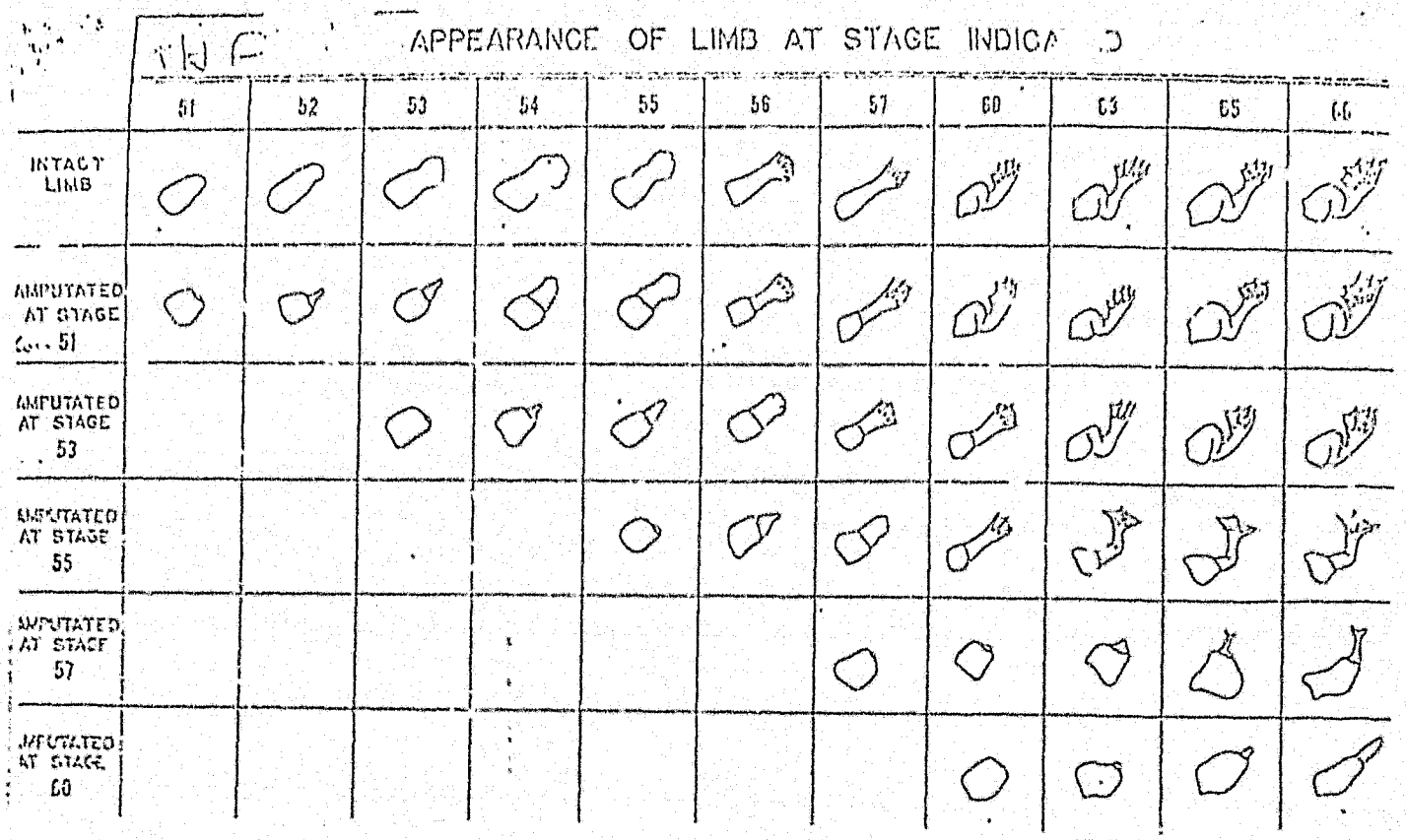


Figure 1.20 *Results of Dent's experiments in which hindlimb buds of Xenopus laevis were amputated at various stages of development.*

The results of Dent's experiments are also summarised as follows :

Amputated Stage	Number of digits reformed
51	5
53	4
55	3
57	2
60	1

It will be noticed that the later in the development the limb is cut off, the less regeneration takes place.

Dent studied the gross morphology only of the limb. The author of this dissertation studied the cartilage development of the limb before and after treatment with 5-BUdR and 5-FUdR using stains and clearing the tissue for cartilage visibility.

The results correlated will with those of Dent in that after stage 53 chemical treatment did not cause limb abnormalities. Dent finds that at this stage a normal limb does not regenerate i.e. all limb parts are determined at this stage.

1.4.1.2. The apical ectodermal ridge (AER)

Saunders (1948) and Zwilling (1961) believe that the AER is indispensable to the proximo-distal outgrowth of the limb. The AER is a thickening of the ectoderm along the edges of the flattened limb bud. See Figure 1.21 below. (It will be recalled that the limb is a core of mesoderm surrounded by ectoderm.)

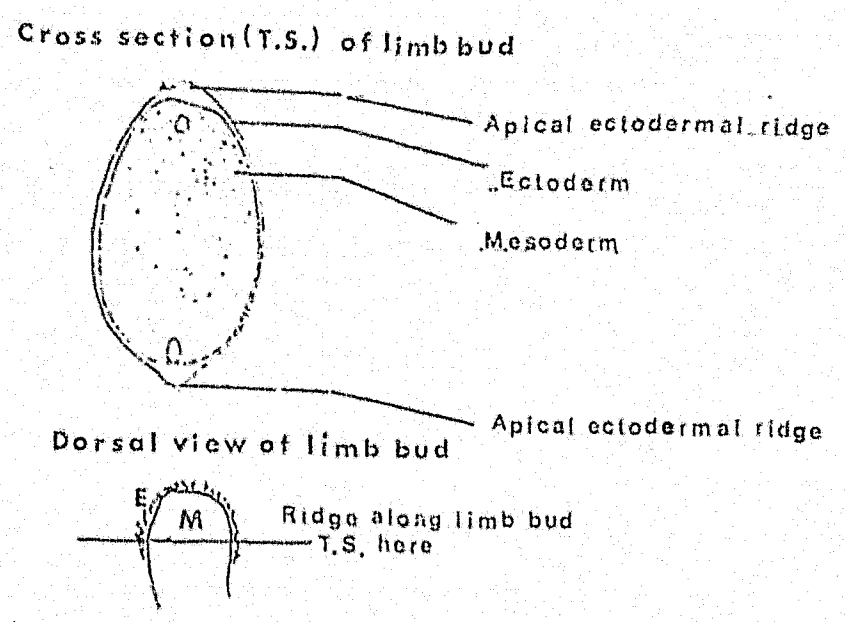


Figure 1.21 *Cross-section of an advanced chick limb bud showing the apical ectodermal ridge. (After Balinsky 1981.)*

The cells of this ridge contain more RNA and glycogen than the surrounding epidermal cells and a high content of alkaline phosphatase. These biochemical properties indicate an active metabolism (Balinsky 1981). If the AER of a three day chick embryo is removed, the distal parts of the limb fail to develop (Saunders 1948). Likewise if the ectoderm covering the limb bud in a chick embryo is removed and replaced by epidermis from another part of the body, an apical ridge does not form and proximo-distal outgrowth is inhibited.

Amphibians are not normally considered to have an AER, but Tarin and Sturdee (1971) found a structure at NF stage 50 which they consider to be the counterpart of the AER, a narrow band of thickened epidermis which runs around the tip of the bud and extends a short distance proximally on both the dorsal and ventral sides of the bud. Histochemical studies revealed that RNA, glycogen and alkaline phosphatase which were characteristic of all the other AER's of other vertebrate groups were not present in significantly larger amounts in the "suspected ridge" of the Amphibia (Tarin and Sturdee 1973). Although histologically this appears to be a ridge it does not have the biochemical properties of a true AER.

1.4.1.3. Final determination of the proximo-distal axis

The proximo-distal axis is capable of reversal, at least until the stages just prior to the appearance of the limb bud, as shown by Hamburger (1938), Chaube (1959), Reuss and Saunders (1965) and MacCabe and Saunders (1971) that is in the prospective limb bud region. there is a proximo-distal axis which can be reversed to form a normal bud until the stage when the limb bud is visible. This was shown by experiments ir which the mesodermal component of the left leg primordium (prospective bud) at stage 16 (seventeen to twenty somites) in the chick embryo was grafted to the right flank of a host chick embryo of the same age, but the proximo-distal axis was reversed i.e. the inside of the prospective limb bud now faced outwards. See Figure 1.22 below.

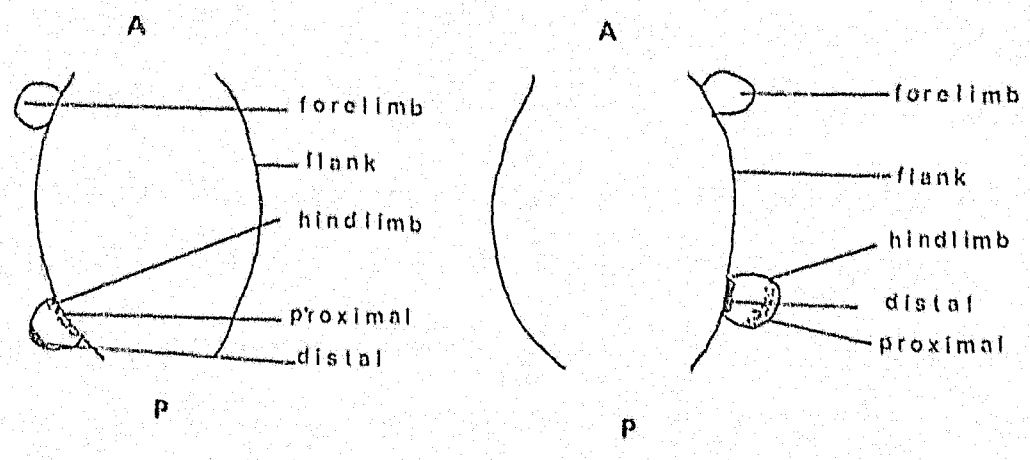


Figure 1.22 *Sketch showing reversal of the proximo-distal axis in the chick embryo. (For convenience the limb is shown as a bulge.)*

The ectoderm of the host's flank covered the graft and formed an ectodermal ridge which then induced proximo-distal outgrowth and the formation of a leg with right symmetry i.e. the original proximal tissue formed distal parts and the original distal tissue formed proximal parts.

1.4.2. The antero-posterior axis (first toe to last toe)

This axis is finally determined early in development, unlike the proximo-distal axis. This can be shown in an experiment in which limb ectoderm is removed and the mesoderm is rotated to change antero-posterior polarity. Prospective limb mesoderm is then allowed to develop in combination with flank ectoderm. (The flank is that region of the body wall between the forelimb and the hindlimb.) The resulting appendages conform to the original antero-posterior polarity of the mesoderm, regardless of the orientation of the graft with respect to the major axes of the host. Thus even if the limb is back to front, the mesoderm which would have developed the first toe will still develop the first toe, but it will now point towards the rear of the animal. If the ectoderm is reversed through 180^{o} the mesoderm develops according to its original orientation i.e. the first toe points fowards. This confirms the role of the mesoderm in determining the antero-posterior axis. It will be recalled that the proximo-distal axis is determined by the AER which is ectodermal (see section 1.4.1.3.) (Zwilling 1956). A major control in antero-posterior determination seems to be a zone of the mesoderm situated at the posterior end of the limb 'ud known as the ZPA (Zone of Polarising Activity), Saunders and Gasseling (1968), Balcuns et al (1970) in Saunders (1972). For the location of this area see Figure 1.23 on page 21.

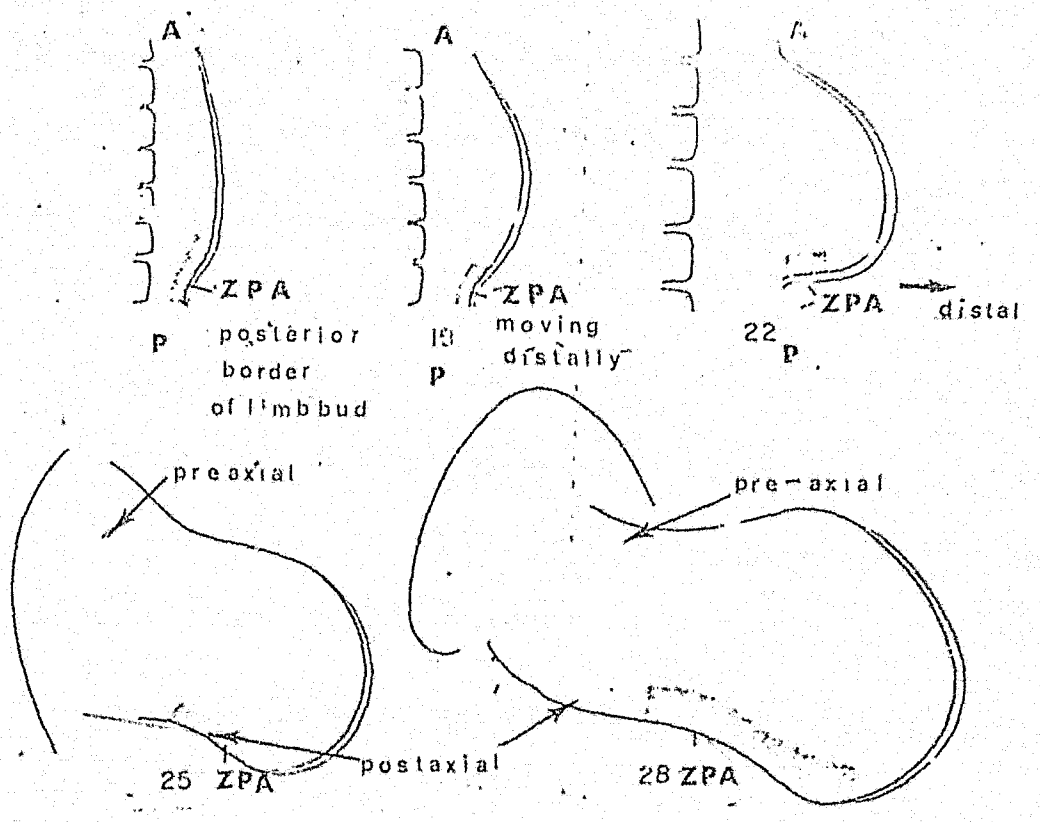


Figure 1.23 *Distribution of the ZPA in the right wing bud of the chick embryo (after Saunders 1972).*

The properties of the ZPA were revealed by Gasseling (Saunders and Gasseling 1968) when mesoderm from the zone below the apical ridge in the ZPA was grafted to the preaxial apex of a host right wing bud. The portion of the apical ridge next to the graft (taken from the region where the ZPA is found) induced the outgrowth of a supernumerary limb (extra limb), in the preaxial mesoderm (mesoderm in front of the ZPA shown in Figure 1.24 below). In Figure 1.24 below an experiment is shown in which a section of the preaxial border of the wing bud at its junction with the body wall is replaced with a similarly sized implant from the ZPA. It was from this that a limb with mirror image symmetry was induced.

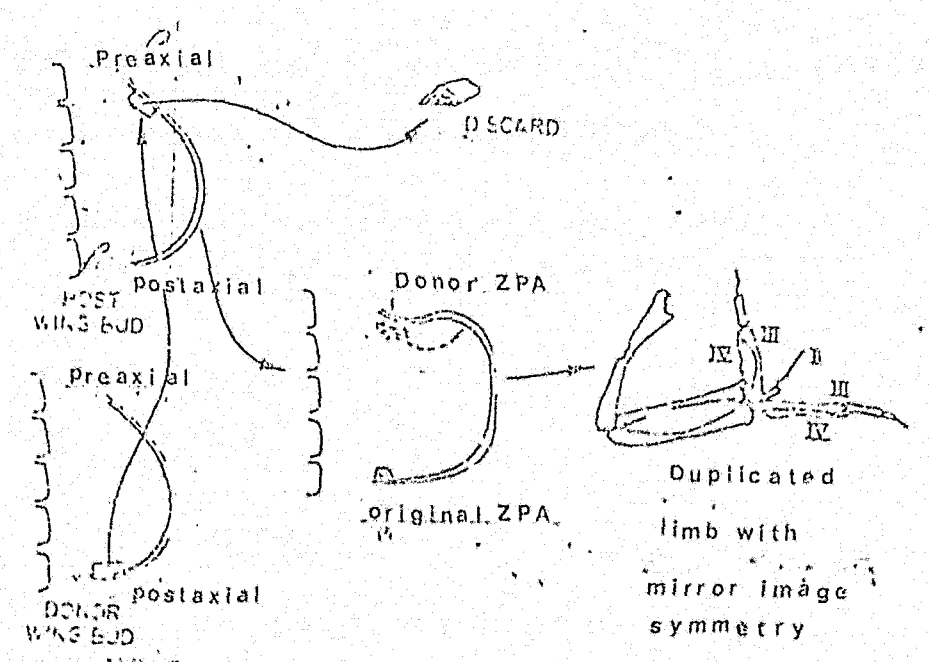


Figure 1.24 *Experiment showing replacement of preaxial mesoderm with ZPA, and the resulting mirror image symmetry. After Saunders (1972)*

The activity of the ZPA is first detected in grafts from the tissues of the postaxial border of the limb bud (HH stage 17, Hamburger and Hamilton (1951) Appendix B). As the limb bud elongates, the polarising power of the ZPA becomes restricted to the posterior margin of the bud near its junction with the body wall. Thereafter it is found progressively more distally along the margin of the lengthening bud and it diminishes near the body wall. By HH stage 28 the polarising activity is shown with low frequency and is not detected after that.

Slack (1977a) confirmed the presence of a ZPA in the axolotl (Amphibian). He found that the flank tissue posterior to the prospective forelimb bud determines the antero-posterior axis. He did a series of transplants, by moving the limb bud tissue to a distinct site on the flank, where in most cases a forelimb is formed. When the same grafts were placed on the head, the forelimb did not grow. However, if a wide strip of flank tissue was grafted along with the anterior disc (presumptive limb tissue), limbs did form. As this flank tissue is the position of the ZPA, it must play a role in the onset of limb formation.

Cameron and Fallon (1977) investigated the hindlimb of Xenopus laevis for the presence of a ZPA. They rotated hindlimb bud tips through 180° on the proximo-distal axis and returned them to the stump. (See Figure 1.25 on p.23).

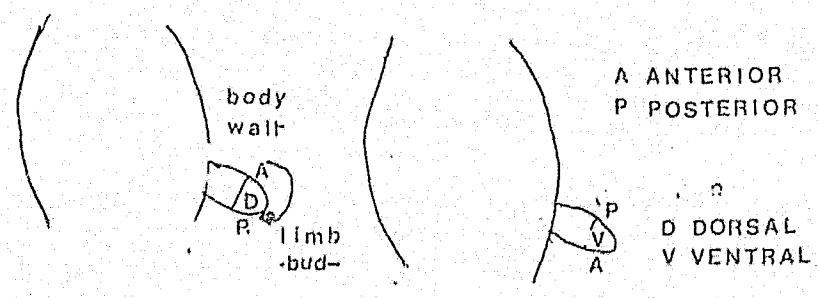


Figure 1.25 *Sketch showing the rotation of the hindlimb bud by Cameron and Fallon.*

Supernumerary limbs (two limbs from one stump) were induced in the preaxial stump tissues and the most preaxial digit always formed next to the grafted postaxial tissue. When they removed the presumptive ZPA and did the same experiment, the incidence of supernumerary limb formation was drastically reduced.

Slack (1977b) tries to explain the presence of this ZPA in terms of a monotonic gradient (one gradient only). According to his point of view the flank defines the high point of the gradient, thus determining the overall antero-posterior polarity, while the level of gradient determines the pattern of the limb. A shallow gradient will suppress the pattern while a deeper gradient will evoke a double structure in the centre of the limb and an even deeper one will cause formation of two limbs. Fallon and Crosby (1977). See Figure 1.26 below.

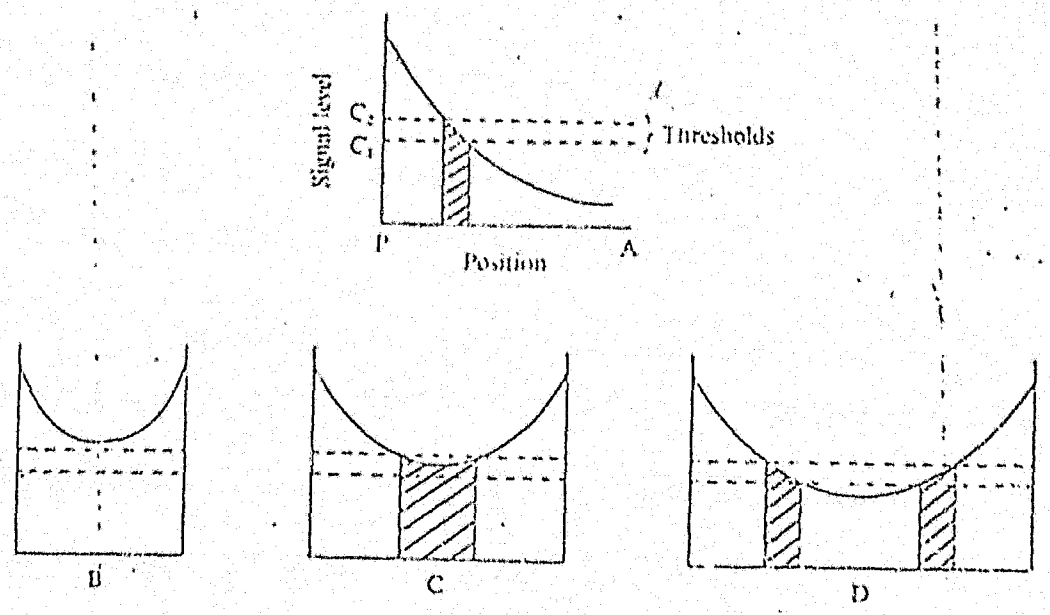


Figure 1.26 *Sketch showing the Gradient Theory of Slack (Slack 1977b)*

A. shows the model for the specification of the normal limb.
B. shows the suppression of the limb in the case of a shallow gradient.
C. shows the double fusion in the centre of the limb.
D. shows formation of two mirror image limbs.

1.4.3. The dorso-ventral axis

The dorso-ventral axis distinguishes the top of the limb from the bottom of the limb. It seems to be the axis which is established first (Chaube 1959). It also seems to be under ectodermal control as, when the limb bud mesoderm of a chick forelimb is dissociated into a cell suspension, re-aggregated and placed in an ectodermal jacket, the resulting limb shows no antero-posterior polarity i.e. as would be expected, the different digits are not able to be distinguished in the correct order but the differences in feather distribution correspond to the dorso-ventrality of the ectodermal jacket (Saunders 1972).

Therefore, imposition of antero-posterior polarity by implantation of the ZPA in either the anterior or posterior end of the recombinant does not alter dorso-ventrality which still conforms to that of ectoderm. Therefore, whereas the AER and proximo-distal and dorso-ventral polarity are under ectodermal control the antero-posterior polarity is under mesodermal control.

1.5 Theories involving cell patterning and its relation to limb development

Many theories have been proposed to account for cell patterning in the limb. Some of these theories explain cell patterning in the proximo-distal direction, while others explain cell patterning in the antero-posterior direction.

Of those theories which propose patterning in the proximo-distal direction, the most well-known is that of Wolpert (1969), of which follows a detailed discussion. There are three important processes occurring during limb development, namely morphogenesis, pattern formation and differentiation. Wolpert (1969) distinguishes between pattern formation and morphogenesis.

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During pattern formation, the spatial organisation of cellular differentiation is specified i.e. the muscle and cartilage are placed in their correct positions in the limb. During morphogenesis the shape of the limb is moulded i.e. the wing shape or the leg shape. It is obvious that pattern formation precedes morphogenesis.

A further process occurring, distinct from pattern formation and morphogenesis, is differentiation, which will be discussed in detail in section 1.7. During differentiation, an initial group of unspecialised cells changes to form specialised tissue i.e. muscle or cartilage.

A theory is proposed for pattern formation of the limb using the concept of "positional information" (Wolpert 1969, 1981). This theory proposes that the path of differentiation followed by the cell depends on its position in the limb prior to differentiation. A good example illustrating this is an experiment in which a piece of proximal tissue from the hindlimb (near the thigh) is transferred to the distal tip of the wing bud in the chick embryo. It develops into a toe. It is still histologically hindlimb tissue, but its position causes it to form the more distal hindlimb structure i.e. the toe. The theory proposes that each cell has a positional value and interprets this accordingly.

Wolpert (1981) proposes three methods for specifying positional information:

As one method of informing each cell of its position, Wolpert suggests a monotonic gradient i.e. a gradient in one direction with a source at the high point and a sink at the low point as shown in Figure 1.27.

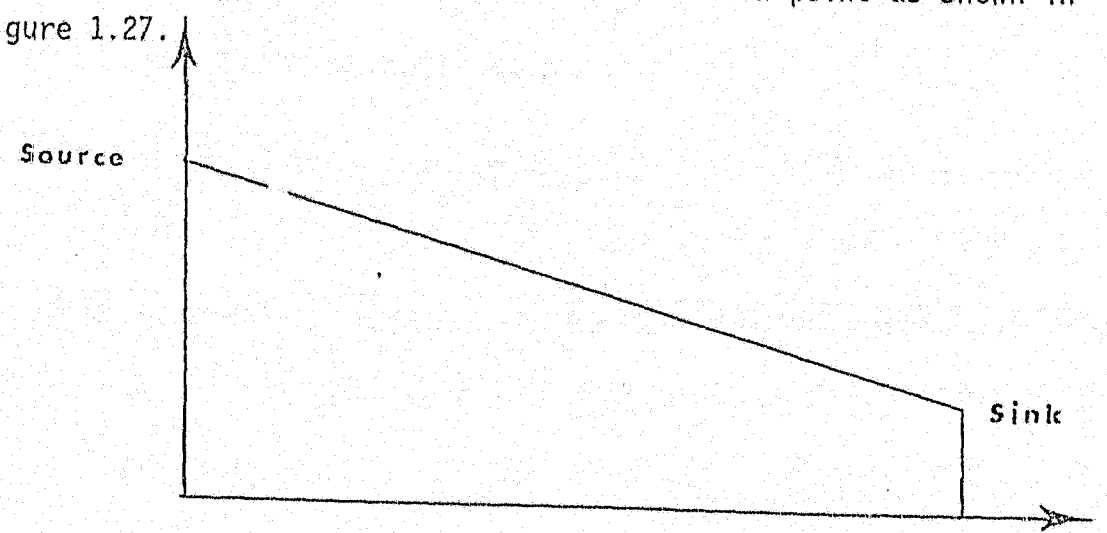

Figure 1.27 *Sketch indicating a monotonic gradient i.e. a gradient in one direction with a source at the high point and a sink at the low point.*

According to this theory the source releases a diffusible molecule and if this is broken down at a rate proportional to the concentration i.e. the higher the concentration the greater the number of molecules are broken down, then the concentration provides a measure of the cell's distance from the source. The greater the number of molecules that are broken down, the nearer to the source the cell is. The source must be of constant concentration forming a boundary at the highest point of the gradient.

As a second method suggested by Wolpert, instead of using a chemical to form the gradient one could use a time zone.
The cells could measure how long they spend in a certain area, which Wolpert calls the "progress zone". This is a zone near the tip of the limb bud which is 350 μ wide. Its existence depends on the AER. The AER permits rather than directs the pattern (Wilby 1977). Cells proliferate in the progress zone by cell division and cells overflow from the progress zone becoming fixed in positional value as they cross its proximal boundary. The cells first emerging from the progress zone would differentiate the structural characteristics of the proximal limb levels. (Later cells having counted more time in the progress zone would generate more distal positional values.) If the AER i.e. progress zone, is removed only those cells already specified by their sojourn in the progress zone form the limb and the resulting limb is defective. When the cells leave the progress zone only then do they differentiate. Below is a sketch showing a cell in the progress zone at various stages of limb development.

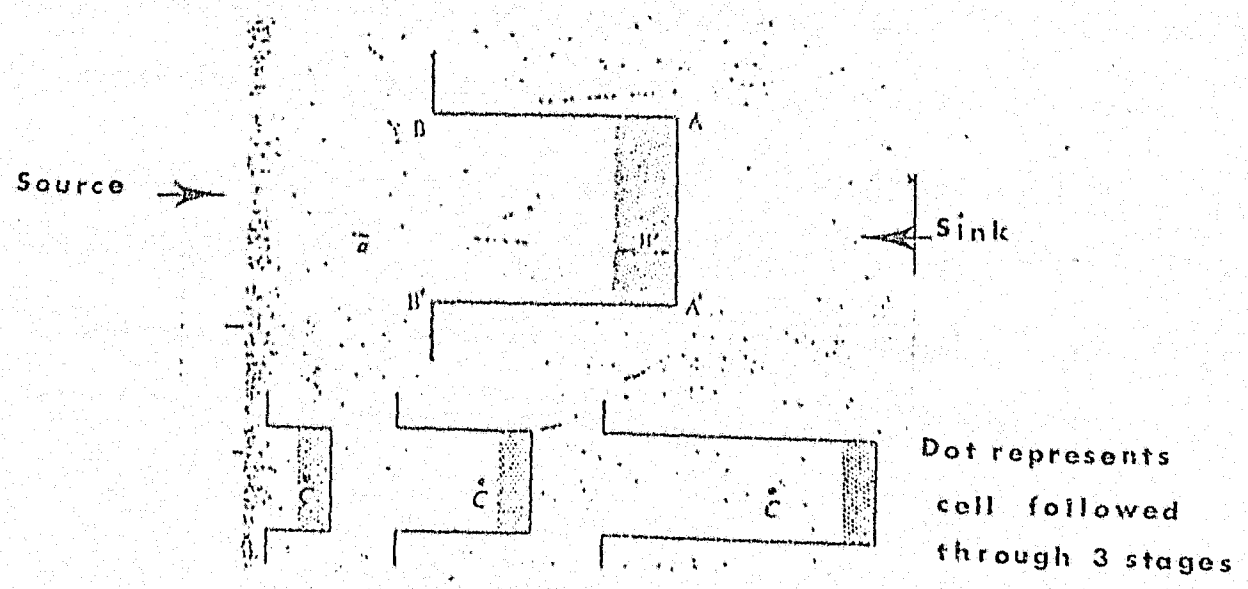

Figure 1.28 *The progress zone at various stages of limb development (Summerbell et al 1973)*

The third way in which cells could determine their position is by direct transfer of positional value from one cell to another. This is shown during the process of induction during development of the embryo. The neural plate of the amphibian embryo is induced by the direct transfer of positional information from the underlying mesoderm.

To illustrate his theory of positional information clearly, Wolpert (1969) uses the French flag (see Figure 1.29 below).

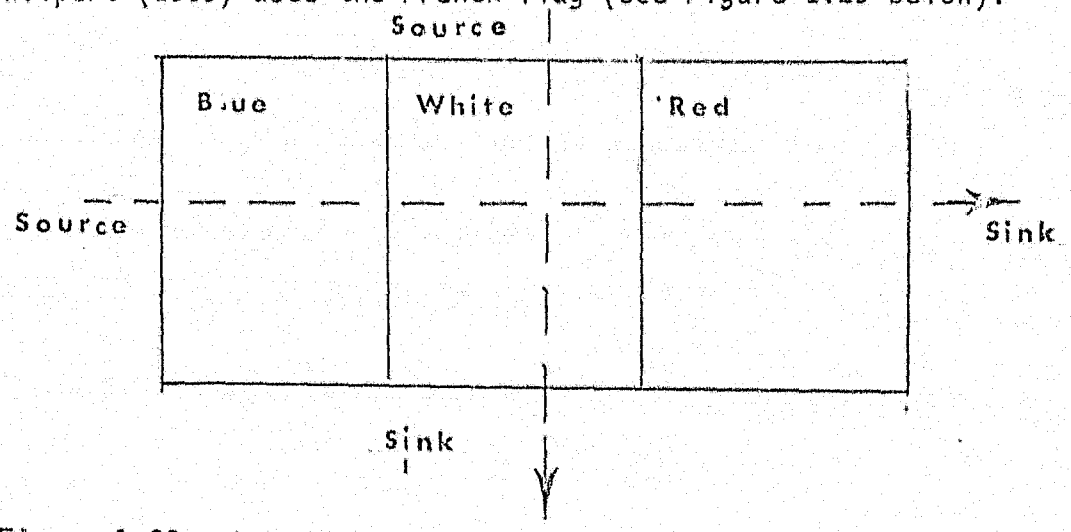

Figure 1.29 *The French flag*

The blue zone could be regarded as the highest concentration while the white could be lower in concentration and the red could be the lowest. The concentrations have definite thresholds, i.e. there are no in between concentrations such as pink or light blue. (See sketch in Figure 1.30 below.)

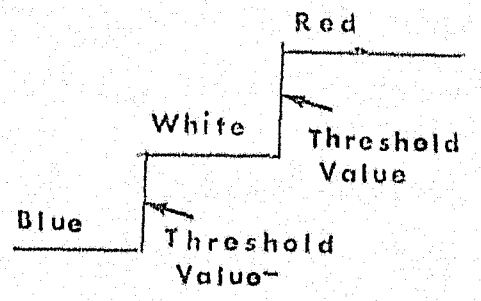

Figure 1.30 *Sketch showing the concentration threshold*

The cell could determine its position by means of the monotonic gradient already mentioned, or it could use a bipolar gradient or morphogen. One gradient could run from the blue to the red, and the other gradient could run from the top of the flag to the bottom, at right angles to the first gradient.

Where these two gradients cross would mark the position of the cell i.e. there would be a proximo-distal gradient and an antero-posterior gradient. One other way in which a cell could determine its position is suggested by Goodwin and Cohen (1969) in which each cell knows its position by measuring the difference in phase between two propagating events sent out from a source. These propagating events could be chemical morphogens.

Cells interpret their position and show a genetic response as is shown in Figure 1.31 below. The Figure is a sketch of the French and American flags, which can be used to illustrate further the experiment discussed in Wolpert (1981) on page 25. (a) and (b) show the flags. A portion is removed from the middle of the French flag in (c) and transplanted to the front end of the American flag in (d) and vice versa. It will be noted that, while the flags remain genetically true in (c) and (f), American flag tissue remains American and French flag tissue remains French, they behave according to their new position by forming the portion of the flag they would have formed at that position in their flag of origin.

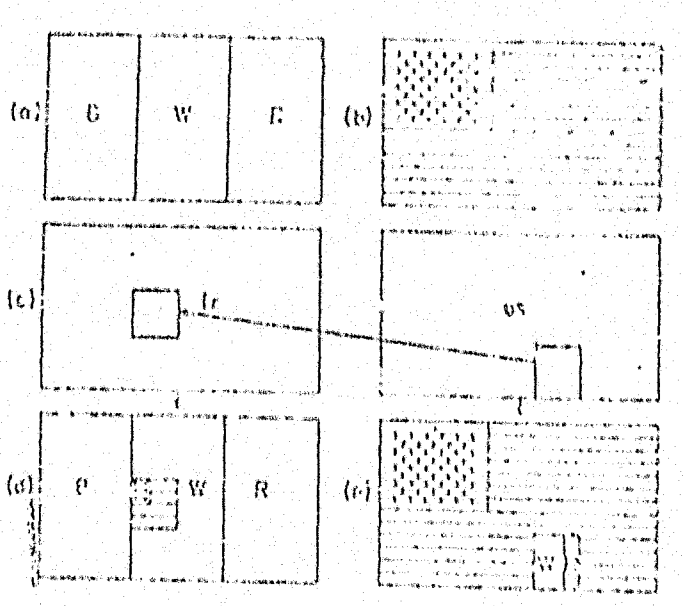

Figure 1.31 *Transplantation between the French flag and the American flag*

The experiment on page 25, in which thigh tissue formed a toe when transplanted to the wing bud of the chick embryo, is a practical illustration of the theory discussed above.

The "positional" theory can also be used to describe recovery of the limb after an injury. Two methods are suggested by Wolpert i.e. morphallaxis or epimorphosis. In morphallaxis the limb remodels the remaining tissue and forms a smaller but complete limb, whereas in epimorphosis there is proliferation of the cells at the cut end to give rise to a new limb bud which forms the missing parts.

Searls (1967) illustrated the phenomenon of "positional information" by showing that when cells were placed in the centre of the limb they formed cartilage, but when cells were placed on the periphery of the limb they formed muscle.

Faber (1976) uses the bipolar gradient theory of Wolpert and applies it to amphibian limb development. The boundaries of the gradient lie at the girdle skeleton proximally and at the digits distally. The distal end of the gradient is defined by the AER. The high point of the gradient is distal and the low point proximal. If parts of the limb which fall in the centre of the gradient are removed, the gradient becomes too steep, as there is a shorter distance from the proximal to the distal end. The limb therefore grows again to spread the gradient over a longer distance. This process is called intercalation i.e. filling in the missing parts (morphallaxis). see Figure 1.32 below.

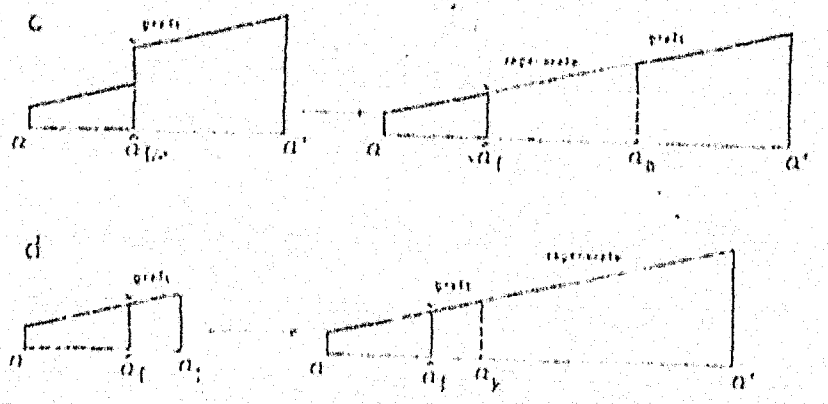

Figure 1.32 *Experiment by Faber (1976) illustrating intercalation (morphallaxis)*

α_f Mid-femur level
α Sink
α' Source
α_a Ankle.

An elegant model for intercalation has been described by French, Bryant and Bryant (1976). According to the model the limb is in the form of a cone, decreasing in diameter distally. At various points along the length of the cone a definite distance apart are circles, A, B, C, D, E and F. On each circle's diameter are twelve points. A to F represents the proximo-distal axis of the limb, while 0 to 12 represents the antero-posterior and dorso-ventral axes of the limb. Figure 1.33 below illustrates this model.

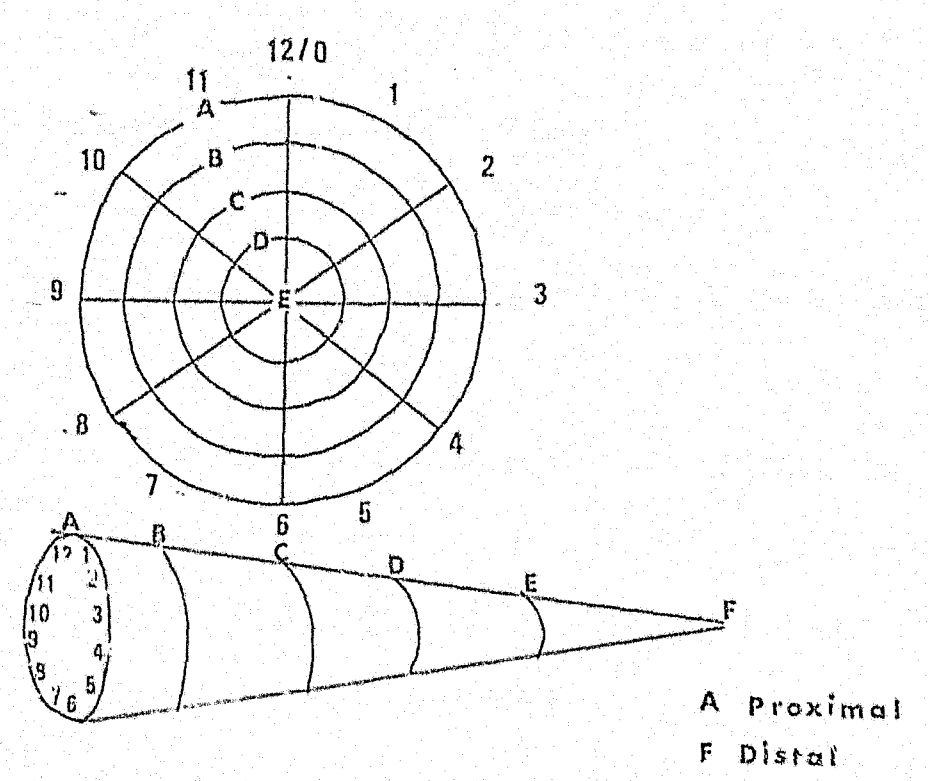

Figure 1.33 *The radial (A to F) and circular sequences (0 to 12) from the model of French, Bryant and Bryant (1976)* and Bryant(1977)

There are two rules in the model for regeneration of the limb when tissue has been removed.

1. Shortest intercalation rule

When cells with normally non-adjacent positional values in either radial or circular sequence are brought together, growth occurs to intercalate the missing positional values i.e. if circular levels 4, 5 and 6 are removed, the missing parts must be intercalated between them. The circular sequence is continuous, therefore there are two possible values which could develop between the two non-adjacent values i.e. the shortest set is 3(4, 5) 6 and the longest set is 3(2, 1, 12, 11, 10, 8, 8, 7,) 6.

2. Complete circle rule

From any given radial position, transformation to form all the more central (distal) radial values can occur, provided that a complete set of positional values in the circular sequence (0 to 12) is either exposed by amputation or generated by intercalation i.e. if D, E are removed the levels left are A, B, C, F and the levels D and E must be intercalated between them.

This intercalation theory has been illustrated experimentally by Iten and Bryant (1975) and Stocum (1975) in the newts Notophthalmus viridiscens and Amblystoma maculata. The forearm bud was transplanted to the upper arm of the same animal, omitting the middle section of the arm. See Figure 1.34 below. There was considerable dedifferentiation of the stump of each of the transplanted sections and intercalation occurred to replace all the missing proximo-distal levels.

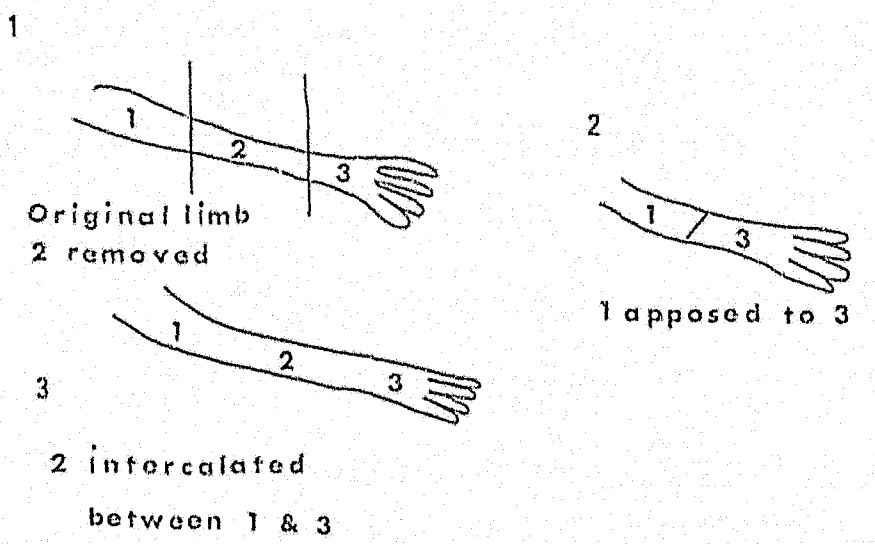

Figure 1.34 *Sketch showing the transplant experiment of Iten and Bryant (1975) and Stocum (1975)*

This experiment was also interpreted graphically by Faber (1976) as shown in Figure 1.31 on page 28.

Maden (1977) developed a theory along the lines of French, Bryant and Bryant (1976), incorporating the "positional information" theory of Wolpert (1969), to explain regeneration in the amphibian limb. He gives the epithelium surrounding the limb a value of 0 and all the other cells inside the epithelium a value higher than 0. See Figure 1.35 on p32

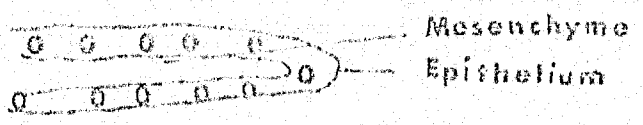

Figure 1.35 *Skotel showing Meinhe's model*

According to this theory, based on mathematical formulae, when the difference between two cells is higher than 1.12 the cells will differentiate. On the other hand the cells dedifferentiate when their difference is lower than this value and this initiates regeneration, by causing mitosis to occur. Mitosis usually ceases with the onset of differentiation. According to this theory, cells display their positional values in order to compare them and this ties up with Wolpert's third theory of cells communicating positional values to each other.

MacWilliams (1978) devised a theory using positional information to try to explain the shape of the limb. He suggested that the pattern formation was controlled by several gradients of "morphogens" and invokes the role of "allosteric proteins" which bind the "morphogens". By complex mathematical formulae this model predicts the formation of a variety of simple shapes, simply by varying the concentrations of the "morphogens". Some of the shapes produced by this mathematical model resembled limbs. According to this model the concentration of the "allosteric proteins" varies with respect to their position in the limb. If one adds more proteins to the model the shapes can be made more complex. A modification of his model can be used to explain repeating structures.

Ede and Law (1969) attempted a computer simulation of the limb in order to describe pattern formation. This was improved upon by Wilby and Ede (1975) using localised cell-cell interaction to give the cartilage pattern in any limb shape. In the model, the cells modify their metabolism irreversibly at critical threshold levels of diffusible morphogen, which may be made or destroyed by cells. Cartilage elements are initiated as single cells and expand centrifugally to full-size developing sequentially along the antero-posterior axis. The final computer pattern gives a good approximation of the final limb pattern.

1.6 How the cells in the limb bud communicate with one another

As discussed in Section 1.5, it has been postulated that there must be some sort of signal which tells the limb which structure to form and what the final shape is to be. In order to achieve the final shape, the cells must communicate with one another.

One of the most well-studied cases of cell communication in the limb is the epithelial-mesenchymal interaction.

Balinsky (1929, 1931, 1933) and Filatow (1930 a and b, 1932), in a study on the limb development of newts, found that the outgrowth of the mesoderm was inhibited if it did not have contact with the limb epidermis. This has been confirmed by Tschumi (1957), who showed that after the initial outgrowth of the limb, further development of the limb bud depended on an epithelial-mesenchymal interaction. He placed the naked limb bud mesenchyme i.e. with epidermis removed, into the abdominal wall of the same or another tadpole. This abdominal wall consists of an epidermis and a pigmented peritoneum with parallel bundles of muscle fibres and some connective tissue lying in between.

In one series of experiments, he placed the naked limb bud mesenchyme between the muscles and epidermis, so that it was in contact with the differentiated non-limb epidermis. In the second series of experiments he placed the naked limb bud mesenchyme between the peritoneum and the muscles so that it would be isolated from the epidermis. The controls were transplanted to the same sites with their limb bud epithelium intact. The location of the buds in the experiment is shown in Figure 1.36 below.

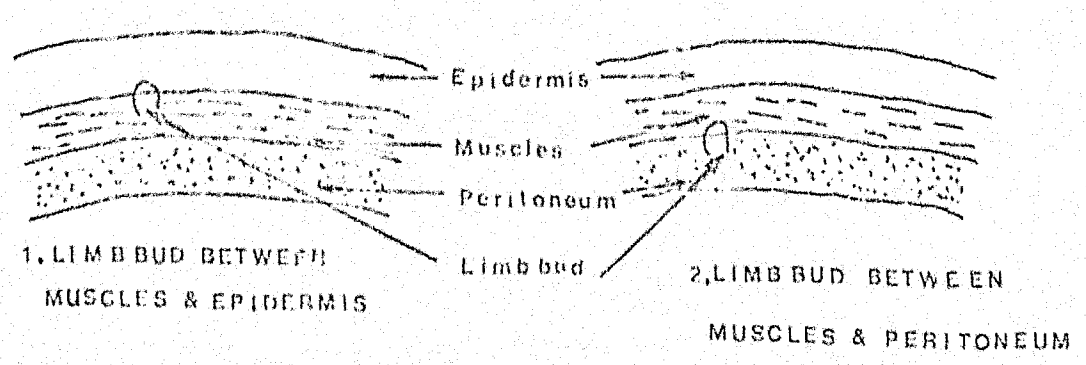

Figure 1.36 *Sketch showing the location of the limb buds in Tschumi's experiment*

The experimental limbs without epidermis, transplanted between the epidermis and mesenchyme, did not establish a new epidermis and did not establish contact with the non-limb epidermis in the wall of the abdomen. In the second case, as there was no epidermis between the peritoneum and muscles, the limb bud also developed without epidermal contact. In these two cases, elements did not develop, that were more distal to those presumptive regions already present in the mesenchyme at the start of the experiment i.e. according to the fate maps based on size, see Figure 1.19. Ishumi thus concluded that further distal outgrowth of the mesenchyme depended on the epithelial mesenchymal contact.

1.7 Cytodifferentiation

Differentiation is the path the cell takes to final development in response to its positional information. The limb begins its development as a multi-potential mesodermal core which can form chondroblasts, myoblasts or fibroblasts (presumptive cartilage, muscle or connective tissue). One way in which these equipotential cells could decide whether to form cartilage or muscle is suggested by Caplan (1977) in which he found that a high concentration of NAD (nicotinamide adenine dinucleotide) in chick limbs causes the cells to become myoblasts while a low concentration of NAD causes cartilage formation. The internal pool size of NAD is regulated by the vascular system. There are two types of vascular zone in the avian limb. The heavily vascularised zone forms myogenic cells while the lightly vascularised zone forms cartilage. Differentiation is characterised by the formation of proteins specific to the specialised function of the cell. This is in addition to the proteins that the cells need to survive, which are common to all cells. In a well-studied case of a differentiating organ, the pancreas, Rutter et al (1968) divide the process of differentiation into a series of well-defined stages. The above authors worked on the pancreas of the chick embryo, but as the present project is mainly a study of cartilage in limb development the stages of differentiation in the pancreas will be compared to those of the cartilage wherever possible.

The cells begin as undifferentiated cells when synthesis of "luxury" or cell-specific proteins, either for the pancreas or limb cartilage, is zero. This is called the undifferentiated state, see Figure 1.37.

The cells are subsequently converted to cells with "pancreatic potential" or "chondrogenic potential" in the primary regulatory event, see Figure 1.37. In the case of the pancreas, although pancreas-specific proteins such as insulin and lipase are not now present, they are present at a concentration which is 10^3 - 10^4 fold lower than that found in the fully differentiated pancreas cell. However, their level is significantly higher than that found in non-pancreatic cells. This state of pancreatic cell development is referred to by Rutter et al (1968) as the protodifferentiated state, see Figure 1.37. In the cartilage cell, the protodifferentiated state is characterised by the first appearance of chondroitin sulphate (which can be detected by alcian blue and chlorantine fast red with which it gives a distinct blue stain).

The conversion of the protodifferentiated cells to the fully differentiated state occurs as the second regulatory event, see Figure 1.37. This second regulatory event is preceded by a terminal cell division which is followed by a 10^3 - 10^4 fold increase in specific pancreatic proteins i.e. lipase and insulin. In the cartilage there is an increase in the production of chondroitin sulphate as a result of a dramatic increase in the concentration of the chondrogenic enzymes. (A teminal cell division is not necessarily the last cell division as the fully differentiated catilage cells continue dividing to achieve growth of the cartilage.)

Rutter et al (1968) also describes a tertiary regulatory event in which the enzyme concentration in the pancreas can be modulated by external factors such as diet or hormones. This is the stage for the maintenance of the differentiated state.

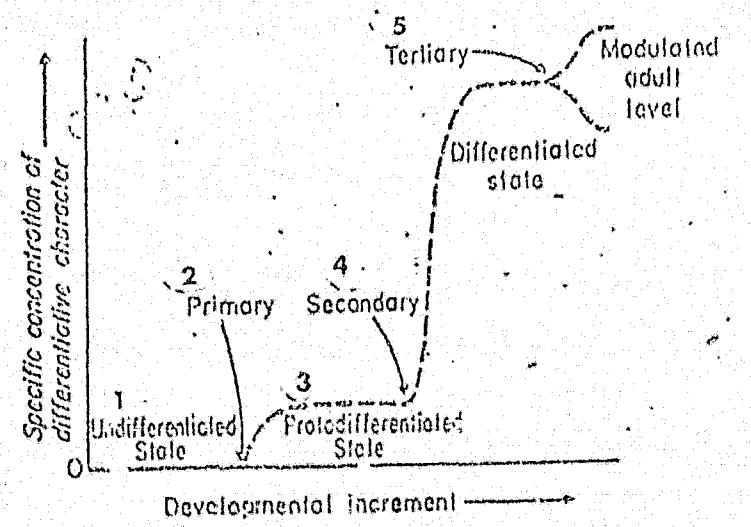

Figure 1.37 *Stages of differentiation of a cell (Rutter et al 1968)*

1.7.1. A detailed discussion of a differentiated cell type, namely cartilage

As seen in Figure 1.38, cartilage consists of cells called chondrocytes and a matrix surrounding them. The matrix consists of collagenous fibres, chondroitin sulphate, chondromucoid, albumoid and 70% water. The cartilage is enclosed in a fibrous bag, the perichondrium consisting mainly of collagenous fibres. This layer is not immediately obvious after cartilage growth ceases. The chondrocytes found in the matrix tend towards a spherical shape in the centre of the cartilage. However, at the edges near the perichondrium, the cells are relatively young and flattened.

To mark the beginning of cartilage development, mesenchyme cells enlarge into crowded vesicular cells in a mucinoid fluid. (Procartilage stage.) Thin plates of matrix then appear between the cells and enclose them. The cells are now called chondrocytes. Mesenchyme surrounding this mass of cartilage becomes compressed and is known as the perichondrium. In the mature cartilage the chondrocytes are found singly or in groups in spaces in the matrix. The spaces are known as lacunae.

Cartilage grows in one of two ways :

1. Interstitial growth

This is the internal growth of the cartilage, taking place by the continued growth of matrix between the chondrocytes, pushing them further apart.

2. Appositional growth

This type of growth takes place from the perichondrium. The innermost cells of the perichondrium specialise into chondroblasts and deposit matrix about themselves. These become overlaid by newer cells and matrix added from the perichondrium. As the cells get buried deeper in the matrix they undergo interstitial growth. (Arey 1968.)

In the limb itself, the first external sign that cartilage is forming is the condensation of cartilage cells (chondrocytes at this stage known as chondroblasts). In the tadpole this stage is referred to by Nieuwkoop and Faber (1967) as the procartilage stage. In the chick wing, these condensations are arranged in the pattern of the future limb in which the prospective humerus etc are indicated. Indeed Ede and Agerbak (1968) compare this to the Talpid^3 chick limb (a mutant of the chick which exhibits the phenomenon of polydactyly). These authors found that at the above stage many more condensations than usual are found, which go on to form more than the usual number of digits. According to Ede and Flint (1972) the cartilage condensations expand by the inclusion of more and more undifferentiated cells at the periphery. The condensations then merge to form areas of precartilage corresponding to the various elements of the limb to be formed. These authors claim that this is a good argument for the increasing mobility of the chondrocytes changing the pattern formation in the Talpid^3 chick limb. Due to the greater adhesiveness, larger less separate condensations form, the breadth of the paddle is larger thus more digits form.

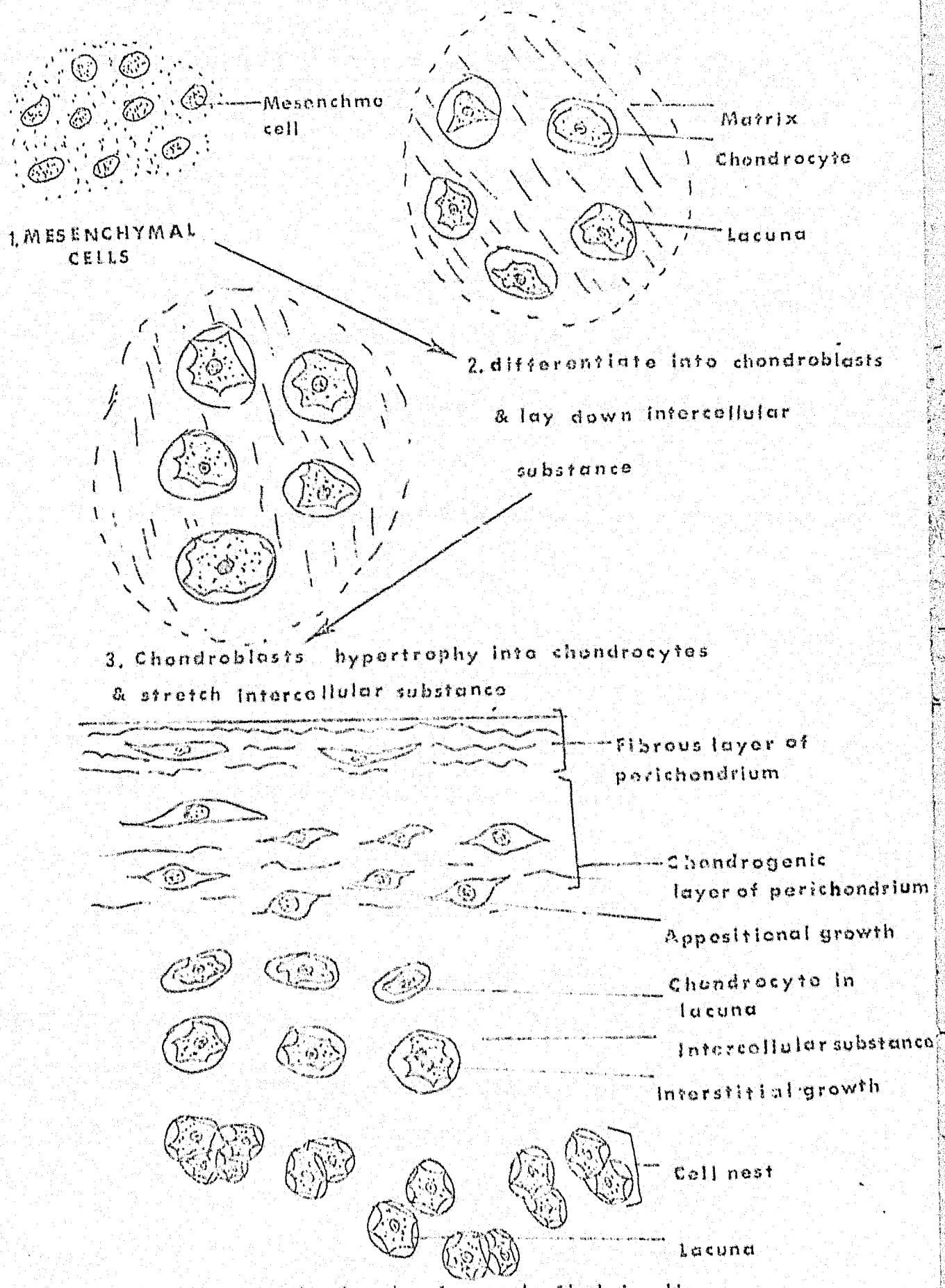

Figure 1.38 *The stages of cartilage development (Ham 1974)*

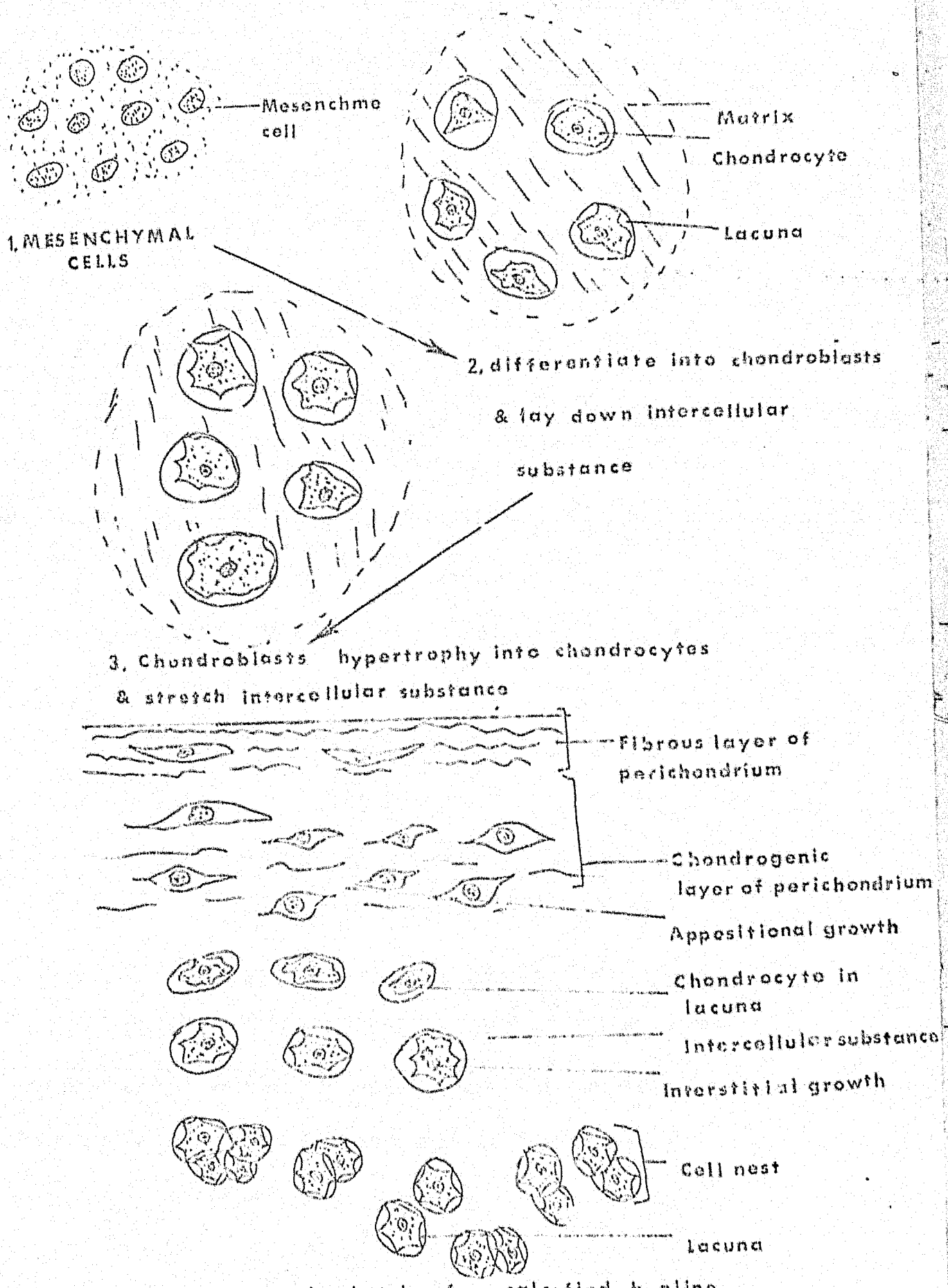

Figure 1.38 *The stages of cartilage development (Ham 1974)*

1.8 How differentiation and cell division can be affected by the use of 5-FUdR and 5-BUdR

5-Bromodeoxyuridine (5-BUdR) is found to act as a replacement for the base thymidine, in the DNA molecule. As a result the differentiation process of cells is affected (Wilt and Anderson 1972). 5-Fluorodeoxyuridine (5-FUdR) was first observed as an inhibitor of the mitosis of cells in culture. The reason for this inhibition was that it prevented the production of thymidylate synthetase, an enzyme required for the production of endogenous thymidine. As this thymidine is required for the replication of DNA, mitosis was inhibited (Conrad and Ruddle 1972). In the present study, the effects of these two drugs, both singly and in combination, have been analysed, to gain further understanding of early limb development in Xenopus laevis tadpoles.

1.8.1. Structure of DNA

As both of these drugs affect the nucleic acid DNA in some way, it will be useful to look at the structure of DNA. The effects of the drugs will be studied in section 1.8.3. and 1.8.4.

DNA is made up of purine and pyrimidine bases, deoxyribose sugar and phosphoric acid. (See Figure 1.39 below.)

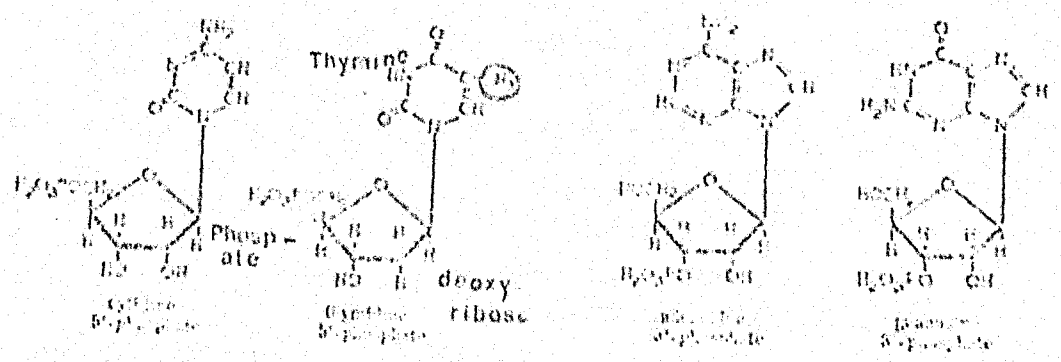

Figure 1.39 *Structure of the deoxyribonucleotides of DNA*

The purines are adenine and guanine, while the pyrimidines are cytosine and thymine. The respective purine or pyrimidine is condensed with deoxypentose sugar to form a nucleoside i.e. guanosine, adenosine, cytidine, thymidine. The phosphoric ester of the nucleoside is a deoxyribonucleotide (see Figure 1.39 above) i.e. adenylate, cytidylate, thymidylate, guanylate.

These four phosphoric esters are twisted onto two spiral strands which intertwine to form the double helix of DNA (see Figure 1.40).

1.8.2. Replication of DNA

Each strand of DNA consists of a particular sequence of purines or pyrimidines, with a purine on the one chain pairing with the complementary pyrimidine on the other chain. The adenine pairs with the cytosine and the guanine pairs with the thymine. During replication, the two strands dissociate and each one serves as a template for the synthesis of two new complementary chains of DNA. When the new chain is being synthesised, adenosine picks up its complementary nucleotide thymine and guanosine picks up its complementary nucleotide cytidine (Davidson 1969).

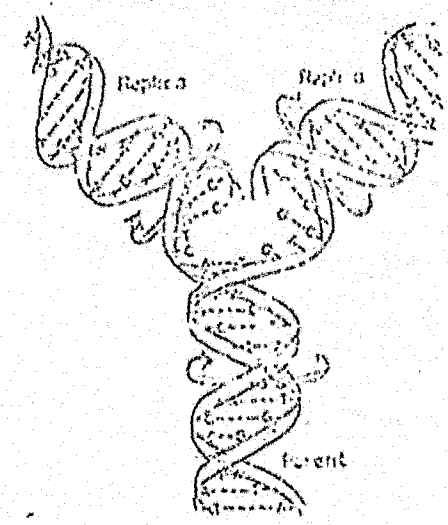

Figure 1.40 *Replication of DNA*

1.8.3. 5-Fluorodeoxyuridine

5-Fluorodeoxyuridine has a similar structure to the nucleoside thymidine (pyrimidine + deoxyribose sugar), see Figures 1.41 and 1.42 on p.41 The only difference is that the CH_3 group of the thymidine molecule is replaced by a fluorine atom. 5-Fluoro-deoxyuridine is referred to as 5-FUdR.