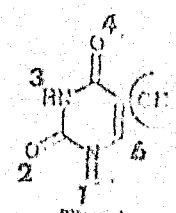
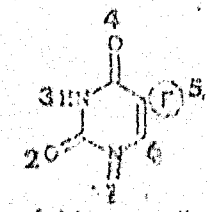


Thymine

CH_3 = 2.03 Å

λ_{H+} = 264 mμ

pK_a = 9.82



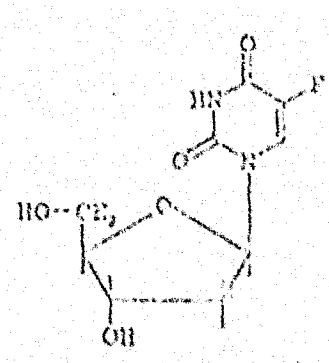
5-Fluorouracil

F = 1.35 Å

λ_{H+} = 266 mμ

pK_a = 8.15

Figure 1.41 *Comparison of thymine and 5-Fluorouracil*



5-Fluoro-2'-deoxyuridine

Figure 1.42 *Comparison of thymidine and 5-fluorodeoxyuridine (nucleoside)*

5-FUdr inhibits the enzyme thymidylate synthetase. This is the enzyme which in the production of endogenous thymidine converts dUMP (uridine monophosphate) to dTMP (thymidine monophosphate), indicated in Figure 1.43 below. In the normal metabolic cycle the thymidine monophosphate is then converted to thymidine triphosphate (nucleotide) and in this form enters the DNA. It can be seen therefore that if thymidylate synthetase is inhibited, endogenous thymidine is not produced and in the absence of thymidine DNA cannot replicate. This, of course, prevents mitosis, as has been observed experimentally (Toliver and Simon 1967). Addition of external thymidine can reverse the effects of 5-FUdR because DNA replication (requiring thymidine) can again take place.

The actual reaction taking place during the formation of dTMP from dUMP is shown below and in Figure 1.43 at (1). The methyl group in the reaction below is transferred from $S_1$10 methylene tetrahydrofolate (CH_2TF) to the 5 position of the pyrimidine ring of dUMP forming dTMP + dihydrofolate.

dUMP + CH_2TF ⟶ dTMP + DHF

CH_2TF: tetrahydrofolate.

DHF: dihydrofolate

The reaction is shown in its context in the metabolic scheme in Figure 1.43 below.

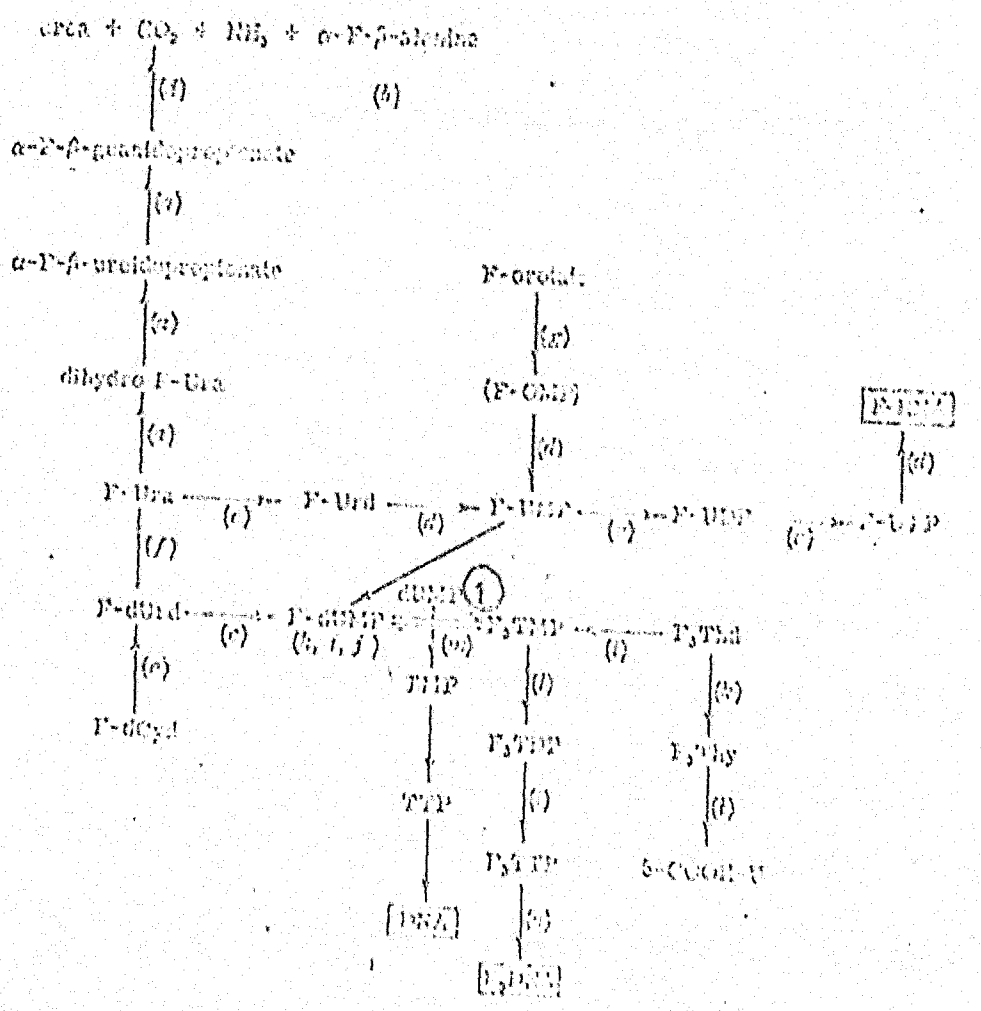


Figure 1.43 *Metabolism of thymidine showing the [illegible] block at 1*

The actual blockage of the activity of the enzyme thymidylate synthetase occurs as follows :
dUMP normally binds the enzyme thymidylate synthetase (TSase) (1 molecule of dUMP to one molecule of thymidylate synthetase). The inhibitor 5-FUdR binds with this enzyme in the ratio two molecules of 5-FUdR to one molecule of thymidylate synthetase (Heidelberger 1965). This is shown in Figure 1.44 below.

$$TSase + 1\ dUMP \rightleftharpoons TSase\text{-}(dUMP)_1$$

$$TSase + 2FdUMP \rightleftharpoons TSase\text{-}(FdUMP)_2$$

Figure 1.44 *Reaction showing the binding of thymidylate synthetase to 5-FUdR*

Once the enzyme has been bound by dUMP (normal reaction) it is used to convert dUMP to dTMP by facilitating the transfer of a methyl group from 5, 10 methylene tetrahydrofolate to the 5 position of the pyrimidine ring of dUMP (Hurlan and Heidelberger 1961). Methylene tetrahydrofolate is required for the inhibition of this reaction by 5-FUdR (in the form FdUMP) as, in its absence, FdUMP binds poorly to the enzyme. The transfer of the CH_3 group from the methylene tetrahydrofolate to the dUMP is shown in Figure 1.45 on page 44

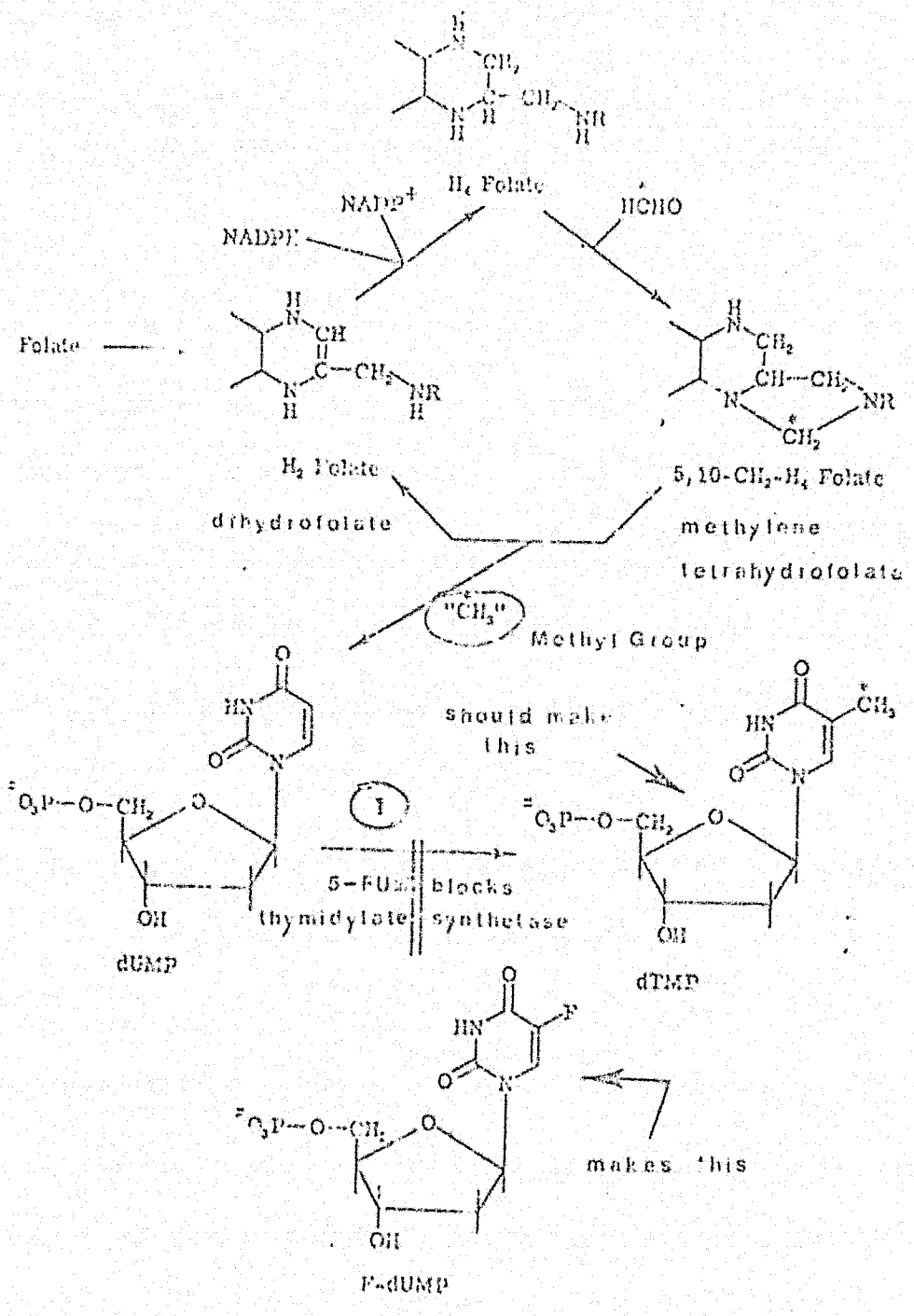


Figure 1.45 *Transfer of CH_3 from CH_2FH_4 to dUMP.*

A further scheme of the 5-FUdR block is shown below with 5-FUdR entering the cycle at (1) and forming FdUMP instead of thymine being able to form dTMP at (2). The FUdR enters the cycle as 5-FdR and is metabolised to FH_2 as shown in Figure 1.46 on page 45

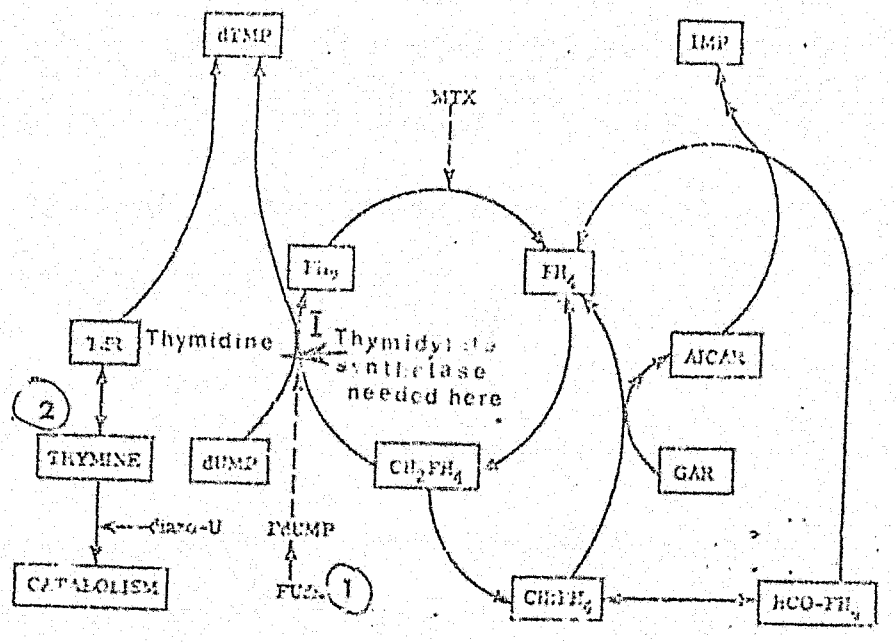


Figure 1.46 *Sketch showing the uptake of 5-FUdR and its further incorporation into the inhibition cycle. The inhibition site is marked I.*

In conclusion, 5-FUdR inhibits the enzyme thymidylate synthetase by competing for its binding site and in so doing prevents the formation of thymidine, thus preventing DNA replication. This causes an inhibition of mitosis.

1.8.4. 5-Bromodeoxyuridine

5-Bromodeoxyuridine differs from 5-FUdR in that a bromine atom rather than a fluorine atom is found at position 5 (see Figure 1.41) as shown in Figure 1.47 below.

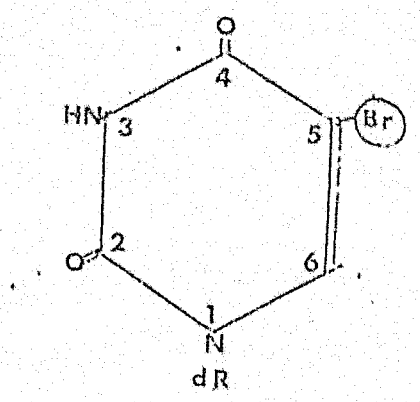
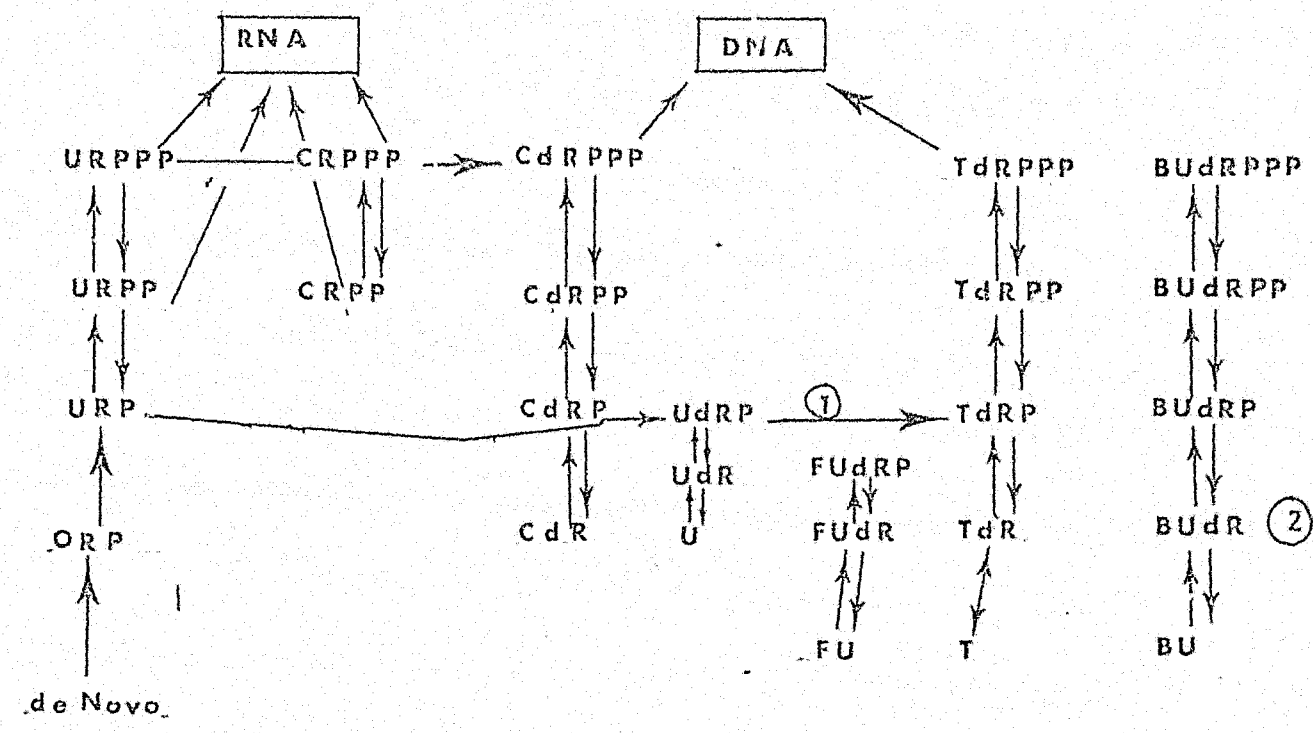


Figure 1.47 *Structure of 5-Bromodeoxyuridine (5-BUdR)*

5-BUdR replaces thymidine in the DNA, making the DNA heavier than normal DNA. Figure 1.48 below shows the mode of entry of the 5-BUdR into the DNA at the position marked 2.

For comparison the position marked 1 again shows the 5-FUdR block. DNA containing 5-BUdR prevents differentiation in cells in culture although the cells grow normally. This will be further discussed in section 1.8.5. below.



① Block in Thymidylate Synthesis

Figure 1.48 *Entry of 5-BUdR into DNA* (Szybalski 1962)

1.8.5. Effects of 5-BUdR and 5-FUdR on cells in culture

Although cells with 5-BUdR in their DNA are fully functional i.e. they continue growing and dividing, the production of "luxury proteins" (proteins for the specialised functioning of the cell e.g. myosin in muscle) is prevented. This can be reversed, as when the 5-BUdR is removed the cells proceed to differentiate normally. Likewise the effects may be reversed if thymidine is added to replace the shortage of endogenous thymidine. Stockdale et al (1964) showed that myogenic cells (prospective muscle cells) in the presence of 5-BUdR failed to fuse and form multinucleated myotubes prior to becoming muscle.

Simon (1963) reported that HeLa cells in culture with both 5-BUdR and 5-FUdR divided only once and incorporated slightly more 5-BUdR than did cells in 5-BUdR alone. It could be suggested that this is due to the lack of thymidine caused by 5-FUdR in the DNA, (which the cells replace with 5-BUdR). The one cell division is the final one in the presence of 5-FUdR.

1.8.5.1. The effects of 5-FUdR on cells and organisms when administered alone, with special reference to concentration

Owing to the cost of 5-FUdR, prudent use was made in the administration of this drug. As a first approximation to set a working concentration of 5-FUdR, it was decided to follow the dosage concentrations for Chinese Hamster cells which were worked out by Conrad and Ruddle (1972). They showed that when Chinese Hamster cells were grown in concentrations of 0,00005 µg/ml 5-FUdR, there was no reduction in the rate of mitosis and hence no reduction in thymidylate activity. In concentrations of 0,0001 µg/ml 5-FUdR, the rate of mitosis initially slowed down, stabilised and then returned to normal, showing an initial reduction in thymidylate activity. When these workers used a concentration of 0,001 µg/ml 5-FUdR, the rate of cell division was slowed down permanently and did not return to normal. Thymidylate synthetase activity was reduced to 20% of its original level.

Another worker (Ferguson 1978) who worked on the teratological effects of 5-FUdR in Wister rats, injected 0,1 µg 5-FUdR per mg. of body weight.

In the present study, tadpoles were maintained continuously in water, and concentrations ranging from 0,2 - 20 µg/ml of 5-FUdR were added to the water. These concentrations were 2000 times greater than the concentrations of 0,001 µg/ml that were effective in tissue culture and 200 times greater than the concentrations injected into the Wister rats. Furthermore, while the rate of uptake of the 5-FUdR may have been relatively lower, this was compensated for by the continuous application of 5-FUdR.

1.8.5.2. Effects of 5-BUdR on cells and organisms when administered alone, with special reference to concentration

In the present project a concentration of 10 ug/ml 5-BUdR was used. This was higher than that used in most tissue culture experiments as the tadpoles were swum in the solution and it was assumed that the chemical could be less toxic with this method than in tissue culture as the tadpoles were only getting a fresh solution of 5-BUdR once weekly.

While 5-BUdR affects differentiation, it does not affect cell division and growth as shown by Bishoff and Holtzer (1970). The fact that thymidine competes with 5-BUdR to enter the DNA was shown by Stockdale et al (1964) where excess thymidine added to the culture medium prevented the uptake of 5-BUdR.

Abbott and Holtzer (1968) examined the effects of 5-BUdR on chondrocytes taken from chick vertebral cartilage. The concentration used was 20 ug/ml. The resulting chondrocytes had bizarre shapes and the clones consisted of widely scattered fibroblastic cells i.e. true condensations required for cartilage formation were not seen. The mucopolysaccharide required for the matrix was destroyed.

Lasher and Cahn (1969) in their studies of cartilage cells found that a concentration of 10^{-4}M was toxic to the cells, while 10^{-5}M was not toxic, but prevented differentiation.

Agnish and Kochhar (1976) showed a relationship between the developmental stage of mice embryos and their sensitivity to 5-BUdR. From each embryo they removed the limbs and grew them in cell culture, one on normal medium and one on 5-BUdR. They found the following:

1. At a concentration of 2 ug/ml 5-BUdR, stage 26 to 29 embryos (eleven day) showed a complete suppression of chondrogenesis.
2. Mid-eleventh day embryos needed 10 to 25 ug/ml 5-BUdR to achieve the same effect.

3. If twelve and thirteen day embryos were used the effects were milder no matter how high the concentration of 5-BUdR was.

The effectiveness of the drug coincides with the various stages of cartilage differentiation, as the first signs of differentiation were apparent only in the late eleventh day embryo and after differentiation has taken place, the drug has no more effect.

Abbott and Holtzer (1968), Coleman et al (1970), (1968), Lasher and Cahn (1969) found that 5-FUdR had a proximo-distal effect. The scapula, first to differentiate, became increasingly resistant to the drug, while the more distal radius and ulna retained sensitivity throughout. This is in keeping with Tschumi (1957) who shows that differentiation proceeds in a proximo-distal direction.

Sala and Rizotti (1975) used stage 40 or 47 (NF stage) tadpoles (Xenopus laevis) and exposed them to 5-BUdR by injecting it into the coelomic cavity (2ul of 5 x 10^{-2}M). The most severe effects were noticed on stage 40 tadpoles reduction in pigmentation, swelling in the anterior portion of the head, curvature of the tail and accelerated cardiac rhythm. The effects were less severe in the stage 47 tadpoles and at stage 51 no external modifications were seen. Histologically, however, all the animals had suffered abnormalities to some degree.

In conclusion 5-BUdR is found to affect the differentiation of cells in culture and the severity of the effect is related to concentration.

1.9 Analysis of DNA by density gradient analytical ultracentrifugation

In the present study DNA was made heavy by swimming tadpoles in a solution of 5-BUdR. This heavy DNA was analysed by Density Gradient Ultracentrifugation. This technique is a means of measuring the amount of 5-BUdR that has entered the DNA due to the fact that the 5-BUdR makes the DNA heavy. Meselson and Stahl (1958) performed the classic experiment using this technique on DNA labelled with ^{15}N heavy hydrogen. If several species of DNA of different density are placed in an ultracentrifuge cell in a solution of caesium chloride and spun for twelve hours or more at 20,000 to 50,000 revolutions per second. As the DNA absorbs ultraviolet light at 260μm, ultraviolet photographs will display these various bands.

Hanawalt (1968) has shown that the buoyant density of DNA containing 5-BUdR increases by 0,9 gcm^{-3}, while Weil et al (1955) have shown that in the presence of 5-FUdR even more 5-BUdR is taken up as shown by the fact that the buoyant density of the DNA increases. Further, by this method one can distinguish between very heavy DNA which has two strands substituted with 5-BUdR and hybrid DNA, which is lighter as only one strand has been substituted with 5-BUdR. The hybrid bands of DNA are found between the normal DNA and the heavy DNA on the photographs. Vinograd (1963) has postulated the use of marker DNA bands using DNA of known density to determine the density of the various bands of DNA. Each DNA species forms a band at the position where the CsCl (caesium chloride) density equals the buoyant density of that species in the ultracentrifuge cell. The CsCl spins down forming a varied density solution throughout the cell, the densest solution being at the base of the cell. The various solutions of DNA stabilise in position in the cell when their density is counterbalanced by the density of the CsCl at a particular point in the cell. This takes up to 12 hours to achieve. At this point bands can be seen on the ultraviolet photographs.

Figure 1.49 below shows a cell with the various densities of CsCl.

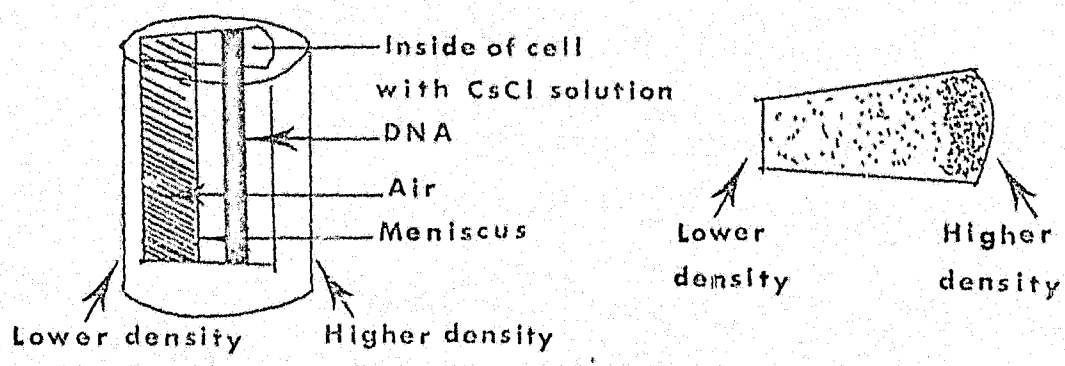


Figure 1.49 *CsCl in the ultracentrifuge cell*

Figure 1.50 on p.51 shows a typical result of an ultracentrifuge ultraviolet photograph.

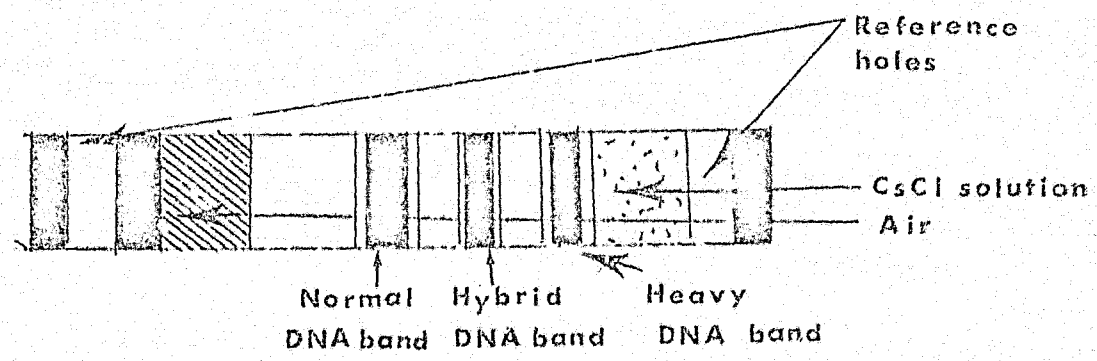


Figure 1.50 *Sketch of ultraviolet photograph showing various DNA bands*

1.10 Objectives of this study

The hindlimb of Xenopus laevis appears to develop in a proximo-distal direction (Tschumi 1957) under the influence of the apical ectodermal ridge. The zone of polarising activity (ZPA) appears to influence the antero-posterior patterning of the limb during its development (Saunders and Gasseling 1968). Of the three limb axes, proximo-distal, antero-posterior and dorso-ventral, the dorso-ventral axis is established first, followed by the antero-posterior axis and lastly the proximo-distal axis is determined.

The differentiation of the hindlimb tissues, muscle, cartilage and connective tissue, appears to be determined by the cell's position which the cell determines according to a gradient of a particular chemical or it could determine its position according to the amount of time it has spent in the "progress zone" (Wolpert 1981).

The present study used 5-BUdR and 5-FUdR to study the above processes further. As 5-BUdR prevents differentiation and 5-FUdR prevents cell division, the hindlimb development was likely to be affected in various ways. These effects could increase our knowledge of the processes of cell differentiation and cell patterning in the Xenopus laevis hindlimb.

2. METHODS AND MATERIALS

2.1 Experiments investigating the effects of 5-BUdR and 5-FUdR on the shape and patterning of the Xenopus laevis hindlimb

2.1.1. Tadpole breeding and rearing

For the experiments in which the tadpoles were swum in 5-BUdR and 5-FUdR a reliable, regular supply of tadpoles was required. Xenopus laevis responds well to life in captivity, in that it breeds all year round when kept at warm temperatures and treated with a regular dose of breeding hormone (Pregnyl). The tadpoles respond equally well by growing and metamorphosing as long as the temperature is kept constantly warm. For these reasons Xenopus laevis was chosen as the amphibian for this study.

Xenopus laevis tadpoles were bred from adults supplied by Jonkershoek Island Fish Hatchery, Stellenbosch. Females and males were isolated and fed on chopped beef liver twice weekly in summer. When breeding became more difficult, in winter, they were fed daily. The water was changed after feeding and the frogs were kept at a temperature of 25°C, which is conducive to breeding. Breeding was induced by Pregnyl (Organon). A solution was made up of 4500 units in 9ml water, about 500 units/ml. The male was given 2 doses of 0,3ml, about 300 units in total. As breeding became more difficult to induce during the winter, it was necessary to prime the males daily for two weeks with the above dosage until the black nuptial pads appeared. The females did not require increased doses. The hormone was injected through the dorsal lymph sac (Gurdon 1967). The frogs were then placed in a laying tank in shallow water on a mesh. The eggs dropped through the mesh and were collected the following morning and placed in shallow trays at 25°C. At this temperature they developed to stage 43 in three days (Nieuwkoop and Faber 1967). At about this stage they started feeding and were fed on Liquifry No. 2 (Liquifry Company Ltd, Dorking), about ten drops daily to sixty tadpoles in two litres of water. The tadpoles were thinned out constantly to prevent growth inhibition by overcrowding. The water was changed regularly.

2.1.2. In vivo experiments in which Xenopus laevis tadpoles were swum in 5-BUdR and 5-FUdR and analysed for growth deformities

The following experiments were set up to test the effects of 5-BUdR and 5-FUdR on Xenopus laevis tadpole growth. A weekly analysis was carried out under the dissecting microscope and hindlimb deformities were noted. (See section 3.1 for pictures.) The hindlimb was therefore focussed on for further experimentation.

2.1.2.1. Pilot experiments

The following initial experiments were set up :

a) Experiment started at stage 43 (NF stage)

Solution	Volume (ml)	Number of tadpoles (2 dishes, 6 tadpoles per dish)
5-BUdR 10 ug/ml + 5-FUdr 0,2 ug/ml	200	2 x 6
5-FUdR 0,2 ug/ml	200	2 x 6
5-BUdR 100 ug/ml	200	2 x 6
5-BUdR 10 ug/ml	200	2 x 6
5-BUdR 1 ug/ml	200	2 x 6
5-BUdR 0,1 ug/ml	200	2 x 6
Water	200	2 x 6

b) Experiment started at stage 46 (NF stage)

Solution	Volume (ml)	Number of tadpoles (2 dishes, 6 tadpoles per dish)
5-BUdR 10 ug/ml	200	2 x 6
5-BUdR 10 ug/ml + 5-FUdR 0,2 ug/ml	200	2 x 6
5-FUdR 20 ug/ml	200	2 x 6
Water	200	2 x 6

2.1.2.2. Further experiments using Xenopus laevis tadpoles swum in 5-FUdR

On the basis of the results of the pilot experiments in 2.1.2.1. further experiments were set up in which the tadpoles were swum only in 5-FUdR as 5-BUdR was noted not to have had any externally visible deforming effects on the hindlimb.

The following experiments were set up :

Stage (Experiment begun)	Solution	Concentration µg/ml	Volume ml	Number of tadpoles (2 dishes, 6 tadpoles per dish)
0	FUdR	10	200	6 x 2
46	FUdR	20	200	6 x 1
46	FUdR	10	200	6 x 1
46	FUdR	10	200	6 x 2
47	FUdR	20	200	6 x 2
48	FUdR	20	200	5 x 1
48	FUdR	10	200	6 x 1
49	FUdR	20	200	6 x 1
49	FUdR	10	200	6 x 1
50	FUdR	20	200	6 x 2
52	FUdR	20	200	6 x 2
43	Water	--	200	6 x 4

(The tadpole stages used can be seen in Appendix A.)

In order to study the deformed limbs in further detail, and for the purposes of clearer photography, the tadpoles were fixed and the limbs stained, removed and mounted for photography (whole mount).

The tadpoles were fixed in Karnovsky's fixative (Karnovsky 1965) as this was found to be less damaging than the formaldehyde mentioned in the same article.

Karnovsky's Fixative

4g of paraformaldehyde are dissolved in 46ml water. The temperature is raised to 60°C in a water bath and 1M NaOH is added dropwise until the solution clears.

Add 50 ml buffer solution C and 4ml 25% glutaraldehyde. Titrate at room temperature to pH 7,2 - 7,4 using 1N HCl.

Buffer C = 41,5ml soln A + 8,5ml soln B.
Soln A = NaH_2PO_4 H_2O (monosodium phosphate) 2,26%
Soln B = NaOH (sodium hydroxide) 2,52%.
Washing solution 50% buffer + 50% glutaraldehyde solution.
The tadpoles were fixed for 24 hours and then washed for 24 hours. They were then transferred to acid alcohol for 24 hours (1% HCl in 70% ethyl alcohol) (Mahoney 1973). They were then stained for one week in 1% Victoria Blue in acid alcohol. They were then differentiated in several changes of acid alcohol until the desired colour was obtained. When differentiation was complete they were transferred to 90% alcohol for twelve hours. They were then dehydrated thoroughly in several changes of absolute alcohol for a few hours. When completely dehydrated they were cleared in methyl benzoate and stored in fresh methyl benzoate. This method stained the cartilage blue and made the limb transparent so that the cartilage could be clearly seen. The limbs were now whole mounted and photographed having been severed from the animal. The preliminary experiments were photographed on the whole animal (see 3.1). These whole mounts were analysed by means of a deformity index (see section 3.2).

2.2 Radioactive thymidine uptake in the presence of 5-FUdR by Xenopus laevis tadpole hindlimbs

As 5-FUdR is known to depress the uptake of thymidine (by decreasing DNA replication which reduces the requirement for thymidine), it was decided to test this by swimming Xenopus laevis tadpoles in radioactive thymidine (^{3}H-thymidine). The experiments were set up as follows :

Control

One dish of 200ml capacity with six stage 49 tadpoles which were swum in the solution of 20 ug/ml 5-FUdR for one week. They were unfed for three days then fed on liquifry No. 2 for four days.

Experiment

One dish of 200ml capacity with six stage 49 tadpoles which were swum in the solution of 20 µg/ml 5-FUdR + 5 µCi/ml thymidine for one week. They were unfed for three days, then fed on liquifry No. 2 for four days.

The limbs of the tadpoles were removed and weighed as were the tails. They were than placed singly into clean glass scintillation vials containing 1ml of soluene which was used to solubilise the tissue. The vials were immediately capped and incubated at 45°C in a water bath for eight hours to assist the solubilisation process. 10ml of a specially prepared Toluene based mixture was added to each vial. The vials were recapped and thoroughly shaken. The vials were then placed in the dark in a refrigerator at approximately 6°C for twelve hours.

The vials were then counted in a Packard Tri-Carb Scintillation Counter (Series 3,000) for fifty minutes each. Counts of radioactivity were registered on all three channels, each channel having a window setting of 50 - 1000 and a gain setting of 53% for tritium. The counts per fifty minutes were then reduced to counts/min/mg. (Price 1973.) The results are analysed in section 3.3.

2.3 Buoyant density gradient analysis of 5-BUdR DNA

It was decided to analyse the amount of 5-BUdR entering the DNA using buoyant density gradient analysis and to compare this to the uptake of 5-BUdR by the DNA in the presence of 5-FUdR. In the presence of 5-BUdR DNA should become heavy and in the presence of 5-BUdR and 5-FUdR DNA should become heavier still as the lack of thymidine should cause the uptake of more 5-BUdR. The tadpoles were swum in the solutions as follows :

a) 100 stage 41 (NF stage) Xenopus laevis tadpoles in 200ml water for three days at 21°C. These tadpoles were killed at stage 43.

b) 100 stage 43 (NF stage) Xenopus laevis tadpoles in 200ml of 0,1 mg/ml 5-BUdR for five hours at 21°C.

c) 100 stage 43 (NF stage) Xenopus laevis tadpoles in 200ml 0,1 mg/ml 5-BUdR and 0,0002 mg/ml 5-FUdR (0,2 µg/ml as 0,001 mg = 1 µg) for five hours at 21°C.

The DNA was then extracted from the tadpoles and analysed by buoyant density ultracentrifugation.

2.3.1. DNA extraction

DNA extraction was done essentially according to the method of Marmur (1961) and Grossman, Goldring and Marmur (1969).

a) 100 tadpoles were killed in 15 ml of ice cold EDTA solution (Ethylene Diamine Tetra-acetic Acid, Disodium Salt, Merck Analytical Reagent). EDTA solution : 0,1M EDTA + 0,1M NaCl. The solution was adjusted to pH 8. The tadpoles were homogenised in this solution by means of an ultra-turrax homogeniser. During this process, the test tube with the solution was placed in a beaker of ice (Ledoux and Huart 1967).

b) The cells were lysed in Sarkosyl NL 30, Sarkosyl NL 97 or Sarkosyl O (samples by kind donation of Ciba-Geigy). The Sarkosyl was used at a concentration of 1,3%. The mixture was placed in a 60°C water bath for ten minutes and then allowed to cool to room temperature (Marmur 1961). Sarkosyl is a new detergent used by Grossman, Goldring and Marmur (1969) instead of SDS (sodium dodecyl sulphate) used by most earlier workers. SDS is usually used to dissociate the protein from the nucleic acid but the amide derivative, sodium dodecyl sarcosinate, is now used due to its high solubility in concentrated CsCl. Probably the hydrocarbon chain of sodium dodecyl sulphate competes for hydrophobic bonds. The anionic detergent appears to inhibit nucleases and the negative charge prevents interaction with nucleic acids (Noll and Stutz 1968). On comparing DNA yields for the different detergents, Sarkosyl was found to give a higher yield than SDS. Of the three Sarkosyl NL 30 was used instead of the Sarkosyl NL 97 used by both of the above groups of research workers, as it gave a better DNA yield. Sarkosyl surfactants are high molecular weight carboxylic acids or their own sodium salts of the form : $CH_3 (CH_2)_n CON (CH_3) CH_2 - COOH$ (or $COONa$).
They are modified fatty acids in which the hydrocarbon chain is interrupted by an amidomethyl group ($-CONCH_3-$). They are the amide derivatives of SDS (also known as sodium lauryl sulphate). This modification improves the solubility and crystallinity of the molecule, enhances the acidity of the carboxylic acid group and increases the absorption characteristics.

They are soluble in most organic solvents and are appreciably soluble at mildly acidic pH's. They foam less than SDS (Handbook - Geigy Industrial Chemicals).

c) Sodium perchlorate (pure reagent) was added at a high concentration to separate the protein from the nucleic acid (Marmur 1961). 1,838 g were added to the lysate thus forming a 5M solution which was then adjusted to pH 5.

d) The solution was deproteinised by shaking in 30ml chloroform : isoamyl alcohol 24 : 1 (isoamyl alcohol 3 methyl butanol $C_5H_{11}OH$ pure reagent, Merck). The solution was shaken gently for thirty minutes in a stoppered flask.
The chloroform causes surface denaturation of the protein, while isoamyl alcohol reduces foaming, aids the separation and maintains the stability of the layers of the centrifuged deproteinised solution (Marmur 1961).

e) The emulsion was separated into three layers by a ten minute centrifugation at 2000 rpm. The upper aqueous layer containing the nucleic acids was removed with a Pasteur pipette. The protein interface and chloroform layer were discarded.

f) Further deproteinisation was accomplished with pronase _Streptomyces griseus_, Miles-Seravac (Grossman, Goldring and Marmur 1969)).
It was made up as follows :
300mg dissolved in 300ml water and placed in a water bath at 37°C for forty-five minutes. The solution was then adjusted to pH 5 with concentrated HCl and left at room temperature for forty-five minutes. Solid Tris Buffer (Seravac) was then used to adjust the pH to 7 and the enzyme was used at a final concentration of 1 mg/ml and allowed to act for three hours at 37°C (Stern 1968). As well as digesting protein, pronase partly digests itself (McCarthy 1968).

g) The lysate was dialysed against SSC for three hours at room temperature and then overnight at 4°C (Grossman, Goldring and Marmur 1969). SSC (standard saline citrate) - 0,15M NaCl + 0,015M Trisodium Citrate (2-Hydrate) (Analytical Reagent, Merck).

h) The lysate was treated with Ribonuclease (RNase) (Bovine Ribonuclease Grade II 5 x crystallised Miles-Seravac). The enzyme was used at a final concentration of 50 µg/ml and allowed to act at 37°C for three hours (Marmur 1961). RNase also partly digests itself (McCarthy 1968). The RNase was made up as follows (Kalf and Grece 1968) :
At a concentration of 1 mg/ml in acetate buffer pH 4,5 and incubated at 37°C for thirty minutes. Acetate buffer - 0,001M sodium acetate (trihydrate) (Analytical Reagent BDH) + 0,001M EDTA. pH was adjusted with concentrated glacial acetic acid. The solution was heated at 80°C for ten minutes to inactivate any DNase.

i) The lysate was dialysed at room temperature for four hours and then overnight at 4°C (Marmur 1961). It was dialysed against 0,2% SSC + 0,15M NaCl at a pH of 5.
The dialysis tubing was handled only with rubber gloves, as enzymes on the hands could denature the DNA. Dialysis removes the detergent, enzymes and the prod[illegible]f enzyme degradation (Kalf and Grece 1968).

j) The nucleic acids were precipated by addition of absolute ethyl alcohol (Kalf and Grece 1968). The DNA was then centrifuged down and dried in a vacuum for two hours and redissolved in 3ml SSC. A few drops of chloroform were added and it was stored at 4°C.

2.3.2. Buoyant analytical density gradient ultracentrifugation

The extracted DNA was analysed by buoyant density ultracentrifugation.

2.3.2.1. Theory of analytical density [illegible]dient ultracentrifugation

As 5-BUdR has been found to enter the DNA, and as bromine has a higher molecular weight than the methyl group which it replaces, the buoyant density of the substituted DNA has been found to increase and this principle has been used by Meselson, Stahl and Vinograd (1957) to study molecular weights and partial specific volumes of macromolecules.

h) The lysate was treated with Ribonuclease (RNase) (Bovine Ribonuclease Grade II 5 x crystallised Miles-Seravac). The enzyme was used at a final concentration of 50 µg/ml and allowed to act at 37°C for three hours (Marmur 1961). RNase also partly digests itself (McCarthy 1968). The RNase was made up as follows (Kalf and Grece 1968) :
At a concentration of 1 mg/ml in acetate buffer pH 4,5 and incubated at 37°C for thirty minutes. Acetate buffer - 0,001M sodium acetate (trihydrate) (Analytical Reagent BDH) + 0,001M EDTA. pH was adjusted with concentrated glacial acetic acid. The solution was heated at 80°C for ten minutes to inactivate any DNase.

i) The lysate was dialysed at room temperature for four hours and then overnight at 4°C (Marmur 1961). It was dialysed against 0,2% SSC + 0,15M NaCl at a pH of 5.
The dialysis tubing was handled only with rubber gloves, as enzymes on the hands could denature the DNA. Dialysis removes the detergent, enzymes and the products of enzyme degradation (Kalf and Grece 1968).

j) The nucleic acids were precipated by addition of absolute ethyl alcohol (Kalf and Grece 1968). The DNA was then centrifuged down and dried in a vacuum for two hours and redissolved in 3ml SSC. A few drops of chloroform were added and it was stored at 4°C.

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The method involves an observation of the equilibrium distribution of the macromolecular material in a density gradient, itself at equilibrium. The density gradient is established by the sedimentation of a low molecular weight solute in a solution subject to a constant centrifugal field. A solution of low molecular weight is centrifuged until equilibrium is closely approached. The opposing tendencies of sedimentation and diffusion have then produced a stable concentration gradient of the low molecular weight solute. The initial concentration of low molecular weight solute, the centrifug field strength and the length of the liquid column are chosen so that the range of density at equilibrium encompasses the effective density of the macromolecular material. At equilibrium, the macromolecules reach positions at which the density of the surrounding liquid is equal to their own, thus macromolecules of different density band at different positions in the ultracentrifuge cell (Meselson, Stahl and Vinograd 1957, and Beckman Review). As the molecules sediment towards the bottom of the cell a transparent solvent solution region is created and the image of this portion of the cell becomes darker. Where the ultraviolet light is absorbed by the DNA light bands will be seen. By means of a densitometer the image on the film can be converted into a plot of optical density versus distance from the axis of rotation (Meselson and Stahl 1958, Beckman 1962).

The advantage of this optical system is that concentrations of 0,001% of DNA can be detected which is of the order of magnitude likely to be found in the experimental samples (Beckman Instruction Manual E-IM-3).

2.3.2.2. Method Used

One of the most effective low molecular weight substances found for DNA is CsCl, which combines with it to form the caesium salt. Meselson, Stahl and Vinograd (1957) chose it due to its high solubility, high molecular weight and high density which allows the obtaining of steep gradients, 1,25 to 1,90 g/ml. It also has a low viscosity suitable for rapid sedimentation, is non-toxic to DNA and is soluble in water (Dirks) and salt solutions (Szybalski 1968).

The average buoyant density of DNA in CsCl is +/- 1,7 g/ml and thus a caesium chloride solution was made up at average density 1,699 g/ml at 25°C, the temperature used for the ultracentrifuge runs (Szybalski 1968).

The experimental work was done on a Beckman Model E Analytical Ultracentrifuge. A 30mm charcoal-filled Epon centrepiece was used as CsCl is corrosive to aluminium (Vinograd and Hearst 1962). The longer centrepiece was chosen, as low volumes of DNA of a certain density were more likely to be detected. 0,2 to 0,5 ug can be used (Vinograd and Bruner 1966). A counter-balance of 30 mm was used.

The optical density of the sample was read on a Beckman Ultraviolet Spectrophotometer at 260 µm

The sample was diluted to an optical density of approximately 0,65 with SSC. 2,85 g of CsCl (Optical Grade - Suprapur Merck) were added and the sample made up to a weight of 5,09 g with SSC. The refractive index of the solution was read on an Abbe Refractometer and the density read off from tables (Chervenka 1969).

The upper plain window of the cell was replaced with a 2° negative wedge window to correct for the light scatter caused by the density of the solution (Vinograd and Hearst 1962).

The AN.E Rotor was used and the machine was run at 44 770 rpm in order to allow the sedimentation of the macromolecules in the dense gradient (Meselson, Stahl and Vinograd 1957, Grossman, Goldring and Marmur 1969).

The duration of the run was eighteen hours and photographs were taken at two hourly intervals with an exposure of two minutes.

The photographs were analysed in a Beckman RB Analytrol with a Film Densitometer attachment.

The following three runs were performed :

	Optical Density DNA	Refractive Index CsCl (g/ml)	Density CsCl (g/ml)	Volume used (ml)
1. Water 3 hrs.	0,58	1,3991	1,699	1,3
2. 5-BUdR 5 hrs.	0,52	1,4000	1,699	1,8
3. 5-BUdR + 5-FUdR 5 hrs.	0,46	1,4005	1,720	1,3

The photographs and densitometer traces are shown in section 3.4.

2.4 Investigation into the growth of tadpoles under different methods of feeding

It was decided to investigate the optimum growth conditions for the tadpoles, as far as feeding was concerned, in both large trays (2 litre capacity normally used) and small dishes (200 ml capacity used in this study due to scarcity of 5-FUdR).

Tadpoles were taken at stage 47 (NF stage) and placed into trays of 2 litre capacity and large surface area. Sixty tadpoles were placed in two litres of water as this was the concentration of tadpoles used in the small (200 ml) dishes.

Experiment A

Food	Dose	Number of Tadpoles	Volume of Water (litres)
Commercial baby food (liver and vegetables)	1 g/day for 2 weeks 2 g/day for rest	60	2
Complan	"	60	2
Yeast	"	60	2
Liquifry	"	60	2

Experiment B

Food	Dose	Number of Tadpoles	Volume of Water (litres)
Commercial baby food (liver and vegetables)	1 g/day for 2 weeks 2 g/day for rest	60	2
Fish food (Tetramin)	"	60	2
Yeast	"	60	2
Liquifry	"	60	2
Lettuce	"	60	2

Experiment C

Food	Dose	Number of Tadpoles	Volume of Water (mls)
Commercial baby food (liver and vegetables)	0,16 g/day - 2 weeks 0,32 g/day for rest	6	200
Yeast	"	6	200
Fish Food (Tetramin)	"	6	200
Lettuce	"	6	200
Liquifry	"	6	200

The tadpoles were analysed for rate of growth once weekly by counting the number of animals at any one stage (NF stage). The results are shown in section 3.5.

2.5 Details of the chemicals used and source list of suppliers

1. BUdR, 5-Bromo 2'deoxyuridine, Sigma Chemical Corporation.
2. 5-FUdR, 5-fluoro 2'deoxyuridine kindly donated by Dr. Ram of Roche Products (Pty) Limited.
3. Victoria Blue, 4R Merck, Art 8539.
4. Tetramin, Lopis, Johannesburg, containing fish meat, fish roe, fish liver, crayfish, water plants, mussel meat, brine shrimp, wheat germ, cod liver, insect larvae, kelp.
5. Liver and vegetables, Licence Gerber Prod. Company, USA, Purity Ricket and Colman SA Ltd, containing modified maize starch, liver, soya protein, tomatoes, yeast, onion extract, caramel, iodised salt.
6. Yeast, Anchor Yeast (Pty) Limited, Watt Street, Industria, Johannesburg.
7. Complan, Glaxo Allenbury's SA (Pty) Limited, Manchester Road, Wadeville, containing protein 31g, fat 16g, carbohydrate 44g, calcium 850mg, phosphorus 790mg, sodium 400mg, chloride 750mg, potassium 1100mg, iron 7,7mg, iodine 44ug, Vit A 1100 units, Vit B_1 1,0mg.
8. CsCl, Art 2041 Merck 2525019 extrapur mw. 168,36.
9. Soluene 350 (Packard) is an extremely efficient solubiliser of animal tissue. Since most of the Biological samples containing radioactivity measured by liquid scintillation counting are not readily soluble in the aromatic hydrocarbon based scintillation solution (Toluene) special solubilisers are required to obtain a homogenous system for reproducible measurement of radioactivity.
10. Toluene scintillant is composed of 100ml Permafluor III (Packard) in 1 litre of Toluene (Packard). The resulting solution contains 5,0g PPO and 0,5g bis-MSB per litre. PPO and bis-MSB are highly efficient scintillators which will not form quenching adducts with tissue solubilisers, amines and acids.
11. Toluene mixture. The solubilising mixture was made up as follows :
    Permafluor III Packard : 70%
    Triton X 100 Packard : 30%

Permafluor is a concentrated solution of stable efficient scintillators (PPO and bis-MSB). It does not form quenching adducts with tissue solubiliser, amines and acids. Triton X 100, especially purified for liquid scintillation counting, is an efficient tissue solubiliser, so that the mixture of Permafluor and Triton is an extremely efficient scintillant. This mixture modified from Thornley 1971, was found to be the most efficent method of detecting radioactivity in aqueous biological samples. Samples were incubated in the dark to allow them to equilibrate and to reduce chemiluminescence to a minimum (Packard handbook).

12. Methyl ^{3}H thymidine specific activity 18 Ci/mmol labels DNA..
13. 5,6 ^{3}H Uridine (methyl) specific activity 40,0 Ci/mmol label A.
14. Liquifry No. 2 (Liquifry Company Limited, Dorking).
15. Pregnyl (Organon). This is a mammalian gonadotropin which in the male frog stimulates the growth of the testes, sexual maturityand release of the sperm, as well as the formation of dark nuptial pads on the insides of the forelimbs. The formation of the nuptial pads indicates that the males are in mating condition.
16. EDTA, Ethylene Diamine Tetra - acetic acid, disodium salt, Merck analytical reagent.
17. Sarkosyl NL 30, sarkosyl NL97, sarkosyl O, by kind donation of Ciba Geigy.
18. Isoamyl alcohol = 3 methyl butanol $C_5H_{11}OH$ pure reagent Merck.
19. Pronase, Streptomyces griseus Miles Seravac.
20. Ribonuclease, Bovine Ribonuclease Grade II 5X crystallised Miles Seravac.
21. Sodium Acetate, Analytical Reagent BDH.

3. RESULTS

The hindlimb buds of the tadpoles swum in 5-FUdR were found to be deformed (see section 2.1) either in shape or lacking the full number of digits or both. A method was devised to approximate the degree of deformity of each limb. In the limb each defect was given equal weighting as described on page 73. For convenience, the limbs were divided into three types: adult limbs (56 - 65) were given a count of 91 when normal, medium mature limbs (54 - 56) were given a count of 32 when normal and immature limbs were given a count of 24 when normal. From these normal counts points were removed depending on the type of deformity and the result is referred to as the deformity index for each limb.

3.1 Results of pilot experiments in which Xenopus laevis tadpoles were swum in solutions of 5-FUdR and 5-BUdR

A series of initial experiments revealed the hindlimb deformities on observation of the tadpoles under the dissecting microscope. These tadpoles were subsequently photographed to show the deformities. The photographs of these tadpoles are shown in the next few pages. In section 2.1 the limbs were further analysed by staining the cartilage and taking magnified photographs of the result. Below, in Figure 3.1, is a picture of a tadpole (NF stage 60) with three toes on the left hindlimb and two toes on the right hindlimb.

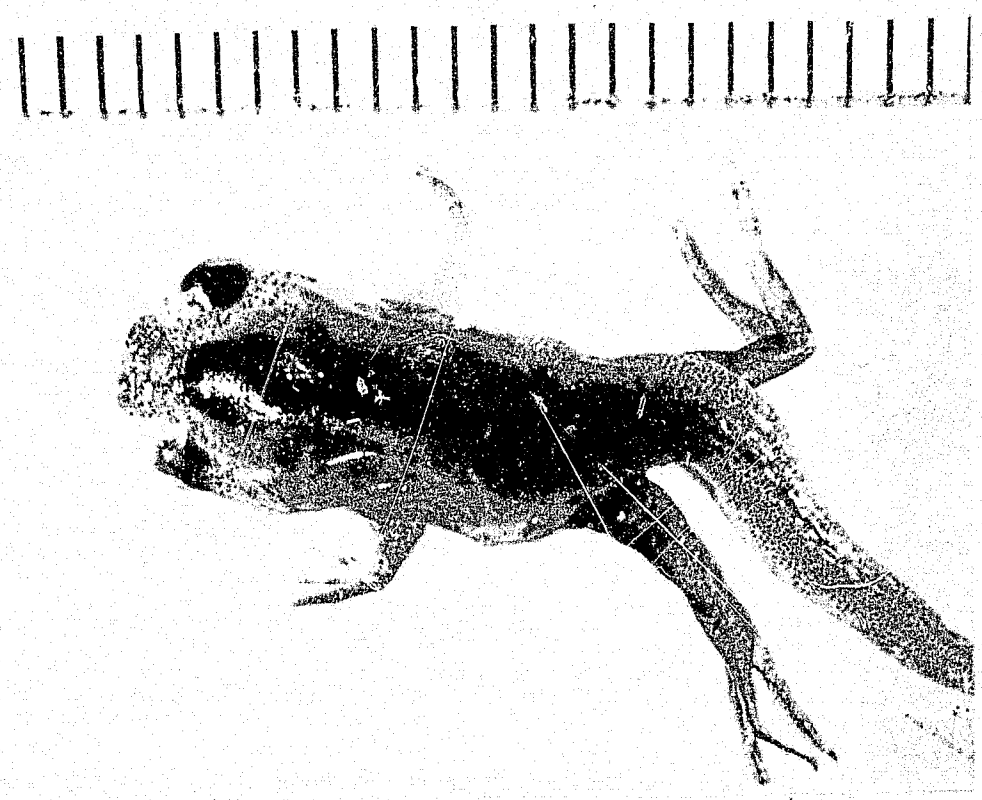


Figure 3.1 *Dorsal view of stage 60 tadpole swum in 0,2 ug/ml 5-FUdR. A claw is visible on the third toe of the left foot.*

In Figure 3.2 below are sketches of the hindlimbs of the above tadpole compared to the sketch of the normal tadpole hindlimb for that stage.

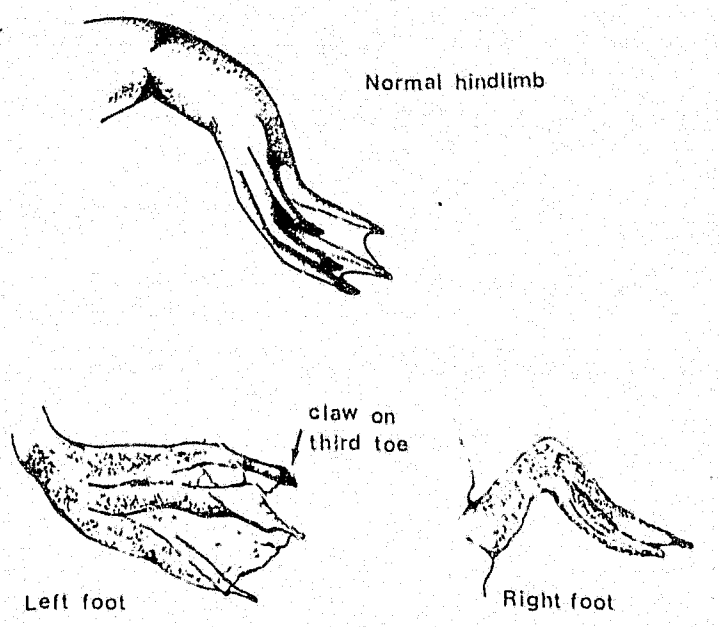


Figure 3.2 *Sketch of the hindlimbs of the tadpole in Figure 3.1 compared to that of the normal stage 60 hindlimb.*

The tadpole in Figure 3.1 can be compared to the normal stage 60 tadpole shown in Figure 3.3 below.

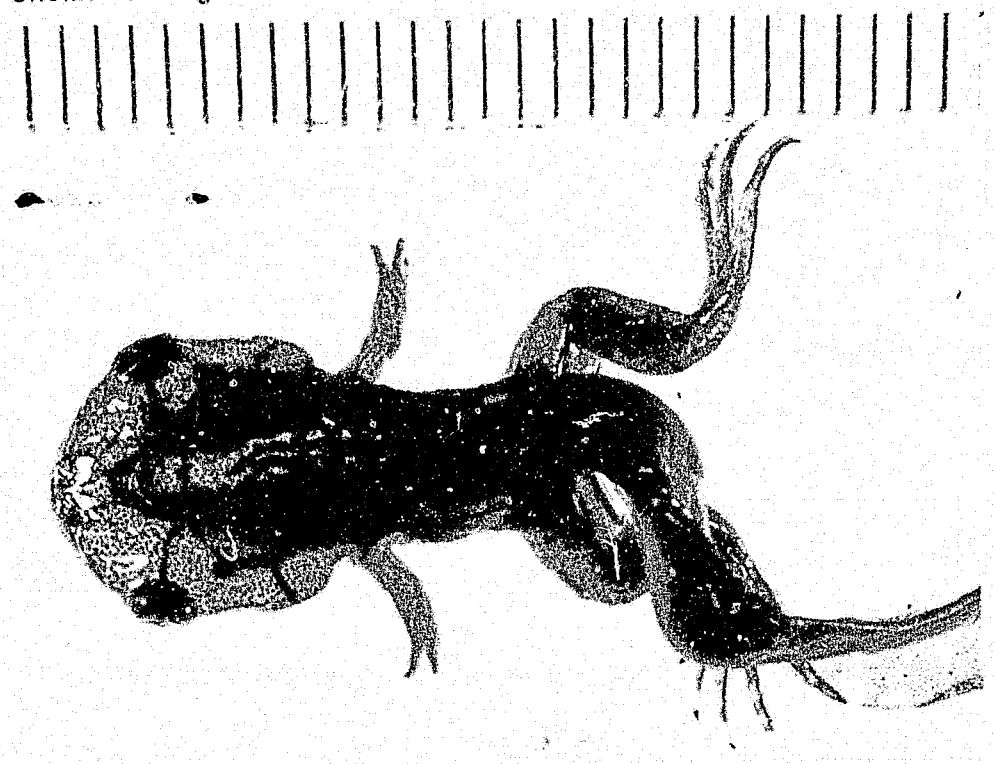


Figure 3.3 *Photograph of normal stage 60 tadpole.*

This type of result was found consistently throughout the various preliminary experiments as will be seen in the following photographs (Figures 3.4 to 3.11). A stage 53 tadpole was seen to have a bifurcated hindlimb bud instead of the normal division into five toes at this stage. See Figure 3.4 below.

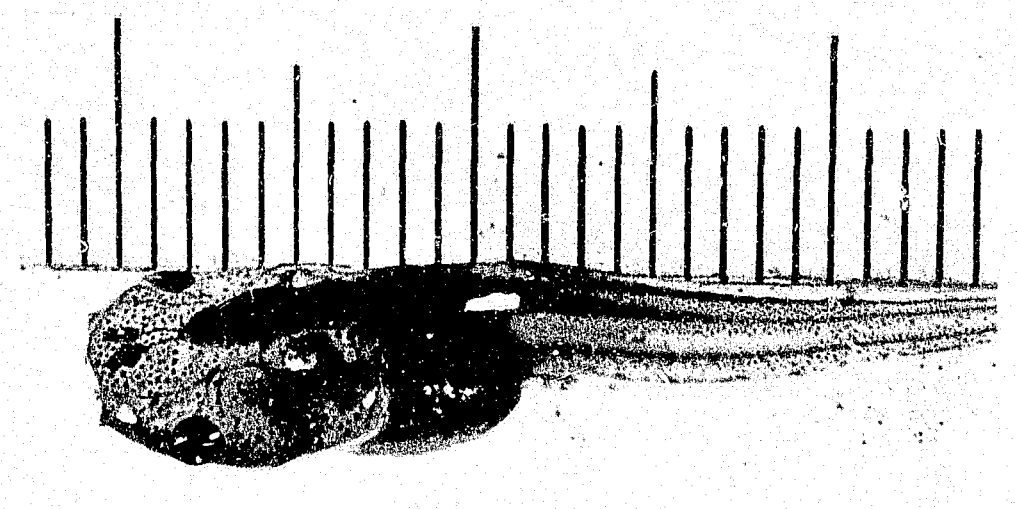
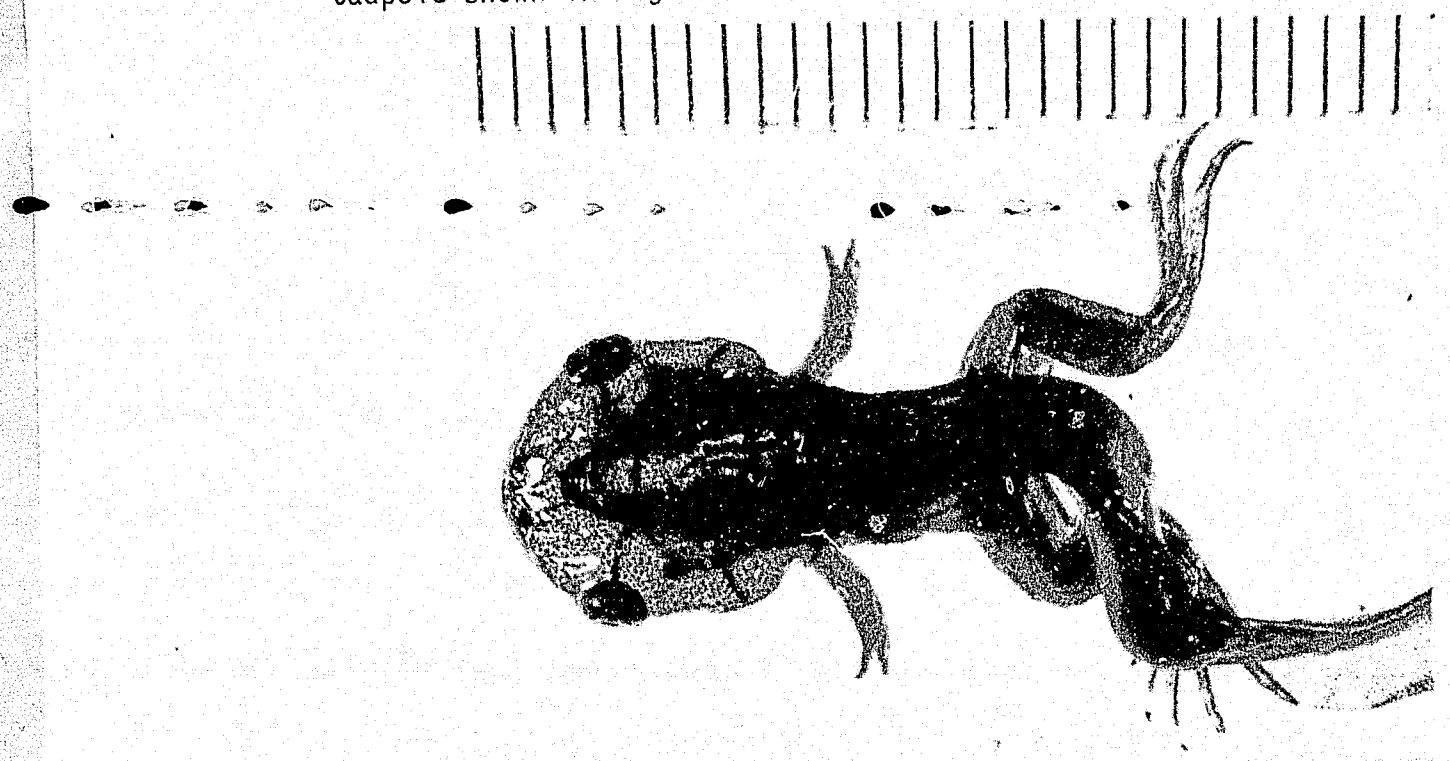


Figure 3.4 *Lateral view of stage 53 tadpole grown in .10μg/ml 5-BUdR + 2μg/ml 5-FUdR.*

The tadpole in Figure 3.1 can be compared to the normal stage 60 tadpole shown in Figure 3.3 below.



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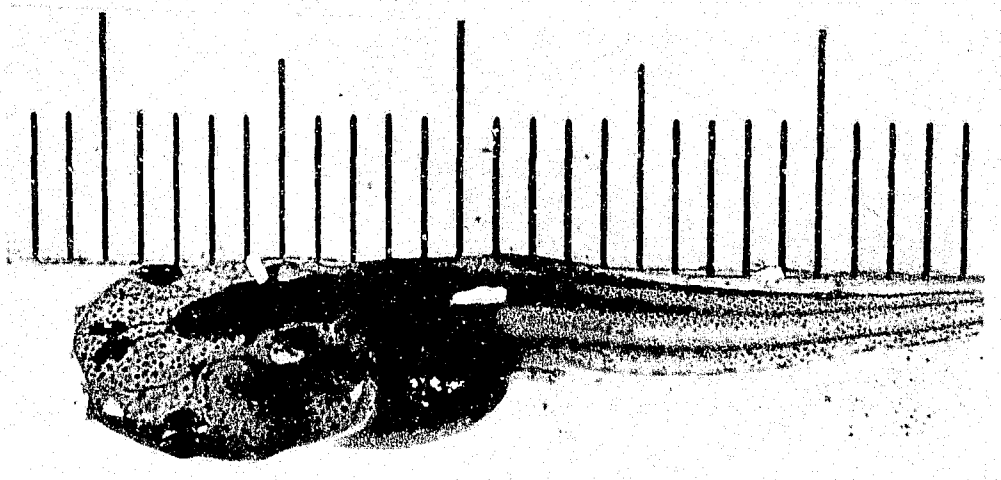


Figure 3.4 *Lateral view of stage 53 tadpole swum in .10µg/ml 5-BUdR + 2µg/ml 5-FUdR.*

The same tadpole was enlarged to show only the abdominal region so that the hindlimb was clearly visible (see Figure 3.5 below).

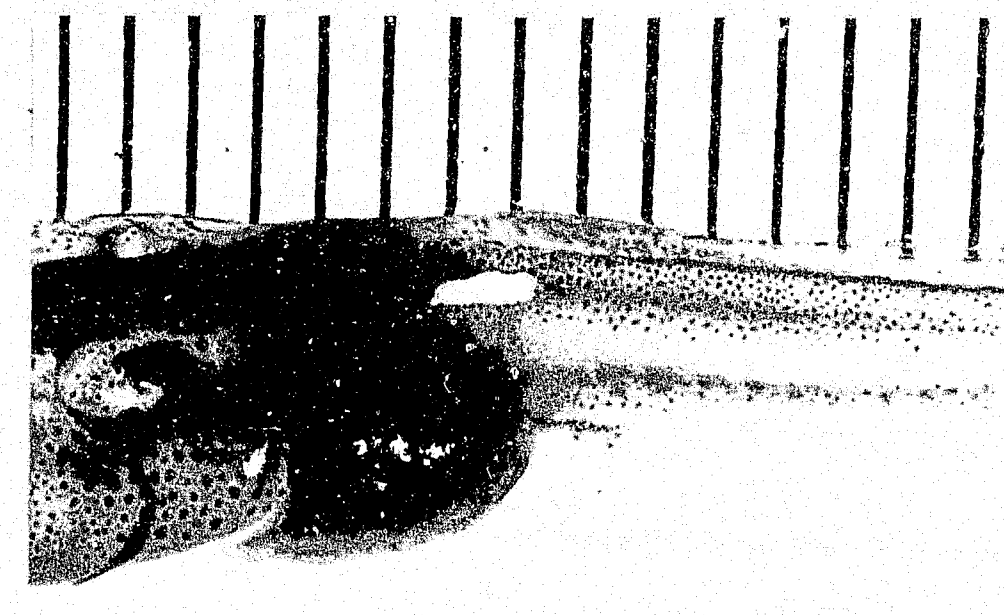


Figure 3.5 *Abdominal region of stage 53 tadpole enlarged to show detail of bifurcated hindlimb.*

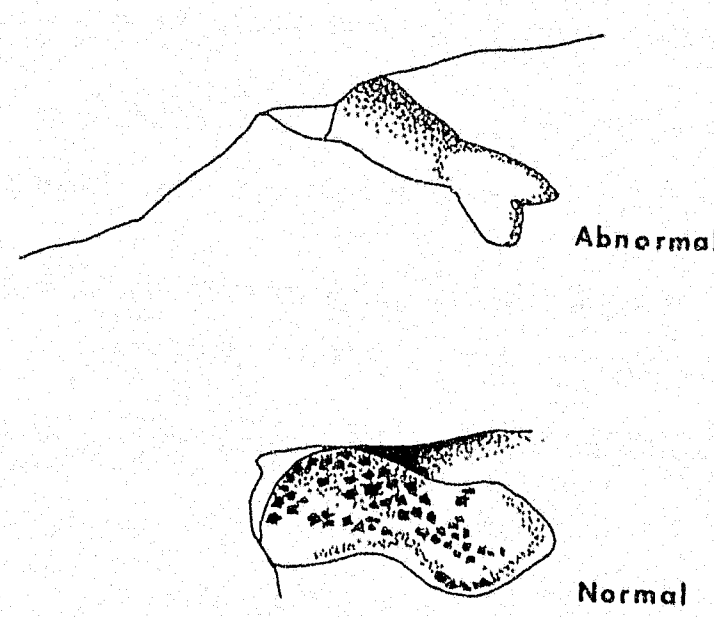


Figure 3.6 *Sketch of the above hindlimb with a normal limb for comparison.*

A photograph of the normal stage 53 tadpole is shown below for comparison to the photograph in Figure 3.4.

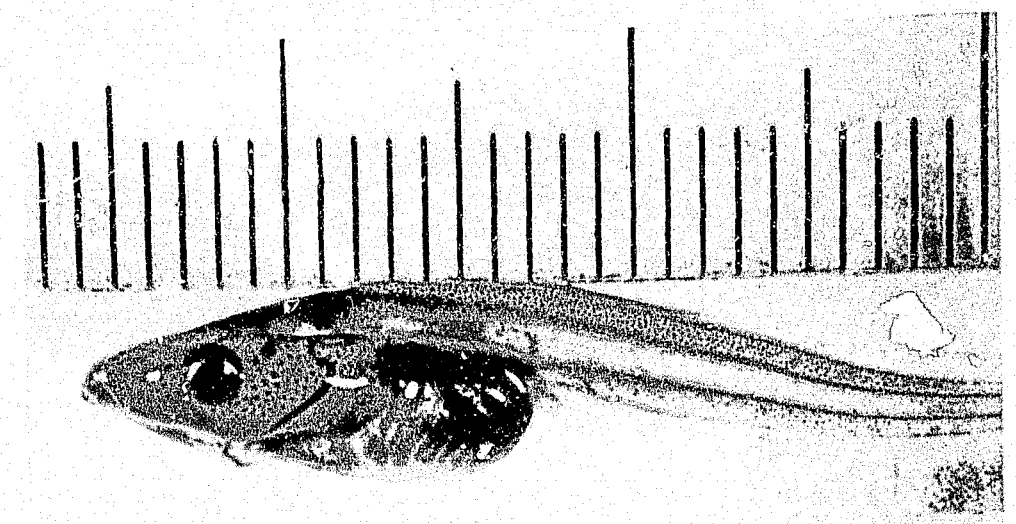


Figure 3.7 *Photograph of normal stage 53 tadpole*

In Figure 3.8 below is a photograph of a stage 57 tadpole with a hindlimb having three toes.

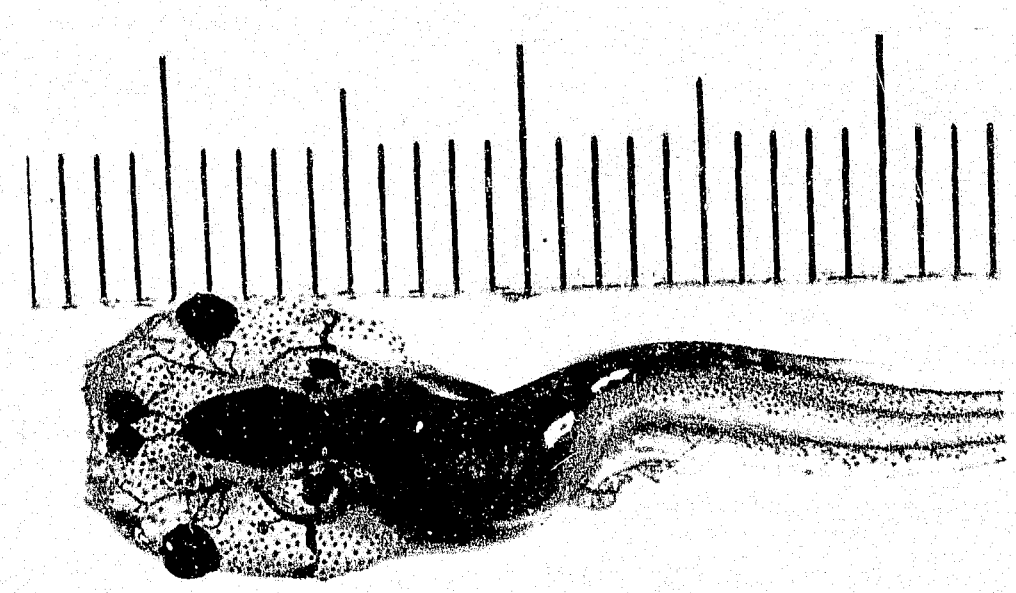


Figure 3.8 *Lateral view of stage 57 tadpole swum in 100µg/ml 5-BUdR + 0,2µg/ml 5-FUdR. Only three toes can be seen.*

The photograph in Figure 3.8 has been enlarged to show the hindlimb more clearly.

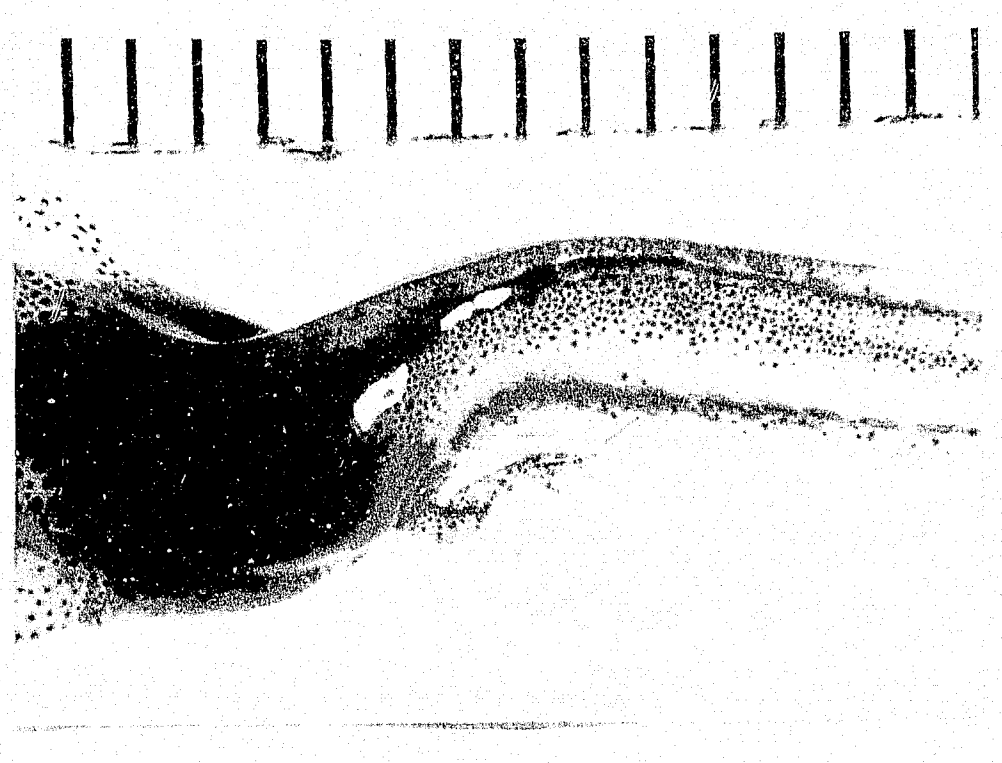


Figure 3.9 *Enlargement of the hindlimb region of the tadpole in Figure 3.8 on page 70. Three toes are clearly seen.*

A sketch is shown in Figure 3.10 below comparing the three-toed stage 57 hindlimb to the normal stage 57 hindlimb.

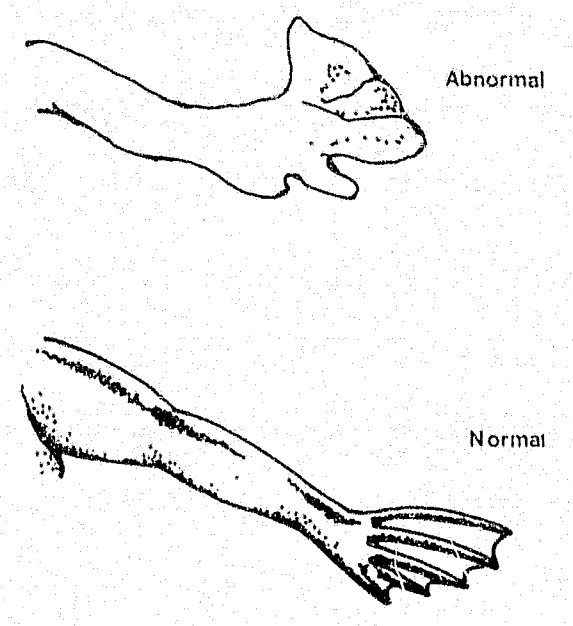


Figure 3.10 *Sketch showing the hindlimb from Figure 3.9 above compared to normal tadpole hindlimb.*

In Figure 3.11 below an enlarged hindlimb of a normal stage 57 tadpole hindlimb is seen for comparison to Figure 3.9.

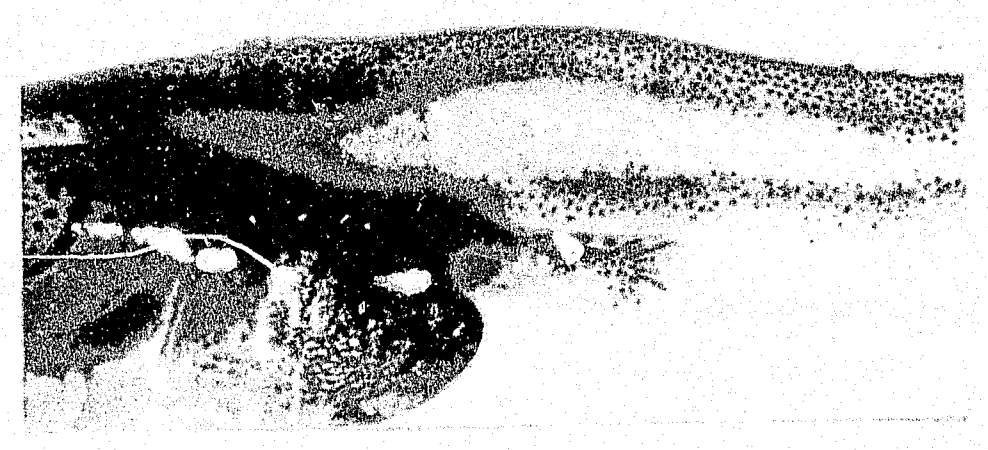


Figure 3.11 *Enlarged hindlimb region of stage 57 tadpole to show normal hindlimb*

These preliminary results provided the basis for further exhaustive experimentation. The hindlimbs were studied in greater detail by the more refined method of cartilage staining (see section 2.1.2.2.) and the limbs were analysed in detail in section 3.2.

3.2 Effect of 5-BUdR and 5-FUdR on tadpole hindlimb development - a detailed analysis

The tadpoles were immersed in solutions of 5-BUdR and 5-FUdR, which were renewed weekly (see section 2.1). The tadpoles were staged and studied each week under the dissecting microscope, in order to detect any deformities in the development of the hindlimb. The progress of the deformity was traced until no further deformity appeared. Subsequently the tadpoles were killed and the hindlimbs stained to make the cartilage clearly visible (see section 2.1).

These stained hindlimbs were photographed. The photographs were analysed in a semi-quantitative manner, according to the degree of the deformity. A penalty number was given which reflected the degree of this deformity.

In order to attain a non-weighted figure (overall) a measure of the degree of deformity with regard to the temporal aspect of the formation of the deformity was adopted. In this approach a score was allocated which reflected the absence or magnitude of the deformity. For convenience the developing hindlimb was classifed into three stages :

1. The most mature limb (adult limb) in which the digits are clearly visible and demarcated and all the cartilaginous elements as would be seen in the adult limb are present. Stages 56 to 65. (See Figures 3.12 and 3.16.)
2. The medium mature limb in which the cartilage elements are not clearly seen but demarcation of toes was visible. Stages 55 to 56. (See Figures 3.13 and 3.17.)
3. Immature limbs in which only a paddle was visible. Only deformities in shape could be detected in these limbs. Stages 54 and younger. (See Figures 3.14 and 3.18.)

In this approach each element was awarded a point of 4 and from this number a penalty of 2 was imposed on a deformed element, while a penalty of 4 was imposed on an absent element. (Toes could be visible)

3.2.1. Adult hindlimb

In this limb according to the point system above a total score of 91 was accumulated in the normal adult limb as follows :

Femur	4	
Tibio-fibula	4	
Tibiale fibiale	4	
Metatarsals	20	for 5 elements = 20
Phalanges	56	2(2x4) + 2(3x4) + 4(4) = 16 + 24 + 16 = 56
Claws	3	(1 per claw)
Total	91	

In a typical deformed adult limb one could calculate a penalty index as follows :

Femur	2	bent (4-2)
Tibio-fibula	2	bent (4-2)
Tibiale fibulare	3	mildly bent (4-1)
Metatarsals	12	two absent (20-2(4))
Phalanges	28	digits 4 and 5 absent (56-4(4)-4(3)
Total	47	

This indicates approximately 50% deformity.

To illustrate these analyses more clearly a series of graphs has been drawn alongside each limb photograph, next to the penalty index analysis. In Figure 3.12 below a normal adult limb is shown on this graph and below it a typical deformed limb as described above with a penalty index of 47.

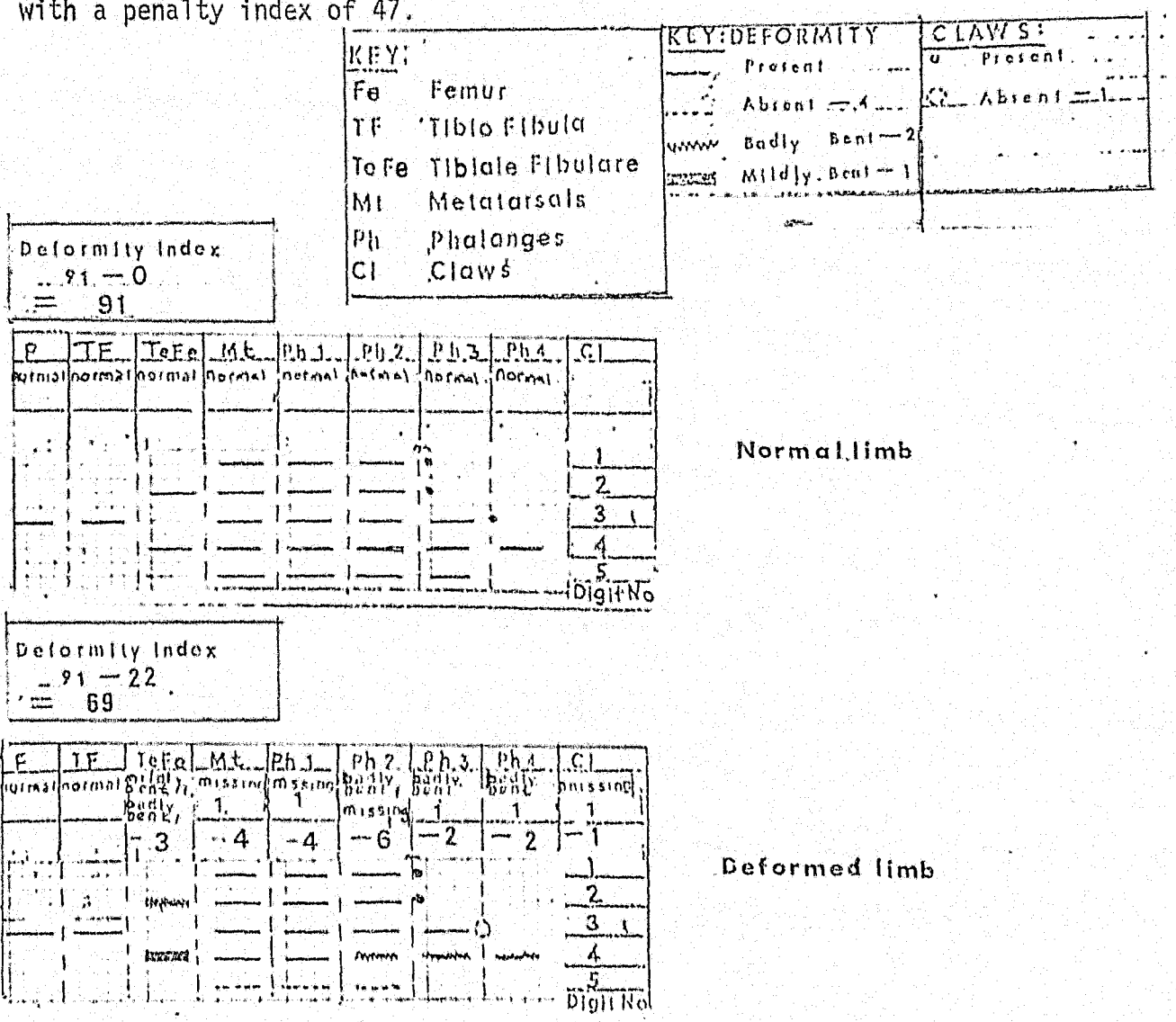


Figure 3.12 *Graphical representation of normal adult hindlimb and a typical deformed hindlimb*

3.2.2. Medium mature hindlimb

In this stage of limb development, although the metatarsals and phalanges were present they were not clearly demarcated and could not easily be counted. For this reason penalties in the distal region of the hindlimb were only given for the presence or absence of toes.

According to the point system a total score of 32 was accumulated for the normal medium mature limb as follows :

Femur	4	
Tibio-fibula	4	
Tibiale fibulare	4	
Toes	20	(5x4)
Total	32	

In a typical deformed medium mature limb one could calculate a deformity index as follows :

Femur	4	
Tibio-fibula	4	
Tibiale fibulare	2	slightly bent
Toes	12	(3x4) 2 toes missing
Total	22	

This indicates approximately 33% deformity.

To illustrate these analyses more clearly, a series of graphs has been drawn alongside each limb photograph, next to the deformity analysis. In Figure 3.13 below is a graphical representation of a normal medium mature limb and below it the typical deformity analysed above.

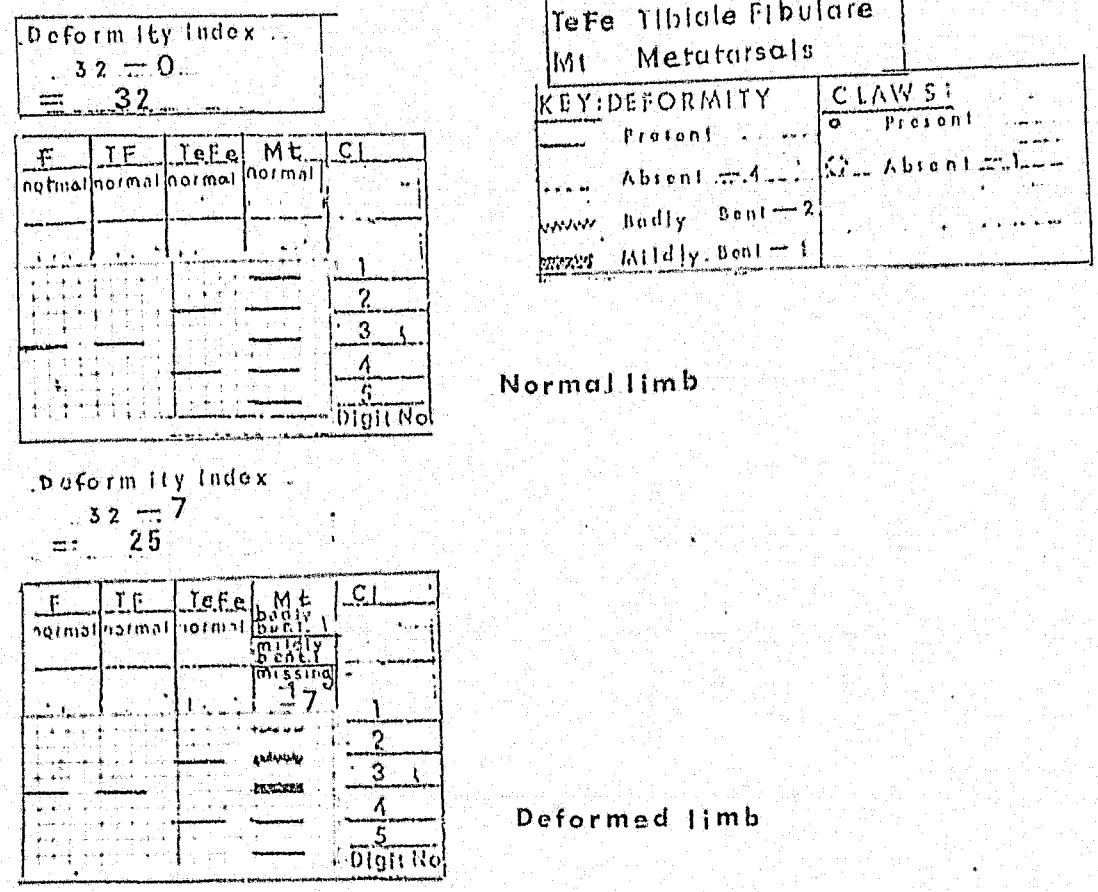


Figure 3.13 *Graphical representation of the normal medium mature hindlimb and a typical deformed hindlimb*

3.2.3. Immature hindlimb

In this stage of limb development deformities in early limb shape were noticed, which often went on to form the typically reduced number of digits in the adult limb. Penalties were given only for shape and possible absence of toes judged by indentations on the paddle at stage 53 only. According to the point system a total of 24 was accumulated for the normal immature limb as follows :

Shape	4	
Toes	20	(5x4)
Total	24	

In a typical deformed immature limb one could calculate a deformity index as follows :

Shape	1	limb very bent
Toes	16	(4x4) stage 53 smaller paddle in width i.e. one toe less
Total	17	

A 28% deformity.

To illustrate these analyses more clearly a series of graphs has been drawn alongside each limb photograph, next to the deformity analysis. In Figure 3.14 below is a sketch of a normal immature limb and below it the deformity analysed above.

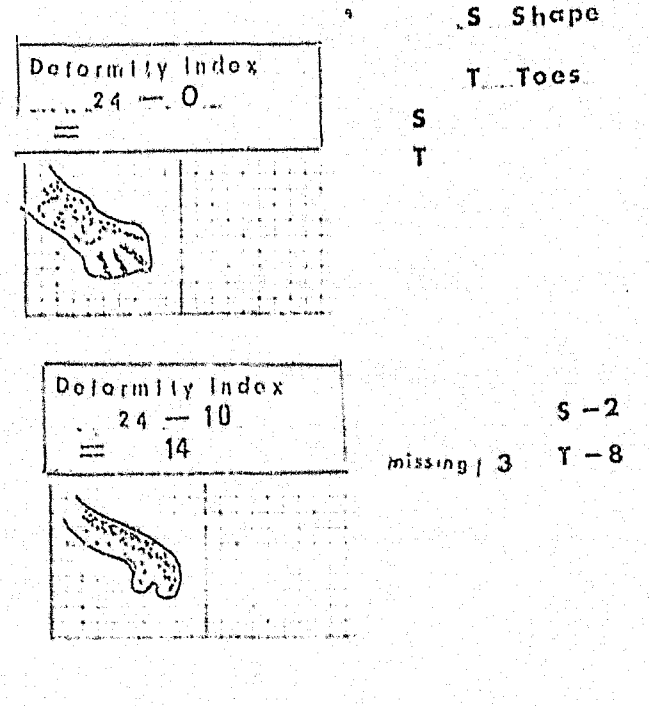


Figure 3.14 *Graphical representation of the normal immature hindlimb and a typical deformed hindlimb*

This detailed analysis is summarised as follows :

There are three classes of limb development for this analysis. The earlier two classes were analysed to show the on-going progress of the deformity while the adult class was a detailed analysis of the deformities themselves. This semi-quantitative approach gave interesting results even though the deformities were not found to be consistent either with stage or concentration.

The detailed analysis of the limb deformities is carried out in 3.2.5. for adult limbs, 3.2.6. for medium mature limbs and 3.2.7. for immature limbs.

The results show limbs with one or more digits missing. In the thirty cases of adult limbs analysed in detail :

Digit 1 missing - 23 - 30 cases
Digit 2 missing - 13 - 30 cases
Digit 3 missing - 6 - 30 cases
Digit 4 missing - 16 - 30 cases
Digit 5 missing - 16 - 30 cases.

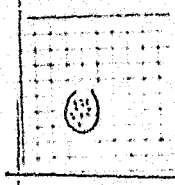
3.2.4. Analysis of normal hindlimbs

Figure 3.15 *Normal hindlimbs*

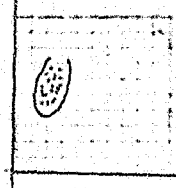
S shape

I loop

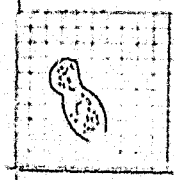
Normal Stage 50



Normal Stage 51



Normal Stage 52

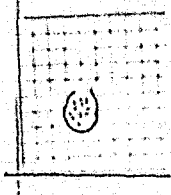


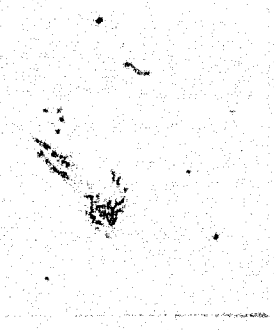
Normal Stage 53

S shape

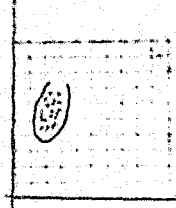
T toes

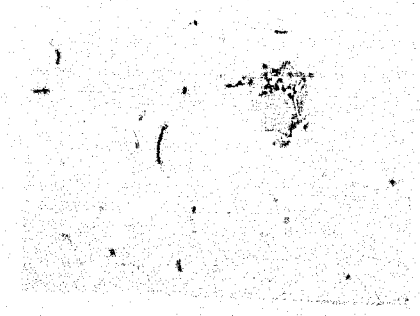
Normal Stage 50



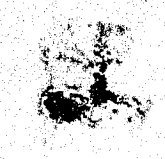


Normal Stage 51





Normal Stage 52



Normal Stage 53

KEY:	
Fe	Femur
TF	Tibio Fibula
TeFe	Tibiale Fibulare
Mt	Metatarsals

KEY: DEFORMITY	
———	Present
- - - -	Absent — 4 —
~~	Badly Bent — 2
~~	Mildly Bent — 1

Normal Stage 54

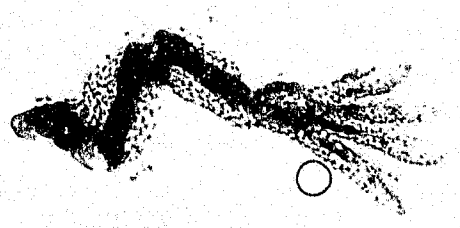
F	TF	TeFe	Mt	Cl
normal	normal	normal	normal	normal
				1
				2
				3
				4
				5
				Digit No.

Normal Stage 55

F	TF	TeFe	Mt	Cl
normal	normal	normal	normal	normal
				1
				2
				3
				4
				5
				Digit No.

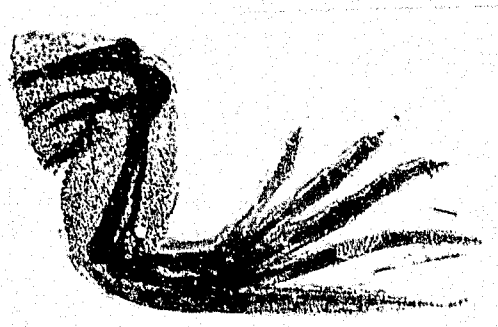
Normal Stage 56

F	TF	TeFe	Mt	Cl
normal	normal	normal	normal	normal
				1
				2
				3
				4
				5
				Digit No.



Normal Stage 57

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	normal	normal	normal	normal	normal	normal
								1
								2
								3
								4
								5
								Digit No.



KEY:

Fe	Femur
TF	Tible Fibula
TeFe	Tiblale Fibulare
Mt	Metatarsals
Ph	Phalanges
Cl.	Claws

KEY: DEFORMITY

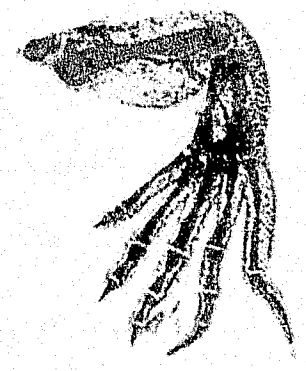
———	Present
----	Absent — 4
~~	Badly Bent — 2
~~	Mildly Bent — 1

Normal Stage 58

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	normal	normal	normal	normal	normal	normal
								1
								2
								3
								4
								5
								Digit No.

Normal Stage 59

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	normal	normal	normal	normal	normal	normal
								1
								2
								3
								4
								5
								Digit No.



3.2.5. Detailed analysis of adult hindlimbs

Figure 3.16 *Adult hindlimbs*

KEY:
Fe Femur
TF Tibio Fibula
TeFo Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

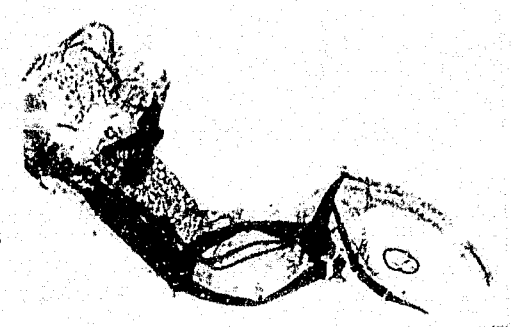
CLAWS:
Present
Absent = 1

Deformity Index
91 − 67
= 24

Start stage 47
0,01 mg/ml 5-BUdR
20 µg/ml 5-FUdR

Left

F	TF	TeFo	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	badly bent −2	missing −14	missing 4 −16	missing 4 −16	missing 3 −12	missing 1 −4	missing 3 −3
								1
								2
								3
								4
								5
								Digit No.

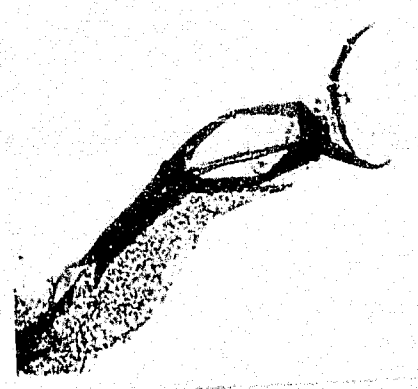


Deformity Index
91 − 67
= 24

Start stage 47
0,01 mg/ml 5-BUdR
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	badly bent −2	missing −14	missing 5 −16	missing 5 −16	missing 3 −12	missing 1 −4	missing 3 −3
								1
								2
								3
								4
								5
								Digit No.



Deformity Index
91 − 87
= 4

Start stage 49
20 µg/ml 5-FUdR

Left

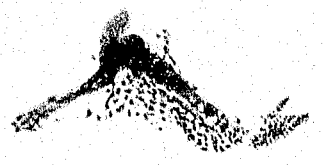
F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal missing −3		missing −4	missing 5 −20	missing 5 −20	missing 5 −20	missing 3 −12	missing 1 −4	missing 3 −3
								1
								2
								3
								4
								5
								Digit No.

Deformity Index
91 − 77
= 14

Start stage 49
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
badly bent −1	badly bent −1	normal	missing 4 −16	missing 5 −20	missing 5 −20	missing 3 −12	missing 1 −4	missing 3 −3
								1
								2
								3
								4
								5
								Digit No.



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
Present
Absent — 4
Badly Bent — 2
Mildly Bent — 1

CLAWS:
Present
Absent — 1

Deformity Index
91 — 73
= 18

Start stage 46
10 µg/ml 5- FUdR

Left

Fe	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	mildly bent	badly bent	missing 3	missing 4	missing 5	missing 3	missing 1	missing 2	
	— 1	— 2	— 16	— 16	— 20	— 12	— 4	— 2	
								1	
								2	
								3	
								4	
								5	
								Digit No	

Deformity Index
91 — 73
= 18

Start stage 46
10 µg/ml 5- FUdR

Right

Fe	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	mildly bent	badly bent	missing 3	missing 4	missing 5	missing 3	missing 1	missing 2	
	— 1	— 2	— 16	— 16	— 20	— 12	— 4	— 2	
								1	
								2	
								3	
								4	
								5	
								Digit No	

Deformity Index
91 — 31
= 60

Start stage 43
0,2 µg/ml 5-FUdR

Left

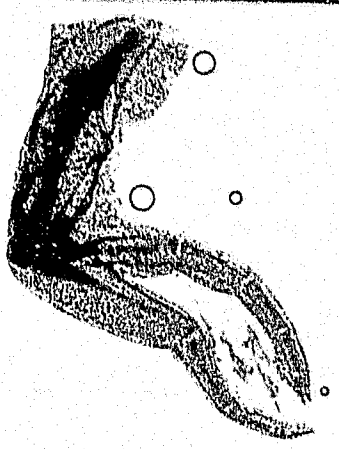
Fe	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	mildly bent	missing 2	missing 2	missing 2	missing 1	normal	missing 1	
		— 2	— 8	— 8	— 8	— 4		— 1	
								1	
								2	
								3	
								4	
								5	
								Digit No	

Deformity Index
91 — 4,5
= 46

Start stage 43
0,2 µg/ml 5-FUdR

Right

Fe	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	badly bent	missing 3	missing 3	missing 3	missing 1	normal	missing 3	
		— 2	— 12	— 12	— 12	— 4		— 3	
								1	
								2	
								3	
								4	
								5	
								Digit No	



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
Present
Absent — 4
Badly Bent — 2
Mildly Bent — 1

CLAWS:
Present
Absent — 1

Deformity Index
91 — 47
= 44

Start stage 48
10 µg / ml 5— FU dR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	Mildly bent	Mildly bent	missing 2	missing 3	missing 4	missing 3	missing 1	missing 1	
	—1	—1	—8	—12	—16	—12	—4	—1	1
									2
									3
									4
									5
									Digit No

Deformity Index
91 — 48
= 43

Start stage 48
10 µg / ml 5— FU dR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	badly bent	missing 2	missing 3	missing 5	missing 3	missing 1	missing 1	
		—2	—8	—12	—20	—12	—4	—1	1
									2
									3
									4
									5
									Digit No

Deformity Index
91 — 56
= 35

Start stage 46
10 µg / ml 5— FU dR

Left

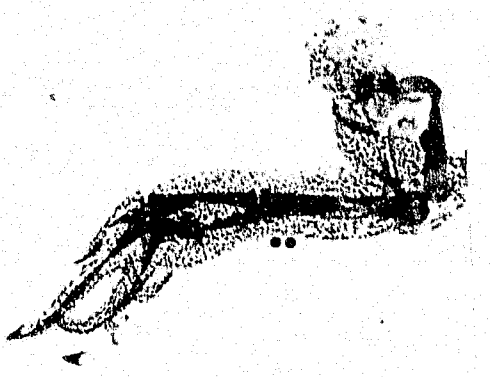
F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 3 missing 2	badly bent 3 missing 2	badly bent 1 missing 3	missing 3	missing 1	missing 2	
			—14	—11	—13	—12	—4	—2	1
									2
									3
									4
									5
									Digit No

Deformity Index
91 — 51
= 40

Start stage 46
10 µg / ml 5— FU dR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 3 missing 2	badly bent 1 missing 2	badly bent 1 missing 3	missing 3	missing 1	missing 1	
			—14	—10	—14	—12	—4	—1	1
									2
									3
									4
									5
									Digit No



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
—— Present
---- Absent = 4
Badly Bent = 2
Mildly Bent = 1

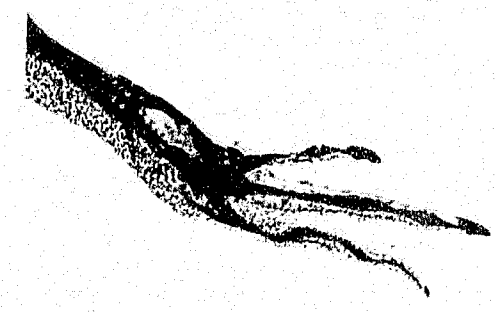
CLAWS:
Present
Absent = 1

Deformity Index
91 — 49
= 42

Start stage 48
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	Badly Bent 3 / Mildly Bent 2 / missing 1	Badly Bent 2 / Mildly Bent 1 / missing	missing 3	missing 3	missing 1	missing
			11	10	12	12	4	
								1
								2
								3
								4
								5
								Digit No



Deformity Index
91 — 49
= 42

Start stage 48
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	Badly Bent 3 / Mildly Bent 2 / missing 1	Badly Bent 2 / Mildly Bent 1 / missing	missing 3	missing 2	missing 1	missing 2
			12	11	12	8	4	2
								1
								2
								3
								4
								5
								Digit No

Deformity Index
91 — 57
= 34

Start stage 46
20 µg/ml 5-FUdR

Left

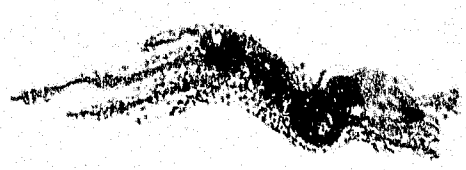
F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	Badly Bent 3 / Mildly Bent 2 / missing 1	Badly Bent 3 / Mildly Bent 2 / missing	Badly Bent 2 / Mildly Bent 1 / missing	missing 3	missing 3	missing 1	missing 3
		1	12	9	16	12	4	3
								1
								2
								3
								4
								5
								Digit No

Deformity Index
91 — 56
= 35

Start stage 46
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	Badly Bent 3 / Mildly Bent 2 / missing 1	Badly Bent 3 / Mildly Bent 2 / missing	Badly Bent 2 / Mildly Bent 1 / missing	missing 4	missing 2	missing 1	missing 3
		2	12	11	16	8	4	3
								1
								2
								3
								4
								5
								Digit No



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

CLAWS:
Present
Absent = 1

Deformity Index
91 — 26
= 65

Start stage 47
0,01 mg/ml 5-BUdR
10ug/ml 5-FUdR

Left

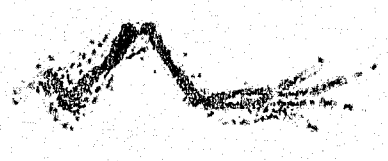
F	TF	TeFe	Mt	Ph1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	missing 1	missing 1	missing 1	missing 2	missing 1	missing 2
			— 4	— 4	— 4	— 8	— 4	— 2
								1
								2
								3
								4
								5
								Digit No

Deformity Index
91 — 26
= 65

Start stage 47
0,01 mg/ml 5-BUdR
10µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	missing 1	missing 1	missing 1	missing 2	missing 1	missing 2
			— 4	— 4	— 4	— 8	— 4	— 2
								1
								2
								3
								4
								5
								Digit No

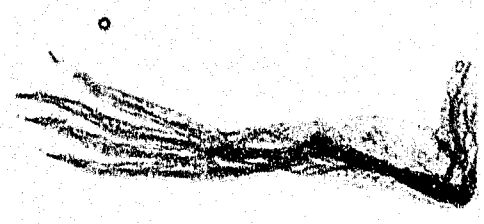


Deformity Index
91 — 26
= 65

Start stage 47
10µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	missing 1	missing 1	badly bent missing	missing 2	missing 1	missing 1
			— 4	— 4	— 5	— 8	— 4	— 1
								1
								2
								3
								4
								5
								Digit No

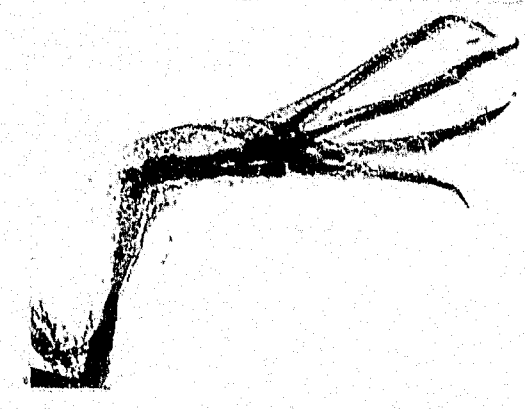


Deformity Index
91 — 26
= 65

Start stage 47
10µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	missing 1	missing 1	badly bent missing	missing 2	missing 1	missing 1
			— 4	— 4	— 5	— 8	— 4	— 1
								1
								2
								3
								4
								5
								Digit No



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
—— Present
.... Absent = 4
wwww Badly Bent = 2
//// Mildly Bent = 1

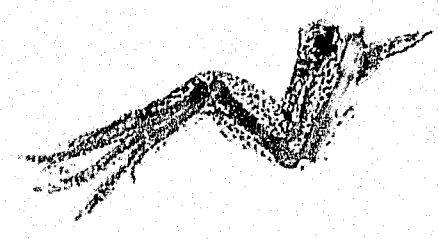
CLAWS:
Present
Absent = 1

Deformity Index
9.1 — 34
= 57

Start stage 52
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	badly bent 4 missing 12	missing 2	badly bent 3 missing 9	missing 1	missing 1	missing 3
			—6	—8	—9	—4	—4	—3
			wwww	----	----			1
		——	——	——	——			2
——	——		——	——	——	——		3
		——	——	——	////	——	----	4
			----	----	----	----		5
								Digit No

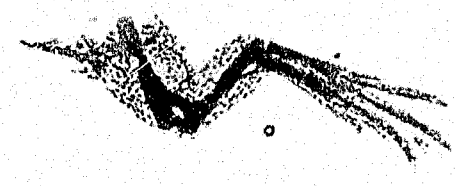


Deformity Index
9.1 — 38
= 53

Start stage 52
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	mildly bent 3 missing 7	missing 2	missing 2	missing 2	missing 1	missing 3
			—7	—8	—8	—8	—4	—3
			////	----	----			1
		——	——	——	——			2
——	——		——	——	——	——		3
		——	////	——	——		----	4
			----	----	----	----		5
								Digit No

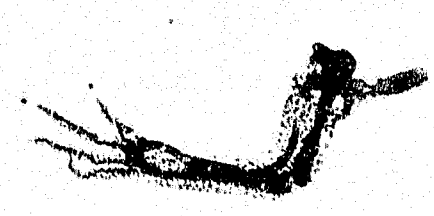


Deformity Index
9.1 — 45
= 46

Start stage 52
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	badly bent 4 missing 12	badly bent 1 missing 6	badly bent 3 missing 14	missing 2	missing 1	missing 1
			—12	—6	—14	—8	—4	—1
			----	----	----			1
		——	wwww	——	——			2
——	——		wwww	——	——	——		3
		——	wwww	——	wwww	----	----	4
			wwww	wwww	----	----		5
								Digit No

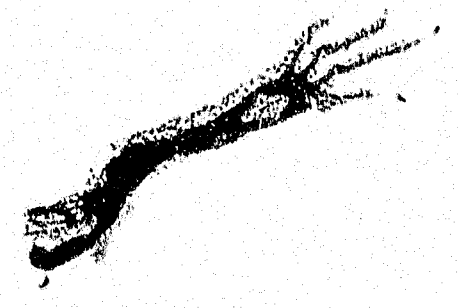


Deformity Index
9.1 — 45
= 46

Start stage 52
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	badly bent 4 missing 12	badly bent 1 missing 6	badly bent 3 missing 14	missing 2	missing 1	missing 1
			—12	—6	—14	—8	—4	—1
			----	----	----			1
		——	wwww	——	——			2
——	——		wwww	——	——	——		3
		——	wwww	——	wwww	----	----	4
			wwww	wwww	----	----		5
								Digit No



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

CLAWS:
Present
Absent = 1

Deformity Index
91 — 39
= 52

Start stage 0
10 ug / ml 5— FU dR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 2 missing	badly bent 1 missing	badly bent 1 missing	missing 2	missing 1	missing 3	
			—8	—6	—10	— 8	— 4	— 3	
								1	
								2	
								3	
								4	
								5	
									Digit No

Deformity Index
91 — 39
= 52

Start stage 0
10 ug / ml 5— FU dR

Right

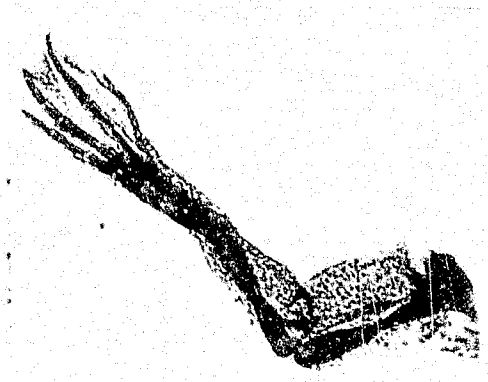
F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 2 missing	badly bent 1 missing	badly bent 1 missing	missing 2	missing 1	missing 3	
			—8	—6	—10	— 8	— 4	— 3	
								1	
								2	
								3	
								4	
								5	
									Digit No

Deformity Index
91 — 30
= 61

Start stage 0
10 µg / ml 5— FU dR

Left

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 1 missing	badly bent 1 missing	badly bent 1 missing	mildly bent missing	missing 1	missing 3	
			—6	—6	—6	—5	— 4	— 3	
								1	
								2	
								3	
								4	
								5	
									Digit No



Deformity Index
91 — 30
= 61

Start stage 0
10 µg / ml 5— FU dR

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl	
normal	normal	normal	badly bent 1 missing	badly bent 1 missing	badly bent 1 missing	mildly bent missing	missing 1	missing 3	
			—6	—6	—6	—5	— 4	— 3	
								1	
								2	
								3	
								4	
								5	
									Digit No

KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals
Ph Phalanges
Cl Claws

KEY: DEFORMITY
—— Present
..... Absent = 4
~~~~ Badly Bent = 2
▨ Mildly Bent = 1

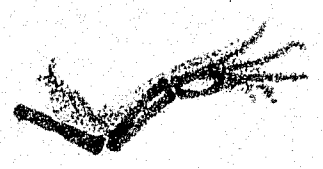
CLAWS:
○ Present
⊘ Absent = 1

Deformity Index
9.1 = 34
= 57

Start stage 52
10 µg / ml 5-FUdR
—

Right

F	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	mildly bent	normal	missing	badly bent	badly bent	missing	missing	missing
	2		2	2	3	2	1	3
	—1		—8	—4	—6	—8	—4	—3
								1
								2
								3
								4
								5
								Digit No



Deformity Index
9.1 = 0
= 91

Start stage 46
10 µg / ml 5-FUdR
—

Left

L	TF	TeFe	Mt	Ph 1	Ph 2	Ph 3	Ph 4	Cl
normal	normal	normal	normal	normal	normal	normal	normal	normal
								1
								2
								3
								4
								5
								Digit No


~~~~

3.2.6. Detailed analysis of medium mature hindlimbs

Figure 3.17 *Medium mature hindlimbs*

2. MEDIUM MATURE LIMBS

KEY:	
Fe	Femur
TF	Tibio Fibula
TeFe	Tibiale Fibulare
Mt	Metatarsals

KEY: DEFORMITY	
———	Present
. . . .	Absent = 4
~~	Badly Bent = 2
≈≈≈	Mildly Bent = 1

Left

Deformity Index
.32 = 12
= 20

start stage 43
0,01 mg/ml 5-BUdR
0,2 µg/ml 5-FUdR

F	TF	TeFe	Mt	
normal	normal	normal	missing 3	
			-12	
				1
				2
				3
				4
				5
				Digit No

Right

Deformity Index
.32 = 12
= 20

start stage 43
0,01 mg/ml 5-BUdR
0,2 µg/ml 5-FUdR

F	TF	TeFe	Mt	
normal	normal	normal	missing 3	
			-12	
				1
				2
				3
				4
				5
				Digit No

Left

Deformity Index
.32 = 13
= 19

start stage 47
10 µg/ml 5-FUdR

F	TF	TeFe	Mt	
normal	mildly bent	normal	missing 3	
	-1		-12	
				1
				2
				3
				4
				5
				Digit No

Right

Deformity Index
32 = 14
= 18

start stage 47
10 µg/ml 5-FUdR

F	TF	TeFe	Mt	
normal	mildly bent	mildly bent	missing 3	
	-1	-1	-12	
				1
				2
				3
				4
				5
				Digit No

2. MEDIUM MATURE LIMBS

KEY:	
Fe	Femur
TF	Tibio Fibula
TeFe	Tibiale Fibulare
Mt	Metatarsals

KEY: DEFORMITY	
———	Present
- - - -	Absent = 4
~~	Badly Bent = 2
≈≈≈≈	Mildly Bent = 1

Left

Deformity Index
.32 = 12
= 20

Start stage 43
0,01 mg/ml 5-BUdR
0,2 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No.
normal	normal	normal	missing 3	
			12	
				1
				2
				3
				4
				5

Right

Deformity Index
.32 = 12
= 20

Start stage 43
0,01 mg/ml 5-BUdR
0,2 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No.
normal	normal	normal	missing 3	
			12	
				1
				2
				3
				4
				5

Left

Deformity Index
.32 = 13
= 19

Start stage 47
10 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No.
normal	mildly bent	normal	missing 3	
	1		12	
				1
				2
				3
				4
				5

Right

Deformity Index
32 = 14
= 18

Start stage 47
10 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No.
normal	mildly bent	mildly bent	missing 3	
	1	1	12	
				1
				2
				3
				4
				5

KEY:

Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals

KEY: DEFORMITY

Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

Deformity Index
32 = 13
= 19

Start stage 52
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	
mildly Bent	Normal	Normal	missing 3	
1			12	
				1
				2
				3
				4
				5
				Digit No

Deformity Index
32 = 13
= 19

Start stage 52
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	
mildly Bent	normal	normal	missing 3	
1			12	
				1
				2
				3
				4
				5
				Digit No

Deformity Index
32 = 8
= 24

Start stage 43
0,2 µg/ml 5-FUdR
0,01 mg/ml 5-BUdR

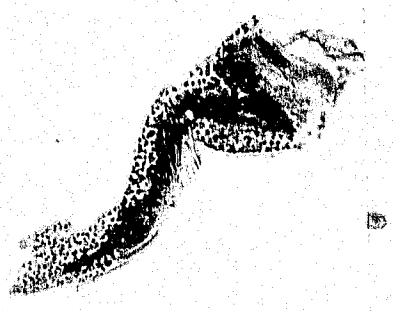
Left

F	TF	TeFe	Mt	
normal	normal	normal	missing 2	
			8	
				1
				2
				3
				4
				5
				Digit No

Deformity Index
32 = 8
= 24

Start stage 43
0,2 µg/ml 5-FUdR
0,01 mg/ml 5-BUdR

Right



F	TF	TeFe	Mt	
normal	normal	normal	missing 2	
			8	
				1
				2
				3
				4
				5
				Digit No

KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals

KEY: DEFORMITY
Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

Deformity Index
.32 = 10
= 16

Start stage 49
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	
badly bent	badly bent	normal	missing	
			3	
-2	-2		-12	
				1
				2
				3
				4
				5
				Digit No

Deformity Index
.32 = 7
= 25

Start stage 49
20 µg/ml 5-FUdR

Right

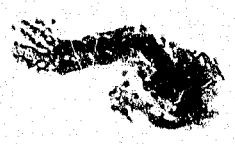
F	TF	TeFe	Mt	
badly bent	mildly bent	normal	missing	
			1	
-2	-1		-4	
				1
				2
				3
				4
				5
				Digit No

Deformity Index
.32 = .6
= 26

Start stage 47
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	
mildly bent	mildly bent	normal	missing	
			1	
-1	-1		-4	
				1
				2
				3
				4
				5
				Digit No



Deformity Index
.32 = .6
= 26

Start stage 47
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	
mildly bent	mildly bent	normal	missing	
			1	
-1	-1		-4	
				1
				2
				3
				4
				5
				Digit No

KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals

KEY: DEFORMITY
Present
Absent —4—
Badly Bent — 2
Mildly Bent — 1

Left

Deformity Index
32 — 14
= 18

Start stage 54
10 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No
normal	Badly Bent	normal	Badly Bent or missing	
	-2-		-12-	1
				2
				3
				4
				5

Right

Deformity Index
32 — 14
= 18

Start stage 54
10 µg/ml 5-FUdR

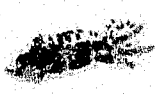
F	TF	TeFe	Mt	Digit No
normal	Badly Bent	normal	Badly Bent or missing	
	-2-		-12-	1
				2
				3
				4
				5

Left

Deformity Index
32 — 4
= 28

Start stage 48
20 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No
normal	normal	normal	missing	
			-4-	1
				2
				3
				4
				5

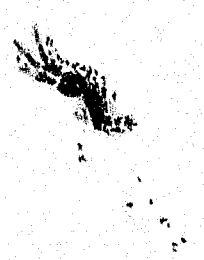


Right

Deformity Index
32 — 4
= 28

Start stage 48
20 µg/ml 5-FUdR

F	TF	TeFe	Mt	Digit No
normal	normal	normal	missing	
			-4-	1
				2
				3
				4
				5



KEY:
Fe Femur
TF Tibio Fibula
TeFe Tibiale Fibulare
Mt Metatarsals

KEY: DEFORMITY
Present
Absent = 4
Badly Bent = 2
Mildly Bent = 1

Deformity Index
.32 = 4
= 28

Start stage 48
20 µg/ml 5-FUdR

Left

F	TF	TeFe	Mt	
normal	normal	normal	missing = 4	1
				2
				3
				4
				5
				Digit No.

Deformity Index
.32 = 4
= 28

Start stage 48
20 µg/ml 5-FUdR

Right

F	TF	TeFe	Mt	
normal	normal	normal	missing = 4	1
				2
				3
				4
				5
				Digit No.