UNIVERSITY OF SOUTHAMPTON

BIOCHEMICAL METAMORPHOSIS IN DEVELOPMENTALLY

RETARDED XENOPUS LAEVIS LARVAE

A Thesis submitted to the University of Southampton

for the degree of Doctor of Philosophy

by

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To my wife

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ABSTRACT

FACULTY OF SCIENCE

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BIOCHEMICAL METAMORPHOSIS IN DEVELOPMENTALLY RETARDED

XENOPUS LAEVIS LARVAE

by Michael James Doyle

Four protein systems, namely haemoglobins, coelomic fluid albumin, lactate dehydrogenase isozymes and lens crystallins were studied electrophoretically in normally metamorphosing and propylthiouracil-treated Xenopus laevis larvae.

Continuous immersion in the goitrogen propylthiouracil arrested development in premetamorphosis but permitted continued growth for at least 15 months. Levels of circulating thyroxine and triiodothyronine in treated tadpoles were depressed to or below normal premetamorphic levels when measured by radioimmunoassay and competitive protein binding assay.

During normal metamorphosis quantitative or qualitative   changes occurred in each protein system so that postmetamorphic protein patterns differed from premetamorphic patterns.   Previous workers have suggested these changes to be an adaptation to postmetamorphic life in Anura. However goitrogen treatment did not prevent these 'biochemical metamorphoses' in many cases.   42% and 50% of treated larvae exhibited metamorphic patterns of haemoglobin and albumin respectively.   All arrested larvae which had continued to grow for at least 2 months had postmetamorphic lactate dehydrogenase isozymes and lens crystallins.

A comparison of the lens crystallins of treated larvae, similar in developmental stage and duration of treatment, but differing in lens size showed crystallin pattern to be correlated with lens diameter and hence body size.

The ability of protein transitions to continue under thyroid blockage suggests that thyroxine and triiodothyronine are not involved as principal controlling factors.   Similar protein changes occur during the embryonic development of many non-metamorphosing vertebrates.   The specialised morphogenetic role for amphibian thyroid hormones which has evolved in such metamorphic events as limb development and tail regression may not have evolved in the protein systems studied.

ACKNOWLEDGEMENTS

ABBREVIATIONS

Ad	Adult
BSA	Bovine serum albumin
$^{\circ}$C	Degrees Celsius
cm	Centimetre (s)
CM-cellulose	Carboxymethyl-cellulose
cpm	Counts per minute
DEAE-cellulose	Diethyl-aminoethyl-cellulose
DIT	Diiodotyrosine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol ("Cleland's Reagent")
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
g	Gram(s)
GH	Growth hormone
GPH	Growth promoting hormone(s)
Gt	Giant
h	Hour(s)
^{3}H	Tritium
Hb	Haemoglobin(s)
IEP	Immunoelectrophoresis
iu	International unit(s)
l	Litre(s)
LDH	Lactate dehydrogenase
M	Molar
mA	Milliamp(s)
mCi	Microcurie(s)
mg	Milligram(s)
min	Minute(s)
MIT	Monoiodotyrosine
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
mRNA	Messenger ribonucleic acid
NF	Nieuwkoop and Faber (1956)
ng	Nanogram(s)
nm	Nanometre(s)
PAGE	Polyacrylamide gel electrophoresis

PBI	Protein-bound iodine
PIH	Prolactin inhibiting hormone
RIA	Radioimmunoassay
RNA	Ribonucleic acid
st	Stage
T3	L-Triiodothyronine
T4	L-Thyroxine
TBG	Thyronine binding globulin(s)
TBPA	Thyronine binding prealbumin
TCA	Tri-chloroacetic acid
TEB	Tris-EDTA-boric acid
TEMED	N:N:N':N'-Tetramethyl-1:2-Diamino-Ethane
TH	Thyroid hormone(s)
TK	Taylor and Kollros (1946)
Tlt	Toadlet
TRH	Thyrotropin releasing hormone
Tris	Tris (hydroxymethyl) methylamine
TSH	Thyroid stimulating hormone
Tyr	Tyrosine
μg	Microgram(s)
μl	Microlitre(s)
v/v	Volume for volume
w/v	Weight for volume

CHAPTER I

INTRODUCTION

The orderly process by which an embryo develops into an adult organism capable of reproducing itself involves interactions between cells, mediated by chemical messengers. At certain stages specific products of certain groups of cells have profound effects on the development of other adjacent cells, as with inductors, or on that of distant cells and tissues, as with hormones. These effects are essential for the coordinated development of the embryo. For instance, in early development induction by the chorda-mesoderm of vertebrates affects the overlying ectoderm and is necessary for the development of the entire nervous system. In later development, the actions of morphogenetic hormones become of similar importance.

Hormones have proved to be the chemical messengers most amenable to study. They can be isolated in some cases as pure compounds and their effects, at all levels of analysis from gross morphological to molecular biochemistry, studied.

A system which has been of continued use, over a period of at least sixty years, is amphibian metamorphosis. A number of vertebrate hormones are able to influence the development of several different tissues and, in the mammal, thyroid hormones have this effect on the development of the central nervous, skeletal, and reproductive systems. But it is in amphibian development that thyroid hormones have evolved a central role in controlling the metamorphosis of most tissues in the body when the larva transforms into the adult.

Thyroid hormones are easily synthesized and this together with the relative ease with which amphibian larvae can be obtained, makes the system an extremely convenient one.

Since the control exerted by thyroid hormones was first recognised there has been a wealth of descriptive and experimental studies, not only on morphology, but also on the biochemical changes which occur during the metamorphic process. In addition, thyrotropin, thyroid releasing hormone, 'prolactin' and 'growth hormone' have also been found to exert influences in the complex process.

Thyroid hormones, like the steroid hormones, are of sufficiently low molecular weight to enter the cell nucleus and evidence is accruing that they react directly with the chromatin in amphibians as well as in mammals (review by Oppenheimer & Surks, 1976).

Because many of the biochemical changes are temporally correlated with the morphological transformation, it has frequently, although sometimes tacitly, been assumed that the biochemical changes are controlled in a similar way to the morphological ones.

For the purposes of this thesis it is important to distinguish between two main types of biochemical change in amphibian metamorphosis. The first class includes biochemical events directly producing major morphological modification. For example, one of the most striking features of larval anuran development is tail regression. Current evidence, reviewed by Dodd and Dodd (1976), suggests the resorptive process is a programmed cell death proximately controlled by thyroxine. Quantitative changes in a number of enzymes, including cathepsin, acid phosphatase, beta - glucuronidase, beta - galactosidase, deoxyribonuclease and collagenase, have been described. These enzymes, possibly produced by macrophages, are the effectors of the ultimate process which is tail regression.

The second class of biochemical change includes those which seem to have adaptive value, in the changing lifestyle of the amphibian, in their own right. An excellent example is found in the synthesis of urea - cycle enzymes. Freshwater tadpoles are able to excrete their nitrogenous wastes as the highly soluble, but toxic, ammonia whereas the terrestrial frog excretes urea. The enzymes of the ornithine - urea cycle make this shift possible. Brown et al (1959) studied the livers of bullfrogs, Rana catesbeiana, during metamorphosis and found a strong correlation between the stage of metamorphosis and the rising titres of the enzymes carbamoylphosphate synthetase, ornithine transcarbamylase and arginosuccinate synthetase.

It is with this second class of metamorphic biochemical change that this thesis is mainly concerned.

In addition to the assumption that such changes are controlled by thyroid hormones it has also been argued that, some at least, are directly adaptive to the animal. In other words, that they change because the metamorphosed amphibian must have an altered biochemical system in order to survive. Generally such arguments have been made with the needs of the typical ranid anurans in mind. The life cycle of these animals usually includes a herbivorous, gill - breathing, aquatic larva which transforms into a terrestrial, carnivorous adult which respires with its lungs. As will be discussed later in this thesis, biochemical metamorphosis also occurs in animals which do not have a profound change in habitat or niche during their life cycle.

Following the experimental manipulation of tadpole morphology by hormone treatment (reviewed by Etkin, 1968) a number of investigators have sought to produce precocious biochemical metamorphosis by treatment, either _in vivo_ or _in vitro_, with exogenous thyroid hormones. Examples are the work of Ledford and Frieden (1973) with serum protein shifts and of Moss and Ingram (1965) and Just and Atkinson (1972) with haemoglobin shifts.

The reverse approach to this analysis is clearly to prevent the production of thyroid hormones and then to study well - defined biochemical systems which are known to change during normal metamorphosis. If the thyroid hormones do directly control the process studied, and if the changes normally occurring are essential to the post-metamorphic frog and non-adaptive in the tadpole, then one would expect the typical tadpole biochemistry to persist for as long as thyroid blockage persists.

It was therefore particularly surprising when Maclean and Turner (1976) found adult-like haemoglobin in quantity in giant _Xenopus laevis_ tadpoles which had been developmentally - arrested in early hind limb development by treatment with the anti - thyroid compound 6 - n - propyl - 2 - thiouracil. Some variation in the proportion of tadpole haemoglobins remaining was apparent suggesting that body size or duration of treatment, both of which also varied, could be of importance. The giant tadpoles were morphologically in premetamorphosis (Etkin, 1968) but, at least with respect to haemoglobins, were adult-like. Both the concepts of control by thyroid hormone and the obligatory adaptiveness of the biochemical transition appeared to be refuted or, at least, in need of modification.

It was possible that the haemoglobins might be a special case.
Both a Xenopus laevis adult (Ewer, 1959) and Rana temporaria tadpoles
(Manwell and Baker 1971) have been found to occur naturally without
detectable haemoglobin.   Also, experimentally induced anaemia in which
nearly 100 per cent of the erythrocytes of bullfrog tadpoles were lysed
by phenylhydrazine injection (Dewitt et al, 1972) was tolerated for 16
days before a population of immature erythrocytes appeared in the cir-
culation.   Flores and Frieden (1968) also working with phenylhydrazine-
injected bullfrog tadpoles found animals with red cell counts of less
than 1% normal could survive for several weeks.   The untreated tadpole
required $65^{\pm}15\mu l O_2/h/g$ at $23^{o}C$.   Water at the same temperature con-
tained $55\mu l O_2/ml$ so that providing the oxygen content of the body fluids
could reach equilibrium with the water in an hour the animals could
survive, without strenous activity.   However aside from the question of
how important haemoglobin is to amphibian larvae the question of hormonal
control of normal haemoglobin transition remains.

Not only is the occurrence of such biochemical metamorphosis in
developmentally arrested tadpoles of interest in relation to possible
thyroid control and to the obligatory adaptiveness of the transition.
In addition most experiments on the biochemistry of larval amphibians
involves grouping them in developmental stages according to the normal
table appropriate to the species concerned.   This presupposes that a
group of larvae, externally identical in terms of say, hind leg develop-
ment, are also homogeneous with respect to the biochemical systems which
normally undergo a metamorphic transformation.   It is of great interest,
not only to understand how growth and morphology can be uncoupled by
goitrogen treatment (Turner, 1973), but also to discover to what extent
biochemical and morphological development can be dissociated.

The present work was designed to study comparatively, chemical trans-
formations in four systems which normally change during spontaneous
metamorphosis to obtain a deeper understanding of the problems previously
raised.

The anuran used was the African Clawed Toad, Xenopus laevis Daudin.
It was chosen partly because it can readily be induced to spawn with
injection of chorionic gonadotropin and partly because the methodology of

thyroid blockage with continued, prolonged healthy growth was well established (Maclean and Turner, 1976).

The type of metamorphosis exhibited by Rana catesbeiana is only one of those evolved by the amphibia. Even amongst anurans there is considerable variation from the ranid type of extreme indirect development to direct development in which there is no free - swimming larval stage (Lynn, 1961). The life cycle of X. laevis, which belongs to the family Pipidae, is an intermediate form in which both tadpoles and adults are fully aquatic. In South Africa the adults inhabit muddy pools staying under water for prolonged periods without surfacing for air. Should the pools dry up the adults frequently aestivate in the mud and only rarely migrate over land. The skin must be kept moist for survival.

Many of the biochemical metamorphoses described in detail (review by Frieden and Just, 1970) have been observed in North American ranids. The very interesting ornithine - urea cycle enzyme shifts of the bullfrog do not occur in X. laevis as the adults continue to excrete ammonia (Munro, 1953). Amongst the systems considered when selecting those to be studied were collagen degradation in the skin (Eisen & Gross, 1965), shift from porphyropsin to rhodopsin as visual pigment (Ohtsu et al, 1964), change from carbohydrases to proteases as digestive enzymes (Lipson & Kaltenbach, 1965) and subepidermal gland secretions (Vanable, 1964). The principal criteria were that methods of studying the transitions should be practicable with small quantities of tissue and that preferably, at least preliminary work, should have been published previously on Xenopus laevis.

The four systems selected had received some previous attention so that data on the normal transition in spontaneous metamorphosis were available. The systems were serum proteins, especially albumin (Herner and Frieden, 1960), lens crystallin soluble proteins (Campbell et al, 1968), lactate dehydrogenase isozymes (Kunz and Hearn, 1967) and haemoglobin (Maclean and Jurd, 1971a; Jurd, 1972).

Serum and coelomic fluid protein shifts seemed attractive because of the relatively large volume, of coelomic fluid at least, extractable from a Xenopus normal tadpole. The work of Thornburg et al (1975) on the bullfrog suggested that similar albumin increases would occur in both serum and coelomic fluid.

Haemoglobin had the advantage of being readily extractable from even small tadpoles. In addition there was a stimulating literature on its control during amphibian development (e.g. Forman and Just, 1976 and Hollyfield, 1967 on erythrocyte life spans; Broyles and Frieden (1973) on changing sites of erythropoiesis; Jurd and Maclean (1970) and Maniatis and Ingram (1971c) on the synthesis of tadpole and adult haemoglobins within single red cells).

Both of the above systems had been studied from the point of view of precocious induction by treatment with exogenous thyroid hormones (Ledford and Frieden, 1973; Moss and Ingram, 1965 and 1968b; Just and Atkinson, 1972).

Thyroid control of lens crystallin and lactate dehydrogenase isozyme shifts had not previously been extensively studied experimentally. The crystallins had been assumed to change as a response to lens growth in order to maintain satisfactory refractile or elastic properties (Clayton, 1970) but Polansky and Bennett (1973) had also obtained evidence of T4 involvement.

The thyroid gland of vertebrates contains two distinct endocrine systems which secrete different hormones. In this thesis calcitonin has not been considered and the expression 'thyroid hormones' refers to triiodothyronine (3, 5, 3' - triiodo - L - thyronine) and L - thyroxine (3, 5, 3', 5' - tetraiodothyronine), referred to as T3 and T4 respectively because of the number of iodine atoms in their molecules.

These hormones are produced by the follicular cells of the thyroid gland. The biosynthetic pathway of mammalian thyroid hormones is illustrated below.

THYROID CELL   PLASMA

Thyroglobulin - Tyr
        3   2   1
                    ───────────→ Iodine ←─── Iodide ←─── Iodide

Thyroglobulin - MIT
                    DIT
        4   5   6

Thyroglobulin - T3 ───────→   MIT, DIT   T3
                    T4   T3, T4   T4
                                        Amino acids
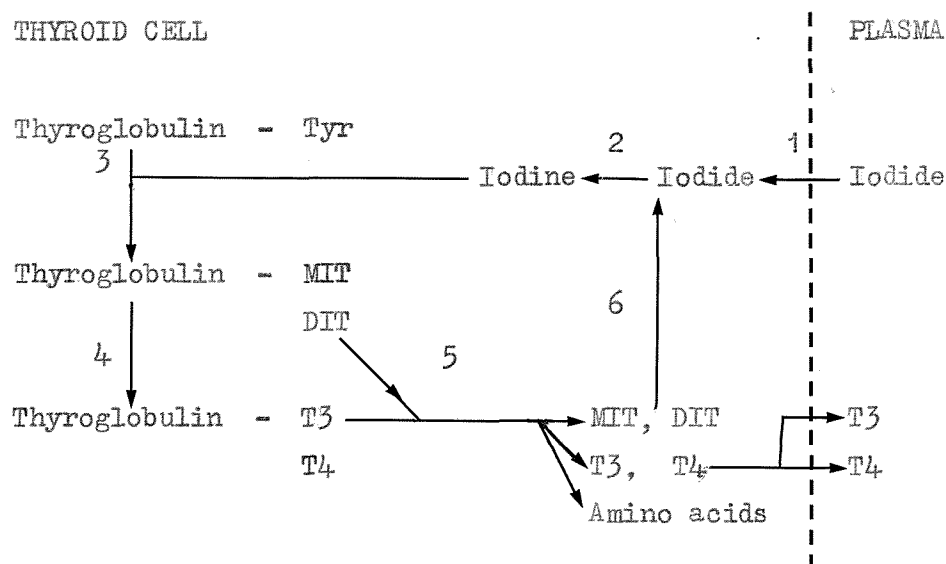
1 = iodide 'carrier' or concentrating mechanism
2 = peroxidase (blocked by propylthiouracil)
3 = tyrosine iodinase
4 = 'coupling' enzyme
5 = protease
6 = dehalogenase   (modified from Varley, Gownlock
MIT is monoiodotyrosine   & Bell, 1976).
DIT is diiodotyrosine

In amphibians basic knowledge of the steps of hormone synthesis is
limited but apparently the process is very similar to that in mammals.
Studies with radioactive iodine have demonstrated that the amphibian
thyroid, like that of all other vertebrates, accumulates and metabolises
iodine. The chemical form of the protein - coupled radioiodine has
been found to be iodotyrosine and a relatively small proportion of
thyroxine and perhaps triiodothyronine (Berg et al, 1959).

The halogenation of thyroglobulin is catalysed by peroxidases in
mammals. Peroxidase activity has been identified in the thyroid gland
of X. laevis by Regard and Mauchamp (1973). The enzyme activity is
found in many components of the follicular cells and first becomes de-
tectable during premetamorphosis. It increases during metamorphic cli-
max.

The proteolytic release of hormone from thyroglobulin has also been
identified in the Xenopus thyroid by Coleman et al (1967) and Regard and
Mauchamp (1973).

As the thyroid gland of <u>Xenopus</u> appears to exhibit similar synthetic activities to that of mammals it is reasonable to assume that the mechanism of goitrogen action is also similar in both groups.

The goitrogen used for the present work was a 0.01% W/V (0.59mM) solution of 6 - n - propyl - 2 - thiouracil. The tadpoles were immersed in the solution when their hind - limbs were present as buds and they became arrested when the digits of the hind limbs had begun to develop. In most cases little further morphological change occurred for periods of up to 15 months during which time the tadpoles continued to grow into gigantic proportions.

A recent cell - free <u>in vitro</u> study by Taurog (1976) has confirmed earlier reports that propylthiouracil inhibits the peroxidase - catalysed iodination of protein and tyrosine. Lynn and Dent (1960) working with larval treefrogs, <u>Hyla versicolor</u>, found that immersion in propylthiouracil solutions, at the same time as a thyroidectomising dose of ^{131}I was being administered, prevented any thyroid damage. This suggests that the goitrogen was also blocking the initial uptake of iodide by the thyroid gland. The perchlorate ion was found to have a similar effect which agrees with work on mammals in which its anti - iodide carrier action has been noted.

The thyroid gland of propylthiouracil - treated larvae becomes goitrous indicating that thyrotopin is produced in abnormal quantities by the pituitary thyrotroph cells in response to a lack of circulating thyroid hormone. Mira - Moser (1972) noted histological criteria typical of surgically thyroidectomised animals in the pituitaries of propylthiouracil - treated <u>Bufo bufo</u>. Goos (1968) found propylthiouracil treatment to inhibit the differentiation of the hypothalmus in <u>Xenopus</u> tadpoles. Etkin (1966) had found hypothalamic differentiation to be stimulated by thyroxine so that Goos' data constitute further evidence for the goitrogenic role of propylthiouracil in <u>X. laevis</u>.

Thus propylthiouracil is well established as a potent goitrogen in amphibians and was an ideal compound with which to arrest morphological development prior to studying the transitions of haemoglobins, albumin, LDH isozymes and lens crystallins.

1.2 Outline of thesis

Chapters 3 to 7 inclusive of this thesis each deal with a section of the experimental work. They are preceded by a general 'Materials and Methods' section in which full details of techniques are given. Each experimental chapter begins with an 'Introduction' which reviews the literature relevant to that chapter and discusses the range of techniques which were considered. The second section of each chapter is headed 'Experimental' and this states the principles of the methods used and lists the experiments performed. Next comes a 'Results' section and finally in the 'Conclusions' the essential information which can be drawn from the results is discussed.

Chapters 2, 3, 4 and 5 are concerned with haemoglobins, coelomic fluid proteins, LDH isozymes and lens crystallins respectively. In each case the normal biochemical transition during spontaneous metamorphosis was studied and compared to that in propylthiouracil - arrested tadpoles.

Chapter 6 sought to confirm that under prolonged goitrogen treatment the tadpoles were unable to synthesise significant amounts of T4 and T3. These hormones were assayed in the coelomic fluids of normal and treated Xenopus laevis.

The final chapter of the thesis, headed 'General Discussion' brings the main conclusions from the experimental chapters together and discusses them in the context of the hormonal control of growth and development in amphibian metamorphosis.

CHAPTER 2
MATERIALS & METHODS

2.1 Nomenclature

2.1a Xenopus laevis developmental stages.

The nomenclature of Nieuwkoop and Faber (1956) in their "Normal Tables of Xenopus laevis (Daudin)" was followed.

In addition reference was made to the metamorphic subdivisions of Etkin (1968). "Premetamorphosis" included all stages from hatching to NF stage 54 inclusive, "prometamorphosis" was from NF stage 55 to 57 inclusive and "climax" began at NF stage 58 (Dodd & Dodd, 1976).

2.2 Biological materials

2.2a Xenopus laevis adults

Deuchar (1975) lists seven subspecies of X. laevis. Following her criteria for distinguishing the subspecies by external mophology the toads used in this work belonged to X.laevis laevis.

Mature adult X. laevis, of unknown origin, were purchased from Harris' Biological Supplies (Weston-super-Mare, Somerset). Some of the animals used to provide fertilised eggs belonged to the colony at the Biology Department, Southampton University and were of various origins. Toads used for spawning were kept in 30 litre glass aquaria with tap water to a depth of about 10cm. They were fed at least three times per week on chopped deep - frozen beef heart and liver. Better results were obtained when toads were fed five times per week and when the diet included fresh liver and Tubifex worms.

2.2b. Injection procedure

Pairs of toads were placed in 33cm diameter glass crystallisation dishes in about 8cm of water. They were left undisturbed for 24h. before beginning injection. The injection routine was basically that described by Billett and Wild (1975). Human chorionic gonadotropin (Chorulon, Organon Laboratories, Morden, Surrey) was dissolved in distilled water and injected into the dorsal lymph sacs. The dosage rates were as follows:

	Male	Female
Day 1	50	100 International
Day 2	100	200 Units

Latterly it was found desirable to prime both males and females 1 week before the above course with 50 i.u. of 'Chorulon'. Eggs were laid within 24 h. of the second injection. The success rate of injection was often less than 25% especially between July and August.

The eggs were collected and placed in aged tap water. It was essential to remove unfertilised and decaying eggs within 48h. of laying to prevent infection spreading throughout the batch.

2.2c. Xenopus laevis larvae - rearing to metamorphosis

Xenopus larvae were kept in aged tapwater in 30 litre glass aquaria with aeration. The water temperature was around 18^oC. The animals were fed thrice - weekly on a suspension of 'Complan' (Glaxo-Farley Foods, Plymouth, Devon). After metamorphosis small toadlets were fed on Tubifex worms. Giant larvae (goitrogen treated) were fed in a similar way.

It is essential to minimise crowding when keeping amphibian larvae. Numbers in excess of 50 larvae per aquarium led to delayed development.

2.2d. Arrest of development with goitrogen.

All the biochemical results obtained here were made with Xenopus larvae placed in an 0.01% W/V (0.59mM) aqueous solution of 6 - n - propyl 2 - thiouracil (Sigma Chemical Co. Ltd., London).

The solid propylthiouracil was dissolved only with difficulty in water at 50^oC with prolonged magnetic stirring. Tadpoles of stage 49 to 51 were placed in this solution, developmental arrest occurring at stage 54. With prolonged immersion (more than 2 months) it was some- times necessary to increase the goitrogen concentration to 0.015% W/V as some larvae entered prometamorphosis. Solutions were changed once in 3 - 4 weeks. The goitrogen treatment was that of Maclean and Turner (1976).

2.2e. Rabbits

Rabbits (Oryctolagus cuniculus (L)) were used to raise antibodies against Xenopus antigens. The rabbits were random - bred New Zealand Whites from the Animal House of the Department of Physiology and Biochemistry, Southampton University.

2.3 Chemical reagents

Unless otherwise stated all chemical reagents were supplied by British Drug Houses Ltd., (Poole, Dorset).

2.4 Water

Unless otherwise stated water used in aqueous solutions and media was glass - distilled.

2.5 Anaesthesia

All operations on both larval and adult Xenopus laevis were performed under general anaesthesia from which the animals were not allowed to recover.

Adults were anaesthetised by immersion for up to 1 h. in 0.2% W/V aqueous solutions of tricaine methane sulphonate (MS 222, Sandoz Products Ltd., London). Complete anaesthesia of larvae took place in about 2 minutes. MS 222 solutions for the latter were made up in Rugh's Ringer.

2.6 Balanced salt solution (Rugh's ringer)

Rugh's amphibian ringer (Rugh, 1962) was used as a basal medium for all biological materials. The solution closely approaches the natural tonicity and plasma electrolyte concentrations of Xenopus plasma (Dessauer, 1961). It has the following composition.

Na Cl	6.60g
K Cl	0.15g
Ca Cl_2 2 H_2O	0.15g
Water	to 1 litre

The pH was adjusted to 7.40 with NaH CO_3. Analytical grade chemicals were used to make the Ringer's solution. Rugh had suggested the use of pH 7.80 but a pH of 7.4 was employed here following Paul's (1970) suggestion that cells survived better in media buffered to a more acid pH. The Ringer's solution was stored at 4^oC.

2.7 Use of thiols

Artificial heterogeneity of protein may be caused by polymerisation

of the proteins. Such polymers result from the formation of disulphide bridges between sulphydryl (-SH) groups and adjacent polypeptide chains.

Riggs, Sullivan & Agee (1964) used 2 - mercapto - ethanol to reduce disulphide bridges and prevent polymerisation. An aqueous solution of this compound was added to protein solutions, buffers and gels to a final concentration of 10mM in a few experiments.

Throughout most of the work dithiothreitol (DTT or "Cleland's Reagent" - Cleland, 1964) was used to a final concentration of 0.01% (0.65mM). DTT was claimed to be more efficacious than 2 - mercapto-ethanol in reducing disulphide bridges because of its low redox potential. No difference was noted between the results obtained with either thiol.

2.8 Extraction of blood from Xenopus laevis

Adult toads were bled by ventricular puncture of the exposed heart while under general anaesthesia. Blood was removed with a syringe, rinsed with Ringer, but no anticoagulent substances were used. If unclotted blood was required to culture erythrocytes or to prepare haemolysates the whole blood was expelled into large volumes of Ringer (1ml of blood in 10 - 15ml. Ringer) in which clotting did not occur.

Tadpoles were lightly anaesthetised and bled by making a ventral incision with fine scissors directly through the ventricle. The tadpole was then placed in 10ml. of Ringer in a tapered centrifuge tube and allowed to bleed for up to 15 min during which time it remained immobile. When erythrocytes from more than one tadpole were pooled the animals were bled into the same tube of Ringer.

It was found preferable to protect tadpole erythrocytes from lysis by the addition of 1.25 mg/ml of bovine serum albumin to the Ringer. Jurd (1972) had found that tadpole red cells survived healthily for only 30 minutes in non-albuminised Ringer whereas adult cells survived for at least 2 hours.

2.9 Washing red blood cells

Prior to haemolysis or incubation, blood cells were washed by three repeated centrifugations of the cell suspension at 500 xg for 5 min. Each centrifugation was followed by resuspension in 12 ml. of Rugh's Ringer containing 1.25 mg/ml of BSA.

The final pellet of cells was suspended in a small volume (usually less than 100 μl for tadpoles) of non-albuminised Ringer.

2.10 Preparation of haemolysates

The resuspended final pellet of blood cells was treated with solid saponin to a final concentration of approximately 1 mg/ml. The tube was agitated vigorously. The cellular debris were removed by centrifugation at 1000 xg for 30 minutes. The clear red supernatant was removed.

2.11 Haemoglobin concentration in haemolysates

The small volumes of haemoglobin solutions obtained from Xenopus larvae made it impracticable to measure haemoglobin concentration spectrophotometrically against a cyanomethaemoglobin standard. Instead it was assumed that all erythrocytes contained equal concentrations of haemoglobin and that equilibration of cell numbers per unit volume would produce solutions of haemoglobin with similar haemoglobin levels.

In a given experiment three aliquots of cell suspension from each sample were counted before the third washing centrifugation. Counting was performed with a haemocytometer counting chamber (Weber & Sons, Lancing, Sussex). After centrifugation the volume of the final haemolysate was adjusted so that each sample was equivalent in cell concentration to that of the least dense sample. Typically this would be 0.7×10^6 cells per ml. for tadpoles. 40 μl of such a solution gave satisfactory bands with polyacrylamide gel electrophorosis (PAGE).

2.12 Conversion to cyanomethaemoglobin

Some workers (e.g. Moss and Ingram, 1968) routinely convert haemolysates from oxyhaemoglobin to cyanomethaemoglobin before analysis. Oxy – and carboxyhaemoglobin are less stable than the cyanomet – form and this could lead to spurious electrophoresis results.

Haemolysates were routinely treated with Drabkin's solution (Drabkin & Austin, 1935) in the present work to convert the sample to cyanmethaemoglobin. Drabkin's solution is prepared as follows:

Na H CO$_3$	10g
KCN	0.05g
K$_3$Fe (CN)$_6$	0.2g
Water	to 100 ml

0.2 volumes of this solution were added to all haemolysates.

2.13 Incubation of red cells with radioisotope

Xenopus erythrocytes were incubated for 3 h. in a medium containing ^{3}H – leucine. The following stock solutions were used (Thomas 1974).

2.13a Amino-acid mixture I

Concentration of each amino-acid in the stock solution was 5 m Mole per l.

L – Alanine	44.5mg	L – iso – leucine	65.6mg
L – Arginine	87.1mg	L – Lysine	73.1mg
L – Asparagine	66.1mg	L – Methionine	74.6mg
L – Aspartic acid	66.6mg	L – Phenylalanine	82.6mg
L – Cysteine	60.6mg	L – Proline	57.6mg
L – Glutamic Acid	73.6mg	L – Serine	52.5mg
Glycine	37.5mg	L – Threonine	59.6mg
L – Glutamine	73.1mg	L – Tryptophane	102.1mg
L – Histidine	77.6mg	L – Valine	58.6mg

The amino acids had been dissolved in Rugh's Ringer and the pH adjusted to 7.40 with Na H CO$_3$. The volume was made up to 100ml. The mixture had been stored in small batches at -20°C.

2.13b Amino-acid mixture II

The concentration of each amino-acid in the stock solution was 2 m Mole per l.

L – Tyrosine	1.8mg
L – Cystine	2.4mg

The amino-acids were dissolved in 50ml Rugh's ringer and the pH

adjusted to 7.40 with Na H CO$_3$.   The mixture had been stored in small batches at -20°C.

2.13c Tritiated leucine stock solution

10 m Ci of L - leucine - 4, 5 - ^{3}H, already dissolved in 10ml. of sterile water, and labelled as 1,000 m Ci per m Mole, were purchased from the Radiochemical Centre, Amersham, Bucks.

2.13d Nucleotide stock solution

Sodium acetate trihydrate	25.00mg
D - Ribose	0.25mg
2 - Deoxy - D - Ribose	0.25mg
Adenylic Acid	0.10mg
Adenine	5.00mg
Guanine Hydrochloride	0.15mg
Thymine	0.15mg
Xanthine	0.15mg
Adenine Triphosphate (ATP)	5.00mg
Cholesterol	0.10mg
Tween 80 (Difco Labs., Detroit, U.S.A.)	2.50mg
Ferric Nitrate monohydrate	0.05mg
Hypoxanthine	0.15mg

The constituents were dissolved in Rugh's amphibian ringer and the volume made up to 10ml.   The solution was stored in 1ml batches at -20°C.

2.13e Vitamin mixture stock solution

Thiamine	5.00mg
Riboflavin	0.50mg
Pantothenate	5.00mg
Pyridoxal	5.00mg
Nicotinamide	5.00mg
Choline	2.50mg
Inositol	10.00mg
Folic acid	5.00mg
Cyanocobalamin	5.00mg
Ascorbic acid	250.00mg

The constituents were dissolved in Rugh's amphibian ringer and the volume made to to 100ml. The vitamin mixture had been stored in small batches at - 20°C.

2.13f Streptomycin sulphate stock solution

A 5 mg/ml solution of streptomycin sulphate (Sigma Chemical Co.Ltd. London) was prepared in Rugh's ringer.

2.13g Benzylpenicillin stock solution

A 3 mg/ml solution of benzylpenicillin (Penicillin-G, sodium salt: Sigma Chemical Co. Ltd., London) was prepared in Rugh's ringer.

2.13h Glucose stock solution

A 50 mg/ml solution of glucose was prepared in Rugh's ringer.

2.13i Short term culture medium

The above stock solutions were passed through Millipore cellulose acetate membrane filters with a pore diameter of 220 nm (Millipore (U.K.) Ltd., Wembley). Such filters are non-toxic and the 220 nm pore diameter precludes the passage of bacteria. The glucose solution was autoclaved for 20 minutes and when cool added to the millipore filtered solutions in a sterile McCartney bottle. The volumes of stock solutions used were as follows:

Amino - Acid mixture I	0.2ml
Amino - Acid mixture II	0.3ml
Nucleotide mixture	0.1ml
Vitamin mixture	0.1ml
Streptomycin sulphate solution	0.1ml
Benzylpenicillin solution	0.1ml
Glucose solution	0.2ml

2.13j Incubation of blood cells

The cell pellet was made up to 2.5ml. with Rugh's ringer containing 54mg. BSA. The cell suspension was pipetted into a sterile culture bottle together with medium and label in the following proportions:

Culture medium	0.4ml
L - Leucine - 4,5 - ^{3}H	0.05ml
Cell Suspension	2.05ml

By bleeding the tadpoles into ringer it was impossible to avoid contamination. The sterile precautions taken above were designed to prevent the growth of micro-organisms during the short 3h. incubation.

The culture bottles were incubated for 3h. at 24°C in a shaker water bath.

2.14 Liquid scintillation counting

^{3}H activity was estimated in gel slices by use of the Inter-technique ABAC SL40 scillination counter. The solubilised gel slices each received 5ml of scintillation fluid in a plastic vial. The scintillation fluid had the following constituents (Bray, 1960).

	Xylene	38.3% V/V
	Dioxan	38.3% V/V
	Ethanol	23.3% V/V
with	PPO (2, 5-Diphenyloxazole)	5g/ litre
	POPOP (1, 4-Di-2 (5-phenyloxazolyl)benzene)	0.1g/ litre

Each vial was counted for 10 minutes and the results expressed as counts per minute (cpm).

2.15 Extraction of lens crystallins

2.15a. Lentectomy

Lenses were removed from anaesthetised tadpoles or tadpoles which had been thawed after storage at - 20°C by making an incision in the cornea while pressing the edges of the eyeball with forceps. The lens was then extruded and could be cleaned of adhering tissues.

2.15b. Measurement of lenses

The diameter of lenses was measured using an eyepiece micrometer graticule in a Nikon stereoscopic microscope. The diameter of the whole lens (including the epithelium) was recorded to the nearest 0.05mm.

2.15c. Homogenisation of lenses

Lenses were homogenised in small volumes (less than 100 µl) of Rugh's amphibian ringer in glass homogenisers. Pooled adult lenses were treated in a 10ml. glass tube homogeniser with a Teflon plunger. Tadpole lenses were ground in a purpose – made homogeniser in which a 2mm diameter concavity had been drilled in the bowl of a solid watch-glass. The glass surface was roughened as was the rounded tip of a 2mm glass rod used as a pestle.

During homogenisation vessels were stood on crushed ice. Homogenates were centrifuged at 1000 x g for 20 minutes and the clear supernatant soluble crystallin solution stored at - $20^{\circ}C$.

DTT was added to a final concentration of 0.003 M to inhibit polymerisation of the soluble crystallins.

2.16. Extraction of lactate dehydrogenase

Whole tadpole hearts (auricle and ventricle) were removed from anaesthetised tadpoles with watchmakers' forceps. In the case of adults a small piece of ventricle was removed. Homogenisation was performed in Rugh's ringer on ice with the homogenisers described above. The homogenates were centrifuged at 1000 x g for 20 minutes and the supernatant stored at - $20^{\circ}C$.

The supernatant was merely an extract of soluble heart proteins. Lactate dehydrogenase activity was identified using a specific staining technique in which sodium lactate was used as a substrate for the isoenzymes.

2.17. Extraction of serum from Xenopus laevis adults and coelomic fluid
from larvae and toadlets.
2.17a. Adult toads

Whole blood was removed from the exposed hearts of anaesthetised toads by ventricular puncture. Anticoagulents were not necessary providing the syringe was rinsed with Rugh's ringer. The blood was expelled into a 10ml. tapered centrifuge tube, stirred and allowed to clot for about 2h. at room temperature before being stored overnight at $4^{\circ}C$. It was then centrifuged at 500 x g for 5 minutes and the super-

natant serum removed. Serum was stored at - 20°C in small batches.

Collecting satisfactory samples of coelomic fluid from the peritoneal cavity of adult toads was difficult because of the great vascularity of the skin and body wall. Coelomic fluids were frequently so heavily contaminated with blood that it was not possible to determine their protein concentrations by colorimetry.

2.17b. Coelomic fluid from larvae and toadlets

Pilot studies were made in an attempt to collect serum from stage 54 larvae. The hearts of anaesthetised tadpoles were exposed and whole blood collected by inserting fine capillary tubes into the conus arteriosus. Although blood was readily collected the problems of clotting, centrifugation and pooling of minute quantities of serum proved too great.

The work of Thornburg et al (1975) on Rana catesbeiana had shown that both serum and coelomic fluid contained the same proteins in similar quantities. Consequently it was decided to collect coelomic fluid from larvae and toadlets.

Anaesthetised larvae were placed ventral side uppermost and blotted dry. A small incision in the body wall ventral to the gut was made with forceps and coelomic fluid collected in capillary tubes. The fluid was pooled and centrifuged to remove detritus.

The skin and body walls of small toadlets were less highly vascularised than those of adults and so it was possible regularly to collect coelomic fluid from the peritoneal cavity without serious haemoglobin contamination.

2.18. Estimation of total protein in solutions

Soluble proteins were estimated by the method of Lowry, et al (1951). Basically four solutions were prepared:

a) 2% $Na_2 CO_3$. 10 H_2O in o.1N NaOH
b) 0.5% $CuSO_4$. 5 H_2O in 1% potassium tartrate

c) 50 : 1 of A:B

d) 1 : 2 of Folin & Ciocaltheu's Reagent : water

However, when preparing solution (b) a white precipitate often formed either immediately or after storage at $4^{O}C$ for 1 or more days. Thus it was found preferable to prepare:

1% Cu SO$_4$ 5H$_2$0

2% Potassium tartrate

both of which were stored for up to 1 week and which were mixed 1 : 1 when it was required to use solution (b) above.

When measuring protein concentrations it is usual to place 5ml of solution (c) in each acetone – rinsed tube followed by 20 μl of protein solution. The solution is vortex-mixed for a few seconds and stood for 10 mins. Then 0.5ml of solution (d) is added, the mixture vortexed and then stood for exactly 20 minutes before measuring its optical absorbance. The absorbance was measured with a 5ml cuvette in a Hitachi – Elmer – Perkin Spectophotometer at a wavelength of 500 nm and slit width of 0.2mm.

However when determining the protein concentration of small and precious samples it was desirable to use less than 20 μl. For much of the work in this thesis the method was scaled – down as follows:

solution (c)   1.4ml

Protein solution   5 μl (measured with Drummond 'Microcaps')

solution (d)   0.15ml

Vortexing and incubation times were as above. This method produced results as repeatable as the normal procedure. A 2ml cuvette was used in the spectrophotometer with a 0.2mm slit width.

Standard lines based upon serial dilutions of crystallised bovine serum albumin in 0.1M saline were prepared on each occas   protein estimations were made. It was not possible to obtain lens crystallins or Xenopus serum proteins in purified crystalline form to act as

standards. The standards were normally 40, 20, 10, 5 and 2.5mg/ml. Lower concentrations of BSA were found to give unpredictable results as suggested by Lowry et al (1951).

2.19. Preparation of antisera to Xenopus antigens

2.19a. Anti - Xenopus adult serum

Xenopus adult serum was collected as described above. Its protein concentration was measured and adjusted to 4 mg/ml. with Rugh's ringer. 0.5ml batches of diluted serum were stored at - 20°C until use.

0.5ml of diluted serum was mixed with 0.8ml of **Freunds** Adjuvant (Difco Labs., Detroit, U.S.A.) by vortexing for 5 minutes. The emulsion was checked by placing a droplet on a water surface. The emulsion was considered satisfactory if the droplet did not disperse. The emulsion was then injected sub-cutaneously into the neck region of a 3 Kg. rabbit dividing the material between three sites. This routine was repeated at weekly intervals until 4 injections had been given. A fifth injection followed 10 days later.

A sample bleeding of 25 ml whole blood was made after a further 10 days. The titre of the antiserum was checked and two more injections at weekly intervals were necessary before a satisfactory titre was acheived.

Bleeding was performed from an ear vein using xylene and warmth from a bench lamp to stimulate local blood flow.

The whole blood was stirred, stood at room temperature for 3h. and then overnight at 4°C before harvesting the antiserum. Small batches of antiserum were stored at - 20°C.

2.19b. Anti-Xenopus adult lens crystallin

The method was similar to that described above. An homogenate of adult lenses was used to prepare a crystallin solution containing 2mg/ml. 0.5ml of this material was emulsified with 0.5ml Freunds Adjuvant.

The injection routine and bleeding were as described above except that an extra injection of a 4mg/ml. crystallin solution was made before removing a large volume (80ml) of blood.

2.19c. Measurement of antiserum titre

The first measurements were made by the Ouchterlony Double Diffusion test. 2ml. of 1% Ionagar in 0.85% W/V saline was poured into each of a number of plastic petri dishes. A central well and six concentric peripheral wells were cut with a cork borer.

Serial dilutions of antigen were prepared beginning with 4mg/ml Xenopus serum and 2mg/ml Xenopus crystallins. The dilutions prepared were 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280 of the originals.

The central well was filled with antiserum and each of the peripheral wells with a different dilution of the appropriate antigen. The dishes were stored at $4^{o}C$ in a moisture-laden atmosphere until the precipitin arcs developed. Unprecipitated protein was leached out by washing for 2 days in 0.1 M saline and 1 day in distilled water. The arcs were then stained with a solution prepared as follows:

Azocarmine	1g
N Acetic acid	450ml
N Sodium acetate	450ml
Glycerol	100ml

Excess unbound stain was removed with 7% acetic acid. The titre of the antiserum was taken as the lowest antigen dilution capable of producing visible precipitin arcs.

The above method was abandoned in favour of precipitin ring tests which were much quicker. Lengths of 2mm. bore glass tubing were drawn out to fine tapers in a flame. Then a small volume of antigen was drawn up followed by the appropriate antiserum such that a clear interface formed between the two. The tip of each tube was sealed and a series prepared each with a different antigen dilution. The tubes were stood for 30 mins. by which time a white precipitate was visible at the interface of some. The lowest antigen dilution which could be precipitated was taken as the titre of the antiserum.

2.20. Protein separation - cellulose acetate electrophoresis

2.20a. Materials

Separations were performed on Celagram 78 x 150mm cellulose acetate membranes (Shandon Southern Ltd., Runcorn, Cheshire) in a Shandon universal electrophoresis tank Model U77, SAE - 3225. Wicks were of 5cm. wide 3 MM chromatography paper (W & R, Balston Ltd., England).

2.20b. Buffers

Initially the buffer used by Thornburg et al (1975) was tried. It was prepared as follows:

Barbitone sodium	10.3g
Barbitone	1.84g
Water	to 1 litre

pH adjusted to 8.6 with 1N Na OH.

However poor resolution of Xenopus serum necessitated preliminary experiments with other buffers, namely;

Tris - EDTA - borate buffer, pH 9.0 (Aronsson and Gronwall, 1957)

Tris	60.5g
EDTA	6.0g
Boric acid	4.6g
Water	to 1 litre

No pH adjustment was necessary.

Barbitone - acetate buffer, pH 8.6 (Owen, 1956).

Sodium acetate	6.5g
Barbitone sodium	8.87g
Barbitone	1.13g
Water	to 1 litre

No pH adjustment was necessary.

Both of these buffers had been recommended for use with cellulose acetate by Sargent (1969).

2.20c. Electrophoresis procedure

The Celagram strip was dropped onto the surface of the running buffer so that only its lower surface was in contact with the buffer. After soaking, the strip was blotted lightly between sheets of filter paper. The strip was then placed immediately in the electrophoresis tank, wicks fitted and the current switched on. Samples were then applied one-third of the way along from the cathode end. Four samples could be applied per strip if 1cm. origins were used. The samples were applied as an even smear with a 10 µl Microcap (Drummond Scientific Co., U.S.A.). The current applied was 6 mA total giving a potential difference of 95 volts. This was maintained for between 55 and 75 mins in different experiments.

The protein zones were then fixed by immersion of the strip for 2 min in 10% W/V aqueous TCA. Staining was with Ponceau S (see below).

2.21 Protein separation - polyacrylamide gel electrophoresis (PAGE)

2.21a. Gelling Solutions - Tris-Glycine/Tris-HCl discontinuous system.

For work with lens crystallins, LDH isozymes and serum proteins the method used was modified from Davis (1964). The latter's method was simplified by omitting a gel in which samples can be applied and in some cases by omitting the 'stacking gel' used to concentrate the samples before separation begins.

The following solutions were prepared and stored at $4^{o}C$ in the dark:

a) 48ml of N HCl, 36.6g of tris, 0.23ml of TEMED and water to 100ml. The pH of this solution is 8.9.

b) 28.0g of acrylamide, 0.735g methylene-bis-acrylamide and water to 200ml.

The following solutions were prepared and used immediately:

1) Separation Gel Solution A: one part of (a) above mixed with two parts of (c) and one part of water.

2) Separation Gel Catalyst Solution B: 0.1g of ammonium persulphate in 100 ml. water.

Equal volumes of (1) and (2) were mixed when gelling would begin within 10 minutes.

To prepare a stacking gel solution the following solutions were used:

c) 48ml of N HCl, 5.7g of tris, 0.46ml of TEMED and water to 100ml.

d) 10.0g of acrylamide, 2.5g methylene-bis-acrylamide and water to 100ml.

e) 0.35g of ammonium persulphate in 100ml water.

The gel was prepared by mixing the solutions in the proportions 1 part of (c), 2 parts of (d), 3 parts of (e) and 2 parts of water.

2.21b. Gelling solutions ——— Tris-EDTA-Boric acid continuous system.

To separate hameoglobins a continuous system was used (Jurd 1972). The gels contained 10% acrylamide and 0.2% methylene-bis-acrylamide. Stacking gels were used with the slab apparatus only. The preparation of the separation gel was as follows:

Smithies T.E.B. buffer, pH 8.6	10.30ml
Glycerol	1.20ml
TEMED	0.015ml
Acrylamide	1.25g
Methylene-bis-acrylamide	0.025g

The catalyst was a 1% W/V aqueous solution of ammonium persulphate freshly prepared. 0.5ml of catalyst was added to 11.515ml of acrylamide solution. Gelling was then complete in about 20 minutes. The 10%

glycerol was added to reduce diffusion of the protein bands (Matioli & Niewisch, 1965).

The stacking gel was prepared as follows:

Smithies TEB buffer, pH 8.6	10.30ml
Glycerol	1.20ml
TEMED	0.015ml
Acrylamide	0.425g
Methylene-bis-acrylamide	0.045g

Catalysis was induced by the addition of 1ml of a 1% ammonium persulphate solution.

Throughout most of the work a 0.003M solution of DTT was used instead of water when making gels.

2.21.c. Buffers

Tris-Glycine Buffer, pH8.3 (Davis, 1964).

Tris	6.0g
Glycine	28.8g
Water	to 1 litre

This was stored in the dark at 4°C until use when it was diluted 9 : 1 of water : buffer. 0.1g of DTT per litre was added.

Tris-EDTA-Boric Acid Buffer, pH 8.6 (Smithies, 1955).

Tris	10.9g
EDTA, disodium salt	0.6g
Boric acid	3.1g
Water	to 1 litre

The pH was adjusted to 8.6 with 1 N Na OH and 0.1g of DTT per litre was added.

2.21.d. Disc gel electrophoretic procedure

Disc gel electrophoresis employed the Shandon spparatus.
Precision bore glass tubes $2\frac{1}{2}$" long and 5mm internal diameter were
rinsed in 1/200 aqueous solution of Photoflo (Kodak Ltd., London)
to facilitate gel removal.   The tubes were marked 1cm from their upper
ends and the lower ends were sealed with plastic caps before being stood
vertically in a rack.   The polyacrylamide solution with catalyst was
pipetted into each tube up to the mark, excluding air bubbles.
Immediately, the polyacrylamide was overlaid with a 0.5cm. layer of
water applied slowly, to avoid surface mixing, with an 'Agla' micrometer
syringe (Burroughs Wellcome & Co, London).   The water layer speeds
gelling by excluding air and prevents the formation of a meniscus on
the gel surface which would lead to distorted protein bands.

When gelling was complete (45min. was always allowed) the water
layer was decanted.   The gel tubes were then placed in the Shandon
apparatus and both reservoirs filled with buffer.   Only the upper buffer
contained the expensive DTT.   The apparatus was pre-run to remove
oxidising persulphate ions from the protein front (Mitchell, 1967).   In
the case of discontinuous buffer systems pre-running was for $\frac{1}{2}$h. at 3mA
/gel whereas with the continuous system 1h. was allowed.

Protein samples received a drop of 80% W/V aqueous sucrose contain-
ing bromophenol blue.   This additive increased the specific gravity of
the sample preventing it from floating up from the gel surface and the
dye showed the position of the buffer front in the gel during electo-
phoresis.

Proteins were always run at pH's greater than 8.2 so that they were
negatively charged.   Thus the lower electrode was made anodal and a
constant current of from 2.5 to 3mA/gel supplied by a Shandon power
pack.   Separation took from 1 - 2h. following an initial concentration
period of 10 min. at 1.5 to 2mA/gel.   The gels were removed from the
glass tubes by rimming, that is, by squirting water between the gel and
tube with a syringe and needle.   The proteins were fixed and stained as
described below.

2.21.e. Slab gel electrophoretic procedure

Vertical slab PAGE (Akroyd, 1967) employed the Raven apparatus (Raven Scientific Company, Haverhill, Suffolk).

The two sheets of glass were rinsed in a 1/200 Photoflo solution and were air dried. Plastic spacer strips 2mm thick were sealed at the edges with vaseline and the sheets were clamped together with stationary clips. The lower edge of the trough thus formed was sealed by pushing the plates into a strip of plasticine on the bench. The plasticine also held the trough vertically while gelling took place.

The lower acrylamide solution with catalyst was then pipetted into the trough to within 2cm of the top. No water overlay was necessary as a meniscus formed only at the extreme edges against the spacer strips. After 45min the stacking gel solution with catalyst was pipetted in and while still liquid a well-forming template was forced into the solution. The wells formed were either 3mm or 12.5mm wide and 16mm deep. After a further gelling period of 45 min the template was removed and the wells rinsed several times with running buffer. The plates, complete with gel, were removed from the plasticine and sealed into the Raven apparatus with vaseline and stationary clips. The upper and lower reservoirs were filled with buffer, the lower electrode being made the anode. Samples were applied with a 1ml syringe together with 80% sucrose and bromophenol blue after a prerunning period of 21 m A for 1h. The samples were concentrated at the interface between the stacking and separation gels by $\frac{1}{2}$h at $1\frac{1}{2}$ m A/ sample followed by separation for 3h. at $3\frac{1}{2}$ m A/sample. The gel was removed by rimming and was fixed and stained as described below.

2.22 Radioactivity in gels

Unfixed disc polyacrylamide gels containing tritiated leucine were placed in aluminium troughs 9cm long and 0.4cm radius and surrounded by crushed dry ice for 15min. The ice did not touch the gel and the latter was kept straight while freezing.

Gel slicing employed the Mickle gel slicer (Mickle Laboratory Eng., Co., Guildford, Surrey). A piece of wet filter paper was placed on the carrier to which the frozen gel adhered. Two drops of water were added to the gel/filter paper interface to facilitate freezing.

Starting a known distance from the origin serial 0.8mm slices were taken through the region containing red haemoglobin bands. Each slice was separated with a scapel and placed in a numbered vial containing 0.1 ml of a 1 : 7 mixture of N.C.S. (Amersham-Searle, Illinois, U.S.A.). Tissue solubiliser : toluene. The slice was shaken and the vial stored overnight at $4^{o}C$ prior to liquid scintillation counting.

2.23. Protein separation - qualitative immuno-electrophoresis (I.E.P)
2.23a. Materials

In this method a combination of the usual agar electrophoretic technique and the immunodiffusion technique of Ouchterlony (1958) is employed.

For lens crystallins two parts of Aronsson and Gronwall buffer were mixed with one part of water. This diluted buffer was used to prepare a 1% W/V solution of agarose. Xenopus coelomic fluids and sera were similarly separated except that the 1% agarose solution was prepared with a barbitol buffer, pH 8.6. The original work was done with Ionagar (Oxoid Ltd , London) but as this product has been discontinued later work used Litex HSA (International Enzymes, Windsor, Berks). The appropriate weight of solid agarose was added to the buffer and boiled with continuous mixing over a bunsen flame until the solid had dissolved. The solution was then kept at $55^{o}C$ in a water bath until use.

Clean glass microscope slides, 24 x 76mm were labelled with a diamond marker and placed on a levelling table. Each slide received 2ml of agarose solution. When gelled the slides were stored at $4^{o}C$ in a moist atmosphere for up to 3 days before use.

2.23b. Buffers

Tris - EDTA - Boric Acid Buffer , pH 9.0 (Aronsson and Gronwall, 1957).

Tris	60.5g
EDTA, disodium salt	6.0g
Boric acid	4.6g
Water	to 1 litre

pH adjustment was unnecessary but sodium azide to a final concentration of 0.01% was added to inhibit bacterial growth. The buffer was stored in the dark at 4°C.

Barbitol buffer, pH 8.6

Barbitone sodium	10.3g
Barbitone	1.84g
Sodium azide	0.1g
Water	to 1 litre

2.23c. Electrophoretic procedure

Just before use the slits for the antiserum troughs and wells for the antigen samples were punched in the agarose using the Shandon Template Cutter. The plugs of agar in the wells were removed by suction and 1 µl of antigen solution was applied with a Hamilton micro-syringe. The slides were placed in a Shandon Universal Electrophoresis Tank the wells of which were filled with undiluted TEB buffer (lens crystallins) or barbital buffer (coelomic fluids). A single thickness of filter paper was attached to each end of a slide for a wick. The cathode end of the slide was that farthest from the antigen well. A constant current was supplied by a Shandon power pack for 1h. sufficient to provide an initial potential difference of 180 volts. Up to 8 slides could be run in the electrophoresis tank at the same time.

The strip of agarose in the central trough was then removed and the trough filled with the appropriate antiserum. The slides were left in a moist atmosphere for at least 24 h. before washing and staining.

2.23d. Washing slides

The slides were washed for 2 days to remove unprecipitated protein with several changes of 0.1 M saline containing sodium azide to a final concentration of 0.01% W/V. Then salt crystals were removed by washing with several changes of distilled water for 12h. The precipitin lines were stained using the general protein stains described below.

2.24. Protein separation ——— quantitative two-dimensional immunoelectrophoresis (IEP)

The method employed was basically that described by Axelsen, Krøll and Weeke (1973) as 'crossed immunoelectrophoresis'. It was used with both lens crystallin and coelomic fluid antigens, the principal difference being in the buffers chosen.

2.24a. Materials - 1st dimension

As in qualitative IEP for lens crystallins a 1% Litex solution was prepared using 2 : 1 diluted, water: TEB buffer. With coelomic fluid and Xenopus serum the 1% Litex solution was made with a barbital buffer, pH 8.6. 15ml. of agarose solution at 55°C was pipetted onto a level glass plate, 100mm X 100mm. After gelling, up to 4 antigen wells were punched with a cork borer, the plugs of agarose being removed by suction. Either 10 μl or 20 μl antigen samples were used in either 4mm. or 6mm. diameter wells respectively. Electrophoresis was performed in a water-cooled electrophoresis tank (Shandon Southern Ltd.).

2.24.b. Materials-2nd dimension

This separation was performed on either 25mm X 100mm glass plates (lens crystallins) or 100mm. x 100mm. plates (coelomic fluids). The plates were levelled before pouring agar. Shandon Universal troughs were used with the appropriate buffer and placed in a 4°C refrigerator before and during use.

2.24.c. Buffers

For lens crystallins the Aronsson and Gronwell, pH 9.0 TEB buffer was used again.

Coelomic fluids and Xenopus sera were electrophoresed with a barbitol buffer, pH 8.6, the composition of which is given above.

2.24d. Electrophoretic procedure - 1st dimension

Antigen samples were placed in the wells using a 'Finnpipette' precision sampler (Buckley Membranes Ltd., Great Missenden, Bucks). adjusted to 10 μl or 20 μl capacity, with disposable plastic tips. With lens crystallins the separation was run at a constant voltage of 100 volts for $2\frac{1}{4}$h. whereas with coelomic fluids the same voltage was

applied for $2\frac{3}{4}$h. After this the plate was removed and laid over a template card. The latter enabled one to cut longitudinal slices of agarose gel with a microtome knife. Each strip was 10mm x 100mm and contained a single antigen well and its separated sample. Each strip was slid gently onto the 2nd. dimension glass plate and arranged to lie approximately one-third of the width from one side.

2.24e. Electrophoretic procedure - 2nd dimension

For lens crystallins a tube containing 6 ml. of 1% Litex in diluted TEB buffer at 55°C received 200 μl of anti-Xenopus adult crystallin serum. The agarose solution was mixed thoroughly and allowed to return to 55°C in a water bath. In the case of coelomic fluid electrophoresis 13ml. of 1% Litex in barbitol buffer received 23.3 μl of anti-Xenopus adult serum proteins serum and after mixing was returned to 55°C. Approximately two-thirds of the agarose solution was poured onto the anodal side and the remaining one-third onto the cathodal side of the plates. The original agar strip was abutted by the antiserum - containing agar but not covered.

Two such plates could be run together in each Shandon Universal electrophoresis trough containing the appropriate buffer and double thickness filter paper wicks. Constant voltages of 50 volts for 20h. (lens crystallins) or 21h. (coelomic fluids) at 4°C were used.

2.24f. Drying and washing plates

Each plate was covered with a single filter paper excluding air bubbles. A 1cm thickness of filter paper and a 100g. weight were then placed above for 3h. The plate was then air dried at room temperature overnight before being washed in saline for 2 days and in distilled water for 12h. The proteins precipitated were stained with Coomasie Brilliant Blue (lens crystallins) or with nigrisin (coelomic fluids) as described below.

2.24g. Quantitation of precipitin peaks

The coelomic fluid stained plates were placed in the carrier of a photographic enlarger and projected at an enlargement of X4 onto white paper. The albumin peaks were traced, cut out and weighed on an Oertling precision balance. This method is superior to taking peak dimensions if the peaks are sometimes skewed.

2.25 Comparison of the results of polyacrylamide gel electrophoretic and immunoelectrophoretic separations of lens crystallins

To correlate lens crystallin bands appearing on polyacrylamide gels with the precipitin peaks obtained by I.E.P., proteins were eluted from sliced gels and re-electrophoresed by I.E.P.

Whole adult lens crystallin solutions were separated as described on polyacrylamide disc gels using a Tris- HCl/Tris-glycine discontinuous buffer system. A single gel was then rapidly stained to identify the positions of the protein bands. The remaining gels were then sliced with a razor blade so that the required protein bands were contained in 3mm thick slices. Slices from different gels containing similar bands were pooled in tapered 10ml tubes, and the protein eluted by three changes of Rugh's ringer at 4°C over an 18hour period. The eluates were dialysed at 4°C, with magnetic stirring for 36h. against 1/60 aqueous Rugh's ringer. The dialysis tubing was Visking Tubing 8/32 (The Scientific Instrument Centre Ltd., London). The dialysed eluates were then freeze-dried after determining their protein concentrations by the Lowry method and their volumes gravimetrically. The lyophilised solids were reconstituted with distilled water to give final concentrations of 6mg protein/ml.

The purity of the eluates was checked by electrophoresing a sample by P.A.G.E. and then further samples were subjected to qualitative I.E.P. using the appropriate anti-serum. Thus protein from a single PAGE band could be correlated with the precipitin arcs obtained by I.E.P.

2.26 Protein staining

2.26a. General protein stains

In the earlier work with polyacrylamide gels and immunoelectrophoresis, proteins were stained with a 1% W/V solution of Napthalene Black 12B in 7% V/V glacial acetic acid. Staining was usually complete after $\frac{1}{2}$h. Destaining was acheived by repeated changes of 7% acetic acid or by electrophoresis in a glass trough containing platinum electrodes. Although the latter method destained gels in hours rather than days the protein bands were sometimes blurred.

Latterly, especially for quantitative immunoelectrophoresis a 0.5% W/V solution of Coomasie Brilliant Blue R-250 was prepared as follows:

Coomasie Brilliant Blue R-250	5g
Ethanol, 100%	450ml
Glacial acetic acid	100ml
Water	450ml

The mixture was stood overnight and then filtered. Staining was normally complete in one hour. Destaining was acheived by repeated washes with the ethanol: acetic acid : water solvent mixture. The process was critical as the stain was easily removed from the faintest precipitin peaks. Johansson and Malmquist (1971) claimed that Coomasie Brilliant Blue is some three times more sensitive thanNaphthalene Black 12B.

The precipitin peaks obtained with two-dimensional I.E.P. of coelomic fluids were so faint as to require an even more sensitive protein stain. Acetic nigrisin was employed (Feinstein, 1976).

Nigrisin (G.T. Gurr, Ltd., London, N.W.9.)	1g
12% V/V Acetic acid	500 ml
1.6% W/V Sodium acetate	500 ml

Destaining was by repeated changes of 3% acetic acid.

With cellulose acetate membranes, Ponceau S (Sargent,1969) was used as a general protein stain. The fixed membrane was floated on to a solution of 0.20% W/V Ponceau S in 3% W/V aqueous TCA. Penetration of the dye into protein was apparent within seconds. The strip was then immersed in the dye solution for a further 10 mins. After staining the strips were washed with 5% V/V acetic acid until the background was white.

2.26b. Rapid protein stain

In experiments where it was desired to elute proteins from gels a marker gel was stained rapidly to identify the bands before slicing. The method used was that of Allen, Spicer and Zehr (1976).

The gels were incubated for 12 minutes at 65°C in a Coomasie Brilliant Blue solution of the following composition.

0.2% W/V Coomasie brilliant blue	45ml
100% Ethanol	45ml
Glacial acetic acid	10ml

Destaining was performed by two washes, each lasting 6 minutes at 65°C with the following solution.

100% Ethanol	25ml
Glacial acetic acid	10ml
Water	65ml

2.26c. Specific protein staining - haemoglobin

The ortho-dianisidine technique of O'Brien (1961) was used. Stock solutions were prepared as follows:

100mg Ortho-dianisidine (Eastman Organic Chemicals, New York). in 70ml. ethanol.

Acetate buffer, pH 4.6, 0.1M prepared as follows:
102ml 0.1N Acetic acid
98ml 0.1N Sodium acetate

30% Hydrogen peroxide aqueous.

Immediately before use the staining solution was made up as follows:

0 - dianisidine solution	4.0ml
Acetate buffer	1.0ml
Hydrogen peroxide	0.2ml
Water	1.5ml

Gels were immersed in the solution for up to 2 h. when haemoglobin bands were stained a brown colour. All staining was carried out in a fume cupboard as 0-dianisidine is highly carcinogenic.

2.26d. Specific protein staining - lactate dehydrogenase

The method of Dietz and Lubrano (1967) for polyacrylamide gels was followed. The stock solutions were as follows:

Stock solution (stored at 4°C)	Amount used
Sodium lactate (Sigma) 1M	1.0ml
Nicotinamide adenine dinucleotide, 10mg/ml	1.0ml
NaCl, 0.1M	1.0ml
MgCl$_2$, 5mM	1.0ml
Phosphate buffer, 0.5M, pH 7.4	2.5ml
Nitroblue tetrazolium, 1mg/ml	2.5ml
Phenazine methosulfate, 1mg/ml	0.25ml

The NAD solution was not kept for more than 1 week.

The 1 M sodium lactate solution was prepared from a lactic acid (Na salt) solution containing 60% sodium lactate.

The polyacrylamide gels were incubated in the staining solution for from 1 to 2 h. at 37°C by which time the LDH bands appeared a violet colour. The gels were washed three times in water and fixed in 7% V/V acetic acid.

2.27. Concentration of samples by freeze drying

It was frequently necessary to equilibrate the concentrations of proteins contained in solutions from different larval stages. The method employed was to measure the protein content of the solution by the Lowry method and to determine its volume gravimetrically. Thus the total protein contents of the sample could be calculated.

The dilute samples were then freeze dried in an Edwards Model EF03 Refrigerated freeze dryer (Edwards Vacuum Components, Crawley, Sussex). The samples were placed in glass tubes and centrifuged while freezing. They were then dried completely over a 3h period. Problems arose when the dried solids were attracted out of the tubes by static electricity. It was found desirable to cover the drying tubes with Parafilm (American Can Co., Wisconsin, U.S.A.), perforated by two small pinpricks, to prevent

loss of material.

2.28. Densitometric scanning of polyacrylamide gels

Both disc and slab gels were scanned using the Chromoscan Mk II (Joyce, Loeble & Co. Ltd., Gateshead-on-Tyne). For slab gels the thin-layer attachment was used. In both cases the transmission mode was employed with the appropriate filters to enable the scanner to be balanced. The results were recorded as a pen trace.

2.29. Photography

Gels etc. were photographed on Kodak Panatomic -X film using a Practika single-lens-reflex camera fitted with 55mm standard lens and a 3 dioptre accessory lens. Illumination was transmitted from a Kodak Coldlight Series 2 Illuminator (Kodak Ltd., London).

2.30. Thyroid hormone assays

2.30.a. Automatic pipetting

The sensitive hormone assays used here depend upon repeatably precise pipetting of small volumes. Oxford Precision Samplers (Oxford Instrument Co., Oxford) of 5, 10, 20, 25, 50 & 100μl volumes and an Eppendorf sampler (W. Sarsteat, U.K., Ltd.) of 1000 μl volume were used with disposable plastic tips. Preliminary trials showed that unless special precautions were taken the accuracy of these samplers was poor. This was checked gravimetrically by dispensing aliquots of water into preweighed vials. The following points are of importance:

a) the plastic tips must be wetted by pipetting several aliquots of the substance with a given tip before pipetting an aliquot in an experiment.

b) the pipette plunger must be released slowly and smoothly when filling.

c) the outside of the tip must be blotted dry after filling.

The preliminary trials showed that, with care, the samplers have a precision of about 1% standard deviation.

2.30b. Radiochemical Centre R.I.A. for T3

The lyophilised reagents were reconstituted according to the manufacturer's instructions. They were as follows:

a) Anti-T3 serum containing 9.5 mg/ml. sodium barbitone.

b) $T_3 - ^{125}I$, approx. 5 µCi (at date of manufacture) and also containing 9.5mg/ml. sodium barbitone.

c) T3 standards in serum containing 0.10, 0.69, 2.01 and 5.11 ng T3/ml.

d) Adsorbent powder suspension also containing 3.8 mg/ml. sodium barbitone.

The assays were performed in special polystyrene test tubes supplied by the kit manufacturer. Although the manufacturer recommended the use of 50 µl aliquots of standards and samples, 25 µl aliquots were found to give excellent repeatability with the standards.

25 µl aliquots of the standards and 25 µl aliquots of samples were pipetted in duplicate into the test tubes. Then 200 µl aliquots of $^{125}I - T3$ solution were pipetted and mixed by 1 second on a vortex mixer. Finally 200 µl aliquots of the antiserum solution were pipetted into all tubes and mixed by vortexing for 1 second. The tubes were capped and incubated in a water bath at $37^{o}C$. for 1h.

1000 µl of the adsorbent suspension was pipetted into all tubes, stirring the suspension continuously. The tubes were capped and all placed on a single rotator (Blood Cell Suspension Mixer, Matburn Surgical Equipt. Ltd., Lancing, Sussex). for 1h. at room temperature (less than $25^{o}C$). The tubes were allowed to stand for at least 10 mins before removing 1000 µl of the supernatant from each tube and placing it into a counting tube of known background. Counting was performed on the Thyrimeter gamma counter.

The count rates for each standard were plotted on linear graph paper against the concentration of T3. Using the mean of duplicate count rates for the unknowns, their T3 concentrations were read off from the standard curve.

2.30c. Radiochemical centre R.I.A. for T4.

The lyophilised reagents were reconstituted according to the
manufacturer's instructions. They were as follows:

a) Anti-T4 serum,
b) T4 - ^{125}I, approx. 6 µCi (at date of manufacture).
c) T4 standards in human serum containing 0.15, 4.10, 10.8
   and 19.2 µg T4/100ml
d) Adsorbent powder suspension

The remainder of the procedure was as described for the Radio-
chemical Centre T3-R.I.A.

2.30.d. Competitive protein - binding assay for T4 on miniature
anion-exchange columns (Bauer et al, 1970).

This method was supplied in Kit Form as the 'Tetralute' method for
T4 (Ames Company, Slough, Bucks).

The lyophilised reagents were reconstituted according to the
manufacturer's instructions. The reagents were as follows:

a) T4 - ^{125}I, approx. 2.5 µCi (at date of manufacture) in 0.1N NaOH
b) T4 standard in human serum - 17.6 µg T4/100ml
c) eluting reagent containing human thyroxine-binding globulin
d) buffer pH8.6 containing 15.4 g/l of sodium barbital and
   2.7 g/l of barbital.

The manufacturers' stress the need to use copper-free water as a
solvent. This was confirmed by Higgs and Eales (1973) who found that
double glass distilled water was inadequate whereas triple - distilled
water was satisfactory. In the present work the water was once glass
distilled and then twice deionised before being stored in glass.

The miniature Sephadex columns were drained free of 0.1 N Na OH
(pH 13) by decanting. Seven drops of the T4-^{125}I reagent were added
directly onto the column. The manufacturers recommended the use of
100 µl aliquots of sample serum. Preliminary trials indicated that
the method was satisfactory with as little as 20 µl. This volume was
added directly to the column and mixed with the ^{125}I-T4 by swirling gently.

The column was drained and eluted with 4ml of barbital buffer pH 8.6.
After draining, the radioactivity of the column was counted in the
'Thyrimeter' gamma counter. The above steps were repeated with each
column containing a sample. A standard curve was prepared using 5, 10
20 and 50 μl aliquots of the reference T4 standard in serum. All
columns were swirled gently and 1.0ml of the TBG - containing eluting
reagent added. At least 5 min were allowed to elapse before the column
was eluted with a further 4ml of barbital buffer, pH8.6. When the
column had drained it was counted again in the 'Thyrimeter'. The percen-
tage retention was calculated as follows:

$$\% \text{ retention} = \frac{\text{Final activity of column}}{\text{Initial activity of column}} \times 100$$

Calibration curves were prepared with the percentage retention as
the ordinate and the known concentrations of T4 as the abscissa. The
values of T4 in the unknowns could then be read off.

Higgs and Eales (1973), who were also working with T4 titres lower
than those normally found in humans were able to steepen the lower part
of the % retention against T4 concentration curve by using a more
dilute eluting reagent. Accordingly in the present work the 30mg of
lyophilised reagent supplied were diluted with 30ml pH8.6 barbital buffer
rather than 15ml as recommended. Higgs and Eales had found a 1:30
dilution preferable to the 1:16 used for human determinations.

2.31. Counting ^{125}I radioactivity

In all thyroid hormone assays activity was counted with the Ames
Thyrimeter. This contains a 2" sodium iodide (thallium - activated)
detector with a 'through well' crystal. The detector is followed by a
pulse height discriminator and a digital computation system. The
instrument was designed especially for use with miniature Sephadex
columns and includes a memory device which enables the initial activity
count to be memorised until the final activity count is made. The
percentage retention is computed automatically. The Thyrimeter can
also be used as a counter for ^{125}I activity contained in any tube
similar in size to the miniature columns. Counts are expressed auto-
matically as thousands of counts per minute (KCPM). Three counting

times are available.   These are 6 sec., 1 min., and 2 min. with maximum counts of 999, 99.9 and 9.99 KCPM respectively.

The control experiment for non-specific binding of T4 in the R.I.A., which involved higher count rates, was counted with a Beckman 'Biogamma' counter.

CHAPTER 3

Multiple haemoglobin transition in developmentally
- retarded Xenopus laevis larvae

3.1 INTRODUCTION

Multiple haemoglobins are of widespread occurrence in animals (Gratzer and Allison, 1960) and a transition of haemoglobin types has been shown to occur in a number of amphibians during development. The functional significance of the shift is not entirely clear. In those anurans which have an aquatic tadpole and a terrestrial adult it might be expected that the physiological properties of the tadpole and adult haemoglobins would differ and that each would be adaptive. McCutcheon (1936) and Riggs (1951) found the haemoglobins of Rana catesbeiana tadpoles to exhibit no Bohr shift and to have a higher oxygen affinity than that of the adult haemoglobin. Tadpole blood has a large "loading capacity" whereas the blood of the adult has a lesser "loading capacity" but a greater "unloading capacity". Bennett and Frieden (1962) have argued that these properties may be considered adaptive from the tadpole's need for a superior oxygen-capturing mechanism as it relies upon dissolved oxygen in its aquatic environment. The molecular transition was thought to accompany the loss of gills and development of the lungs at metamorphosis. However amphibians with aquatic adults such as X. laevis and neotenous adults such as axolotls Ambystoma mexicanum also have marked haemoglobin shifts (Maclean and Jurd, 1971 a and b). In the case of Xenopus both tadpoles and adults possess functional lungs (review by Deucher, 1975) while Fletcher and Myant (1960) showed that oxygen consumption does not increase during the metamorphosis of captive animals of this species. In the axolotl though oxygen consumption did increase during triiodothyronine-induced metamorphosis (Gahlenbeck and Bartels, 1970). Another objection to the argument that the haemoglobin transition is necessitated by a change from an aquatic to terrestrial existence comes from the work of Brown and Dewitt (1970) on the newt Notophthalmus (Triturus) viridescens. This species metamorphoses from an aquatic gill-bearing larva to a terrestrial subadult eft which breathes with lungs. A "second metamorphosis" precedes its final return to the water as a breeding adult which still uses the lungs. The first metamorphosis only is accompanied by a haemoglobin transition. This case would appear to correlate the haemoglobin shift with the transition from gill - to lung - respiration rather than to life on land. However the

argument does not apply to Xenopus laevis. It is conceivable that cutaneous respiration is of greater importance in Xenopus larvae than in Notophlalmus although the second metamorphosis of the latter is accompanied by a reversion to the larval type of skin. It seems possible that a haemoglobin transition is a common feature of amphibian development which has been exploited as a preadaptation by those species which have greatly different respiratory needs in the post-metamorphic form.

The temporal correlation between the haemoglobin transition and morphological metamorphosis has naturally led researchers to seek evidence for common control mechanisms. The term 'metamorphosis' is used in this thesis in the sense of Etkin (1964) and Dent (1968). Metamorphosis is any post - embryonic change in non-reproductive structures by which a larva adapted to one mode of life is readapted for a different mode of life. The change takes place in a time period which is short by comparison to the whole developmental period and is essentially a preparation for, not a response to, the new environment. The haemoglobin change in amphibia appears to fit this definition although the previous paragraph has detailed some reservations as to its adaptiveness.

Maclean and Jurd (1971 a) studied the haemoglobins of developing X.laevis by carboxymethyl - cellulose column chromatography and polyacrylamide gel electrophoresis (PAGE). In tadpoles, two haemoglobins (HbF_1 and HbF_2) were found. HbF_2 was present in smaller amounts. In addition a third fraction, HbA_1 was present in tadpoles in small quantities but became the dominant fraction of adults. In adults HbF_1 and HbF_2 were absent while a new fraction HbA_2 appeared. The latter was not a polymer of HbA_1 and comprised only 5 to 10% of the total adult haemoglobin. More detail of the transition was given by Jurd (1972).

Several similar basic studies have been carried out on the bullfrog, Rana catesbeiana together with more detailed experimental analyses. Baglioni and Sparks (1963) using starch gel electrophoresis showed 3 Hbs in tadpoles and 4 different ones in adults. Hamada et al (1964) showed three components in each of tadpole and adult using DEAE - and CM - cellulose column chromatography. Moss and Ingram (1965) resolved five

haemoglobin components in tadpoles by PAGE and five components in the adult frog. Wise (1970) obtained similar results and confirmed immunologically that there was no polypeptide chain of common antigen-icity between tadpole and frog haemoglobins. Manwell and Baker (1971) have cited the results of Elzinga (1961) which showed that considerable individual variation occurs in the number of haemoglobin bands observed in R. catesbeiana tadpoles. This was not so in Xenopus laevis (Jurd, 1972 and this thesis).

There is some disagreement about the timing of the bullfrog haemoglobin transition. Theil (1967) and Moss and Ingram (1968a) found it to occur during tail regression whereas Trader et al (1963) and Benbassat (1970) observed its completion up to 10 weeks after foreleg emergence. In X. laevis, Jurd (1972) had shown the transition to be a gradual one beginning at stage 43 (the earliest studied) and being completed in small toadlets.

Haemoglobin transitions have been described in several other anurans including Rana pipiens (Benbassat, 1970), Rana grylio and R. heckscheri (Herner and Frieden, 1961), R. clamitans (Dessauer et al, 1957), and R. esculenta (Chieffi et al, 1960). Only R. catesbeiana and R. pipiens have been analysed in any depth.

The cellular mechanisms involved in the transition are discussed in detail in Chapter 8 of this thesis. These mechanisms include changes in erythropoietic site (Broyles and Frieden, 1973), changes in the popula-tions of circulating red blood cells (Dewitt, 1968) and changes in the nature of globins and haemoglobins synthesised (Theil, 1967, Maclean and Jurd , 1971 a, Just and Atkinson, 1972).

The effect of treatment with exogenous thyroid hormones on red cell populations (Hollyfield, 1967; Dewitt, 1968; Ducibella, 1974b) and upon globin and haemoglobin synthesis (Moss and Ingram, 1968b; Hirayama, 1967; McMahon and Dewitt, 1968; Just and Atkinson, 1972; Maniatis and Ingram, 1972) has been studied and this work will also be discussed in Chapter 8. The general conclusion has been that most changes observed during the spontaneous haemoglobin transition can be partially mimicked by TH treat-ment. However, Frieden and Just (1970) and Just and Atkinson (1972)

have pointed out that the resemblance is not complete. When premeta-
morphic bullfrog tadpoles were injected with T3 adult haemoglobin
appeared precociously in haemolysates 12 days later. However larval
haemoglobins were also present. Both larval and adult haemoglobins
continue to be synthesised _in vivo_ as indicated by the incorporation of
labeled amino acids. Hormone administration caused an increase in
amino acid incorporation, perhaps by altering pool sizes, but most of
the increased incorporation was into larval Hb. Just and Atkinson
(1972) speculated that in normal metamorphosis cells containing pre-
dominantly larval haemoglobin may be selectively removed from circula-
tion. There has been no evidence that this process can be produced by
T H   treatment.

The work of Maclean and Turner (1976) on propylthiouracil -
retarded giant _Xenopus_ larvae showed three animals to contain predom-
inantly adult haemoglobin. As it is assumed that the effect of the
goitrogen is solely to prevent thyroid hormone synthesis, with no direct
effect on haemoglobin synthesis, this was further evidence that TH does
not act as a primary signal in the transition. Maclean and Turner (1976)
speculated that the haemoglobin transition might be determined by
chronological age, or size rather than by the thyroid gland.

In the present work propylthiouracil was also used to prevent TH
synthesis and provided an alternative approach to hormone treatment in
investigating the control mechanisms of the haemoglobin transition.
Larval and adult haemoglobins were separated by polyacrylamide gel
electrophoresis because it was felt that this method would provide the
best resolution of the small haemolysates collected from individual
retarded tadpoles.

Three main problems attended this work. They were as follows:

    a) identification of protein bands as haemoglobins,
    b) identification of a given haemoglobin band as, for
       example, Hb A_1.
    c) assessing the relative concentrations of the bands.

These problems will be discussed in the Experimental section.

FIGURE 3/1

The haemoglobin transition during the normal metamorphosis of Xenopus laevis.

A) Diagrams showing the nomenclature used.

B) Polyacrylamide disc gels which had been stained with o-dianisidine to identify haemoglobin and then restained with Naphthalene Black 12B.

    i) Normal st. 53 tadpoles (pool of 3 animals)

   ii) Normal st. 57-58 tadpoles (pool of 3 animals)

  iii) Toadlet (2 cm snout to vent length)

   iv) Adult female toad

These gels have been aligned with reference to the Hb Al and Hb Fl bands.

NORMAL
St.53
HAEMOLYSATE

ADULT
HAEMOLYSATE

Hb F2
Hb A1
Hb F1
Hb F3

Hb A2
Hb A1

A

+

i   ii   iii   iv

F2
A1
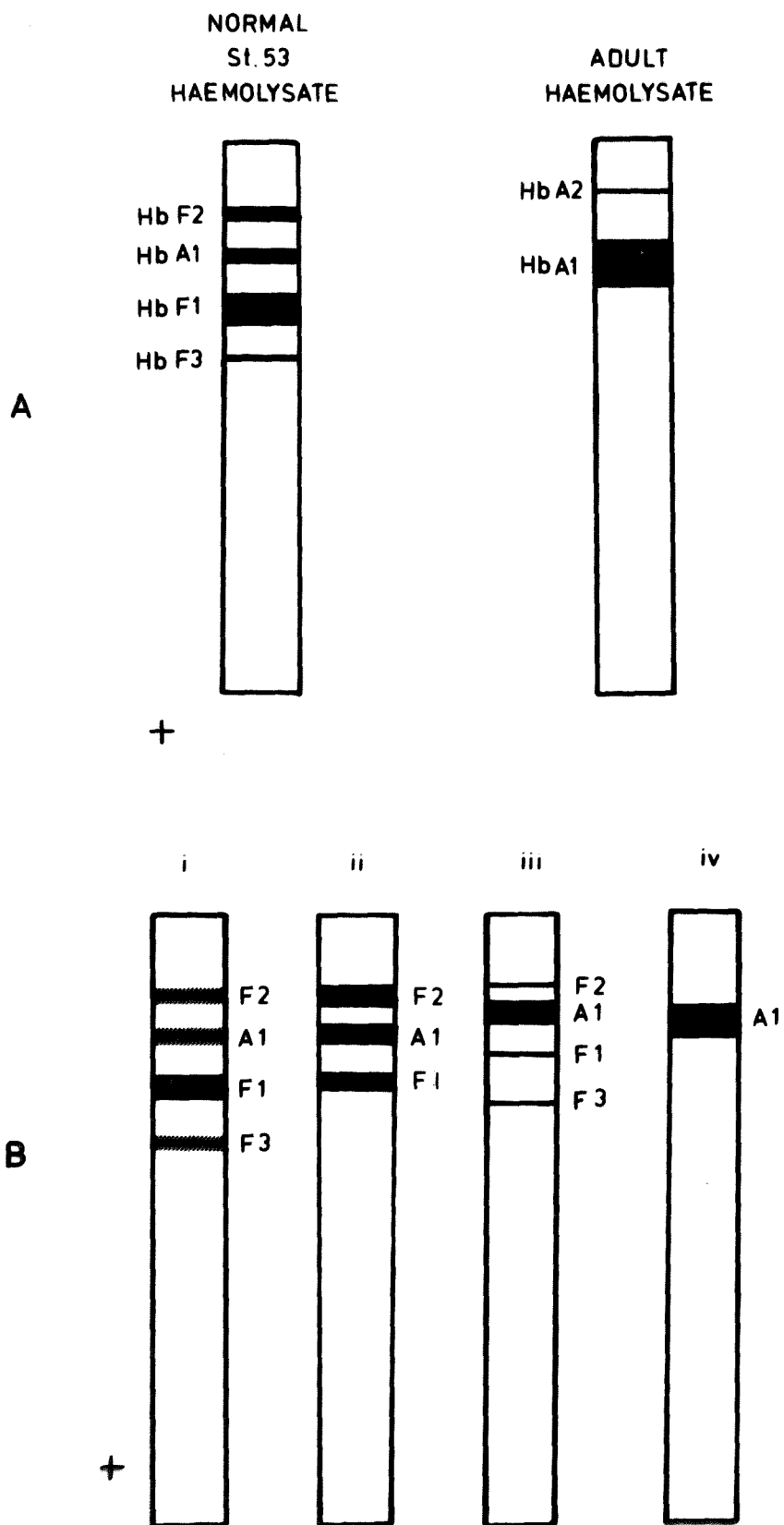F1
F3

F2
A1
F1

F2
A1
F1
F3

A1

B

+

FIGURE 3/2

Haemoglobin patterns of <u>Xenopus</u> larvae which had been reared in
propylthiouracil.

A) Haemoglobin patterns of larvae which had been goitrogen
treated for either $8\frac{1}{2}$ or 15 weeks.

a), b) & c). Replicate separations of the haemolysate
from a st. 54 tadpole which had been immersed in
propylthiouracil for 15 weeks (wet weight 1.37 g).
"ADULT Hb"

d), e) & f). Replicate separations of the haemolysate
from 2 pooled st. 55 tadpoles immersed in
propylthiouracil for $8\frac{1}{2}$ weeks (0.56 g and 0.39 g
wet weight). "TADPOLE Hb".

These samples were separated on a polyacrylamide slab gel
and stained with o-dianisidine and Naphthalene Black 12 B.

B & C). Polyacrylamide gels showing that minor haemoglobin
fractions, visible on disc gels, may not be visible on a slab.
Both methods were used with haemolysates from the same animals.

a) Normal st. 57/58 tadpoles (pool of 4 animals)
b) & c) Replicate separations of the haemolysate from
a st. 56 tadpole immersed in propylthiouracil for
41 weeks (1.85 g wet weight) "ADULT Hb"
d) Toadlet (1.9 cm snout to vent length)
e) Toadlet (1.8 cm snout to vent length)
f) Adult female toad.

The disc gels were aligned with reference to the Hb A1 and
Hb F1 bands.
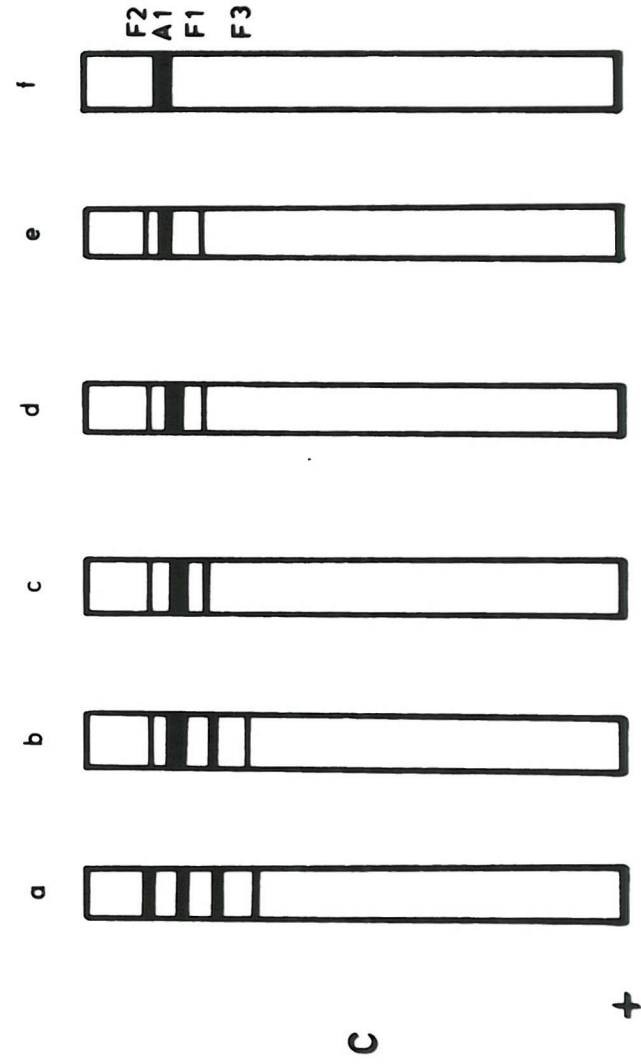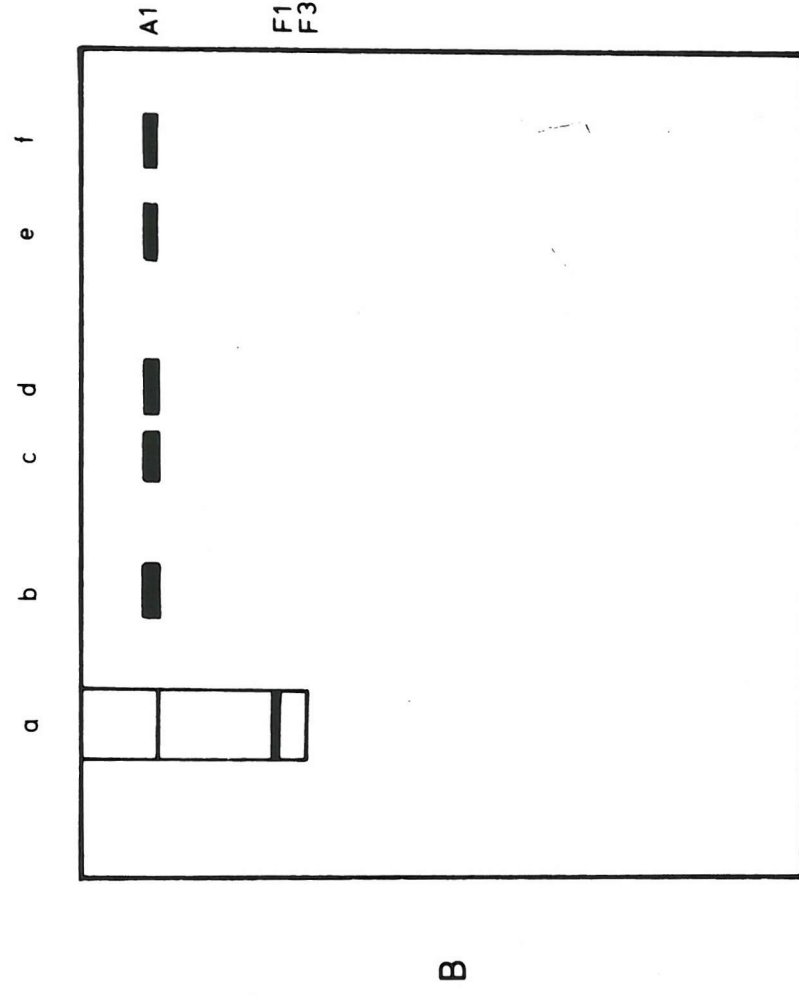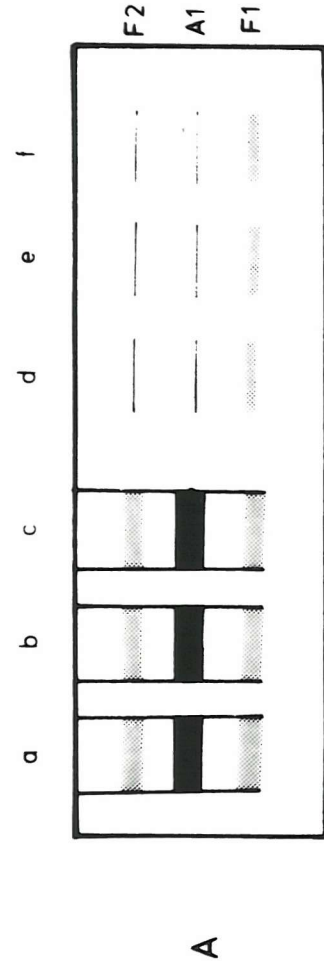
A variety of goitrogen - retarded tadpoles were used individually so that the effect of stage arrest, chronological age and size upon the haemoglobin transition could be assessed.

3.2 EXPERIMENTAL

A similar experimental procedure was adhered to in all experiments. The haemolysates were reduced by the addition of dithiothreitol (Cleland, 1964) to prevent polymerisation by S - S bonding. Adult anuran haemolysates are particularly likely to form polymers (Riggs, Sullivan and Agee, 1964: Maclean and Jurd, 1971a). Dithiothreitol was also added to the gel buffer and running buffer. Following Maclean and Jurd (1971a) the haemoglobin solutions were converted to the stable cyanmet form by the addition of Drabkin's solution. The additional precaution of pre-running the electrophoresis apparatus to remove persulphate ions, used as a gel catalyst, was taken. Mitchell (1967) has pointed out a possible source of artifacts if such oxidising ions are present during electrophoresis.

In all cases the haemolysates were of too small a volume for their haemoglobin concentrations to be determined spectrophotometrically against a cyanomet - haemoglobin standard. During the earlier part of the thesis work a scanning densitometer was not available so that comparisons were only semi-quantitative. The nature of the haemoglobin transition in <u>X. laevis</u> made this satisfactory as normal premetamorphic larval haemolysates are predominated by HbF_1 while HbA_1 is only a minor component. In adults HbA_1 is the major component while HbF_1 is no longer visible. Thus it was only necessary that samples under comparison should not be so different in concentration that a minor fraction, just visible on the electropherogram of a concentrated sample, would be invisible with a relatively dilute sample. In earlier experiments the samples in a given experiment were diluted to approximately the same degree of redness. In later work a haemocytometer red cell count was made after the final cell wash and then the volumes adjusted to equilibrate cell concentrations. Assuming the haemoglobin content of the cells to be approximately equal this method ought to ensure haemolysates of similar concentration (problem 'c' above).

All electrophoresis was made on polyacrylamide gels using a continuous buffer system with a TEB (tris - EDTA - boric acid) buffer of

pH8.6. Disc gels were used for the greatest resolution but even when apparently identical gels are run in the same apparatus at the same time the mobility of a given band on different gels varies. This made positive identification of the Hb bands difficult (problem 'b' above). Thus the vertical slab apparatus was also used in which all samples in a given experiment were separated on the same gel. The mobilities of a band, common to several samples, were found to be the same. Identification was therefore simple as control haemolysates of premetamorphic blood or adult blood were always run. The resolution of the slab method was poorer than with discs.

The method of bleeding the tadpoles and of preparing the haemolysates clearly makes contamination with non-haemoglobin proteins likely. In the more concentrated solutions red bands could be seen on the gels but in most cases the o - dianisidine staining method of O'Brien (1961) was used. This method is not specific for haemoglobin as it detects pseudoperoxidase activity which can be shown by some enzymes such as catalases. In practice, o - dianisidine is widely used as a haemoglobin stain for the following reasons:

i)   major Hb bands, such as HbF_1 and HbA_1 are often visible unstained and show the location of bands with the mobilities of haemoglobins before faint bands are detected by staining.

ii)   it is unlikely that any other protein in haemolysates would have a similar mobility to known haemoglobins and be present in similar concentrations,

iii)   any contaminants from the body fluids would be expected to be variable whereas multiple haemoglobin patterns are fairly consistent at a given stage of development.

Thus problem 'a' above was resolved. Unfortunately the pale yellow colour produced by o-dianisidine soon fades and does not give good photographic records. After identifying the bands the gels were usually restained with the general protein stain Naphthalene Black 12B for photography and storage. In practice extraneous protein bands never appeared in the region of the haemoglobins.

FIGURE 3/3

Haemoglobin patterns of propylthiouracil treated <u>Xenopus</u> larvae

A) Polyacrylamide slab gel separation of haemolysates from:

    a) & b) Replicate samples of st. 54 treated tadpole
          haemoglobin (treated for $15\frac{1}{2}$ weeks; wet weight
          1.11 g). "ADULT Hb"

    c) Normal st. 54/55 tadpoles (pool of 5 animals)

B) Polyacrylamide disc and slab gel separations of
   haemolysates from:

    a) St. 55 treated for $14\frac{1}{2}$ weeks."INTERMEDIATE Hb"

    b) St. 54 treated for 15 weeks."ADULT Hb"

    c) St. 54 treated for $15\frac{1}{2}$ weeks. "ADULT Hb"

    d) Adult female toad

    e) St. 54 treated for $16\frac{1}{2}$ weeks. "TADPOLE Hb"

    f) St. 54 treated for 17 weeks. "TADPOLE Hb"
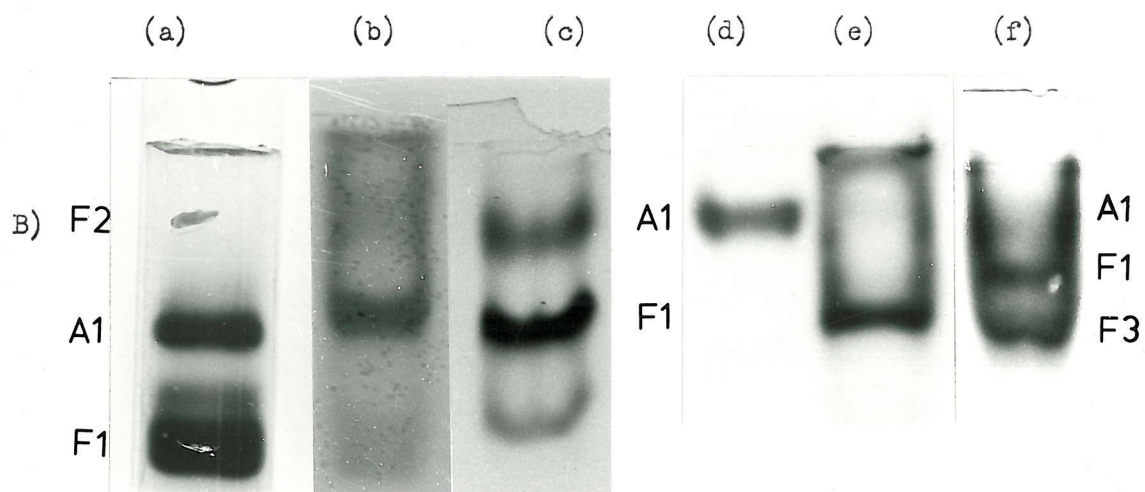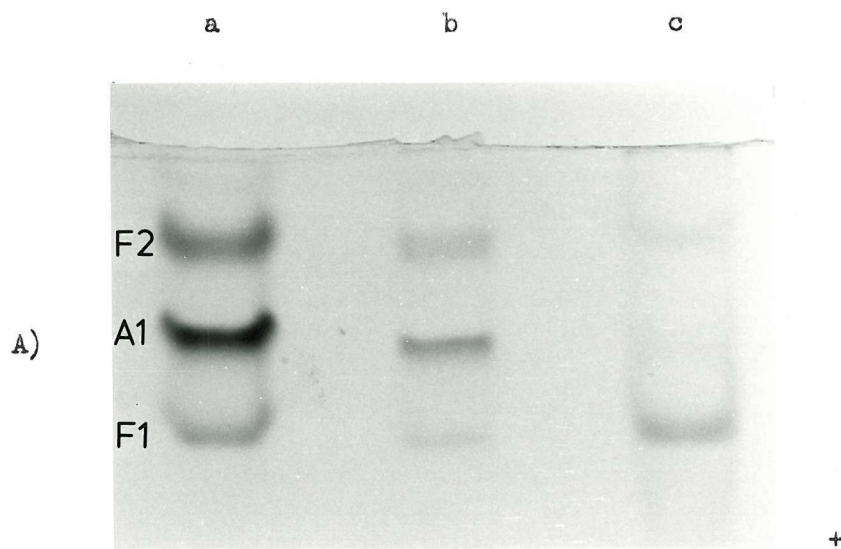
To measure the relative rates of de novo protein synthesis in circulating red cells in vitro experiments were carried out. The incorporation of ^{3}H - leucine into the haemoglobins was used as a parameter of protein synthesis during a 3h. incubation period. The cells were then lysed, electrophoresed, the gels sliced and the slices counted for radioactivity. Replicate gels were stained for haemoglobin to correlate the radioactivity counts with the haemo-globin fractions.

Table 3/1 lists the goitrogen - retarded larvae which were used to provide haemolysates. Details of stage of arrest, weight and duration of goitrogen immersion appear,

3.3 RESULTS

The normal haemoglobin transition is shown in Fig. 3/1 together with the nomenclature used to identify the individual haemoglobin bands. These results were obtained with untreated X. laevis tadpoles, toadlets and adult toads. These gels differ slightly from those of Jurd (1972). He studied stages 43, 45, 48, 61 and toadlets of 1.4, 1.8, 2.9 and 7.5 cm mouth to anus lengths. The main difference is that band HbA_1 is more marked in Fig. 1 in stage 57/58 gel than at stage 61 in Jurd's work. Jurd (1972) noted the presence of a fourth Hb band in tadpoles which was more variable than the others and which was more anodal. He called this "t_4" but in the present thesis to avoid confusion with the abbreviation for thyroxine it will be called HbF_3. Jurd found this to be absent from toadlets but was able to detect the HbA_2 fraction in very small quantities in toadlets.

In the present work stage 53 and stages 57/58 were studied because the former represented the earliest stage at which propylthiouracil - treated tadpoles were arrested while the latter filled in the post - arrest period. With Jurd's work data on most of the post - arrest haemoglobin shift was available.

Fig. 3/3 shows gels obtained with two treated tadpoles in which the HbA_1 band was predominant. These haemoglobin patterns were classed as 'adult' although more haemoglobin in the tadpole fractions was present than in the gels illustrated by Maclean and Turner (1976).

FIGURE 3/4

The pattern of tritiated leucine incorporation into the haemoglobins of propylthiouracil – treated <u>Xenopus laevis</u> larvae. Two stage 55 giant tadpoles were used to provide erythrocytes. The tadpoles had been immersed in 0.01% propylthiouracil solutions for $13\frac{1}{2}$ weeks.

a) ^{3}H – leucine counts against gel slice number

b) A replicate gel stained with o – dianisidine to show the positions of the haemoglobin bands.
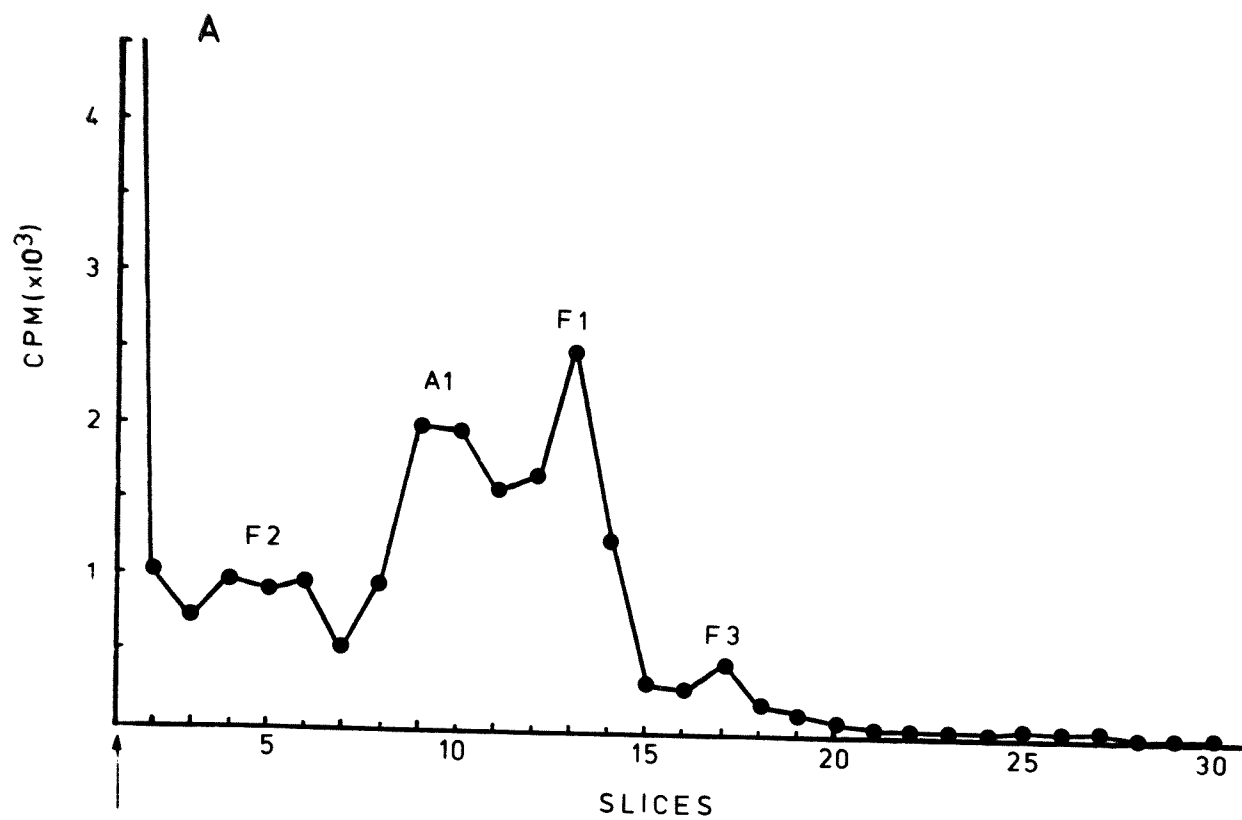
Experiments with other giant tadpole haemolysates made it clear that
the degree of transition was very variable.   To facilitate comparison
the electropherograms were categorised as 'adult', 'intermediate' and
'tadpole'.

"Tadpole" - when HbF_1 was much darker (i.e., more concentrated)
than HbA_1.

"Intermediate" -   when bands HbA_1 and HbF_1 were more or less
equal in intensity.

"Adult" - when band HbA_1 was the major band with the other bands,
especially HbF_1, very faint.

Examples of gels within each category are as follows: "tadpole" -
(Fig 3/1, normal stage 53 haemolysate; Fig. 3/6, gel iv, giant
tadpole haemolysate).

"Intermediate" - (Fig 3/1, normal stage 57/58 haemolysate; Fig 3/6,
gels ii and iii, giant tadpole haemolysates).

"Adult" - (Fig 3/1, normal adult toad and toadlet haemolysates,
Fig 3/6, gel vii, giant tadpole haemolysate).

The results obtained when the haemolysates of 24 propylthiouracil
treated tadpoles were electrophoresed and categorised are given in Table
3/1.

Table 3/1.   Summary of haemoglobin patterns obtained with propyl-
thiouracil - treated X. laevis tadpoles.   Stage of arrest, weight,
length and duration of treatment are also shown.   The electropherograms
are categorised as "tadpole", "intermediate" and "adult" as defined in the
text.

Haemoglobin Pattern	Stage	Wet Weight (g)	Length (cm)	Duration of immersion in propylthiouracil (weeks)
Tadpole	55	0.48	3.4	8
Tadpole	55	0.63	4.1	8
Intermediate	55	1.17	5.8	14.5
Tadpole	55	0.56	4.3	8.5

(cont'd)

Table 3/1 (cont'd)

Haemoglobin Pattern	Stage	Wet Weight (g)	Length (cm)	Duration of immersion in propylthiouracil (weeks)
Tadpole	55	0.39	3.6	8.5
Adult	54	1.37	6.7	15
Adult	54	1.11	-	15.5
Tadpole	54	1.17	5.9	16.5
Tadpole	54	1.25	-	17
Tadpole	55	1.62	-	13.5
Tadpole	55	1.95	-	13.5
Intermediate	54	1.27	-	29
Intermediate	55	0.61	-	55
Intermediate	56	1.16	-	55
Intermediate	56	1.30	-	55
Intermediate	58	1.38	-	40
Intermediate	58	1.39	-	40
Tadpole	58	1.80	-	40
Adult	56	1.85	-	41
Tadpole	53	0.90	-	60
Tadpole	56	1.59	-	66
Tadpole	56	0.95	-	42
Tadpole	54	0.59	-	18
Tadpole	53	0.62	-	60

Total number of animals examined: 24 (100%)

Total number of 'tadpole' patterns: 14 (58.4%)

Total number of 'intermediate' patterns: 7 (29.2%)

Total number of 'adult' patterns: 3 (12.4%)

That the visibility of the tadpole Hb bands in biochemically metamorphosing animals can be affected by the polyacrylamide method used, discs or slab, is shown in Fig 3/2, b and c. Haemolysates from a propylthiouracil-treated tadpole and from two toadlets were separated by both methods. On the slab only the HbA_1 fraction is visible but on the disc gels the tadpole fractions HbF_1 and HbF_2 can also be seen. However the HbF_1 band is faint in each case so that the classification of these electropherograms as "adult" would not be affected.

FIGURE 3/5

The pattern of tritiated leucine incorporation into the haemoglobins of propylthiouracil – treated <u>Xenopus</u> <u>laevis</u> larvae.   One stage 54 giant tadpole was used to provide erythrocytes.   It had been immersed in 0.01% propylthiouracil solutions for 29 weeks.

a) ^{3}H – leucine counts against gel slice number.

b)   A replicate gel stained with Naphthalene Black 12B to show the positions of the haemoglobin bands.   These bands had been identified as haemoglobins by their red colour before staining.
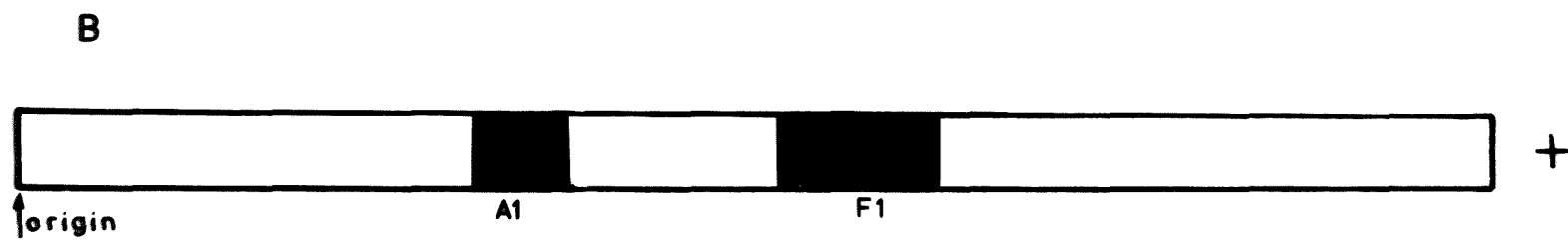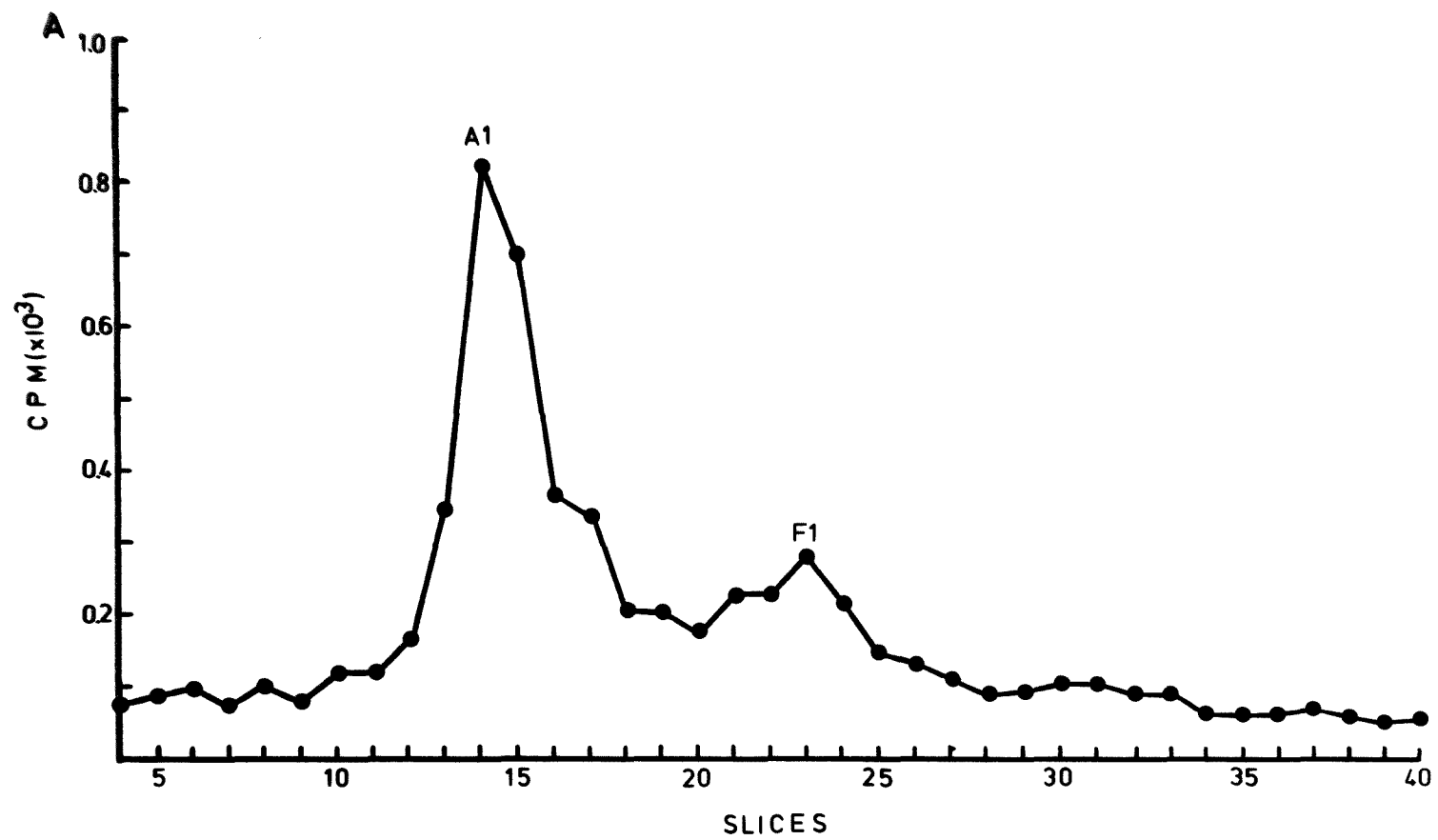
FIGURE 3/6

Variation in the haemoglobin patterns of propylthiouracil treated
tadpoles and normal <u>Xenopus</u> toadlets

    A) Slab gel electrophoretic separation of haemolysates from
        treated tadpoles and a normal adult <u>Xenopus</u>. All tadpoles
        had been treated for 40 weeks.

        i) Adult female toad
       ii) Treated tadpole (st. 58; 1.38 g wet weight).
            "INTERMEDIATE Hb"
      iii) Treated tadpole (st. 58; 1.39 g wet weight).
            "INTERMEDIATE Hb"
       iv) Treated tadpole (st. 58, 1.80 g wet weight).
            "TADPOLE Hb"
        v) Adult female toad
       vi) Normal stage 57/58 tadpoles (pool of 4 animals)
      vii) Treated tadpole (st. 56; 1.85 g wet weight). "ADULT Hb"

    B) Disc gel separations of haemolysates from normal <u>Xenopus</u>
        toadlets. The gels are aligned with reference to the
        Hb A1 and Hb F1 bands

     viii) 2.0 cm Toadlet (1.15 g wet weight)
       ix) 1.9 cm Toadlet (0.87 g wet weight)
        x) 1.8 cm Toadlet (0.69 g wet weight)
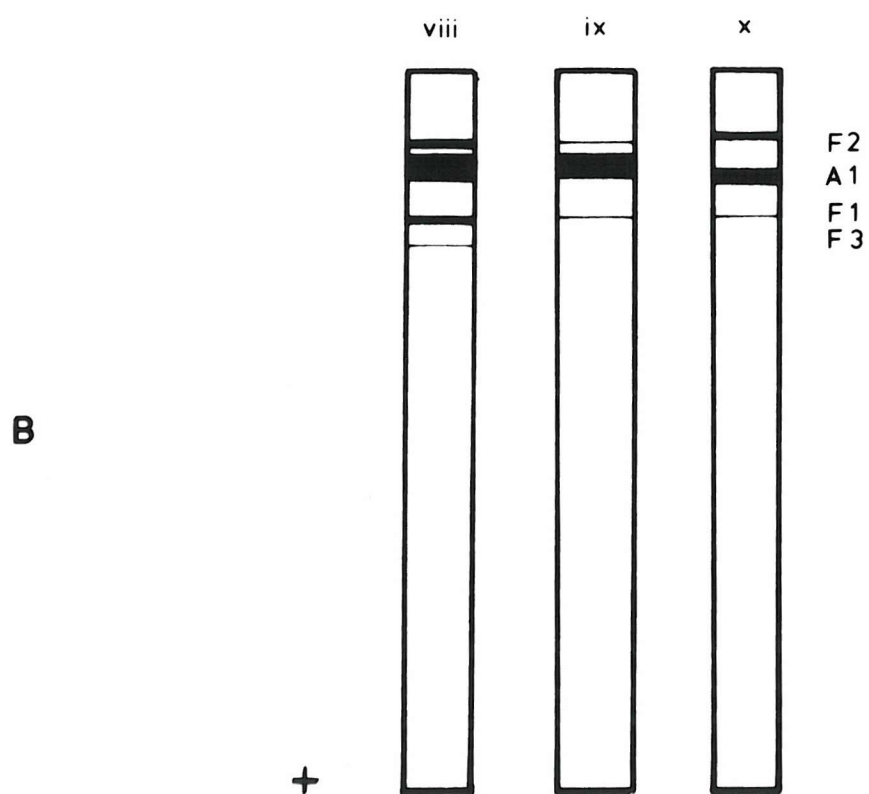
Originally two groups of goitrogen - treated tadpoles were
generously supplied by Dr. S. Turner. At the time of the experiment
illustrated in Fig. 3/2a they had been treated for $8\frac{1}{2}$ or 15 weeks
respectively. Both haemolysates were separated on the same slab with
normal stage 54/55 haemoglobin as a control. The latter enabled the
single band visible with the $8\frac{1}{2}$ week material to be identified as HbF_1.
The lower resolution of the slab was insufficient to show the slower
tadpole fractions with the low total haemoglobin concentration necessary
in this experiment. The 15 week haemolysate gave an 'adult' pattern
suggesting the transition might occur between $8\frac{1}{2}$ and 15 weeks of
goitrogen treatment.

It would clearly be possible for an arrested tadpole to have begun
synthesising mainly adult haemoglobin but for the HbA_1 fraction to be
in low concentration in haemolysates by comparison with a persisting
high level of HbF_1. To discover whether larvae arrested for less than
15 weeks were already synthesising mainly adult haemoglobin amino acid
incorporation experiments were performed. The experiments illustrated
by Figs 3/4 and 3/5 compared the incorporation of ^3H - leucine into the
haemoglobins of a stage 54 giant (treated for 29 weeks) with that into
the haemoglobins of two pooled stage 55 giants (treated for $13\frac{1}{2}$ weeks).
The radioactivity associated with slices of gels prepared by separately
electrophoresing the haemolysates of in vitro red cell cultures is graphed
in the figures. Replicate gels were run in each case to enable the
radioactivity peaks to be correlated with haemoglobin bands.

Fig 3/4 shows that in the $13\frac{1}{2}$ week haemolysate some leucine had
been incorporated into all four haemoglobin fractions during the 3h.
incubation period. Approximately 28% of the labeled leucine (estimated
from the area under the curve) had been incorporated into HbF_1 while 31%
was in HbA_1. The o-dianisidine stained gel showed bands in the HbA_1,
HbF_1 and HbF_3 positions with a very faint HbF_2. This gel was classi-
fied as "tadpole pattern" in Table 3/1 and that conclusion was confirmed
by the incorporation of leucine predominantly into HbF_1.

The other set of gels obtained with the 29 week haemolysate was
quite different with regard to leucine incorporation (Fig 3/5). In
this case 39% of the leucine had been incorporated into HbA_1 while the
proportion in HbF_1 had declined to 19% . The stained gel showed only

FIGURE 3/7

The haemoglobin pattern categories of propylthiouracil treated
larvae grouped as follows:

a) stage of arrest classes

b) wet weight classes

c) treatment duration classes.

The haemoglobin categories are defined on page 54.

'Tadpole' haemoglobin

'Intermediate' haemoglobin

'Adult' haemoglobin
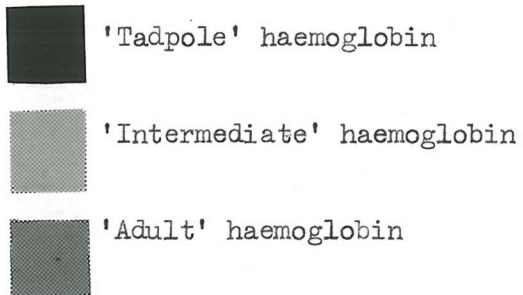
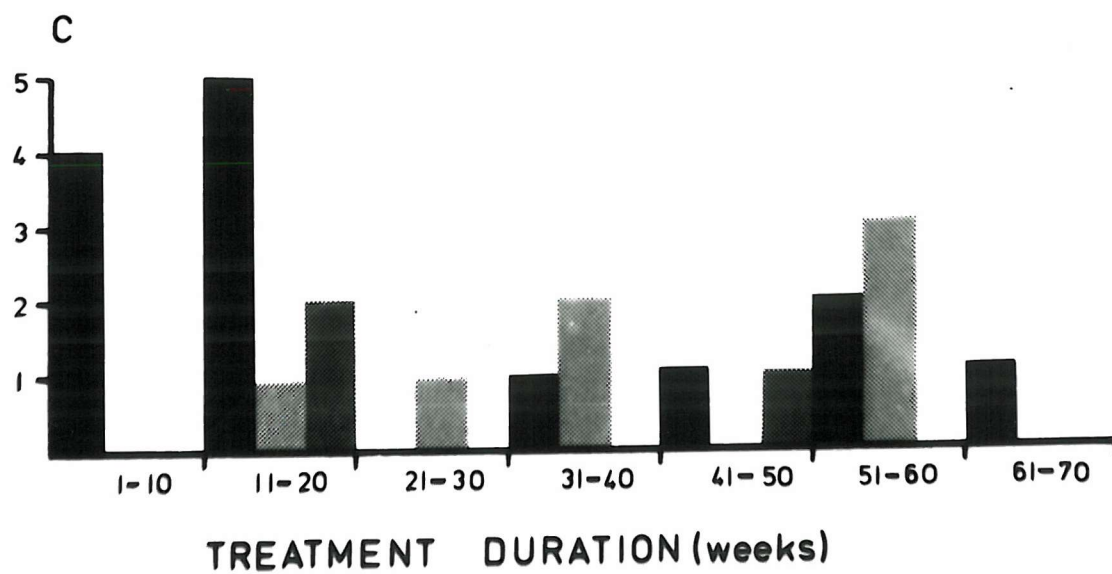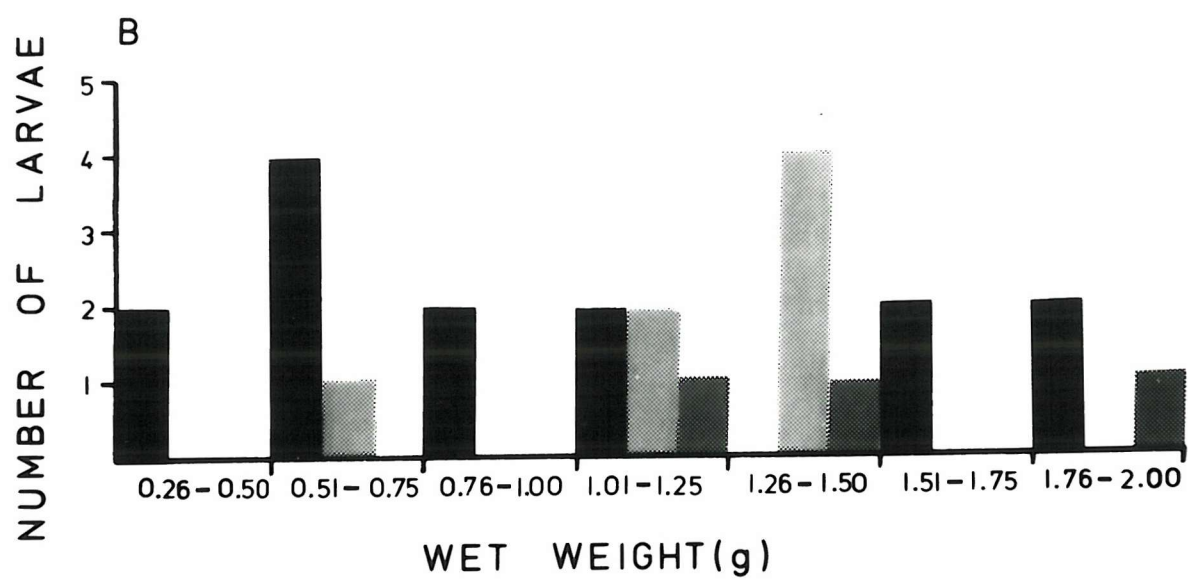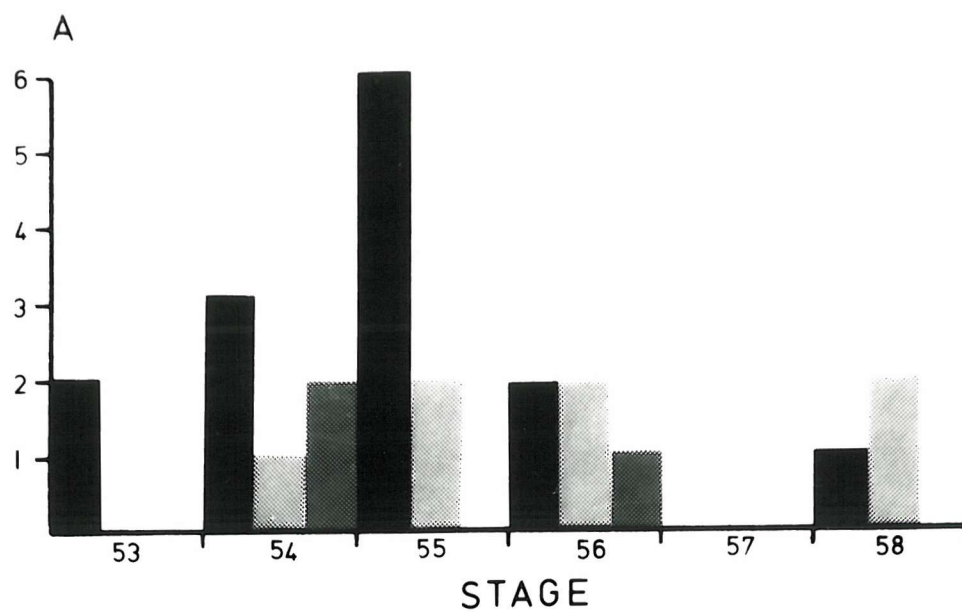the HbA$_1$ and HbF$_1$ bands but was classified as "intermediate" because the HbA$_1$ band was very dark although narrower than the HbF$_1$. Again this was supported by the amino acid incorporation results.

On the basis of these experiments the switch to predominantly adult haemoglobin seemed to occur between 13.5 and 29 weeks of goitrogen treatment. However a further experiment, illustrated in Fig 3/6a, indicated that variation could occur even after 40 weeks of treatment with propylthiouracil. In this case, 4 larvae from the same batch of eggs and reared in the same aquarium were bled separately and the haemolysates electrophoresed on slab gels with normal adult toad and normal stage 57/58 haemoglobin as controls. Three of the larvae were clearly tadpole - like in Hb pattern whereas the fourth was adult-like as it showed only the HbA$_1$ fraction. These larvae had been retarded less effectively by 0.01% propylthiouracil and 3 had developed to stage 58. However these 3 were all tadpole-like in Hb pattern whereas the fourth, arrested at stage 56, was adult-like in this respect.

The variation noted amongst tadpoles arrested for 40 weeks by propylthiouracil treatment was also observed in the haemoglobin patterns of three newly metamorphosed toadlets (Fig 3/6b). All three possessed principally HbA$_1$ but there was considerable variation in the relative concentrations of the tadpole fractions remaining.

The results given in Table 3/1 were arranged as histograms in which the number of larvae with each haemoglobin pattern was plotted against stage of arrest, weight or duration of immersion (Fig 3/7). There was no convincing evidence for a correlation between any of these factors and haemoglobin pattern. Only in the case of body weight was there a trend suggesting that heavier larvae were more likely to possess "adult" haemoglobins than light animals. Thus when all the data collected were considered, chronological age or duration of goitrogen-treatment as the larvae were all of similar age when first treated, appeared to have no consistent effect.

3.4 CONCLUSIONS

The most interesting observation was that 12% of goitrogen-arrested X. laevis larvae had continued biochemical metamorphosis and showed

adult haemoglobin patterns. This supported the data of Maclean and Turner (1976). The three animals with adult haemoglobins were developmentally arrested at stage 54 to 56 and had been treated for from 15 to 41 weeks. Had the latter animal been allowed to develop normally it would have been a large juvenile toad and so the goitrogen was clearly preventing TH synthesis most effectively. Three other tadpoles had been arrested less effectively and had developed slowly to stage 58 when killed after 40 weeks of treatment. However one of these still possessed the "tadpole" haemoglobin pattern whereas the other 2 were classed as "intermediate". A fourth animal from the same batch had been more effectively retarded at stage 56 and yet had an "adult" haemoglobin electropherogram. It is therefore unlikely that the great variation in degree of haemoglobin transition observed can be attributed to differences in the effectiveness of the goitrogen in depressing circulating TH levels in different animals.

The amino acid incorporation experiments showed that a stage 54 tadpole arrested for 29 weeks was actively synthesising HbA_1 haemoglobin. The level of incorporation into HbF_1 had dropped severely. This larva was classed as "intermediate" on the basis of its electropherogram but it was clearly synthesising adult-type protein. Thus if all larvae with either "adult" or "intermediate" haemoglobin patterns are grouped together 41% of arrested animals had entered the haemoglobin transition phase.

Nevertheless thyroid gland blockage clearly retarded the haemoglobin transition severely. Two larvae had been arrested in stage 53 for 60 weeks, by which time they might have been expected to be breeding adults if untreated, and yet they still exhibited typical "tadpole" haemoglobin patterns.

It therefore seems that the thyroid hormones are involved in the haemoglobin transition but that the involvement may be indirect. Maclean and Jurd (1972) have discussed the role of erythropoietin in mammals and have noted that many of its effects are similar to those produced by treatment with exogenous thyroid hormone inamphibia. Mammalian erythropoietin is active in amphibians so that the process may be more complicated than sometimes assumed. The involvement of prolactin in amphibian development is discussed in Chapter 8.

If the thyroid hormones are involved it must be that they have a long-term "triggering" effect on erythropoietic differentiation or that the minute levels of hormone which may still be in circulation exert a progressive or "stoichiometric" effect (Etkin, 1964a) over prolonged periods of time. It may also be that the erythropoietic cells have either a lower TH "threshold level" (Kollros, 1961) or a lower total thyroxine requirement as suggested by Ducibella (1974a) for the erythropoietic tissues of the axolotl. These possibilities and the validity of the concepts involved will be discussed in Chapter 8. It is interesting to note that the three arrested larvae examined by Maclean and Turner (1976) had been goitrogen-treated for over 18 months and were heavier at 2.06 ± 0.17g wet weight than any studied in this thesis. Thus the possibility remains that if arrested for a sufficiently long period all Xenopus larvae might complete the haemoglobin transition.

The considerable variation in haemoglobin pattern observed in treated larvae made it difficult to assess the effects of chronological age, size and developmental stage. There was certainly no clear cut evidence that any of these factors had an important effect. The only trend noted was that animals with large body sizes were more likely to have begun the transition but there were exceptions. The results are not simply explicable by assuming that as arrested tadpoles grow larger their oxygen requirements necessitate a haemoglobin transition which is induced by physiological stress as has been suggested for the control of mammalian liver and kidney regeneration (Goss, 1964).

That Xenopus larvae are able to survive with exclusively or predominantly the adult haemoglobin adds further doubt, to that previously expressed, that the transition is necessitated by a change of respiratory requirement after metamorphosis.

CHAPTER 4

The coelomic fluid albumin transition of developmentally -
retarded Xenopus laevis larvae

4.1 INTRODUCTION

Changes in coelomic fluid total protein concentrations and in the
relative concentration of albumin was the second system chosen, as a
biochemical marker of development.   As will be shown below serum and
plasma protein transitions, during normal metamorphoses, have been
studied in a number of amphibians including X. laevis.   Preliminary
attempts to work with serum showed that collection of adequate volumes
from single propylthiouracil-treated X. laevis larvae was extremely
difficult.   The variation in the haemoglobin transitions of different
treated tadpoles, described in Chapter 3, indicated that the pooling of
sera would be likely to obscure the nature of the serum transition under
goitrogen blockage.   Thornburg et al (1975) had studied protein transi-
tion at metamorphosis in both the sera and coelomic fluids of Rana
catesbeiana.   Both fluids were separated electrophoretically on
cellulose acetate membranes at various stages of metamorphosis.   Bands
of identical mobility in both the albumin and globulin regions were
observed leading those authors to conclude that the serum was the source
of coelomic fluid proteins and that quantitative changes in the hepatic
synthesis of proteins, such as albumin, were reflected in both fluids.
A similar conclusion was drawn with the plasma proteins of Rana esculenta
by Reichel (1970) who showed them to be immunologically identical to
the serum proteins.

The coelomic derivatives of amphibians are the peritoneal and
pericardeal cavities (Hyman, 1942).   Thornburg et al (1975) compared
fluids from both sources and found them to be similar.   In the present
work fluids of peritoneal origin only were compared.   Further comparison
was made with the serum proteins of post-metamorphic toadlets and adults.

That quantitative metamorphic changes in proteins of seral origin
in X. laevis were likely to be useful as a marker was suggested by the
work of Herner and Frieden (1960).   Those authors found total serum
protein concentrations to increase during larval development and meta-
morphosis in Rana grylio, R. catesbeiana, R. heckscheri and X. laevis.

In addition, paper electrophoresis showed the distribution of proteins to change, most of the increased total protein being accounted for by albumin with concommitant rises in the albumin/globulin ratios. In the Ranids a redistribution of globulins also occurred such that those of intermediate mobility declined in favour of faster and slower - migrating globulins. In Xenopus slow - moving globulins predominated throughout.

Most detailed work has been carried out on the North American bullfrog R. catesbeiana. Bueker (1961) compared the mobilities of serum proteins from mouse, bullfrog and R. pipiens sera. In the bullfrog, proteins migrating on paper electropherograms with mobilities similar to mouse albumin, alpha-, beta and gamma- globulins were found, but albumin comprised 21% of the total compared to 53% in the mouse. In R. pipiens a fraction with a similar mobility to mouse albumin was lacking. Richmond (1968) separated bullfrog serum on polyacrylamide gels and found 20 to 40 components. The number of molecular species and the concentrations of alpha- and beta- globulins changed during development whereas albumin remained fairly constant until the appearance of the forelegs when concentration increased markedly. Feldhoff (1971) used polyacrylamide gels to quantify the albumin increase in bullfrog plasma and found it to increase from 0.0039 mg albumin /0.10 mg total plasma protein in early tadpole stages to 0.0179 mg/0.10 mg in climax larvae. During the entire metamorphic period there was a 15-fold increase in albumin. Nagano, Shimada and Shukuya (1972) characterized bullfrog serum albumin and used the purified protein to quantify the albumin concentration changes observed in bullfrog development (Nagano, Shimada and Shukuya, 1973). Tadpole serum contained 0.6 mg/ml albumin and this increased steadily during subsequent development to plateau at about 5.5 mg/ml. in the young adult.

In other anurans Dessauer and Fox (1956) compared paper electrophoretic mobilities of human and Rana clamitans serum proteins. Again, albumin - like and globulin - like mobilities were observed, the alpha- and gamma- globulins being predominant.

Chen (1967) compared the sera of eleven European amphibian species by PAGE and extended the work in a later paper (Chen, 1970). In R. temporaria, Bombina variegatus, and Bufo bufo, proteins with the specific staining reactions of albumin, transferrin, glycoprotein, lipo-

protein and possibly haptoglobins were identified.   In all species the albumin fraction increased during metamorphosis resulting in a two - to five - fold increase in the albumin/globulin ratio.   Other serum proteins shown to increase in concentration during anuran metamorphosis are ceruloplasmin (Inaba and Frieden, 1967) transferrin (Frieden and Just, 1970) and the immunoglobulins (Marchalonis, 1971).

The urodeles have been shown to possess more variable serum proteins in terms of electrophoretic mobilities than anurans.   For example, Ambystoma maculatum was shown by Gleason and Friedberg (1953) to lack a fastmoving albumin - like component.   The same was true of Necturus maculosus.   However Chen (1967) seperated sera from Triturus cristatus, T. alpestris, T. vulgaris and T. helveticus on polyacrylamide gels and found the fastest - fraction, which was slower than human albumin, to bind Evans Blue.   The latter dye is not completely specific for albumin but is regularly used to mark that protein in mammalian serum.   Galton and Ingbar (1962b) agreed that the serum of Necturus maculosus lacked an albumin - like component on the basis of mobility. Gasser and Durand (1965) in Proteus anguinus and Dessauer and Fox (1956) in Amphiuma means found the fastest component to migrate in the alpha 1- glycoprotein or alpha 2- globulin regions of human serum.

It seems possible that the fastest component of sera which lack albumin has a similar physiological function to albumin.   Ambystoma tigrinum mavortium lacks albumin but at metamorphosis the proportion of alpha - globulin (fast moving) to total globulins increases from 0.34 to 0.75 (Hahn, 1962).   Ambystoma mexicanum also lacks an albumin - like fraction (Gasser and Durand, 1965, Charlemagne, 1967).   The latter author found few qualitative differences between larvae and adults, the increase in protein concentration noted apparently being contributed to equally by all components.   However Ducibella (1974a) in the same species, detected the appearance of a new slow-moving fraction in both neotenous and transformed adults.   The premature appearance of the adult pattern was induced by immersion in thyroxine solutions.

Other species which possess albumins and which show an increase in the serum concentration of this protein are Salamandra salamandra and Pleurodeles waltii.   In Salamandra the increase at metamorphosis is very marked (Gasser, 1964) but in Pleurodeles the level in larvae was already as great in metamorphosed Salamandra and little further increase was found by

cellulose acetate electrophoresis. Chalumeau - le Foulgoc (1967) compared pre-metamorphic and adult serum of _Pleurodeles waltii_ by qualitative IEP . New precipitin arcs appeared but this could well be explained by concentration effects.

In addition to the evidence of Ducibella (1974b) mentioned above that thyroxine can initiate the serum protein shift in one urodeles, there is further evidence from one other source. Galton and Ingbar (1962b) administered a variety of thyroid hormone analogues to _Necturus maculosus_ larvae but no response was elicited.

In contrast, the increased albumin synthesis of the bullfrog tadpole under T_3 treatment was thoroughly investigated by Ledford and Frieden (1973). These workers concluded that the increased albumin synthesis of normal metamorphosis is caused by endogenous thyroid hormone levels. This confirmed the earlier work of Herner and Frieden (1960), who found increased albumin levels in T_3 - treated tadpoles of _Rana grylio_, _R. heckscheri_ and _R. catesbeiana_ comparable to those in spontaneously metamorphosing individuals. No publications on thyroid hormone stimulation of serum protein synthesis in _Xenopus_ tadpoles have appeared to date. The stimulation of hepatic synthesis of serum albumin by T_3 (Tata, 1967) is discussed in Chapter 8.

It has been assumed by several authors (e.g. Hahn, 1962) that the increase in serum protein concentration during anuran metamorphosis serves to increase water regulation, in the kidneys and tissues, by exerting an osmotic influence and by promoting blood pressure through plasma volume expansion and increased viscosity. Frieden _et al_ (1957) pointed out the similarity between the protein concentration of anuran tadpoles and fresh-water fishes. However this argument seems less convincing in the metamorphosis of the wholly - aquatic adult of _Xenopus_. Roughly similar protein concentration increases take place during the development of birds and mammals also.

4.2 EXPERIMENTAL

From the foregoing 'Introduction' it is apparent that a number of conditions must be satisfied for the coelomic fluid protein transition to be a suitable metamorphic marker.

Firstly there is a nomenclatural problem when dealing with non-mammalian serum proteins. For this reason it was decided initially to electrophorese Xenopus fluids on cellulose acetate membranes. These give a simple pattern showing the major components of mammalian serum whereas polyacrylamide gels give patterns of great complexity. 1 or 2 μl of whole mammalian serum are adequate and the separation is simple and fast.

Serum was collected by the orbital-bleeding of a laboratory brown mouse (Mus musculus) and by the ear vein-bleeding of a rabbit (Oryctolagus cuniculus). 1 μl aliquots of undiluted material were electrophoresed with a pH 8.6 barbital buffer. The zones observed were compared to those of adult Xenopus serum by separating 1 μl aliquots of whole serum on the same cellulose acetate membranes.

As previously mentioned collection of serum from Xenopus tadpoles proved very difficult and it was decided to work with coelomic fluid. Preliminary experiments showed that 1 or 2 μl aliquots were inadequate because of their low total protein concentrations. Even with 5 μl aliquots (near the maximum for cellulose acetate) coelomic fluid protein bands were not visible in premetamorphic samples. By freeze-drying and subsequent redilution the total protein concentration was raised to 6 mg/ml enabling the components to be visualised.

An unsatisfactory separation of Xenopus coelomic fluid globulins was obtained with the pH 8.6 barbital buffer used by Thornburg et al (1975) with Rana fluids. Two alternative buffers, regularly used to separate human globulins, were tried. They were a tris - EDTA - boric acid buffer, pH 9.0 and a barbitone - acetate buffer, pH 8.6. Because of the shortage of tadpole coelomic fluid these trials were made with adult Xenopus serum. The pH 9.0, TEB buffer proved the most satisfactory.

However when concentrated normal - and treated - tadpole coelomic fluids were electrophoresed with that buffer all the protein bands were repeatedly blurred so that only provisional conclusions on the protein transition were possible.

Cellulose acetate electrophoresis was useful only in comparing the

mobilities of <u>Xenopus</u> serum and coelomic fluid proteins with those of mammalian sera to justify the use of mammalian nomenclature.

Table 4/1. Total protein concentrations of <u>Xenopus laevis</u> body fluids during normal metamorphosis. Some larvae were grouped into the metamorphic stages of Gosner (1960).

Code	Animal	Fluid	Total protein concentration (mg/ml)
--	Stage 47-52 pool (Gosner St. I)	Coelomic	2.0
A	Stage 53-55 pool (Gosner St. II)	"	2.9
S	Stage 52-55 pool (2 X 52; 2 X 53; 4 x 54; 1 x 55).	"	2.0
P1	Stage 53-54 pool (2 x 53, 7 x 54)	"	2.2
P2	Stage 52-55 pool (1 x 52; 2 x 53; 4 x 54; 1 x 55)	"	2.2
--	Stage 56-58 pool (Gosner St. III)	"	3.5
--	Stage 59-66 pool (Gosner St. IV)	"	4.0
Tlt A	Toadlet	"	8.0
Tlt B	Toadlet	"	8.0
Tlt C	Toadlet (also used for serum)	"	8.6
Tlt E	Toadlet	"	9.4
AdS	Adult female (also used for serum)	"	7.0
AdT	Adult female	"	8.0
Tlt C	Toadlet (also used for coelomic fluid)	Serum	40.6
Tlt D	Toadlet	"	33.5
AdA	Adult female	"	32.6
AdB	Adult female	"	35.0
AdC	Adult female	"	33.0
AdS	Adult female (also used for coelomic fluid)	"	42.0

The second condition was that both the normal transition and that in treated tadpoles should be quantified. The haemoglobin transition described in Chapter 3 was conveniently qualitative in that spontaneous

metamorphosis was accompanied by a shift from HbF_1 to HbA_1 as the major component. Quantitation was therefore of less importance than in the present system in which the markers are total protein concentration and relative albumin concentration. Total protein was readily measured by the method of Lowry et al (1951). When cellulose acetate electrophoresis proved unsatisfactory it was decided to quantify the components of Xenopus coelomic fluid and serum by quantitative immuno-electrophoresis (IEP).

Table 4/2. Total coelomic fluid protein concentrations, albumin categories and specimen data of propylthiouracil - retarded X. laevis larvae

Code	Stage	Wet weight (g)	Duration of immersion (weeks)	Total protein concentration (mg/ml)	Albumin category (see text)
Gt1	54	0.43	41	0.95	Tadpole
Gt2	53	0.39	41	3.0	Tadpole
Gt3	56	0.93	41	2.0	Adult
Gt5	56	0.78	41	4.3	Tadpole
Gt6	56	1.33	41	12.5	Intermediate
Gt7	55	1.10	41	1.8	Intermediate
Gt9	53	0.62	61	3.0	Tadpole
Gt11	54	0.59	31	2.8	-
Gt12	53	0.90	61	≈ 0	-
Gt13	56	1.59	67	2.7	Adult

The principle of the method is as follows. A solution of soluble proteins (antigens) is electrophoresed in a 1% agarose layer on a glass plate. The strip containing the separated proteins is removed and placed on another plate. A 1% agarose solution containing antiserum raised against the particular antigens is poured so that it abuts the first-dimension strip. Electrophoretic migration of antigens into the antibody containing gel results in specific immunoprecipitation of the antigens by the corresponding antibodies. Each antibody/antigen system results in the formation of an individual precipitate, usually in the form of a peak. The area enclosed by the peak is proportional to the antigen/antibody ratio.

At the start of the electrophoretic run the antigens migrate into the gel in excess of the antibody molecules in the surrounding gel. Small soluble immune complexes are formed which migrate, binding increasing numbers of antibody molecules until sufficient antibody molecules have become bound for precipitation to occur. This point is known as the "equivalence point" (Versey, 1976). From this moment on the position of the precipitate will not alter even if electrophoresis is continued.

The area enclosed by the precipitate will depend upon both the concentrations of the antigen and the antibody in the system. In the present work the buffer system, current, times of separation in both dimensions, antiserum batch and concentration were kept constant in each run. The total protein concentration of each coelomic fluid sample applied has been adjusted to 0.4 mg/ml following a total protein measurement of the undiluted material. 10 µl aliquots of fluid were used in every case. Thus the only variable should be the concentrations of the constituent proteins which is measured by their respective peak areas. 0.4 mg/ml of total protein was chosen as a level less than any likely to be found in coelomic fluid so that concentration by freeze-drying was unnecessary.

The conditions for electrophoresis should be such that the antibodies should not migrate during the run, so that the distribution of antibodies remain constant around the origin. Such electrophoretic separations are usually carried out at pH8.6, at which point all proteins are negatively charged and will migrate towards the anode. Gamma-globulins will possess a weak negative charge but this will be counterbalanced by electroendosmosis if carried out in a 1% agarose gel (Versey, 1976). An antiserum against adult Xenopus serum was used here. The words "antiserum" and "antibodies" as used in this chapter refer only to specific proteins raised by immunisation of a rabbit with Xenopus antigens. They do not refer to any antibodies present in Xenopus body fluids.

The third condition was proof that the proteins of adult Xenopus serum, used both for immunisation and in electrophoretic comparisons, were identical to the coelomic fluid proteins employed as metamorphic

markers. Provisional proof had been obtained by comparing the mobilities of serum and coelomic fluid protein bands in cellulose acetate electrophoresis. Definitive proof was provided by qualitative IEP in which the cross - reactivity and mobilities of antigens in adult serum and coelomic fluid, toadlet serum and coelomic fluid, normal tadpole coelomic fluid and treated tadpole coelomic fluid were compared.

Coelomic fluid and serum donors used in IEP experiments are listed in Table 4/1.

To obtain albumin peaks, high enough for accurate peak area measurements, it was necessary to employ a very low antiserum concentration in the second dimension gel. By trial and error the necessary concentration was found to be 23 µl antiserum (1/640 titre) in 13 ml. of agarose (equivalent to 1.77 µl of antiserum/ml. agarose solution).

A suitable separation time for the equivalence point to be reached was found to be between 18 and 21h. (Fig 4/7). A time of 21h. was used throughout.

The great sensitivity of this method makes repeatable consistency in technique imperative. Each sample was run at least twice, on separate days. If the peak areas agreed closely the two values were averaged. If there was a discrepancy a third experiment was performed and the outlying value, assumed to be due to experimental error, was disregarded.
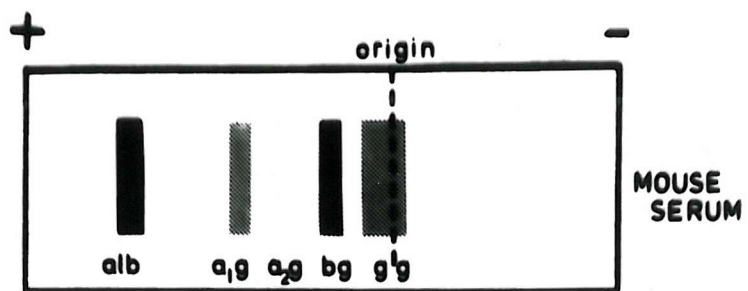
The amount of cross-reactivity obtained with tadpole coelomic fluid was much less than with that of toadlets and adults. Thus it was suspected that a concentration effect was occurring in spite of total protein equilibration. Nagano et al (1975) had shown that the equivalent of alpha- fetoprotein may be present in bullfrog tadpole serum. To check that a major protein fraction, present in tadpole sera, but absent in adults was not causing spurious results, samples of serum and coelomic fluid from adult, normal tadpoles and giant tadpoles were separated on polyacrylamide gels.
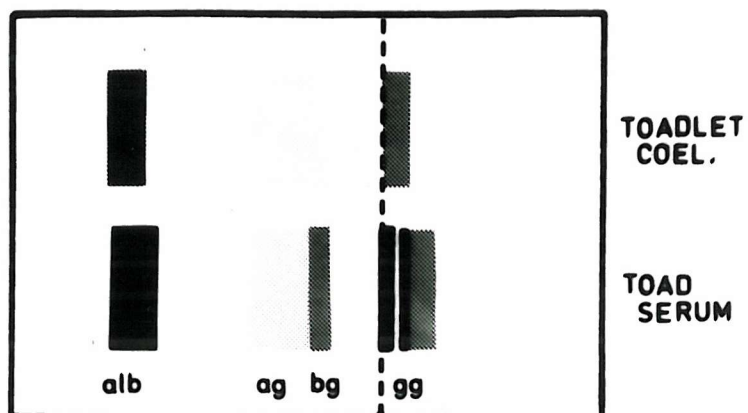
FIGURE 4/1

Cellulose acetate electrophoresis of mammalian serum and
Xenopus serum and coelomic fluid to illustrate the similar
mobilities of the constituent proteins.   A barbital
buffer of pH 8.6 was used in all cases.

a)   Mouse serum (upper membrane) compared to Xenopus
     toadlet coelomic fluid and adult female serum (lower
     membrane)

b)   Rabbit serum compared to Xenopus toadlet coelomic fluid
     and adult female serum.
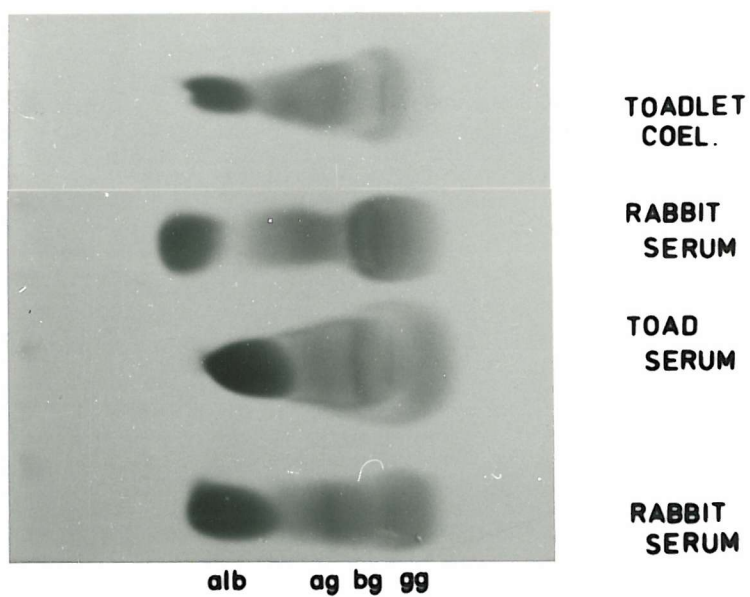
c)   Normal Xenopus tadpole coelomic fluids.

     alb = albumin
     a_1g = alpha 1-globulins
     a_2g = alpha 2-globulins
     bg   = beta-globulins
     gg   = gamma globulins
     g   = globulins

+   origin   −

A

MOUSE SERUM

alb   a₁g a₂g bg g'g

TOADLET COEL.

TOAD SERUM

alb   ag bg gg

B

TOADLET COEL.

RABBIT SERUM

TOAD SERUM

RABBIT SERUM

alb   ag bg gg

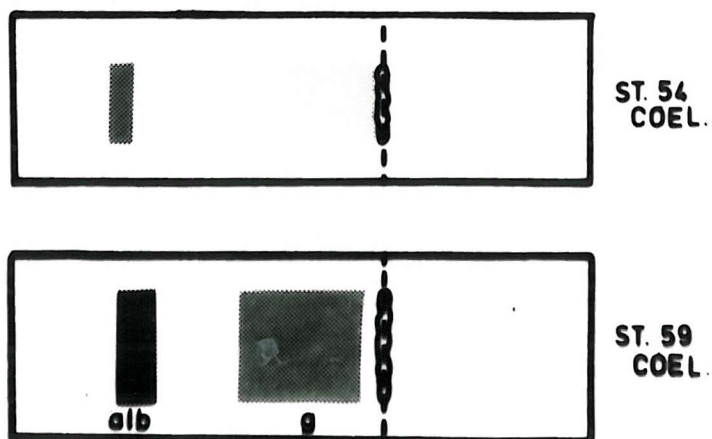**C**

ST. 54 COEL.

ST. 59 COEL.

alb

FIGURE 4/2

Cellulose acetate electrophoresis of <u>Xenopus</u> serum and coelomic
fluid to show the similar mobilities of 'albumin' before and
after metamorphosis in treated and untreated animals. A tris-
EDTA-boric acid buffer of pH 9 was used in all cases.

a) Adult female <u>Xenopus</u> serum

b) Coelomic fluids from normal tadpoles and toadlets
compared with that from propylthiouracil-treated
tadpoles. These fluids had been concentrated to
6 mg/ml total protein.

alb = albumin
ag = alpha-globulins
bg = beta-globulins
gg = gamma-globulins
g = globulins
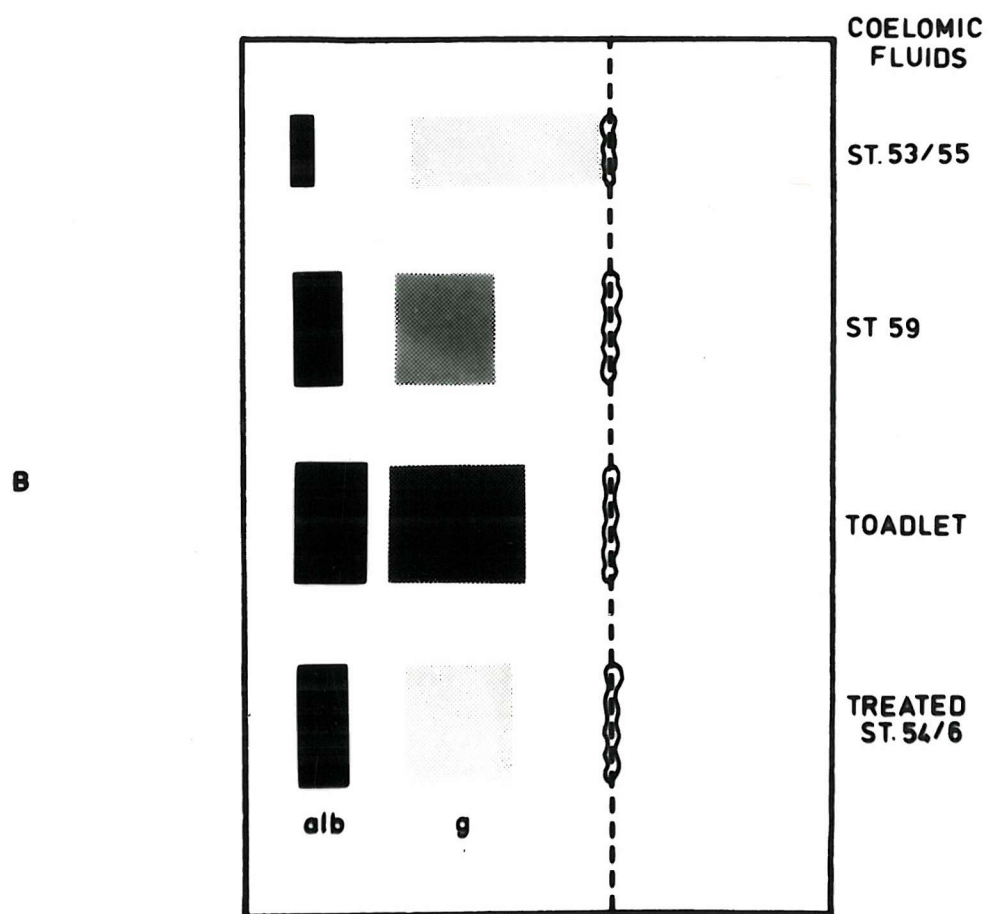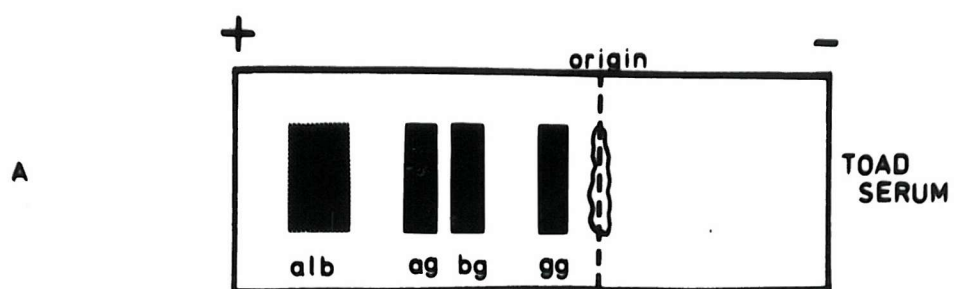
4.3 RESULTS

Fig 4/1 shows the proteins of Xenopus serum and coelomic fluid and those of mammalian serum separated by cellulose acetate electrophoresis. In Fig 4/1a the major components of mouse serum, namely albumin, alpha-1-, alpha 2-, beta - and gamma - globulins are compared with the major components of Xenopus toad and toadlet serum. When separated under identical conditions the fastest - moving Xenopus protein had a similar mobility to muscine albumin. The alpha - globulin region stained with a diffuse colour but towards the cathodal end of this region, in the adult, there was a well-defined band in the position of mouse beta-globulin. Near the origin there were two bands, one anodal and one cathodal, in the mouse gamma - globulin area. Xenopus toadlet serum appeared to lack a well-defined alpha globulin component on cellulose acetate membranes.

In Fig 4/1b the major proteinaceous components of rabbit serum are compared to those of Xenopus serum and coelomic fluid. These samples were separated simultaneously on the same cellulose acetate membrane. The fastest moving Xenopus component had a slightly greater mobility than rabbit albumin. The total protein concentrations of these samples had not been equilibrated which accounts for the faintness of the propylthiouracil - treated tadpole coelomic fluids. It also accounts for the narrower albumin bands of the less concentrated samples. The addition of Evans blue caused distortion of Xenopus and rabbit serum bands but these samples both show 2 components in the alpha - and beta - globulin positions. These are just visible also in tadpole coelomic fluid separations. Near the origin the single gamma - globulin of rabbit serum is matched by 3 components in adult toad serum. These are not visible on the tadpole strips. Toadlet serum was similar to that of adult toads. Separation of rabbit serum were poorer than that with mouse serum using a pH 8.6, barbital buffer.

Fig 4/1c shows further cellulose acetate strips on which tadpole coelomic fluids were separated. Only the fast-moving component was resolved clearly at the protein concentration used.

Some improvement in the separation of Xenopus beta - globulins was observed with the pH 9, TEB buffer. (Fig 4/2a). The diffusely stained region illustrated in Fig 4/1a appeared as 2 distinct bands. Their

FIGURE 4/3

Qualitative immunoelectrophoresis of Xenopus laevis serum and coelomic fluid to demonstrate that identical proteins occur in both fluids. The rabbit antiserum used was raised against adult Xenopus serum.

    i)   Adult female Xenopus serum.
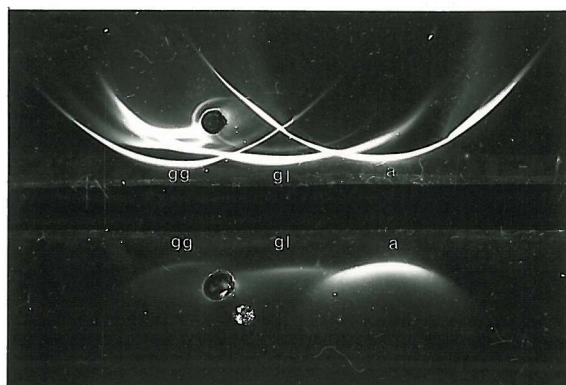
   ii)   Adult female Xenopus coelomic fluid.

  iii)   Toadlet serum.

   iv)   Toadlet coelomic fluid

    v)   Normal stage 54 coelomic fluid (2 mg/ml total protein concentration).

   vi)   Propylthiouracil treated tadpole coelomic fluid (No 6 in Table 4/2 - 12.5 mg/ml total protein concentration).

       a = albumin
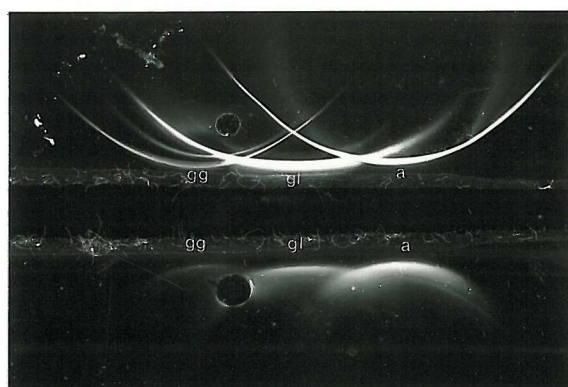
      gl = alpha or beta globulin

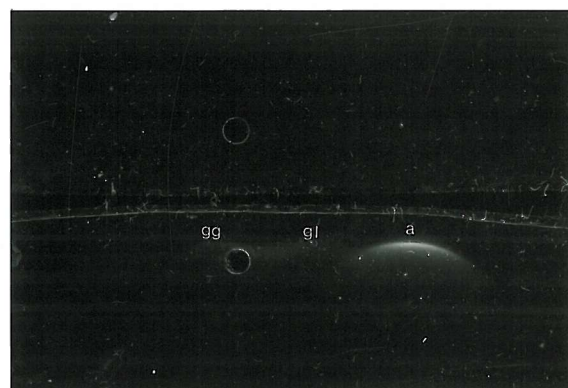   gg = gamma globulin

mobilities were somewhat greater than those of mouse alpha- and beta-globulins but they were in the same region, intermediate between albumin and gamma - globulins.

Fig 4/2 b shows a cellulose acetate membrane on which concentrated coelomic fluid samples (6mg/ml) from normal stage 53/55 and stage 59 tadpoles were compared to samples from propylthiouracil - treated tadpoles and from a toadlet. The improved separation of beta- globulins obtained by using a TEB buffer with adult serum, did not occur with coelomic fluids. The albumin band became broader, suggesting an increased albumin concentration, from stage 53/55 to stage 59 and from the latter to toadlet. The concentration of albumin in the giant tadpole sample was intermediate between normal stage 59 and toadlet and was certainly much greater than in untreated animals of the same external morphology.

The similar mobility of the fastest moving components of <u>Xenopus</u> serum and coelomic fluids suggests them to be the same protein. As this mobility was identical to that of mammalian albumin the mammalian terminology is justified. A similar argument applies to the use of the terms 'alpha-', 'beta-' and 'gamma- globulins'.
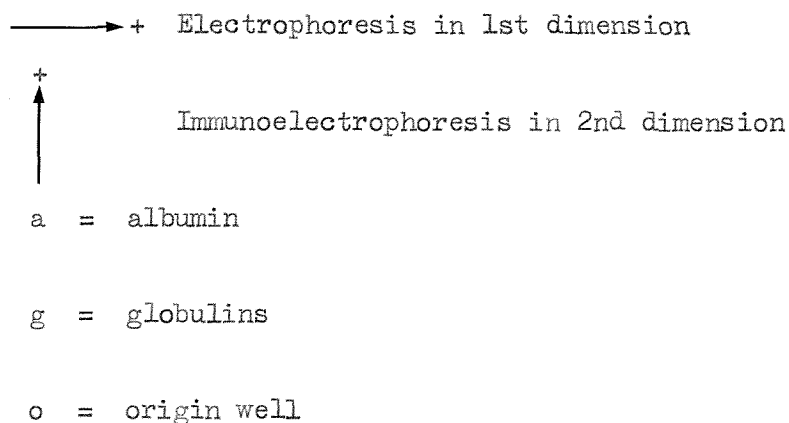
Cellulose acetate electrophoresis did not prove to be a satisfactory method with which to compare normal and treated - tadpole coelomic fluid proteins. Its only importance to the argument presented here is that it proved the use of the terms "albumin" and "globulins" for <u>Xenopus</u> proteins to be justified.

To obtain more rigorous proof that identical proteins occur in both the serum and coelomic fluid of <u>X. laevis</u> qualitative IEP was performed (Fig 4/3). The antiserum employed was raised against adult toad serum and Fig 4/3a shows the antigens precipitated by this anti-serum with toad serum. Amphibian serum proteins have not been thoroughly described and no attempt will be made here to produce a complete nomenclature. It is sufficient to note that there are components in an anodal position (albumin), intermediate positions (alpha- and beta-globulins and in a strongly cathodal position (gamma-globulins). The introductory review to this chapter (Section 4.1) mentioned other amphibian serum proteins, such as transferrin and ceruloplasmin, which

FIGURE 4/4

A comparison of the antigens precipitated by the one- and two-dimensional immunoelectrophoresis of _Xenopus_ serum and coelomic fluid.

a) One-dimensional qualitative IEP of adult female (i) serum and (ii) coelomic fluid.

b) Two-dimensional IEP of adult female serum using a high gel antiserum concentration (500 µl of antiserum in 11 ml of agarose solution) sufficient to precipitate the globulins clearly.

c) Two-dimensional IEP of normal st. 57/58 tadpole coelomic fluid using the same gel antiserum concentration as in Fig. 4/4b.

$\longrightarrow$ + Electrophoresis in 1st dimension

$\uparrow$ Immunoelectrophoresis in 2nd dimension

a = albumin

g = globulins

o = origin well

Figs 4/4b and c are to the same scale. Fig 4/4a was enlarged so that the position of the albumin arc is similar to that of the albumin peaks in 4/4b and c.

i

a)

G   G   A

ii

G   G   A

b)

+

A

G

G

c)

G   G   A

+
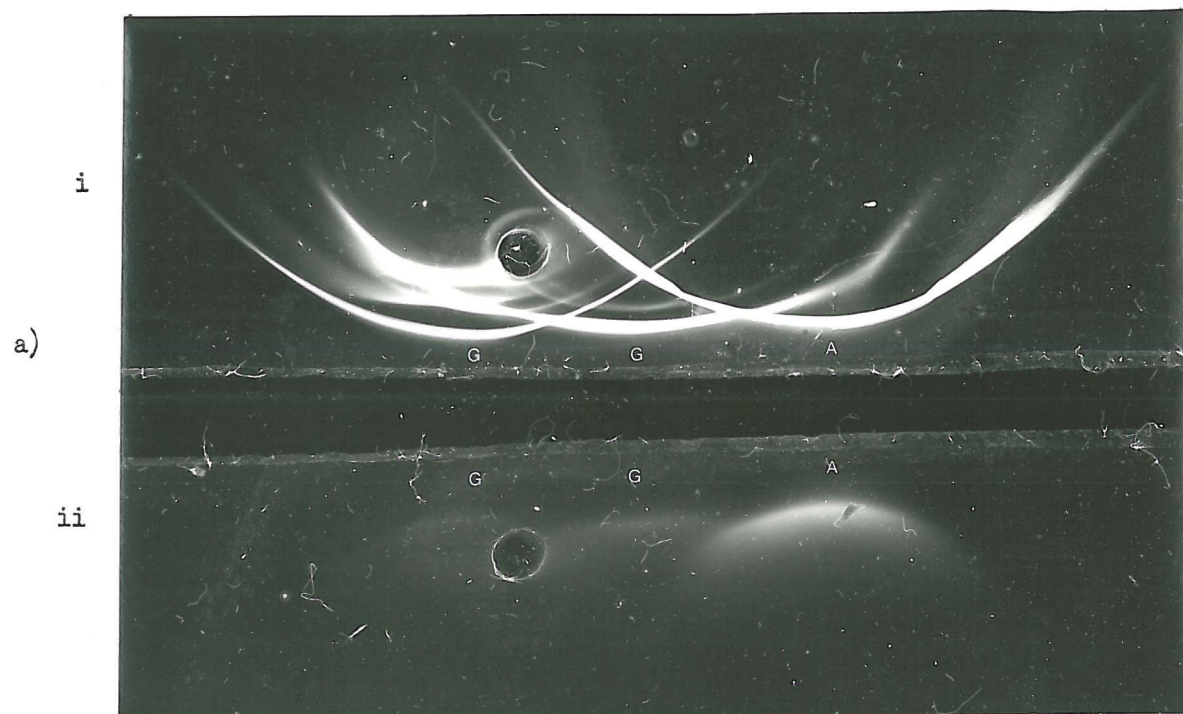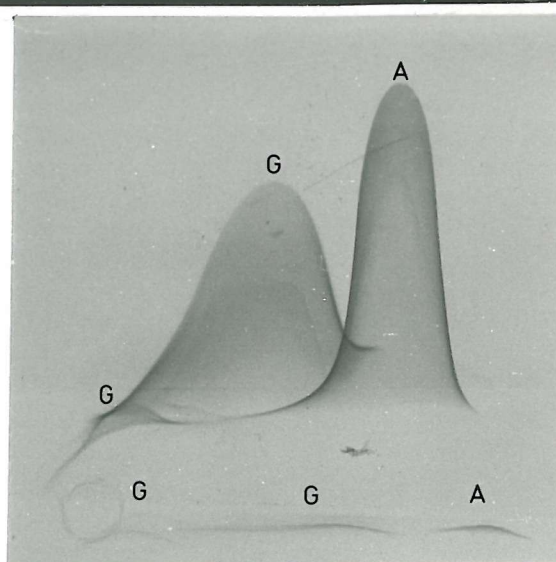
FIGURE 4/5

Polyacrylamide disc gel separations of coelomic fluids from
normal and propylthiouracil treated larvae and serum from an
adult <u>Xenopus</u> toad. No major fraction occurs in tadpole
coelomic fluid which is not also present in adult serum.

The gels are aligned with reference to the albumin band
and were stained for protein with Naphthalene Black 12B.

STAGE 54   TREATED LARVAE   ADULT

albumin

+   COELOMIC FLUIDS   SERUM
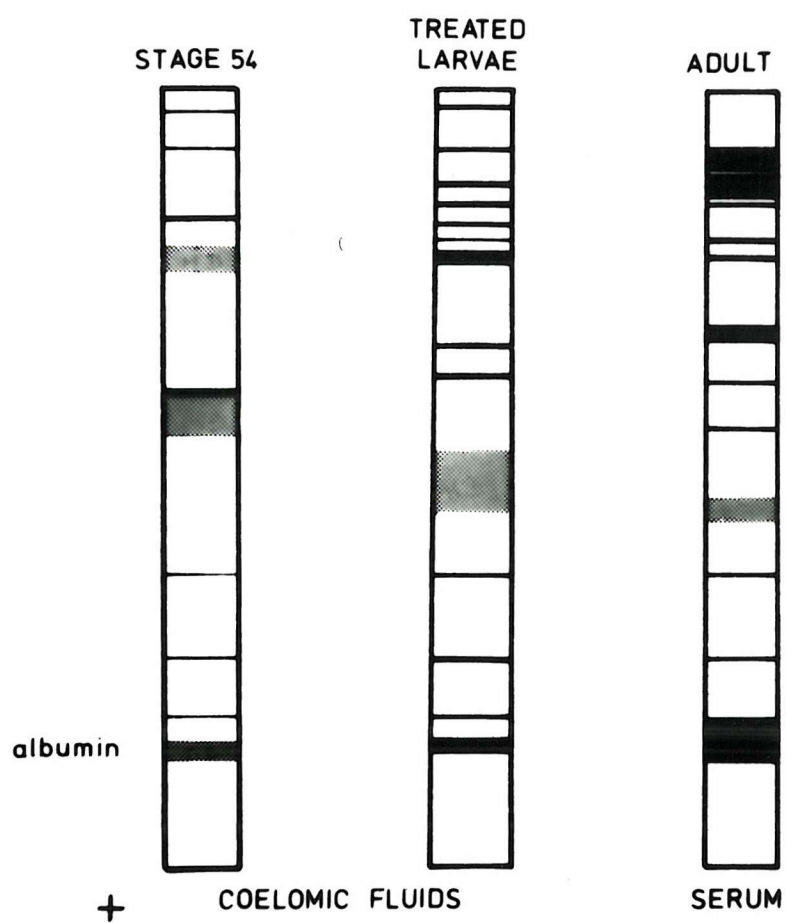
may be precipitated here but which have not been identified.

The purpose of Fig 4/3 is to demonstrate that these major classes of protein are also precipitated in toadlet serum and in coelomic fluid from adult, toadlet and propylthiouracil - treated tadpole. This evidence justifies the use of an antiserum, raised against adult antigens, in the quantification of tadpole antigen transitions. Toad and toadlet serum antigens are similar (Fig 4/3a and c) but increases in concentration of some components close to the cathodal side of the origin evidently occur with growth. This is not attributable merely to a difference in total protein concentrations which were similar. Coelomic fluid, whether from adult, toadlet or propylthiouracil-treated tadpoles, showed arcs in the albumin region and also in the intermediate globulin and cathodal globulin positions.

The absence of stainable precipitin arcs in Fig 4/3e should not be taken to mean that antigens, precipitable by this antiserum were absent from premetamorphic tadpoles. The normal st. 54 tadpole coelomic fluid had a total protein concentration of 2 mg/ml. whereas that of the propyl-thiouracil - treated animal (Fig 4/3f) had a concentration of 12.5 mg/ml. The difference is therefore almost certainly due to the sensitivity of the technique.

The two-dimensional IEP method was more sensitive and showed the major antigenic components to be present in normal tadpole coelomic fluid (Fig 4/4). Fig 4/4a and b compares the proteins precipitated from adult X. laevis serum by the qualitative IEP and two-dimensional IEP methods respectively. Most important for the present work is the well-defined albumin peak. The concentration of gel-antiserum, required to obtain such peaks with adult serum, was too great for peaks to form with tadpole coelomic fluid antigens (Fig 4/4c). However the cross reactivities and mobilities of these proteins proves their presence in normal premetamorphic tadpole fluid. Both adult serum and tadpole coelomic fluid had been approximately adjusted to 3 mg/ml total protein (assuming their protein content to be the same as other similar samples). The lack of well-defined tadpole peaks suggested the presence of a major component or components, absent in adult serum, but present in tadpole coelomic fluid. This would have the effect of producing high total protein values in tadpole fluids but much of this protein would not be

precipitable by the antiserum employed. Fig 4/5 shows PAGE separations of serum and coelomic fluid from adults, a treated tadpole and normal tadpoles. The great sensitivity of the polyacrylamide gel method would be expected to show any proteins present only in tadpoles. The gels are aligned on the fast-moving (albumin) band but no attempt will be made to identify the other bands. From these gels there was no evidence that the Lowry total protein measurements could be affected by transient proteins. The difference in cross-reactivity noted above might be due to the approximate total protein adjustment made in that case but could also be due to the fact that the Lowry method depends on a reaction with peptides rather than whole proteins. Thus, if the coelomic fluid contained large quantities of small polypeptides, which would migrate very quickly on polyacrylamide gels and be lost, spuriously high Lowry values might be obtained. This is a general criticism of the Lowry method but this method is still considered the most accurate for regular use with small samples of body fluid. The PAGE and IEP results suggest that similar proteinaceous components occur throughout development and are directly responsible for the changing Lowry values. Neither PAGE nor IEP showed albumin polymorphism such as had been reported in Rana tigrina by Rao and Lakshmipati (1974).

To produce coelomic fluid protein peaks sufficiently large for measurement it was necessary to use a lower gel - antiserum concentration than in Fig 4/4 experiments. However this also had the effect of clearly precipitating only the albumin peak. It is assumed that the preponderance of albumin in the serum used for immunisation resulted in relatively more numerous antibody being produced by the rabbit against albumin than against any other serum protein. When it became apparent that the increase in albumin concentration would provide an excellent biochemical marker it was decided to work with that protein alone. Thus the globulins will not be considered further in this chapter.

The coelomic fluid and serum samples used for albumin measurements were first assayed for total protein, known from the work of Herner and Frieden (1960) to increase during development. The normal transition is shown in Table 4/1 and Fig 4/6. For some of this work tadpoles were grouped in the metamorphic stages of Gosner (1960). Coelomic fluid total protein increased by a factor of 4 from stage 47/52 premetamorphic tadpoles to post metamorphic toadlets and adults. The increase appeared to be complete soon after metamorphosis as there was little difference

FIGURE 4/6

Albumin indexes and total protein concentrations of _Xenopus_
coelomic fluids and sera. The uncorrected albumin indexes
are directly related to the proportion of albumin in coelomic
fluid or serum.

Coelomic fluid albumin index.

Serum albumin index.

Total protein concentration.
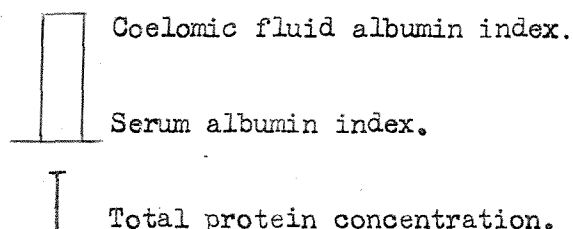
The albumin indexes of treated tadpoles Gts. 1, 2, 5 and 9 are
categorised as 'tadpole' (page 84). Treated tadpoles 6 and 7
are 'intermediate' while 3 and 13 are 'adult'.
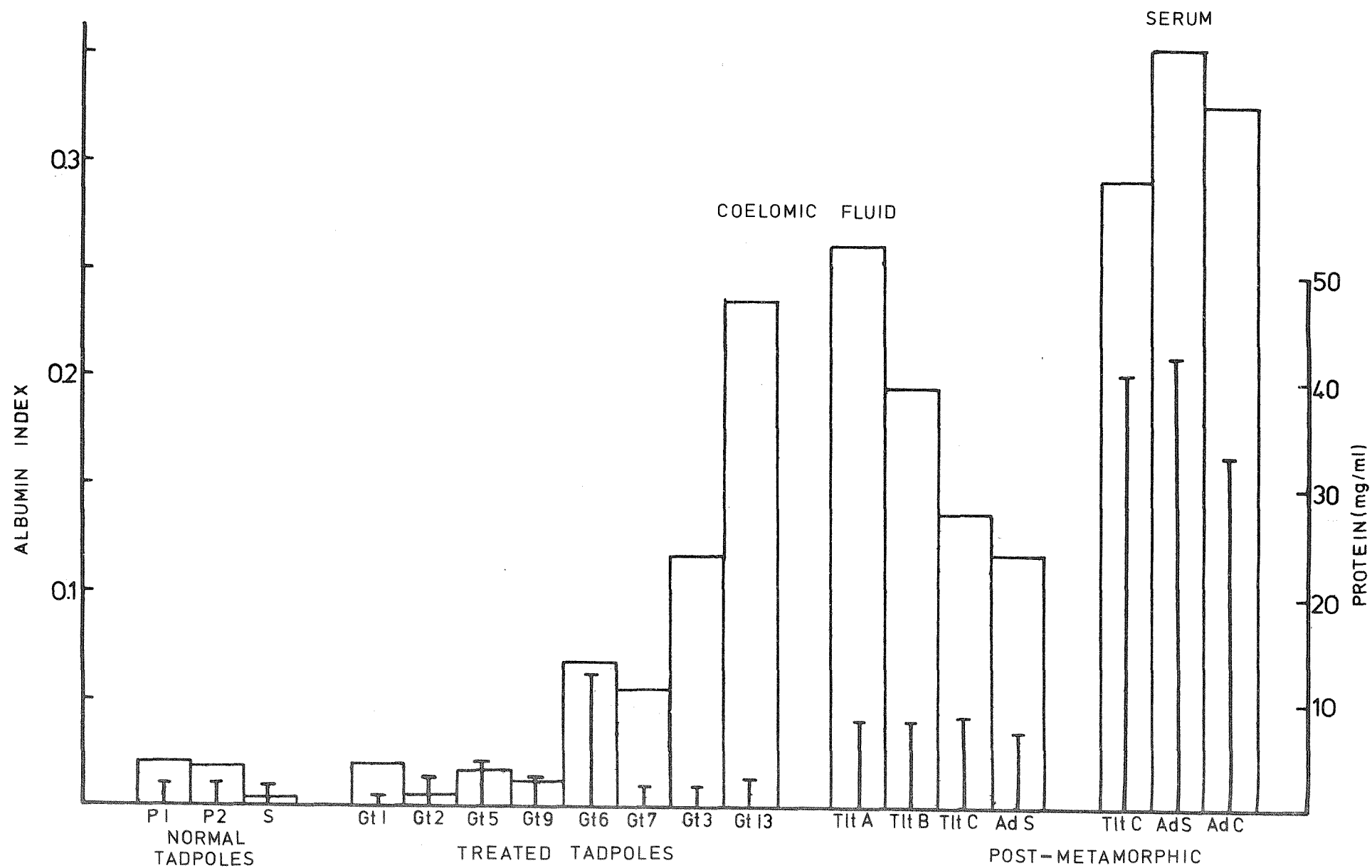
FIGURE 4/7

Two dimensional immunoelectrophoresis of propylthiouracil treated
tadpole coelomic fluid to show that albumin indexes were not
affected by minor variations in the duration of electrophoresis.

Both plates were run with fluid from giant tadpole No. 6 under
identical conditions except for duration of separation.

a) 18 h - albumin index 0.065

b) 21 h - albumin index 0.065

→ + Electrophoresis in 1st dimension

↑
+   Immunoelectrophoresis in 2nd dimension

a = albumin

g = globulins

o = origin
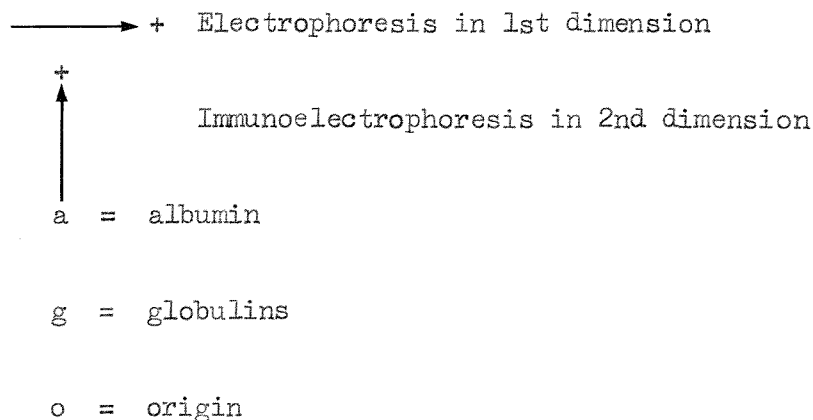
Both Figs. are to the same scale.

a)

b)

between young toadlets and adults. Serum protein concentrations were also similar in both toadlets and adults but were some four times higher at about 32 to 42 mg/ml, than those of coelomic fluid which contained 7 to 9.4 mg/ml total protein. The apparently sudden increase from 4.0 mg/ml in Gosner st. IV coelomic fluid to 8.0 mg/ml in toadlets is explicable by the fact that most of the larvae used to form the stage IV pool were in early climax (NF stages 59-60).

Propylthiouracil-treated tadpoles are compared with respect to coelomic fluid total protein in Fig 4/6 and Table 4/2. Considerable variation is apparent between individual larvae. Particularly noticeable is No. 6 with a concentration of 1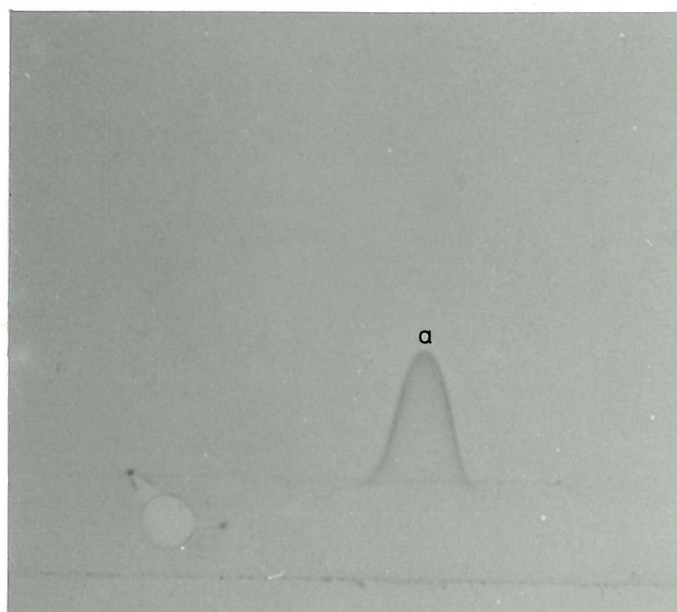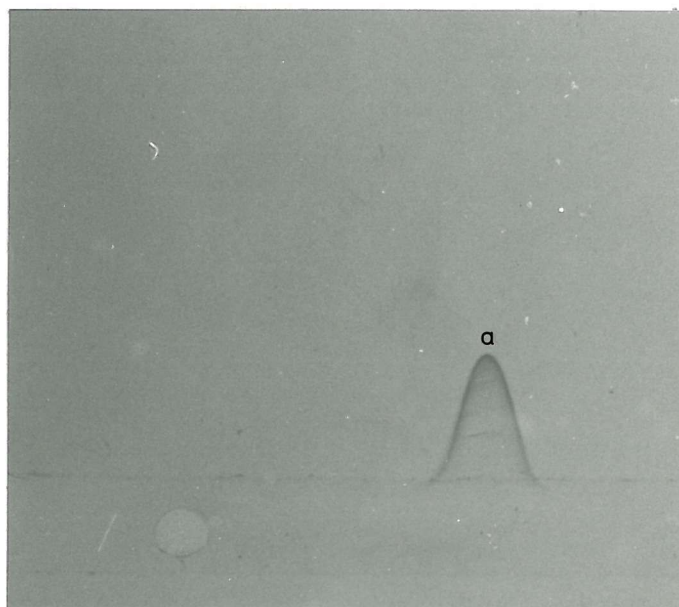2.5 mg/ml - higher than any toadlet or adult sample measured. This result was confirmed by a duplicate Lowry determination and by qualitative IEP of a 1 µl sample (Fig 4/3f). Other treated tadpole coelomic fluids failed to give visible precipitin arcs presumably because of their low concentrations. Variation in total protein was not caused by accidental dilution of samples (by contamination with water from the pharynx) or by concentration due to evaporation. Three other treated tadpoles (Nos, 2, 5 and 9 in Table 4/2) had protein concentrations higher than the control stage 54 value of about 2.5 mg/ml. Their values, namely 3 to 4 mg/ml were encountered in prometamorphic and early climax controls (Table 4/1).

It is important to stress that the parameter expressed as an "albumin index" (related to peak area - Section 2.24g) in two-dimensional IEP and used as a metamorphic marker is directly related to the proportion of the total coelomic fluid which is albumin. It is not possible to compare directly undiluted samples because of the four-fold difference in total protein concentration between premetamorphic and postmetamorphic fluids. The high gel-antiserum concentration required to produce stable precipitin peaks with undiluted postmetamorphic samples on a 100 mm x 100 mm glass plate had been shown to give excessively small peaks with undiluted normal premetamorphic samples. Thus all samples were diluted to a known common total protein concentration of 0.4 mg/ml. This does not alter the proportion of the total protein which is albumin.

The dilution factor used to adjust each sample to the common protein concentration is shown in Table 4/3. Although unnecessary for the study of metamorphic markers, which is the primary concern of this

chapter, the average albumin index for each sample has been multiplied
by the dilution factor. This enables one to estimate the total change
in albumin during metamorphosis and facilitates comparison with the
data of other authors.

The normal transition of coelomic fluid uncorrected albumin indexes
is shown in Table 4/3 and Fig 4/8. Although variation occurred between
the 3 premetamorphic pools the index was always below 0.025 whereas in
toadlets it had increased by a factor of about 10. The single adult
female used was similar to the toadlets in albumin concentration.
Serum albumin indexes were higher than coelomic fluid albumin indexes in
toadlet and adult (Fig 4/9). This shows that the proportion of albumin
in postmetamorphic serum is even higher than that in coelomic fluid.

In Chapter 3 haemoglobin electropherograms were categorised as
'adult', 'intermediate' and 'tadpole' as an aid to investigating the
effects of body size, etc., on the transition. A similar categorisa-
tion is used here. Uncorrected albumin indexes were categorised as
follows:

'Adult' - albumin index greater than 0.119 (the lowest value of a
          post-metamorphic animal).
'Intermediate' - albumin index less than 0.119 but greater than
          0.021 (the highest value found in normal pre-
          metamorphic animal).
'Tadpole' - albumin index less than 0.021.

Examples of 'adult' IEP plates are shown in Fig 4/8 on the same
scale as controls. Giant No. 3 is included because its' index of
0.117 was almost identical to that of an adult female at 0.119. Fig
4/10 compared examples of 'intermediate' and 'tadpole' plates with
normal controls at the same scale of reproduction. Inspection of the
replicated data in Table 4/3 shows the albumin indexes of controls and
experimentals to be very repeatable. The categorisation of albumin
indexes for all 8 giant tadpoles studied appears in Table 4/2 together
with details of developmental stage, weight and immersion duration.
2 tadpoles (25%) were adult, 2 (25%) were 'intermediate' and 4 (50%)
were 'tadpole' according to the albumin index categories. If the 10
values for coelomic fluid total protein shown in Table 4/2 are cate-

FIGURE 4/8

Two dimensional immunoelectrophoresis of <u>Xenopus</u> coelomic fluids
to show, (a) the normal transition of albumin index at
metamorphosis and (b) propylthiouracil treated tadpole coelomic
fluids with 'adult' indexes.

a) Normal 'metamorphic' transition in albumin index

    i.   Normal st. 54 coelomic fluid (0.017)
   ii.   Normal toadlet coelomic fluid (0.236)
 iii.   Normal adult coelomic fluid (0.121)

b) Giant tadpoles with 'adult' albumin indexes (page 84)

   iv.   Gt. No. 3 (0.129)
    v.   Gt. No. 13 (0.204)

⟶ +   Electrophoresis in 1st dimension

+
↑
|
|
|
a = albumin

Immunoelectrophoresis in 2nd dimension

g = globulin

o = origin well
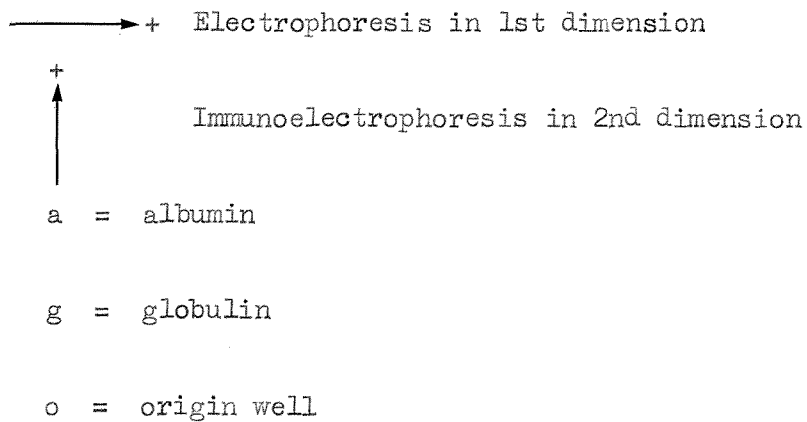
All Figs. are to the same scale
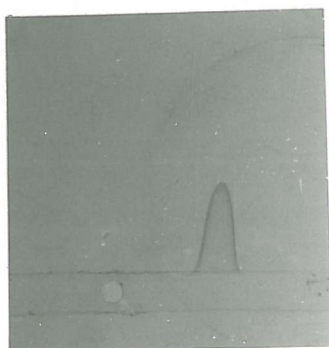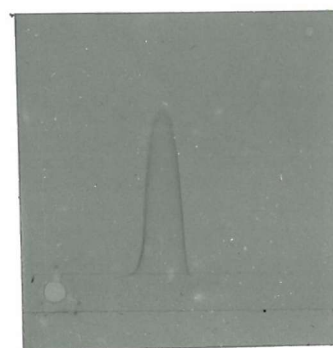
Table 4/3. Albumin indexes of <u>Xenopus laevis</u> body fluids during normal metamorphosis and in propylthiouracil - retarded larvae. Uncorrected indexes are related to the proportion of albumin, and corrected indexes to the total albumin, in a sample.

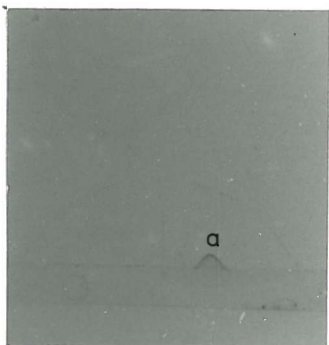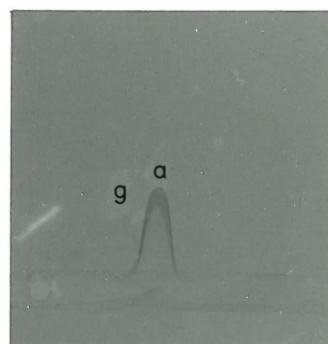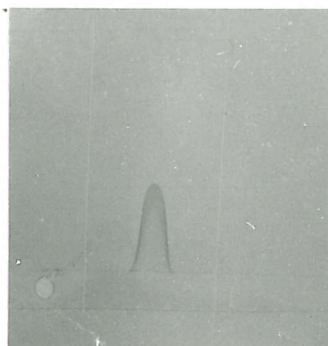| Code	Animal	Fluid	Replicate	Albumin index	Average	Dilution factor	Corrected albumin index
P1	St.53-54 normal	Coelomic	1) 2)	0.022 0.020	0.021	2.3	0.05
P2	St.52-55 normal	"	1) 2) 3)	0.017 0.016 0.022	0.018	2.3	0.04
S	St.52-55 normal	"	1) 2)	0.005 0.006	0.006	2.1	0.01
TltA	Toadlet	"	1) 2)	0.287 0.236	0.262	8.4	2.21
TltB	Toadlet	"	1) 2)	0.194 0.195	0.195	8.4	1.64
TltC	Toadlet	"	1) 2)	0.132 0.143	0.137	9.1	1.24
S	Adult Female	"	1) 2)	0.121 0.116	0.119	7.4	0.88
Gt.1	Treated	"	1) 2)	0.018 0.020	0.019	1.0	0.02
Gt.2	Treated	"	1) 2)	0.005 0.010	0.008	3.2	0.03
Gt.3	Treated	"	1) 2)	0.104 0.129	0.117	2.1	0.25
Gt.5	Treated	"	1) 2)	0.019 0.013	0.016	4.5	0.07
Gt.6	Treated	"	1) 2) 3)	0.072 0.065 0.065	0.067	13.2	0.88
Gt.7	Treated	"	1) 2)	0.056 0.052	0.054	1.9	0.10
Gt.9	Treated	"	1) 2)	0.015 0.010	0.012	3.2	0.04
Gt.13	Treated	"	1) 2)	0.204 0.268	0.236	2.8	0.67
TltC	Toadlet	Serum	1) 2)	0.311 0.274	0.293	42.7	12.5
AdS	Adult Female	"	1) 2) 3)	0.345 0.345 0.362	0.351	44.2	15.5
AdC	Adult Female	"	1) 2) 3)	0.311 0.304 0.367	0.327	34.7	11.4

gorised similarly by comparison with values of about 2.5 mg/ml in normal premetamorphic tadpoles and 8 mg/ml after metamorphosis, then 1 animal (10%) was 'adult', 1 (10%) was 'intermediate' and 8 (80%) were 'tadpole'. Two of the treated larvae which were classified as 'tadpole' because of total protein concentrations between 2 and 3 mg/ml were adult-like by the albumin index criterion. Conversely No. 6 which had a high total protein concentration was 'intermediate' in its' albumin index. The relationship between albumin index and total protein is discussed below.

4.4 CONCLUSIONS

The presence of a fast-moving anodal component, with a mobility similar to the albumin of mouse and rabbit serum, before and after metamorphosis in Xenopus serum and coelomic fluid was confirmed by cellulose acetate electrophoresis. It was shown immunologically that the albumin component of tadpoles is identical to that of adults in both body fluids. Quantitation of coelomic fluid albumin concentrations was achieved by two-dimensional IEP. This technique was highly specific and gave highly repeatable results providing great care was taken with methodology. During normal metamorphosis the uncorrected albumin index increased by a factor of approximately 10 and was therefore an ideal metamorphic marker. Total protein increased by a factor of 4. As this parameter could be affected in many ways it was concluded that the albumin index was likely to be a more meaningful marker. Of 8 propylthiouracil-treated tadpoles, retarded for from 41 to 67 weeks in late premetamorphosis, 50% had albumin indexes substantially higher than premetamorphic controls. 25% of these were definitely adult-like in albumin proportion. Thus in spite of prolonged goitrogen treatment, which had caused arrest at stage 56, these larvae were synthesising albumin in post-metamorphic quantities suggesting that the absence of circulating thyroid hormone was not able to prevent the biochemical transition.

Considerable variation was noted in the category of albumin index as had been demonstrated in the haemoglobin system (Chapter 3). Inspection of Table 4/2 shows no correlation between albumin category and either stage of arrest or duration of goitrogen treatment. Some correlation with body weight is apparent however. The continued

FIGURE 4/9

Two dimensional immunoelectrophoresis of <u>Xenopus</u> toadlet and adult coelomic fluids and sera to show that albumin forms a larger proportion of the total protein in sera than in coelomic fluids.

    a) Toadlet   i. Serum

                   ii. Coelomic fluid (both from same animal).

    b) Adult female

                iii. Serum

                iv. Coelomic fluid (both from same animal)

⟶ +   Electrophoresis in 1st dimension

↑+   Immunoelectrophoresis in 2nd dimension

a = albumin

g = globulins

o = origin well

All Figs. are to the same scale.

i

ii

a)

iii

iv

b)
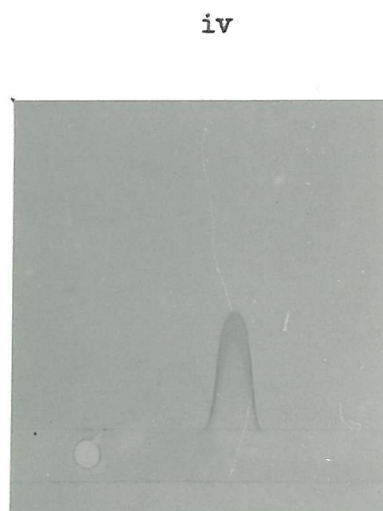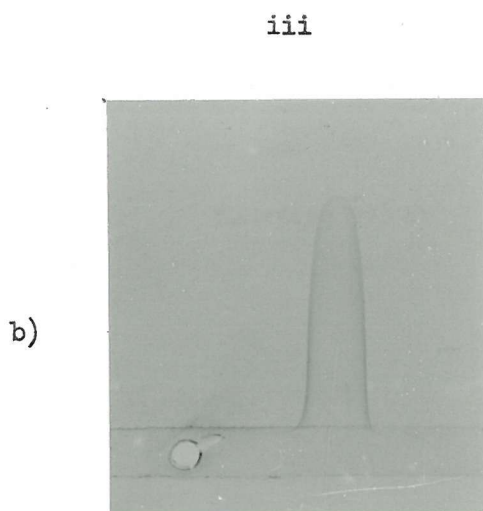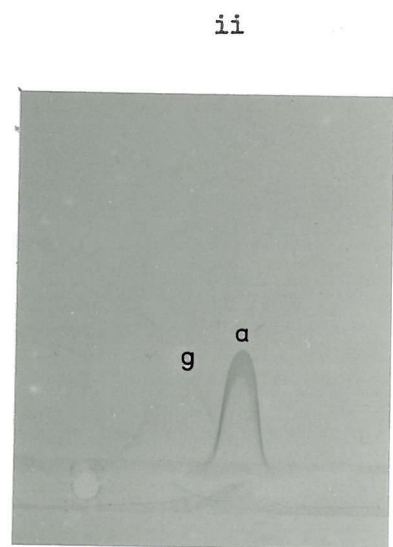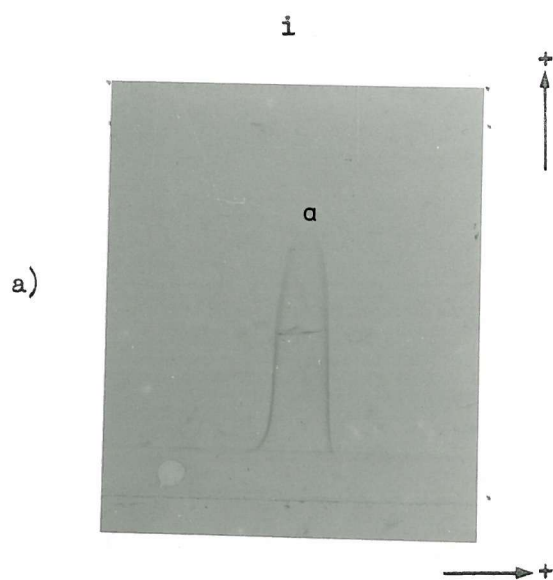
FIGURE 4/10

Two dimensional immunoelectrophoresis of <u>Xenopus</u> coelomic
fluids to show, (a) the normal transition of albumin index at
metamorphosis, (b) propylthiouracil treated tadpoles with
'intermediate' indexes and (c) propylthiouracil treated tadpoles
with 'tadpole' indexes.

a) Normal 'metamorphic' transition in albumin index
    i.   Normal st. 54 coelomic fluid (0.017)
    ii. Normal toadlet coelomic fluid (0.236)

b) Giant tadpoles with 'intermediate' albumin indexes
    (page 84)
    iii. Gt. No. 6 (0.072)
    iv. Gt. No. 7 (0.056)

c) Giant tadpoles with 'tadpole' albumin indexes
    (page 84)
    v.   Gt. No. 1 (0.018)
    vi. Gt. No. 2 (0.010)

$\longrightarrow$ + Electrophoresis in 1st dimension

+

$\uparrow$ Immunoelectrophoresis in 2nd dimension

a = albumin

g = globulin

o = origin well
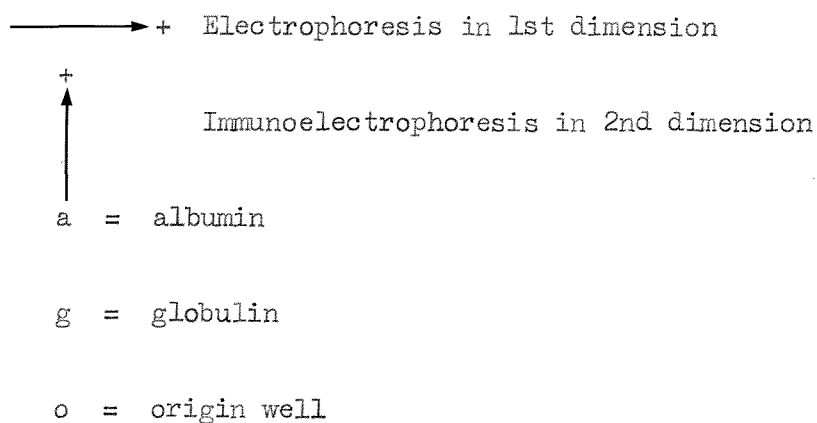
All Figs. are to the same scale
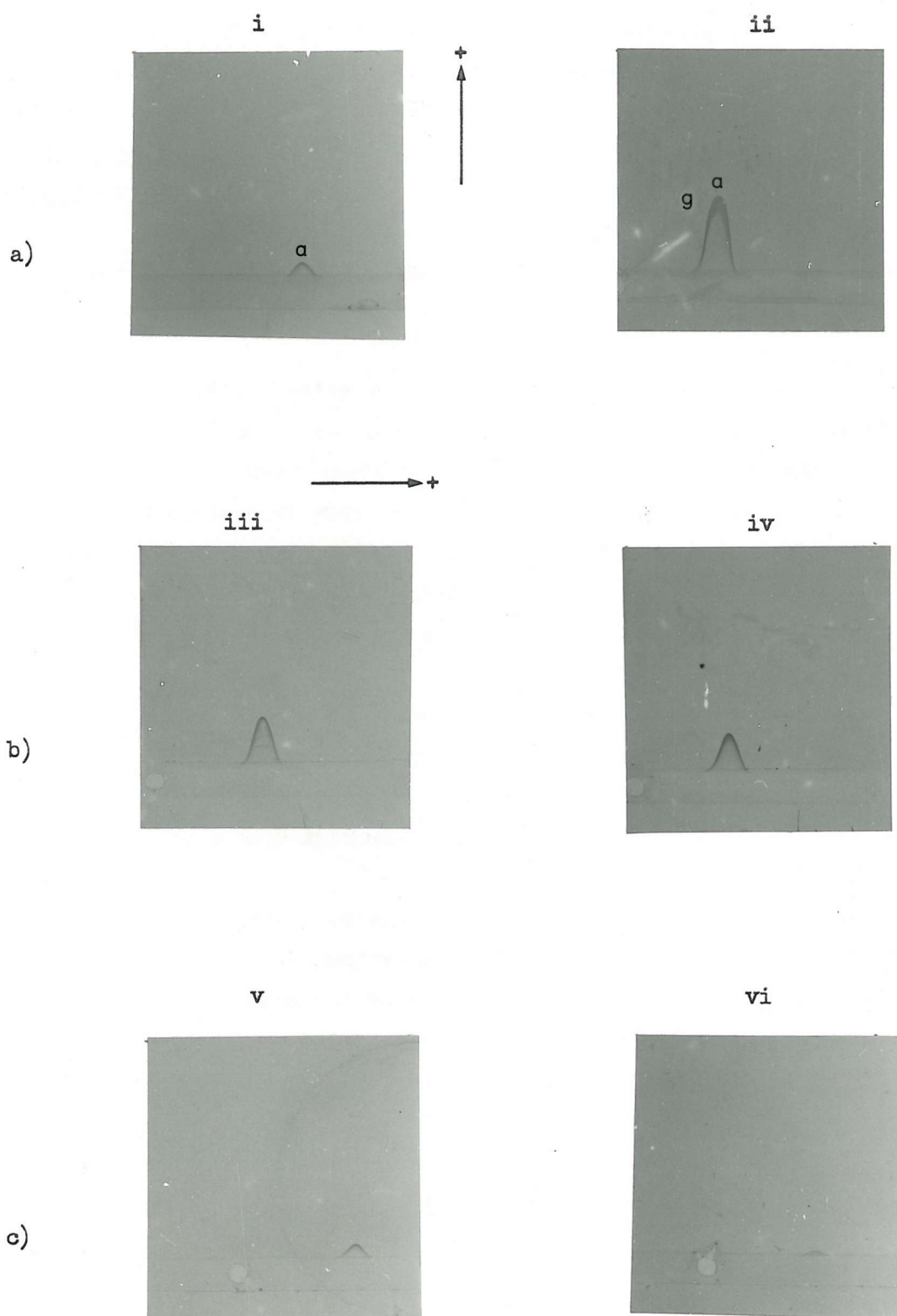
albumin transition of a proportion of retarded larvae is undeniable but a larger quantity of data would be necessary to substantiate this correlation with body wet weight. Some evidence that body size can affect the percentage of serum albumin in the European Midwife toad, Alytes obstetricans, has been obtained by Gueyétant (1970) who found that Alytes larvae grew fatter when raised in groups than when isolated. More recently he has claimed that, at the same stage of development, grouped individuals are bigger and their serum proteins contain up to 20% more albumin than isolated controls (Gueyétant, 1977).

The introductory review to this chapter (section 4.1) showed that in a number of anurans albumin increases account for a large part of the total serum protein concentration increase at metamorphosis. In the present work samples with high uncorrected albumin indexes had albumin forming a greater percentage of the total protein concentration than samples with low albumin indexes. However it was apparent that the relationship between albumin index and total coelomic fluid and serum protein concentration is erratic (Fig 4/6). This variation was observed in control as well as in treated animals. It would be necessary to assay every major proteinaceous component separately to fully understand this relationship and this complexity supports the decision to work mainly with albumin.

However the absolute levels of total protein encountered here agree well with previous published data. Herner and Frieden (1960) found X. laevis tadpoles (unstaged) to have 2 mg/ml total protein in serum. This suggests that in premetamorphic larvae there is little difference between the concentration of serum and coelomic fluid total protein. Herner and Frieden(1960) found a recently metamorphosed toadlet to possess 23 mg/ml and a large adult to possess 35 mg/ml total serum protein. These figures are also within the range of those obtained here (Table 4/1).

In Rana pipiens adults Constantine and Stenroos (1962) found 26.5 mg/ml total serum protein. Just (1972) repeated this work and obtained a value of 33.9 mg/ml in adults. Premetamorphic tadpoles had 2.6 mg/ml in their serum. Just also analysed the pericardial fluid. The pericardium, like the peritoneum, is a coelomic derivative. The level rose from 1.8 mg/ml in premetamorphic tadpoles to 12.9 mg/ml

in adults, a similar increase to that observed here in coelomic fluid.
Thus it seems that during metamorphosis there is an increasing differ-
ence between the total protein concentrations of serum and coelomic
fluid. This was confirmed in Rana catesbeiana by Thornburg et al (1975)
although the difference was less marked. These authors suggested that
a sieving effect occurs at the inflow site by which serum proteins
enter the coelomic fluid. In bullfrog plasma, Feldhoff (1971) found a
metamorphic transition from 14.6 mg/ml to 51.6 mg/ml total protein.
This was accompanied by an approximately 4-fold increase in percentage
albumin. The total albumin concentration increased by a factor of 15.
Using a quantitative immuno-diffusion method Nagano et al (1973) found
bullfrog total serum albumin to increase by a factor of only 9.

In Xenopus the very accurate two-dimensional IEP technique had
shown the increase in total coelomic fluid albumin to be much greater.
Albumin indexes, corrected for dilution, increased from an average of
0.04 in premetamorphic controls to 1.70 after metamorphosis. This is
a 40-fold increase. However the uncorrected albumin index, related to
the percentage albumin in a sample, increased by a factor of only 10.
There is no discrepancy in these figures. For example if the percentage
albumin in a premetamorphic fluid of 2 mg/ml total protein were 5% the
absolute albumin concentration would be 0.1 mg/ml. If the percentage
albumin increased at metamorphosis by a factor of 10 to 50%, in 8 mg/ml
total protein samples, the absolute concentration of albumin would be
4 mg/ml. Thus the absolute albumin concentration would have increased
40-fold.

Xenopus coelomic fluid and bullfrog plasma total protein concen-
trations both increased by a factor of about 4 at metamorphosis (cf.
Feldhoff, 1971). The greater increase in Xenopus total albumin is
accounted for by the more marked increase in percentage albumin observed
here.

CHAPTER 5

Lactate dehydrogenase isozymes in developmentally
retarded Xenopus laevis larvae

5.1 INTRODUCTION

Lactate dehydrogenase isozymes were chosen for study, in the first
instance, because of the theoretical background available on their
nature and genetics, especially in non-amphibian vertebrates. There
have been a number of studies also on amphibian LDH's and most impor-
tantly, 3 papers on Xenopus laevis (Kunz and Kearn, 1967, Claycomb and
Villee, 1971; Kunz, 1973).

LDH occurs in most vertebrate tissues in 5 isozymic forms. These
isozymes are tetramers composed of 2 structurally distinct subunits of
equal size, A and B, each synthesised at a separate gene locus (Markert,
1963). The subunits associate at random, into all possible combina-
tions of 4, to form 5 tetramers which differ in net charge and can
therefore be separated by electrophoresis. The isozymic forms of
lactate dehydrogenase are therefore: LDH_1 = B4; LDH_2 = AB3; LDH_3 =
A2B2; LDH_4 = A3B and LDH_5 = A4 (from most anodal to most cathodal).

The LDH isozymes of various adult X. laevis tissues were separated by
PAGE by Claycomb and Villee (1971). Liver tissue was consistently
found to have 7 bands whereas tissues from the lung, heart and skeletal
muscle revealed 9 isozymes.

A number of other adult amphibians have been studied. Goldberg and
Wuntch (1967) found 3 in Rana pipiens but other species of Rana contained
6 - 8 different LDH isozymes (Moyer et al, 1968). The same authors
reported R. sylvatica and R. virgatipes to have 18 - 25 LDH's. In
developing embryos of Bombina variegata and Triturus alpestris, Chen
(1968) resolved 3 forms. Thus it seems that most Amphibia examined
have either more or fewer than the predicted 5 isozymes and do not fit
the hypothesis of a random aggregation of 2 subunits.

In Rana palustris and R. pipiens Salthe et al (1965) and Salthe
and Kaplan (1966) found that some subunits would not hybridise so that

only "LDH$_1$" and "LDH$_5$" enzymes were found. Manwell and Baker (1971) obtained similar results with the isozymes of R. esculenta and R. temporaria. Tadpoles had primarily the "LDH$_1$" (anodal) type in all tissues, whereas adults had the "LDH$_5$" (cathodal) type as well, especially in muscle and liver. There were only traces of bands intermediate between "LDH$_1$" and "LDH$_5$". Apparently this is due to evolutionary changes in amino acid sequence of the B subunit, rendering polymerisation with the A subunit impossible or unstable.

To explain the large number of isozymes found in some adult amphibia most authors have postulated one or more additional genes coding for one or more new polypeptide subunits (Wright and Moyer, 1966; Adams and Finnegan, 1965). In the brook trout, Salvelinus fontinalis, which like Xenopus can have 9 LDH isozymes in adult tissues, Goldberg, (1965) claimed that at least 3 polypeptide subunits under the control of 3 nonallelic genes were responsible. However he later (Goldberg, 1966) admitted that the pattern could be explained by heterozygosity at one gene locus with the production and aggregation of 3 types of subunit.

Kunz and Hearn's (1967) preliminary work with agar and starch gel electrophoresis showed the Xenopus liver LDH pattern to be somewhat variable. However in heart muscle there were detectable changes during larval development, in both band number and density, which were consistent. On starch, six new cathodal components appeared between a pre- and prometamorphic tadpole pool and adulthood. On agar gels the large cathodal bands of tadpoles were resolvable into a number of narrower bands in the adult, there being nine in total. With PAGE Claycomb and Villee (1971) found the last one of the 9 resolvable isozymes of the adult complement to appear at stage 45 in conflict with Kunz and Hearn's (1967) data. It seems likely that Claycomb and Villee (1971) are correct as the apparent absence of bands in tadpole tissue extracts, with the low resolution agar and starch techniques, could easily be explained by the low concentrations of the proteins concerned. Kunz (1973) again using agar gel electrophoresis found the full complement of 9 isozymes to be present in stage 49 hearts.

In the present work polyacrylamide gel electrophoresis was used for its' great sensitivity. Thus it was not expected that 'new' bands

would appear during metamorphosis. The probable biochemical marker was likely to be a sharp increase in one or more isozyme concentrations relative to the others, which would stay constant during metamorphosis.

5.2 EXPERIMENTAL

The protein solutions used were centrifuged homogenates of whole tadpole pooled hearts. Adult samples were taken from small pieces of ventricle. All tissues were washed thoroughly, with ringer and tris-glycine buffer before homogenisation, to remove blood cells which might contain different LDH isozymes.

To identify LDH the specific staining method of Dietz and Lubrano (1967) was employed. Disc gels only were used as high resolution was necessary with dilute tadpole samples. The discontinuous buffer system of Davis (1964) gave good results. Total LDH per sample was equili-brated only approximately. The wet weight of pooled tadpole hearts was matched with an equivalent weight of adult tissue. Ringer volumes and homogenisation times were then the same in all samples so that it was unlikely that great differences in total LDH enzyme concentration occurred between samples. Finally identical sample volumes, typically 50 to 100 μl, were applied to each gel.

A preliminary experiment showed that the single whole hearts of individual giant tadpoles did not contain enough extractable LDH for satisfactory electrophoresis. The stained gels showed merely a blurred reaction.

The bands on the stained gels were aligned according to the most anodal (fast-moving component) which was always very clear in both tadpoles and adults. During the period that these experiments were performed the Joyce-Loebl densitometer was not available and gels were compared visually. Records were made mainly by accurate drawings as the faint LDH bands of dilute samples did not photograph well.

Three experiments were made to compare the LDH isozymes of normal adult female toads with those of normal tadpoles. Only 2 experiments contained propylthiouracil-treated tadpole heart extracts. The need for 2 or 3 hearts per sample made the system impracticable.

FIGURE 5/1

Lactate dehydrogenase isozymes of normal and propylthiouracil treated
Xenopus laevis separated by polyacrylamide gel electrophoresis.

a)   The normal 'metamorphic' transition

   i.   Normal stage 54/55 LDH
   ii & iii.   Adult female LDH

b)   Comparison of normal and treated Xenopus LDH isozymes

   iv.   Normal st. 54/55 tadpoles (pool A)
   v.   Normal st. 54/55 tadpoles (pool B)
   vi.   Normal st. 54/56 tadpoles
   vii.   Adult female A
   viii.   Adult female B
   ix.   Adult female C
   x.   Propylthiouracil treated tadpoles (3 x st. 54; wet
         weights 1.17, 1.25 and 1.27 g; treated for $16\frac{1}{2}$,
         17 and 21 weeks).
   xi.   Propylthiouracil treated tadpoles (2 x st. 56; wet
         weights 2.13 and 2.24 g; treated for 20 weeks).

The gels are aligned on the most anodal band (No. 1)
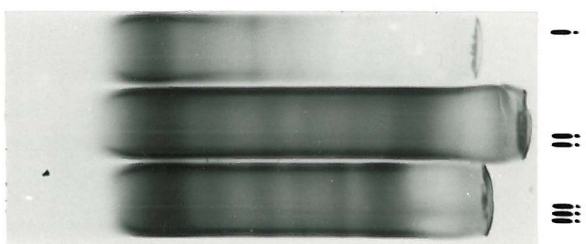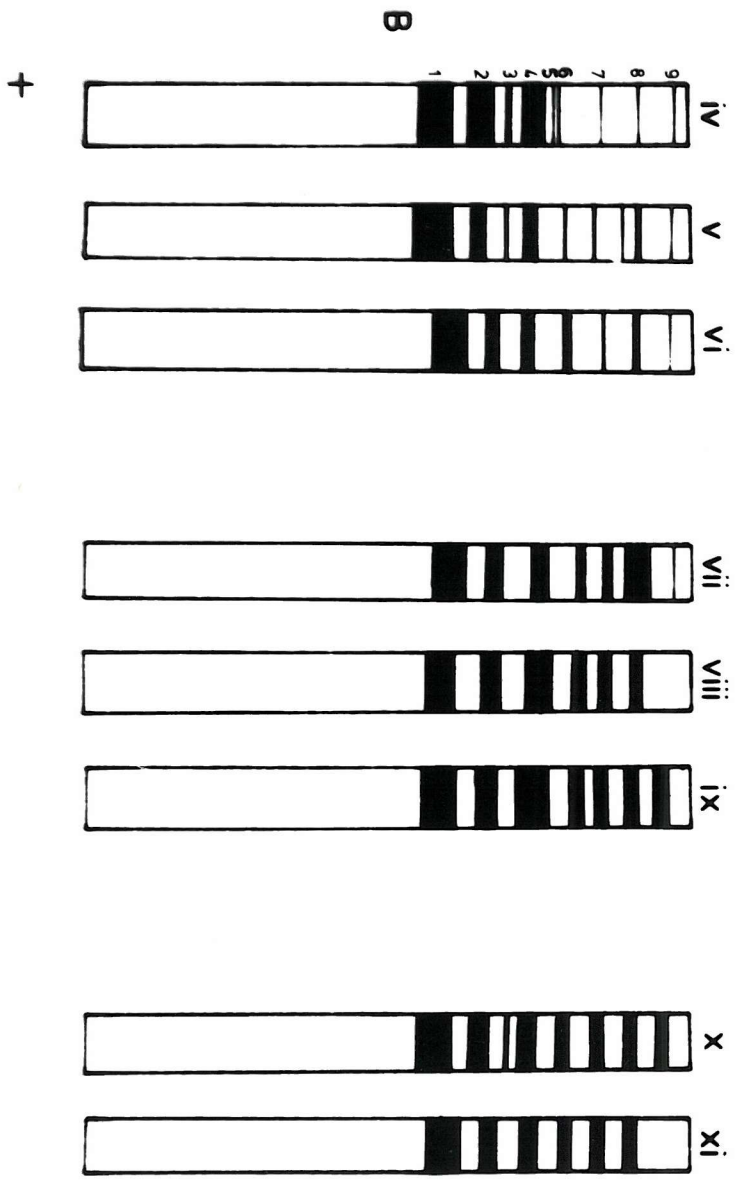
Manwell and Baker (1971) obtained evidence for polymorphism of the B chain of Rana esculenta tadpoles and adults. This was apparent as a variable number of anodal bands depending upon the genetic constitution of the donor. It was not feasible to screen a number of individual normal tadpoles for this complication in the present work. But an experiment was performed with heart extracts from 9 different female toads. The samples were electrophoresed on disc gels at the same time and the stained gels compared. Had a polymorphism been observed it would have complicated the interpretation of the comparison of treated with normal larvae.

5.3 RESULTS

Fig 5/1 illustrates typical adult and tadpole LDH isozyme separations. The 2 giant tadpole pooled-samples are also shown. In Fig. 5/2 the 9 adult heart extracts separated simultaneously are compared. The isozymes were numbered, for convenience only, beginning with No. 1 the most anodal component.

In agreement with the work of Kunz and Hearn (1967) and Claycomb and Villee (1971) 9 bands could be resolved on some tadpole and adult gels. Comparison of the gels photographed in Fig 5/2 shows that bands 3/4 and 8/9 frequently appear fused such that only 7 in total are visible. There was no evidence that a polymorphism of LDH subunits occurs in Xenopus laevis. Also Fig. 5/2 shows the concentrations of the cathodal isozymes to be similar to the anodal bands 2 and 3/4 in all adults. Band 1 was always the most dense.

In normal tadpole separations (Fig 5/1) the cathodal bands were always very faint suggesting that the synthesis or survival of cathodal subunits increases at metamorphosis. This increase in the density of the cathodal isozyme bands (Nos. 5 to 9) was used as a biochemical marker. It was so distinctive as to be usuable qualitatively in practice as no intermediates were observed.

The 2 giant tadpole samples (Fig 5/1) were clearly adult-like in that bands 5 to 9 were as dense as the more anodal fractions.

5.4 CONCLUSIONS

The lactate dehydrogenase isozyme shift was the least convenient
of the 4 protein systems studied in this thesis. The need for 2 or 3
treated-tadpole hearts would have required more material than was
available if the system had been studied further. Nevertheless the
results obtained are striking in that goitrogen-retarded larvae
possessed adult-like LDH patterns in their heart muscle. The marker
used, an increase in the concentration of the more cathodal isozymes
by comparison to the stable concentrations of the anodal components,
was convenient in practice. This increase in cathodal isozymes and
presumably in the cathodal subunit is similar to that observed by
Manwell and Baker (1971) in R. esculenta and R. temporaria normal meta-
morphosis.

Maclean (1976) has discussed the regulation of such LDH shifts
during ontogeny. They may be due to a combination of increased syn-
thesis and increased degradation of subunits. The evidence in non-
amphibian vertebrates suggests a genetic regulation of the 2 subunits.
In addition, Fritz et al (1969) found that LDH half-lives in the rat
were 1.6 days (heart), 31 days (skeletal muscle) and 16 days (liver).
Thus changes in degradation rate may well vary not only from tissue to
tissue in the adult animal but in a single tissue during development.
Claycomb and Villee (1971) used metabolic inhibitors in X. laevis and
suggested that the appearance of new isozyme during development is
controlled by a presynthesised RNA template which was stored until
being translated shortly before the appearance of the new protein.
The mechanisms of translation control and messenger storage were unknown.

However it is also known that direct metabolic enzyme-pattern
responses in LDH can occur in lower vertebrates. Tsugawa (1976)
worked on changes in the LDH patterns of Xenopus cells exposed to
different temperature regimes in vitro. There was a cathodic increase
in isozyme induced by cold adaptation.

Manwell and Baker (1971) pointed out that LDH isozyme shifts at
metamorphosis might be a response to altered oxygen levels. In the
chick embryo Lindy and Rajasalmi (1966) showed that the relative amounts
of A and B chains present can be altered by oxygen level independently

FIGURE 5/2

Comparison by polyacrylamide disc gel electrophoresis of the
lactate dehydrogenase isozymes from the hearts of 9 different
adult female Xenopus laevis.

The gels are aligned on the most cathodal band, No. 9,
60 mg of washed ventricle from each heart were
homogenised in 800 µl ringer.   10 µl of homogenate
were applied to each gel.
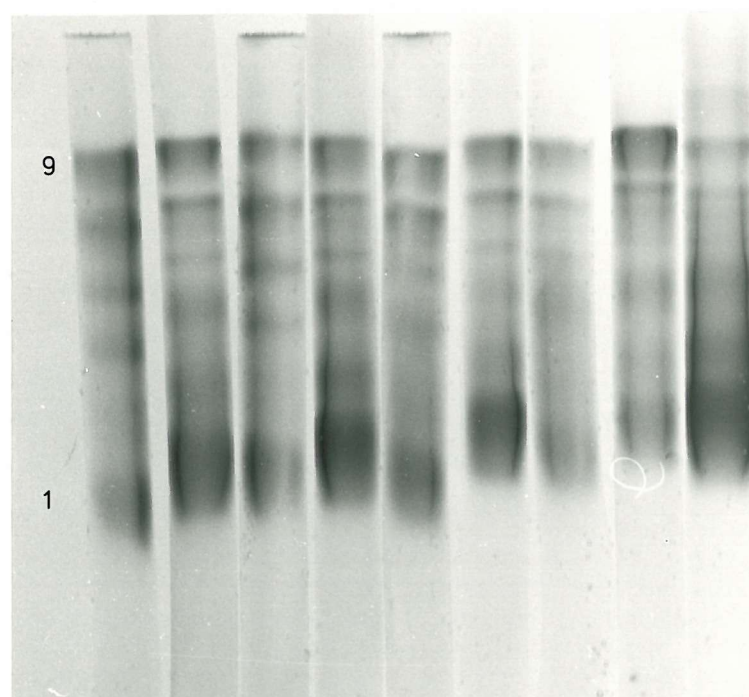
of other embryological events. The complexities of arguments concerning respiratory changes at metamorphosis in anurans have been pointed out in chapter 3. This is emphasised by the fact that both Xenopus and Rana tadpoles have been shown to contain LDH isozymes composed mainly of the anodal subunit (B-type in other vertebrates) which Kaplan (1964) considered to be more suited in its kinetic properties to metabolism in obligatorily aerobic tissues. The ontogenetic LDH transition of the mouse (Markert and Ursprung, 1962) is the opposite, from predominantly LDH_5 (A4) 9 days before birth to predominantly LDH_1 (B4) in adult heart.

There was no evidence in the present work that the LDH transition is primarily controlled by thyroid hormones. In Rana temporaria Lagerspetz et al (1974) found that the thyroid mediated compensatory changes in oxidative metabolism during temperature acclimation but the nature of the molecular enzymes was not investigated. Heinemann and Weber (1966) found the oxygen uptake of Xenopus tails to increase during spontaneous and induced metamorphosis so it is conceivable that metamorphic changes in morphology induced by TH necessitate a change in LDH pattern. In the present work with goitrogen - retarded larvae it seems that this is unlikely as Fletcher and Myant (1960) showed that oxygen consumption was unaffected by propylthiouracil-arrest in X. laevis larvae.

Although well-understood genetically, in vertebrate classes, the control of ontogenetic LDH transitions is obscure. This thesis has not supported the contention that thyroid hormones exert a central role in the control.

A recent isozyme study on quite a different enzyme, lysozyme, makes an interesting comparison. Ostrovsky and Nace (1976) studied changes in the isozyme pattern of Rana pipiens and found 8 isozymes in larvae. In prometamorphic larvae, cathodal bands were absent from polyacrylamide gels. After climax these appear and there is an increase in tissue isozyme concentration. When tadpoles were arrested at Taylor-Kollros stage14, by 0.05% thiouracil, they continued to grow. Their lysozyme isozymes and lysozyme activity were compared with normal stage 14 tadpoles and untreated siblings which had reached TK stage 25. It was found that the cathodal isozymes had not appeared and the electropherograms were identical to stage 14 controls. However the total enzyme

activity had increased as in untreated stage 25 siblings. Thus it seemed
that thyroid blockage had prevented the appearance of the new isozymes
while a compensatory increase occurred in the original isozymes as the
animals increased in size (Ostrovsky, 1976). This appears to be a quite
different situation to that observed in this chapter with propylthiouracil
treatment. It may be that a total LDH activity increase, brought about
only by increased synthesis of the anodal components, is not possible.
The shift in treated larvae may be merely a response to increased size
perhaps mediated by changes in respiration as body volume increases
faster than the skin area involved in cutaneous respiration. There were
insufficient data here to look for a correlation with body size such as
was carried out in Chapter 3 (haemoglobin) and Chapter 4 (coelomic fluid
albumin).

CHAPTER 6

The lens crystallin transition of developmentally
- retarded Xenopus laevis larvae

6.1 INTRODUCTION

The advantages of the vertebrate lens as a system for differen-
tiation studies have been reviewed by Clayton (1970). The lens is an
isolated tissue without blood vessels or stroma. It is composed
entirely of an epithelial layer and fibres derived from that layer.
Thus one is not dealing with a structure containing many different cell
types. As a source of material for electrophoretic studies the lens is
ideal as its' protein content is high (about 35%) and between 80 and 90%
of this consists of the lens structural proteins, the crystallins. The
latter are soluble, whereas the other major class of lens proteins, the
albuminoids, are insoluble. The few remaining proteins are enzymes such
as esterases and LDH (Papaconstantinou, 1967) but these comprise a small
percentage of the total protein.

The crystallins are classified on a basis of decreasing molecular
weight and electrophoretic mobility, as alpha-, beta- and gamma -
crystallin, in the amphibian. All classes of crystallins in vertebrates
are heterogeneous being composed of aggregates of more than one type of
subunit. As a result each major crystallin class has a range of mole-
cular weights and electrophoretic mobilities. Changes in the electro-
phoretic mobility of the individual crystallins during ontogeny is
thought to be due to changes in subunit composition (Clayton 1970).

Although the lens is simple with respect to the number of cell
types the polymeric nature of the crystallins makes them rather complex
biochemically. Little work has been carried out to determine the sub-
unit compositions of the major crystallin classes in Amphibia but work
on other vertebrates demonstrates the propable complexity of subunit com-
positions. For instance, there is evidence for at least four different
peptide chains in chicken alpha-crystallin. In the chicken beta-crys-
tallins, there appear to be from 8 to 10 subunits, depending upon the
method of separation used (Clayton et al, 1968, Clayton, 1969). The
pattern observed after immunoelectrophoresis suggests that at least one
antigenic determinant is distributed throughout the mobility range but
is complexed with a range of constituents which differ both antigenically

and electrophoretically (Clayton and Truman, 1967). Avian lenses do
not contain gamma crystallins but in cattle these low molecular weight
moieties appear to be single protein chains of which there are 5
different types.

Maisel and Goodman (1965) used two-dimensional starch electrophore-
sis to demonstrate the three main crystallin classes of an unnamed frog
species. There were 2 alpha-, 5 beta- and 6 gamma- crystallins. In
Rana pipiens, McDevitt (1967) found four gamma components using DEAE-
cellulose collumns. These were apparently undissociated polymers as
similar results were obtained with PAGE using tris - HCI buffers. The
alpha-crystallins of Rana temporaria, Bufo bufo and Xenopus laevis
adults were dissociated by electrophoresis with 6M urea by De Jong et al
(1976). As in the cow, two types of polypeptide chains were distin -
guished, the acid A- and more basic B- chains. Additional cathodal
bands were also present which were thought to be due to contamination
with beta components. Manwaring (1972) also dissociated Xenopus adult
lens soluble proteins with 8 M urea. Eleven components were observed,
the same number as non-dissociated crystallins separable by PAGE. At
stage 50/52 only 6 subunits were visible. The other 5 bands (3 of which
were the most anodal components of the adult pattern) appeared sequentially
during prometamorphosis and climax. Further work on amphibian crystallin
subunits remains to be carried out. The remainder of this chapter
refers only to crystallin polymers of the three major classes, alpha-,
beta- and gamma-, separable by electrophoresis with buffers lacking urea.

In urodelans, Brahma and van Doorenmaalen (1969) separated Ambystoma
mexicanum and Triturus cristatus crystallins by agar electrophoresis.
All 3 major classes were present. Complete immunological cross-reactivity
between these antigens and a Xenopus laevis lens antiserum was demonstrated
Similarly Nöthiger et al (1971) found complete cross-reactivity purified
R. pipiens and Notophthalmus viridescens gamma-crystallins using the
appropriate antisera.

During embryonic lens formation the cells of the lens vesicle,
nearest the future retina, elongate and eventually become the primary
fibre cells. The primary fibres remain as the nucleus of the adult lens.
Further growth of the embryonic and larval lens results from cells of the
equatorial germinative region of the epithelium elongating to become the
secondary fibres of the lens cortex.

The major crystallin classes are thought to appear, in detectable quantities, sequentially during lens development. Takata et al (1964) produced immunofluorescence data which showed that the gamma-crystallins are characteristic of early fibre development in Triturus viridescens. McDevitt et al (1969) performed similar studies with Rana pipiens and found gamma-crystallins to be present in quantity in the embryonic primary fibres. Some variation according to the technique used, was apparent. The work mentioned involved the labelling of lens sections whereas in Ouchterlony tests (McDevitt, 1968) small quantities of beta-crystallin were also detectable in Shumway Stage 25 embryonic lenses. In Xenopus laevis later work confirmed that the gamma-crystallins were the first to appear (McDevitt and Brahma, 1973).

The appearance of the major crystallin classes and of the various proteins within each class continues throughout larval development and metamorphosis. This means that the system was potentially a useful one for studying biochemical markers of developmentally-retarded larvae in the present work.

McDevitt (1968) continued his immunofluorescence studies and first detected the beta-crystallins in Stage I (Taylor and Kollros, 1946) Rana pipiens larvae. At stage XXIV, the alpha component first appeared and the complement of crystallins was then apparently adult-like. Polansky and Bennett (1970) used cellulose acetate electrophoresis to study the crystallin transformation at metamorphosis in Rana catesbeiana. They found the gamma-crystallins to be in higher concentrations in the nucleus whereas in the cortex the alpha proteins were more apparent. The electropherograms of the whole larval lens protein and adult lens nucleus proteins were similar. The differences apparent at metamorphosis were in the alpha components of the cortex. Both Manwaring (1972) and Brahma and Bours (1972) studied Xenopus laevis. The former author noted only gamma crystallins from stage 36 to 42 after which the beta components were detectable by immunoelectrophoresis. The alpha crystallin arc was first detected in stage 48/50 lenses. The complexity of the immunoelectropherograms in all regions increased through metamorphosis into the adult in which at least 13 antigens were observed. Brahma and Bours (1972) found the relative concentration of the alpha- and beta- classes, as estimated from densitometric scans of thin-layer isoelectric focussing plates, to increase through metamorphosis. The

concentration of beta proteins approximately doubled whereas that of the alphas increased by a factor of fifteen. The gamma component decreased somewhat in total concentration. By way of contrast, McDevitt and Collier (1975) were unable to detect differences between larval and adult individuals of some five North American Salamander species.

The only hypothesis on the adaptive value of the crystallin transition which has appeared is that of Clayton (1970). She argued that, as Goldman (1964) had shown the refractive index of the bovine lens to increase towards the centre, the different crystallin types are required for maintenance of correct refraction as the lens grows in size. Thus it would be predicted that, irrespective of developmental stage and duration of goitrogen treatment, larvae with lenses of equal size would possess similar crystallin patterns. This would seem to suggest an interesting type of gene control being regulated by body size or lens size. This point is discussed in section 6.4 and in Chapter 8.

To separate the small lens crystallin samples obtainable from Xenopus larvae electrophoresis was again the preferred method. Two types were employed, PAGE and IEP (one - and two - dimensional).

6.2 EXPERIMENTAL

Similar basic problems attended this work to those which were discussed in Chapter 3 on haemoglobins. These problems were:

a) identification of the proteins separated electrophoretically as crystallins,
b) identification of a given crystallin band as, for example, band No. 8 (nomenclature is described below),
c) assessing the concentrations of the different crystallins.

The first problem was minimal because, as discussed in section 6.1, over 90% of extractable lens proteins are crystallins. Other proteins are unlikely to be present in detectable concentrations at electrophoretic mobilities similar to those of the major fractions, which must be crystallins.

The soluble lens crystallins studied were all obtained by homogenis-

-ing whole lenses in Rugh's ringer. After centrifugation the supernatant was removed and, if not used immediately, was stored at -20°C in small batches. Most of the PAGE separations included DTT in the crystallin solution, gel and running buffer to prevent protein polymerisation. However, it was found that DTT gives a colour reaction with the Folin and Ciocaltheu reagent used in the Lowry protein determinations. For this reason DTT was not used in the IEP experiments in which the total protein concentrations of all crystallin solutions was measured. This is unlikely to have been important as no difference was detectable between PAGE separations of crystallins with or without DTT.

The first PAGE separations were made on disc gels for greatest resolution. The adult crystallin electropherogram was compared to that figured by Manwaring (1972) and the nomenclature used to identify the bands was worked out. Most of the subsequent experiments were performed with the Raven slab apparatus which made identification of individual bands simpler as adult, normal tadpole and treated-tadpole extracts were separated on the same gel.

Ten PAGE experiments were carried out, 8 of which included crystallins from propylthiouracil-treated tadpoles. The controls used were crystallin solutions from six separate adult females, one adult male and pooled crystallin from normal stage 50/52 tadpoles, normal stage 53 tadpoles, normal stage 54/55 tadpoles and newly metamorphosed toadlets.

At the time when the polyacrylamide gel experiments were performed the modified Lowry protein estimation method (section 2.18), needing only 5 μl of sample, had not been worked out. To obtain crystallin solutions of a sufficiently high concentration to give clear bands it was necessary to homogenise the lenses in small volumes of ringer (about 100 μl). It was decided not to use 20 μl of this for protein estimation. Instead the lenses were weighed and equal wet weights of say, adult and tadpole lenses, were used to prepare homogenates. Although the proportion of soluble protein per lens may vary this was unlikely to cause serious differences in total protein between samples. Particular attention was paid to the width and density of protein bands within a single separation. Thus one can state that a given band or bands contain a large proportion of the total crystallin in that sample if their widths and densities are much higher than those of any other band. To quantify this assessment most gels were scanned densitometrically with the Joyce - Loebl 'Chromo-

scan'. This machine provided pen traces which supported judgements made
visually but, because of variable background staining, full quantifica-
tion was not reliable. General protein stains detect material in the
interband regions which is especially obvious near the origin. The
material causes a variable densitometric baseline such that estimations
of band density by measuring area under the pen-trace peak were un-
reliable. This was especially true with less well-defined peaks which
merged imperceptibly into the back-ground trace such that an arbitrary
definition of the limits of the peak were necessary. This was found to
give poor repeatability with replicates of the same sample.

Both in the present study (section 6.3) and in previous work changes
in lens crystallins during late premetamorphosis and to the end of climax
were mainly quantitative. By concentrating on certain well-defined
bands (section 6.3) adequate markers were found. Both visual and densi-
tometric assessment relied upon comparing the relative density of these
marker bands in treated tadpoles with the same markers of the normal
crystallin transition observed in controls.

Campbell et al (1968) and Manwaring (1972) had used qualitative IEP
to study the appearance of new lens antigens during the metamorphic
period. Manwaring's work suggested that sufficient markers ought to
be available for this more sentitive technique to be useful. In addi-
tion, when an antiserum had been raised by immunising a rabbit with an
adult toad lens homogenate prepared from carefully cleaned lenses, a more
specific method of identifying crystallins was available than with a
general protein stain. The antiserum was tested initially in micro-
capillary tubes and was found to have a titre of 1/320. Qualitative
IEP against adult lens homogenates on Ionagar slides produced a preci-
pitin arc pattern similar to that of Campbell et al (1968). Because
Ionagar is no longer available commercially, later IEP experiments
used Litex HSA agarose. The results obtained with this medium were
somewhat different in minor detail but quite satisfactory for the present
purpose.

The first group of experiments compared the crystallin band and
antigen patterns of treated larvae with those of animals metamorphosing
spontaneously. The treated larvae were raised either alone or in small

groups with about 2.5 l of goitrogen solution per tadpole. To test
Clayton's hypothesis that lens diameter and crystallin transition are
connected further treated larvae were raised under crowded conditions.
About 60 tadpoles were placed in a single aquarium containing 20 l of
0.01% w/v propylthiouracil solution. Some of these died subsequently
but there was approximately 0.4 l of solution per animal during most of
the rearing period. These crowded larvae were arrested as usual by
the goitrogen but their growth was much less than that of uncrowded
specimens. The crowded larvae are referred to subsequently as 'stunted
giants' as they were larger than normal stage 54/56 larvae but smaller
than the uncrowded group which are referred to as 'large giants'.
'Large giants' had lengths of about 5-6.4 cm (0.62-1.10g wet weight)
whereas 'stunted giants' were about 4 cm long and weighed about 0.3 to 0.7g.
Normal stage 54 tadpoles are about 3.5 cm long. The distinction
between these groups was made statistically on the basis of lens diameter
rather than body size _per se_.

Both groups of treated larvae were retarded in late premetamorphosis
and differed mainly in body size. The growth of the lens during normal
metamorphosis was studied by measuring the diameters of a series of
lenses from stage 48/49, 50, 51, 52, 53, 54, 55, 56, 57, 58 and 59 tad-
poles. In addition toadlet and adult toad lenses were measured
immediately after lentectomy. Differences between stage mean diameters
were compared statistically. In addition they were compared to the mean
diameters of the 'stunted' and 'large giant' lenses.

Crystallin solutions from stunted and large giant lenses were
compared by means of qualitative IEP. The crystallin solutions used
had been adjusted to 6 mg/ml total protein. To do this the total protein
concentration of each original supernatant was measured with the scaled-
down Lowry method. The volume of the supernatant was determined
gravimetrically. The solution was then freeze-dried and made up again
with distilled water to the final volume necessary to give a 6 mg/ml
protein solution. The degree of concentration was less than 50% and so
the solutions were not dialysed to decrease the ionic concentration.

It seemed possible that during freeze-drying and reconstitution the
crystallin subunits might dissociate and recombine in an abnormal manner.
A control experiment was performed in which adult toad crystallins were

FIGURE 6/1

Polyacrylamide disc gel electrophoresis of <u>Xenopus</u> lens crystallins

A) Diagram showing the nomenclature used. Asterisks mark
the crystallin bands employed as metamorphic markers

B) Photographs of disc gels

i) Adult female lens crystallins (2 lenses)

ii) St. 55 propylthiouracil treated tadpole (2 lenses).
This animal had a wet weight of 1.17 g.

iii) Two St. 55 propylthiouracil treated tadpoles (4
lenses). These animals had wet weights of 0.48
and 0.63 g.

iv) Normal stage 53 tadpoles (6 lenses)
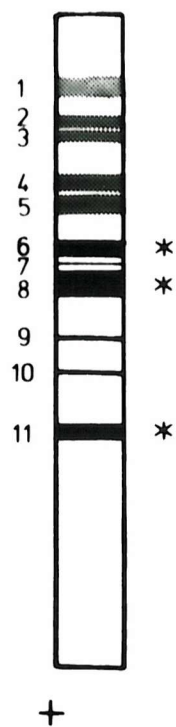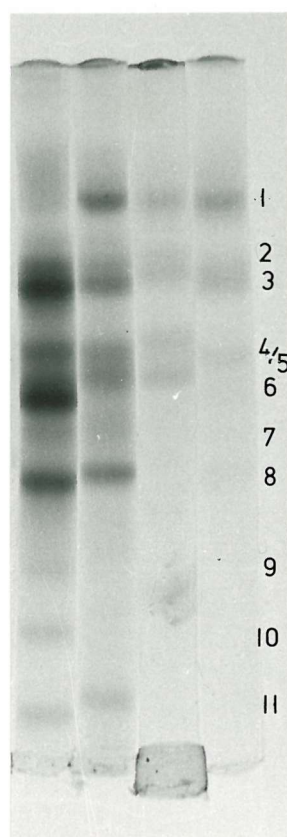
FIGURE 6/2

Polyacrylamide slab gel electrophoresis showing the crystallin
transition during normal metamorphosis.

    A) Premetamorphic tadpole crystallins compared with those
       from adult males

        a) Normal st. 50/52 tadpoles (18 lenses)

        b) Adult males (4 lenses)

    B) Premetamorphic tadpole crystallins compared with those
       from an adult male and an adult female

        c) Normal st. 54/55 tadpoles (12 lenses)

        d) Adult male (1 lens)

        e) Adult female (1 lens)

       The gels are stained with Naphthalene Black 12B.
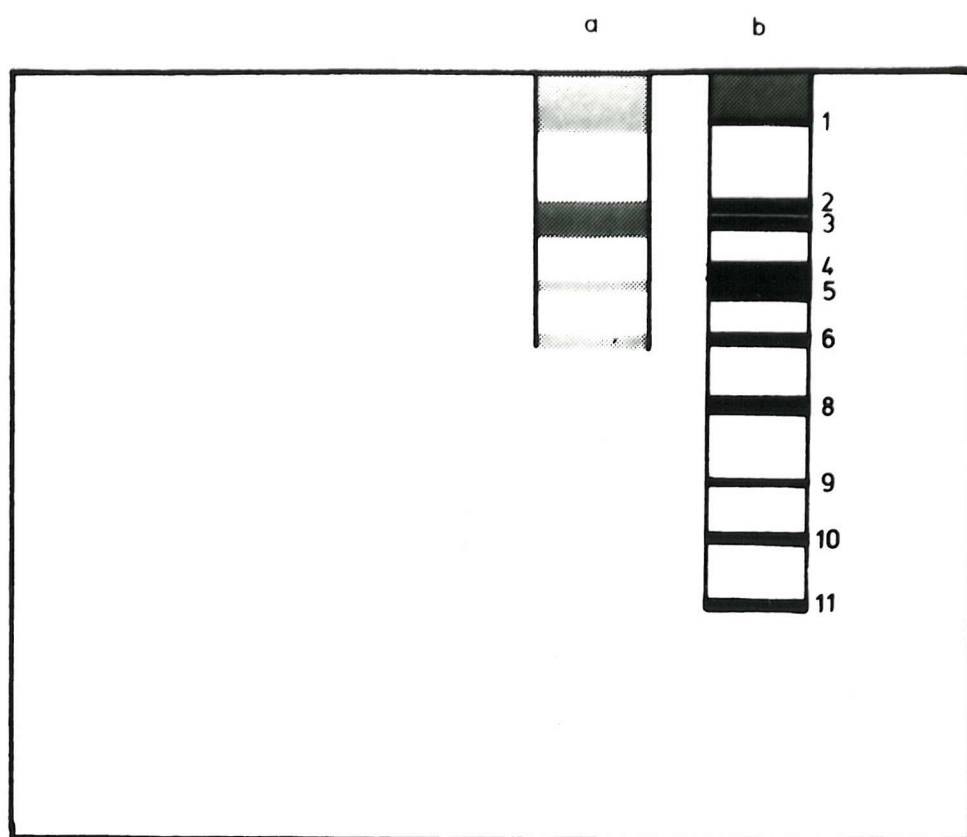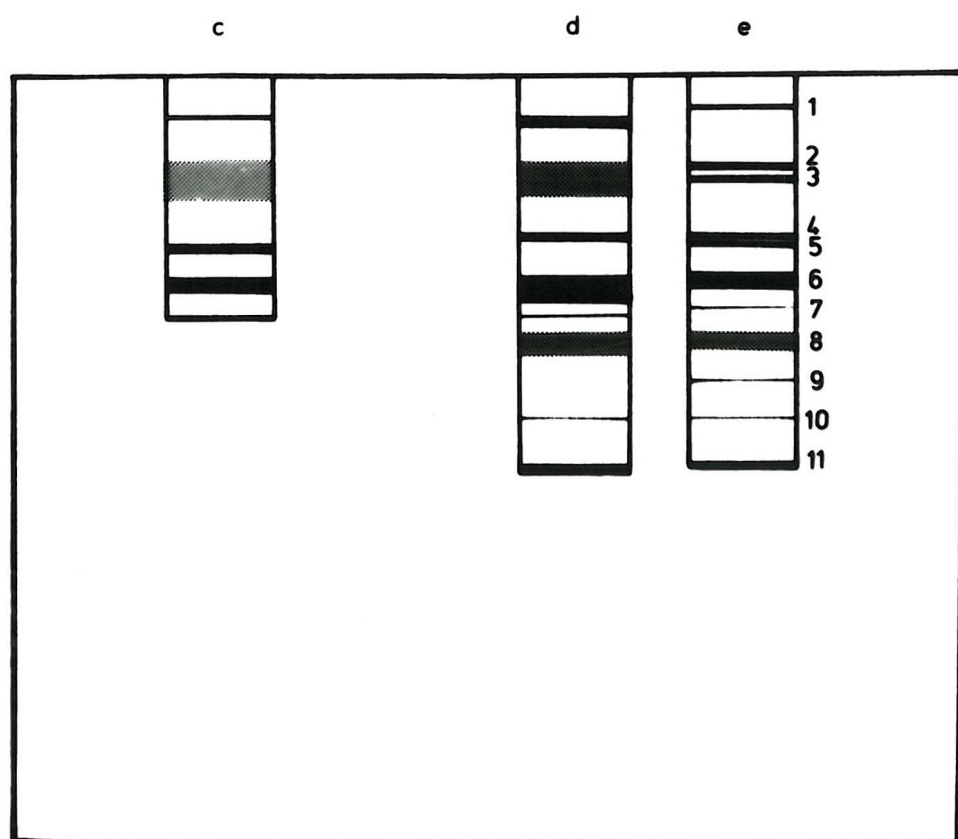
Table 6/1. Propylthiouracil - treated tadpoles used to prepare lens crystallin solution for polyacrylamide gel electrophoresis. Also summarised are the categories of crystallin, haemoglobin and LDH patterns of individual tadpoles. Protein pattern categories are defined in the text.

Stage	Wet Weight (g)	Length (cm)	Lens diameter (mm)	Duration of goitrogen treatment (weeks)	Crystallin pattern category	Haemoglobin pattern category	LDH Pattern category
55	0.63	4.1	-	8)	Intermediate	-	-
55	0.48	3.4	-	8)			
54	1.37	6.7	-	23	Adult	Adult	-
55	0.56	4.3	-	8.5)	Tadpole	Tadpole	-
55	0.39	3.6	-	8.5)			
54	1.11	-	-	23.5	Adult	Adult	-
54	1.17	5.9	-	24)	Adult	Tadpole	Adult
54	1.25	-	-	24.5)			
54	1.27	-	-	29	Adult	Intermediate	Adult
54	0.63	5.0	1.0	41)		-	-
53	0.89	5.4	0.95	41)		-	-
56	0.93	5.9	1.15	41)		Tadpole	-
54	0.62	5.2	1.05	41)	* Adult	-	-
56	0.78	5.5	1.15	41)		-	-
56	1.33	6.4	1.10	41)		-	-
55	1.10	6.4	1.15	41)		-	-
56	0.95	6.0	1.15	41)		-	-
56	1.59	-	1.25	65	Adult	-	-
53	0.90	-	1.05	59	Adult	-	-

* This pooled group was used to provide the 'large giant' crystallin sample used in immunoelectrophoresis.

separated simultaneously on 14 disc polyacrylamide gels. Two gels
were rapid-stained for protein to identify the crystallin band positions.
The unstained gels were cut in two such that the upper sections contained
the 5 more cathodal bands and the bottom sections contained the 6 more
anodal ones. Protein was eluted from the two groups separately and the
eluate was dialysed, freeze dried and made up to a final concentration of
6 mg/ml. The samples were separated again on disc gels. This con-
firmed that random reassociation had not taken place as the gels with
cathodal eluates contained only cathodal bands while gels with anodal
eluates contained only anodal bands.

It was desirable to correlate those bands (Nos. 6, 8 and 11 in
section 6.3) on polyacrylamide gels, which were used as metamorphic
markers, with the antigenic markers detected by IEP. Bands 6, 8 and 11
were located on unstained adult crystallin gels by comparison with
stained gels. These bands were sliced out and the proteins eluted from
a number of slices in each case. After dialysis, freeze-drying and
adjustment to 6 mg/ml total protein the samples were separated by quali-
tative IEP on microscope slides. The precipitin arcs obtained were
compared to the pattern of arcs observed when whole adult crystallin
supernatants were used as antigen solutions.

The two-dimensional IEP method described in Chapter 4 was modified
in an attempt to quantify the differences in antigen concentration
observed in the qualitative IEP experiments. 1% Litex gels with a
tris - EDTA - boric acid buffer of pH 9 were used. Preliminary experi-
ments showed that the formation of peaks was less satisfactory than with
coelomic fluid and serum proteins (Chapter 4). Additional preliminary
experiments were performed to check that the crystallins were not break-
ing down under prolonged electrophoresis and that the antiserum was not
denatured by incubation at $55^{\circ}C$. Finally an antigen - antiserum ratio
was found that would precipitate separate alpha- and beta- crystallin
peaks on two-dimensional plates. Samples of normal stage 54, stage 57
and adult crystallins were compared to those from 'large' and 'stunted'
treated tadpoles.

6.3 RESULTS

Soluble crystallin extracts from adult Xenopus toads show 11 bands
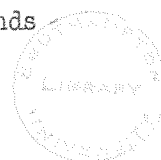
on polyacrylamide gels (Fig 6/b).   This is in agreement with Manwaring
(1972).   However Manwaring drew the bands evenly spaced and of even
width.   This was never the case in the present work.   Bands 7, 9 and
10 are often very faint.   Also bands 2 and 3 and bands 4 and 5
frequently appear to fuse as 2/3 and 4/5.   There is some variation in
minor detail from gel to gel even with a crystallin solution from a
single toad.

Certain features are quite constant in adults and these can be used
as 'landmarks' especially when comparing disc gels within one experiment
or slab gels from different experiments.   Bands 6 and 8   are the
most intense in the middle region of the crystallin electropherogram.
Band 11, the fastest migrating, is also a good reference.   Using these
reference bands it is simple to number the remainder according to the
nomenclature shown in Fig 6/1a.

Bands 6, 8 and 11 (marked with asterisks in Fig 6/1a) were also those
found to change most during development.   Figs 6/1(b) and (c) show that
the relative concentrations of Nos. 6, 8 and 11 are much lower (in this
case they were barely visible but in some gels more prominent faint bands
were present) in normal stage 53 lenses than in adult lenses.

In the same experiment, 2 separate samples extracted from propyl-
thiouracil - treated tadpoles of different body sizes were compared to
the controls.   Fig 6/1 d shows the crystallin bands of the larger giant
(treated for 23 weeks) to be adult-like with respect to the concentration
of Nos 6, 8 and 11.   The smaller treated tadpoles had been development-
ally-arrested for only 8 weeks and were intermediate in crystallin
pattern between normal stage 53 and adult.   Only band 11 had increased
markedly in concentration (Fig. 6/1 e).

The fact that the bands used as "landmarks" for nomenclature were
also the ones which changed most during metamorphosis was a potential
problem.   Thus, most of the subsequent experiments were made on slab
gels which facilitated direct comparison of bands from adjacent samples.

A typical crystallin transition in untreated X. leavis is shown in
Fig 6/2 together with densitometric traces.   In this case bands 8, 9, 10
and 11 were not visible on the stage 50/52 strip when the gel was stained

FIGURE 6/3

Polyacrylamide slab gel electrophoresis and chromoscan traces
of lens crystallins from adult, toadlets, normal tadpoles and
propylthiouracil treated tadpoles.

a) Adult female (2 lenses)

b) Normal st. 54/55 tadpoles (18 lenses)

c) Toadlets (8 lenses)

d) Normal st. 54/56 tadpoles (20 lenses)

e) Adult female (1 lens)

f) St. 54 propylthiouracil treated tadpoles
(6 lenses).
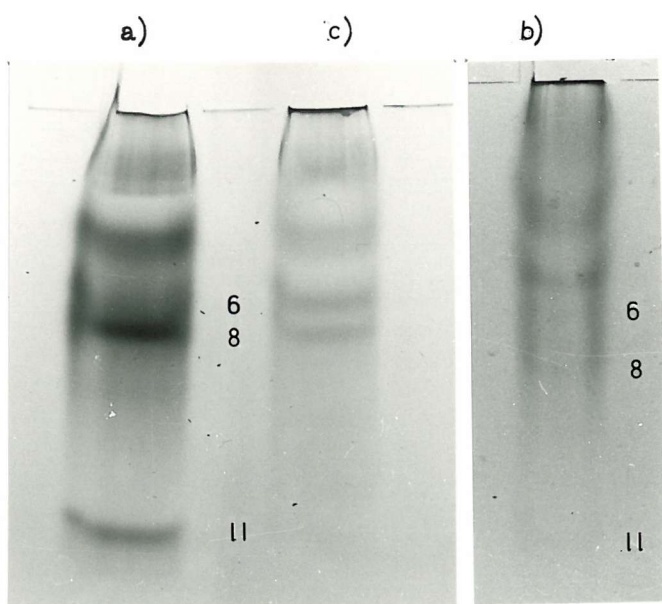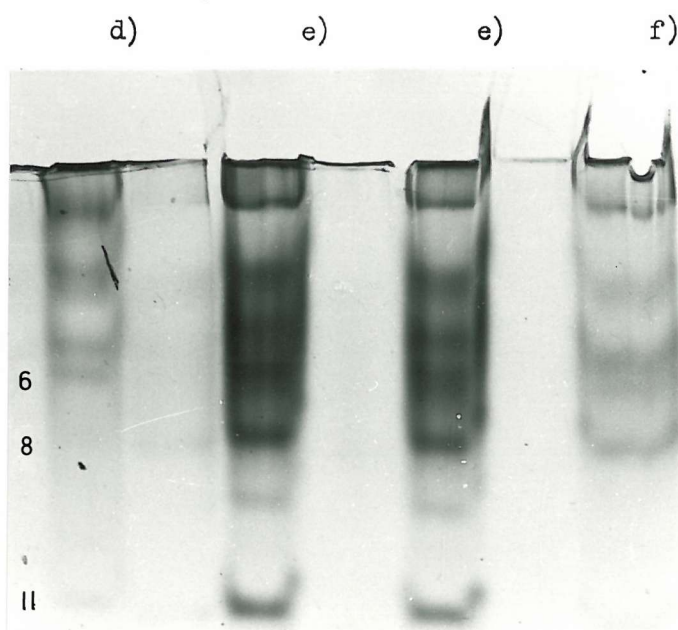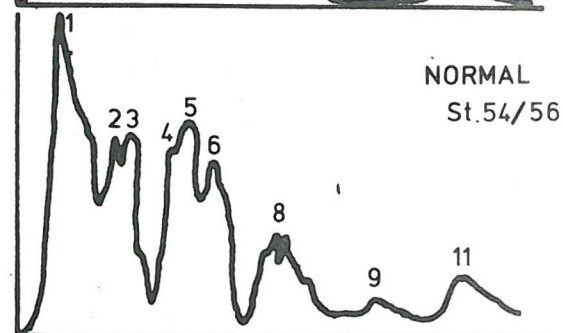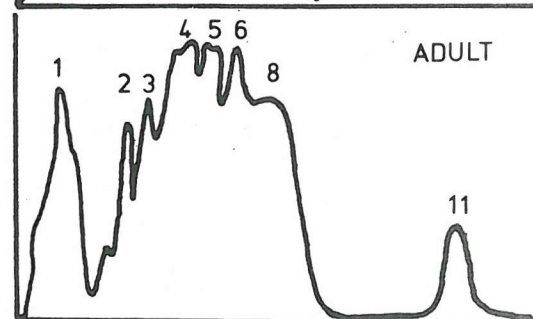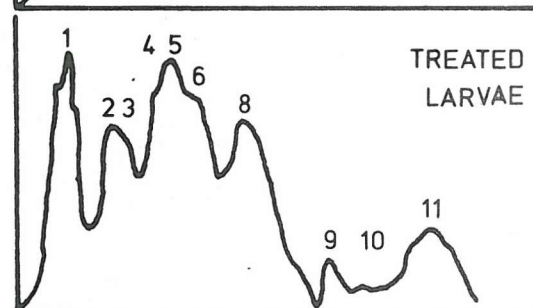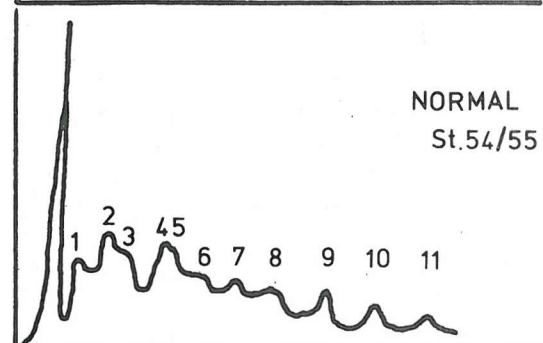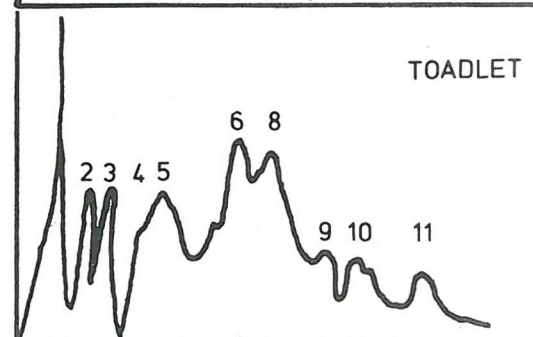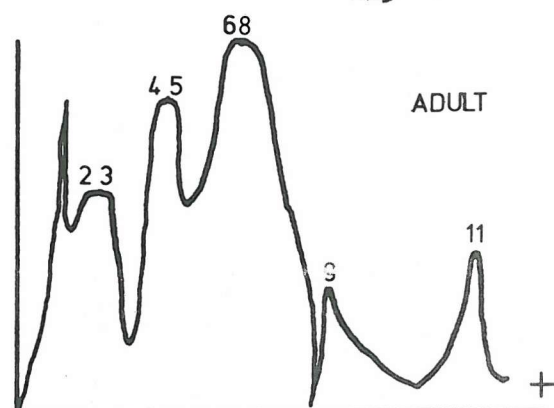
FIGURE 6/4

Comparison of the adult _Xenopus laevis_ lens crystallin antigens precipitated in qualitative immunoelectrophoresis (drawings of typical slides).

a) Ionagar electrophoresis gels.

b) Litex HSA electrophoresis gels.

a = alpha crystallin.

b = beta crystallin.

g = gamma crystallin.

0 = origin (sample well)
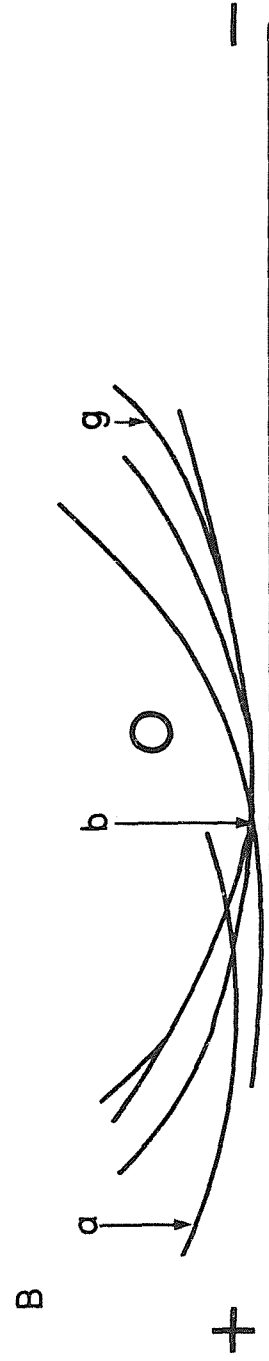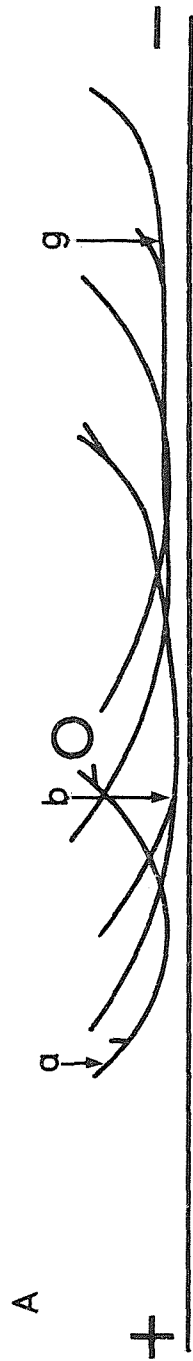
with Naphthalene black. In this experiment the adult crystallins came
from a male toad. A further expriment demonstrated that there was
little difference between the crystallins of an adult male and an adult
female (Fig 6/2). The series of 7 adult toads studied by PAGE showed
no evidence of a lens crystallin polymorphism and it was assumed that
polymorphism did not occur in tadpoles. It was not practicable to
compare the crystallin patterns of individual normal tadpoles.

Table 6/1 lists the treated tadpoles used as crystallin donors in
PAGE experiments. Only 2 crystallin pools used tadpoles immersed in
propylthiouracil for short periods (8 weeks). Neither of these had
completed the crystallin transition. All other crystallin patterns
were of the adult type. A further example is shown in Fig. 6/3. Both
the slab gel and Chromoscan traces show bands 6, 8 and 11 to be adult-
like in relative concentration.

The timing of the normal shift was of interest. Manwaring(1972)
showed that a gradual transition occurred from premetamorphic to climax
larvae. To investigate the problem further samples from an adult
female and normal stage 54/55 tadpoles were compared with a pooled
sample from newly-metamorphosed toadlets (Fig 6/3). The toadlet appears
to be intermediate between the tadpole and adult patterns. In fact,
the gel of a sample from propylthiouracil - treated tadpoles in the same
figure had crystallins 8 and 11 of slightly greater relative concentra-
tion than those of the toadlet.

Immunoelectrophoretic separations of adult toad lens crystallins
gave results similar to those of Campbell et al (1968), when carried out
with 'Ionagar' agar-coated slides (Fig 6/4a). The major classes of
crystallins are labelled. Campbell et al (1968) introduced a complex
numbering system for Xenopus lens antigens but this has not been necessary
here. Their Group 1 is the alpha-crystallin arc which appears to share
no common antigenic determinant with the other crystallins. The alpha-
arc crosses the arcs of the beta- group without fusing with them. Group
5 (sensu Campbell et al) is the beta-group which is a complex one.
Groups 7 and 8 correspond to gamma-crystallins and they are also complex.
It seems that there may be shared antigenic determinants between beta- and
gamma- crystallins (Litex agar slides) but this was not supported by the
Ionagar slides on which the beta- and gamma-arcs appear to cross. There
was no evidence for a common antigenic determinant throughout the mobility

range as in the chick (Clayton and Truman, 1967). Campbell et al (1968) also detected minor components which did not fit the normal classification of mobilities and which were immunologically distinct from the main crystallin groups. These components may have been enzymes detected in spite of their low concentrations by a very potent antiserum. No such precipitin arcs were detected in the present work. On Litex-coated slides the precipitin arc pattern was more compressed even when electrophoresis was carried out for longer times than with Ionagar. The major crystallin classes on Litex agar are shown diagrammatically in Fig 6/4 and photographically in Fig 6/5.

The alpha-crystallin component was found to be absent from normal pre- and pro- metamorphic lens homogenates (Fig 6/5). The antigens precipitated in each case were a single arc in each of the beta- and gamma- positions. The absence of an alpha- component and the lack of complexity of beta- and gamma- crystallins were the metamorphic markers used with IEP to indicate pre-climax stages.

The PAGE results had pointed to the possibility that body size and therefore lens size was an important factor in determining the crystallin pattern. The variation in protein pattern, observed in haemoglobin and albumin, was absent in animals treated for over 8 weeks in uncrowded conditions. To test the correlation with size two groups of treated tadpoles, 'large giants' and 'stunted giants' were used. Lenses removed from these animals were quickly measured before homogenisation and their diameters compared statistically. Both groups had been treated for about 35 to 41 weeks. In addition their diameters were compared statistically with the diameters of lenses of normal tadpoles and toadlets; a) of similar morphological stage to the 'giants' and (b) of similar lens diameters to the 'giants'. The lens diameters appear in Table 6/2 and graphically in Fig 6/6. Lens diameter increased steadily from a mean of 0.47 mm in stage 48/49 to 0.91 mm in stage 59. There was insufficient material available to study later climax stages but a series of young toadlets had a mean lens diameter of 1.02 mm. Sixteen lenses from adult females had a mean diameter of 2.00 mm.

Fig 6/7 shows diagrammatically the statistical comparison of propylthiouracil - treated lens with normal lenses. The 'average stage'

FIGURE 6/5

Qualitative immunoelectrophoresis, using a rabbit anti-adult
Xenopus crystallin serum, of crystallin solutions from normal
Xenopus illustrating the metamorphic antigen transition.

    i)   Adult female crystallins.

    ii) Normal stage 57 crystallins.

   iii) Normal stage 54 crystallins.

    iv) Normal stage 57 crystallins.

           a = alpha crystallin

           b = beta crystallins

           g = gamma crystallins
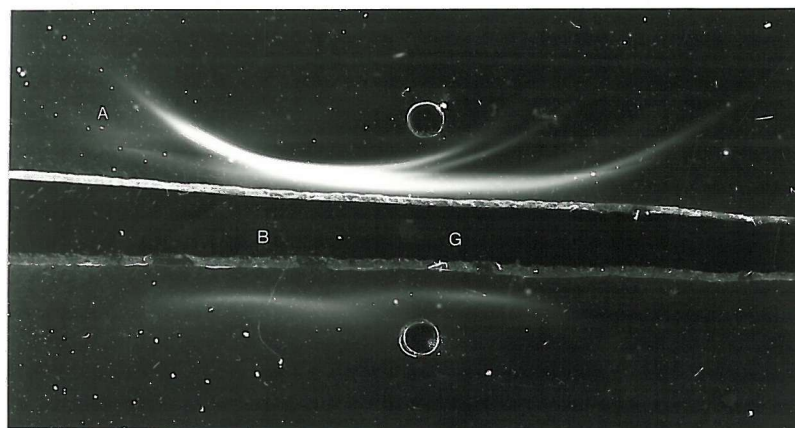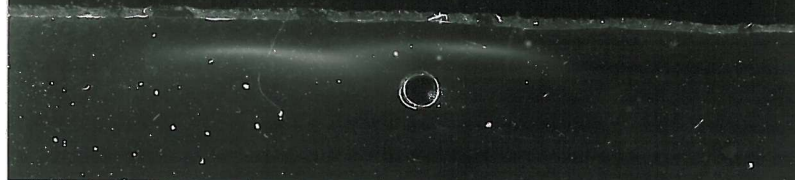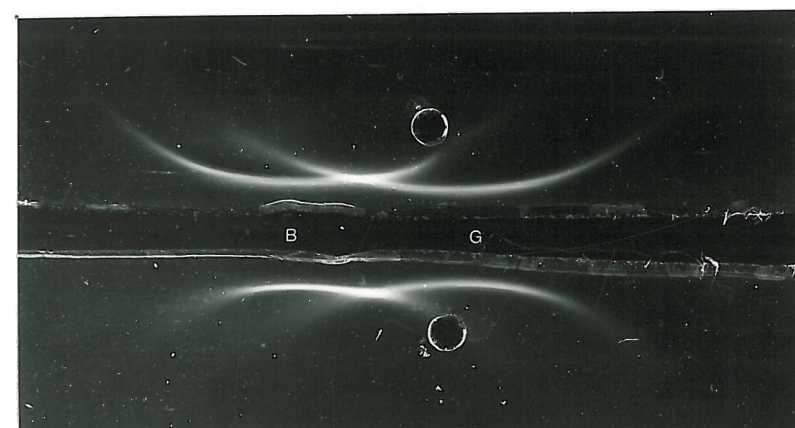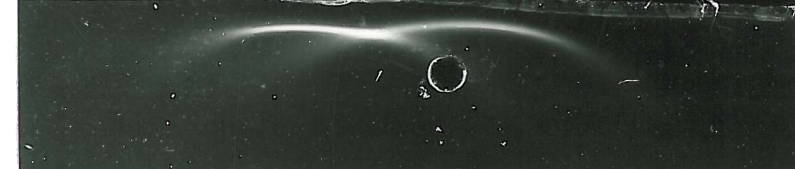
Table 6/2. Lens growth during the development of <u>Xenopus laevis</u> and
the lens diameters of propylthiouracil-treated larvae.

Stage	Mean lens diameter (mm)	Number of Lenses measured	Standard error
48/49	0.47	8	0.01
50	0.52	20	0.01
51	0.51	20	0.01
52	0.53	24	0.01
53	0.55	18	0.01
54	0.66	38	0.01
55	0.68	16	0.01
56	0.73	28	0.01
57	0.77	26	0.01
58	0.85	16	0.01
59	0.91	4	0.02
Toadlets (newly metamorphosed)	1.02	14	0.04
Adult female toads	2.00	16	0.05
Stunted giant tadpoles	0.82	31	0.02
Large giant tadpoles	1.08	16	0.02

FIGURE 6/6

Growth in lens diameter during the normal metamorphosis of Xenopus laevis. This figure is a graphical representation of the mean lens diameters given in Table 6/2 but also shows ranges of values.

●——●Mean lens diameter

Range of lens diameters.
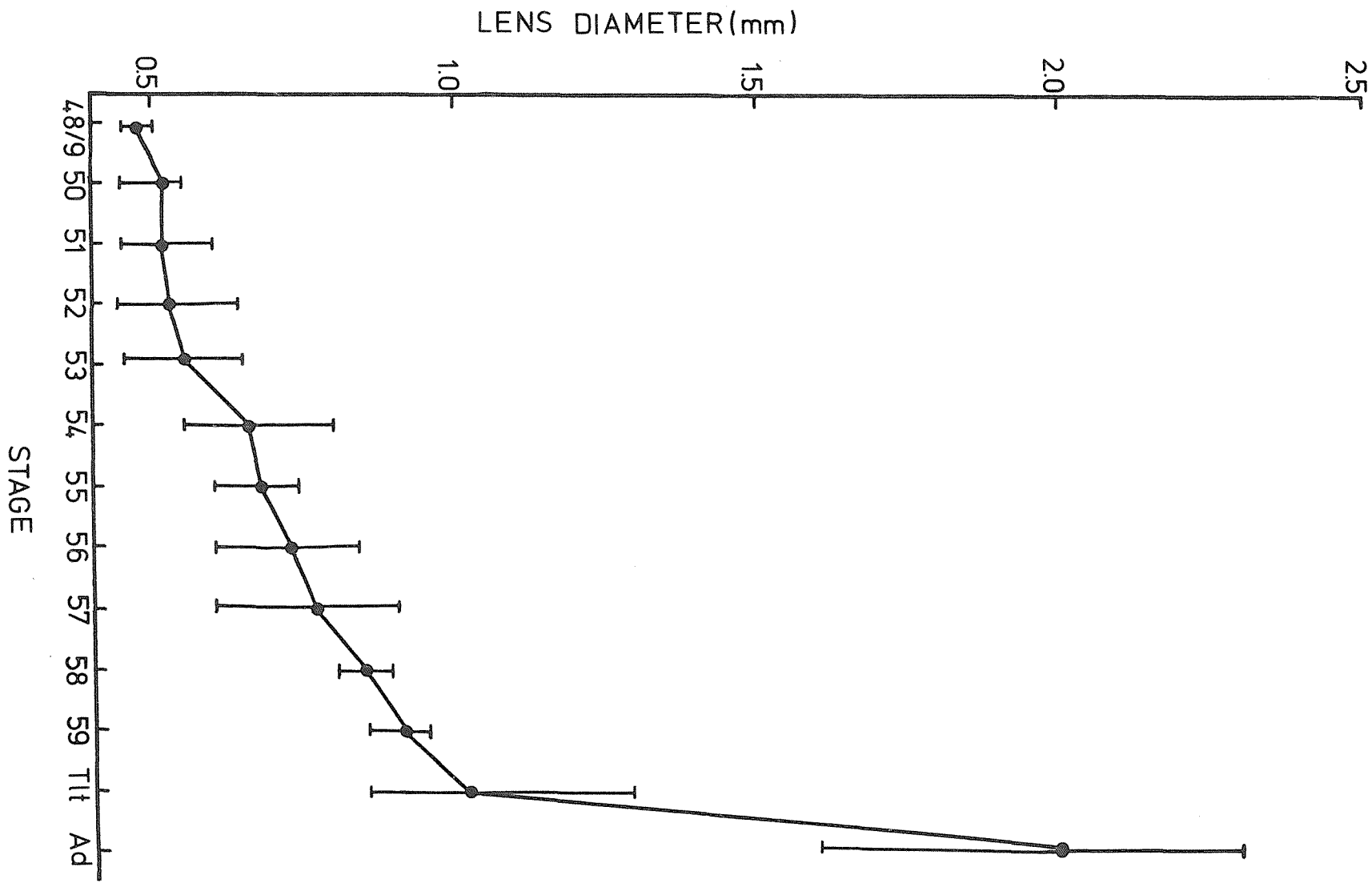
FIGURE 6/7

Summary of the statistical comparison (by "t" test) of the diameters
of lenses from stunted giant treated larvae, large giant treated
larvae and normal <u>Xenopus</u> larvae.

MLD = Mean lens diameter

NS   = Not significantly different (P = > 0.05).

NORMAL ST. 53 TADPOLES
(MLD = 0.55 mm)

NORMAL ST. 54 TADPOLES
(MLD = 0.66 mm)

NORMAL ST. 57 TADPOLES
(MLD = 0.77 mm)

NORMAL ST. 58 TADPOLES
(MLD = 0.85 mm)

NORMAL ST. 59 TADPOLES
(MLD = 0.91 mm)

TOADLETS
(MLD = 1.02 mm)

STUNTED GIANT
TREATED TADPOLES
(Stages : 52 - 55
(Average stage : 53/54
(MLD = 0.82 mm

P = < 0.001

P = < 0.001

P = < 0.02

NS

NS

P = < 0.001

P = < 0.001

NORMAL ST. 55 TADPOLES
(MLD = 0.68 mm)

NORMAL ST. 58 TADPOLES
(MLD = 0.85 mm)

NORMAL ST. 59 TADPOLES
(MLD = 0.91 mm)

TOADLETS
(MLD = 1.02 mm)

ADULTS
(MLD = 2.0 mm)

LARGE GIANT
TREATED TADPOLES
(Stages : 53 - 56
(Average stage : 55
(MLD = 1.08 mm

P = < 0.001

P = < 0.001

P = < 0.001

NS

P = < 0.001

FIGURE 6/8

Qualitative immunoelectrophoresis of lens crystallins from stunted
giant treated tadpoles and from untreated pre and postmetamorphic
<u>Xenopus</u>.

      i)   Stunted giant treated tadpole crystallins

     ii)   Normal stage 54 crystallins

    iii)   Stunted giant treated tadpole crystallins

     iv)   Normal stage 57 crystallins

      v)   Stunted giant treated tadpole crystallins

     vi)   Adult female crystallins

    vii)   Normal stage 54 crystallins

  viii)   Normal stage 57 crystallins

   ix)   Normal stage 57 crystallins

      x)   Adult female crystallins

          a = alpha crystallin

   b = beta crystallins

   g = gamma crystallins
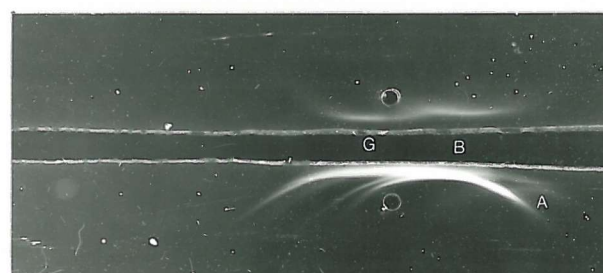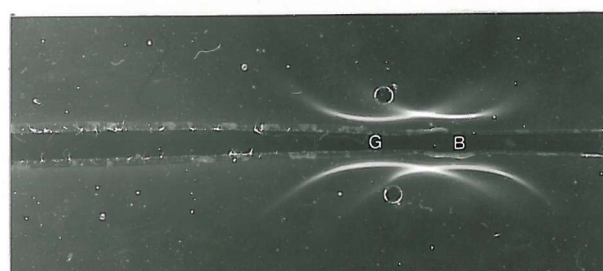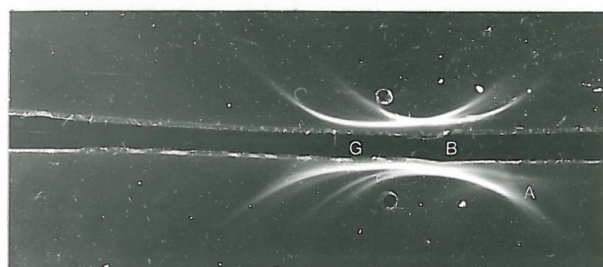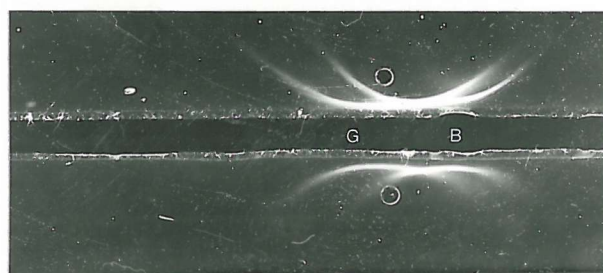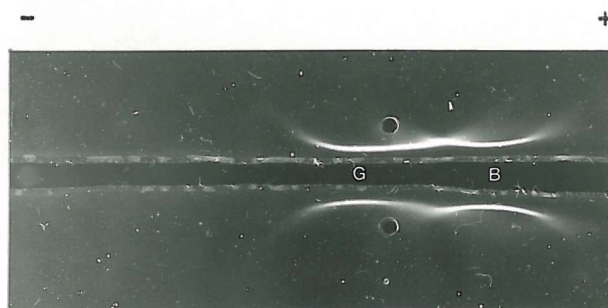
of stunted giants was 53/54 (range 52-55) while the average for large
giants was 55 (range 53-56). Their mean lens diameters of 0.82 mm
and 1.08 mm respectively differed at the 1% level ('t' test). Thus
the degree of crowding had produced the desired significant difference
in lens diameter in two otherwise similar groups of treated tadpoles.

The mean lens diameter of the stunted group was compared
statistically to the mean lens diameters of normal tadpoles of similar
morhpological stage (viz: stages 53 and 54). Both were significantly
different reflecting the fact that even the stunted group had larger
body sizes than untreated controls. Analysis showed the mean lens
diameter of the stunted group to be statistically similar to normal
stage 58 and 59 tadpoles but they were different from toadlet lens
(P=<0.001).

The large giant tadpoles, however, were similar in lens diameter to
normal toadlets but different from both adults and from normal tadpoles
of similar morphology (stage 55).

The comparison by IEP of 'stunted giant' and 'large giant' lens
homogenates with those from normal stage 54 and 57 tadpoles and from
adults is illustrated in Figs 6/8 and 6/9. The markers used were the
presence of the alpha- component and the degree of complexity of the
beta- and gamma- components.

The IEP patterns of stunted giant crystallins varied somewhat from
slide to slide but Fig 6/8 shows that an alpha- component was absent so
that the pattern was not adult-like. However in 2 of the slides illus-
trated additional fused arcs in the beta- and gamma- regions were present
so that the pattern was more like the normal stage 57 than like stage 54.
Thus the IEP pattern, like lens diameter, was appropriate to tadpoles of
normal stage 57/58 tadpoles rather than those of the stage of arrest
(stage 53/54).

Fig 6/9 shows the comparison of large giant crystallins with those
from normal X. laevis. In this case the patterns of large giant and
adult are scarcely separable whereas, because of the presence of
an alpha - component and increased beta- and gamma- crystallin antigen
complexity, the treated tadpole pattern is clearly more advanced than

those of the normal tadpoles. The mean lens diameter of the large giants has already been shown to be similar to that of post-metamorphic Xenopus and the crystallin pattern is similar.

Finally, in Fig 6/9, the patterns of stunted and large giant crystallins is compared directly. This confirms the presence of the alpha-arc and of more beta- and gamma- arcs in the large giant lens. All were absent from stunted giant lenses.

Elution of polyacrylamide bands, 6, 8 and 11 and their subsequent separation by IEP is shown in Fig 6/10. Bands 6 and band 8 proteins each gave a single arc in the beta- crystallin position. The electrophoretic mobility of this arc is most readily compared to the single beta- component of stage 54 and 57 tadpoles also shown in Fig 6/10. The centre of the arc is slightly to the anodal side of the origin. Band 11 produced faint arcs which appear to have their centres closer to the anodal end of the slide. Thus this protein is probably alpha-crystallin.

The eluted proteins were also re-run on polyacrylamide disc gels to check their purity. Eluates 6 and 8 each gave 2 adjacent bands. In each case one band was stronger than the other suggesting that some contamination from an adjacent band had occurred during gel slicing and elution. It is likely that the band 6 eluate was contaminated with protein from band 8 and vice versa. In neither case was there material in the fast-moving band 11 position, when the gels were compared to a control gel on which whole adult crystallins had been separated. A very faint fast-moving band was observed when the band 11 eluate was re-electrophoresed showing that no detectable contamination with the slower moving crystallins had occurred.

The metamorphic markers employed on polyacrylamide gels, band 6 and 8, therefore appear to be beta- crystallins while band 11 is alpha-crystallin. This agrees with the mobilities of these bands on polyacrylamide gels in the first part of these Results.

It appears from PAGE, in which these bands become wider and more darkly staining at metamorphosis, that the crystallins contained increase in relative concentration during the larval and post-metamorphic periods.
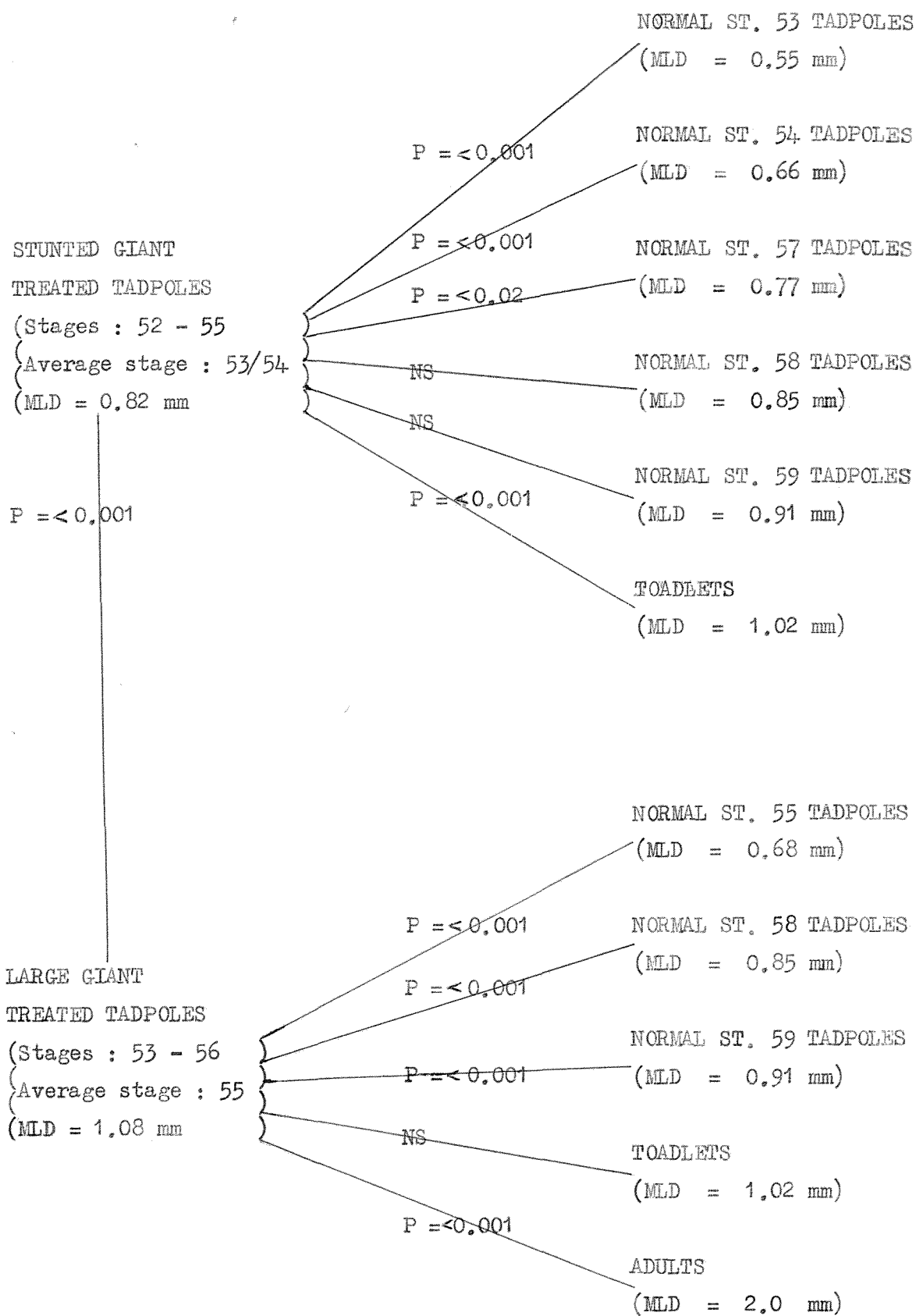
FIGURE 6/9

Qualitative immunoelectrophoresis of lens crystallins from large
giant treated tadpoles and from untreated pre- and
postmetamorphic Xenopus.   A direct comparison of crystallins
from stunted and large giant treated tadpoles is also illustrated.

    i)   Large giant treated tadpole crystallins.

   ii)   Normal stage 54 crystallins.

  iii)   Large giant treated tadpole crystallins.

   iv)   Normal stage 57 crystallins.

    v)   Large giant treated tadpole crystallins.

   vi)   Adult female crystallins.

  vii)   Stunted giant treated tadpole crystallins.

 viii)   Large giant treated tadpole crystallins.

        a = alpha crystallin

        b = beta crystallin

        g = gamma crystallin

124

i)
ii)
iii)
iv)
v)
vi)
vii)
viii)
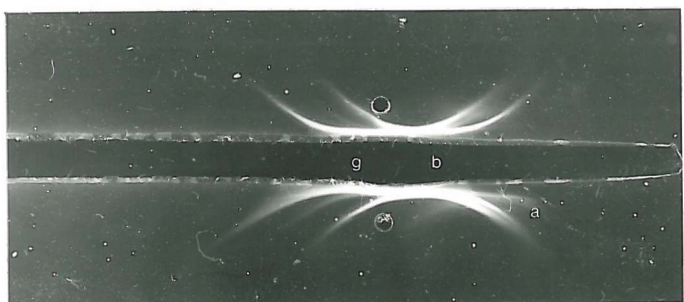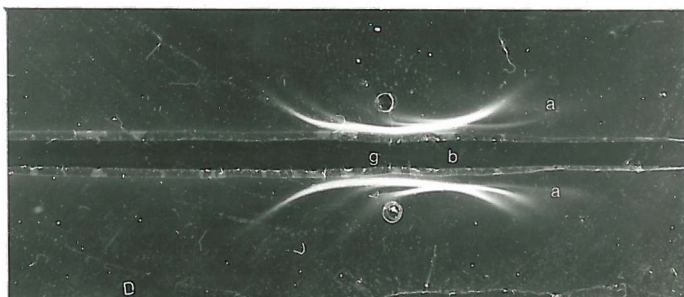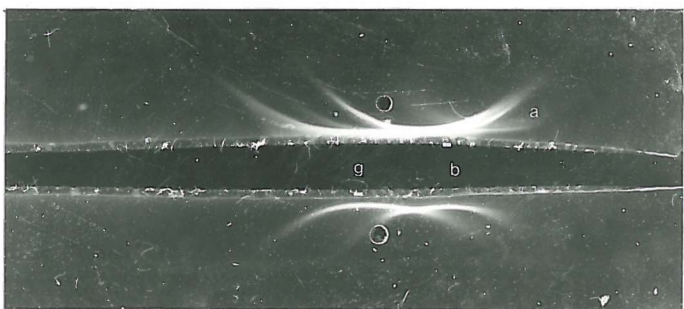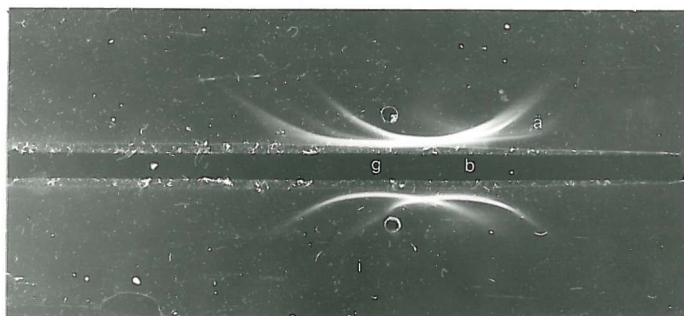
FIGURE 6/10

Correlation between electrophoretic separations of adult <u>Xenopus</u>
lens crystallins by two different methods. Whole lens extracts
were separated by PAGE. Certain crystallin bands were eluted
from the gels and the eluates were electrophoresed by IEP against
a rabbit anti-adult crystallin serum.

    i) PAGE band No. 8.

   ii) PAGE band No. 6.

  iii) PAGE band No. 11.

   iv) PAGE band No. 11.

    v) Normal stage 57 whole lens extract (not eluted)

   vi) Adult <u>Xenopus</u> whole lens extract (not eluted)

          a = alpha crystallins

          b = beta crystallins

          g = gamma crystallins
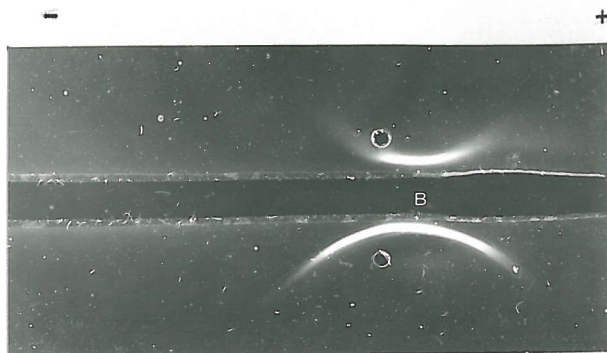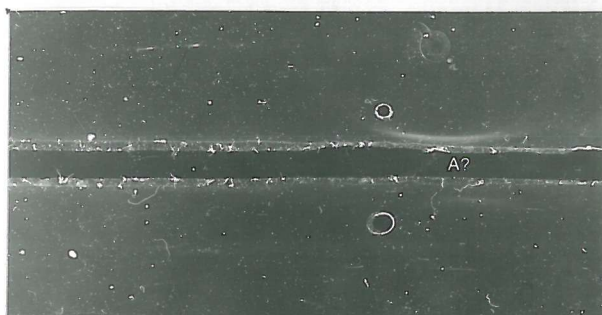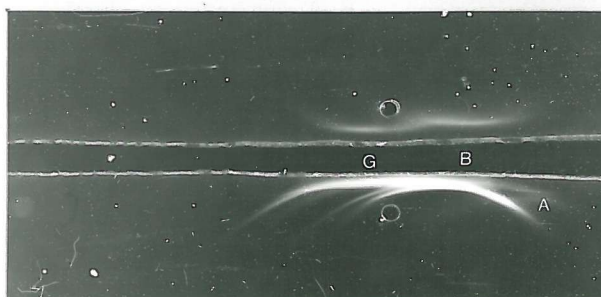
A similar conclusion may be drawn from the increased density and complexity of precipitin arcs and spurs. Two-dimensional IEP provided further evidence that this is so (Figs 6/11 and 6/12). Fig 6/11 shows the appearance of precipitin peaks obtained with adult female lens crystallins. Two groups of peaks are visible. These contain different proteins as the precipitin lines cross in the trough between the peaks. The most anodal peak is taken to be alpha-crystallin. There appear to be 2 or 3 proteins of similar mobility but different concentrations. The beta-crystallin region is more complex and contains at least 4 different proteins of similar mobility. The gamma-crystallins which were poorly precipitated, having migrated towards the cathode in the second-dimension, will not be considered here. The pattern shown was obtained with 2 pools of female crystallins from separate adult donors. However a third pool (Fig 6/11 b) did not exhibit a high alpha-peak. In neither case were there antigens of intermediate mobility between alpha- and beta- crystallins in adults.

Fig 6/11 c - e shows two-dimensional plates obtained with normal stage 59, 57 and 54 tadpoles. Two main differences between tadpoles and adults are noticeable. First, the alpha-peak is higher in the adult than in tadpoles. As the only variable in these experiments was the relative concentrations of individual crystallins, within the 6 mg/ml total protein samples, this indicates a greater alpha-crystallin concentration in adults. Second, an antigen peak of intermediate mobility and high concentration is visible between the alpha- and beta- peaks of tadpole plates but is not visible on adult plates. As the antiserum used was raised against adult crystallins the intermediate antigen must be present in the adult but must be in much lower concentrations than in tadpoles. Its presence on all tadpole plates (each sample was run between 2 and 4 times in separate experiments) suggests that it is not an artifact caused by polymerisation of beta-crystallins. Campbell et al (1968) detected a group of lens antigens, with an electrophoretic mobility intermediate between alpha- and beta- crystallins, in adult Xenopus. They termed this "group 3". This was not detected by qualitative IEP in the present work but the two-dimensional technique is more sensitive.

These two differences were used as biochemical markers when comparing lens antigens from 'large' and 'stunted' propylthiouracil-treated tadpoles (Fig 6/12). The large giant plates were adult-like in that there was a marked alpha-peak with a deep trough between it and the beta-

peaks. Thus, as with qualitative IEP the alpha-crystallin concentration was similar to post-metamorphic controls. There was no precipitin peak of intermediate mobility.

The stunted giant plates also had a well-defined alpha peak which was lower than on large giant plates. The intermediate peak was present but this was somewhat more cathodal than in normal tadpoles. Thus the stunted giant lenses were more tadpole-like than those of the large group. The poorer quality of the two-dimensional separations of lens crystallins, by comparison with the albumin plates of Chapter 4, made full quantification by peak area measurement impossible.

6.4 CONCLUSIONS

The lens crystallin transition observed during normal metamorphosis was an increase in the relative concentration of bands 6, 8 and 11 on polyacrylamide gels. These bands apparently represent fast-moving beta-crystallins (Nos 6 and 8) and alpha-crystallins (No 11). This transition was confirmed by immunoelectrophoresis. Qualitative IEP showed the first appearance of a detectable alpha-component at metamorphosis. This was accompanied by an increase in the number of beta- and gamma-crystallin arcs and spurs. The two-dimensional IEP method also showed that an increase in the concentration of alpha-crystallin accompanies late larval development.

The nature of the electrophoretic patterns observed in adults was generally in agreement with previous reports. Eleven crystallin bands were resolved by PAGE. Brahma and van Doorenmaalen (1969) also showed 11 crystallins on starch gels. Manwaring (1972) showed the same number of components with PAGE. The resolution of lens antigens here was less complete than with the very potent antisera used by Campbell et al (1968) but all the major components were visible. The metamorphic transition was similar to that illustrated by Manwaring (1972) and Billett and Wild (1975).

The main conclusion was that lens crystallin pattern was correlated with lens diameter. All tadpoles which had been treated for over 8 weeks in uncrowded conditions, and which had lenses of about 1.0 mm diameter or larger, had an adult-like crystallin pattern. This correlation was specifically demonstrated with two groups of treated-tadpoles

FIGURE 6/11

Two dimensional immunoelectrophoresis to show the increase in
concentration of alpha crystallin and the decrease in
concentration of an intermediate alpha/beta crystallin during
normal metamorphosis in <u>Xenopus laevis</u>.

a) Adult female lens crystallins

b) Adult female lens crystallins

c) Normal stage 59 lens crystallins

d) Normal stage 57 lens crystallins

e) Normal stage 54 lens crystallins

Each photograph is accompanied by a drawing interpreting the
IEP plate.   Dotted lines were only faintly visible on the
original plates.

The area under each peak is directly proportional to the
concentration of crystallin precipitated.

————▶+   Electrophoresis in 1st dimension

+
|
| Immunoelectrophoresis in 2nd dimension
|
|

a = alpha crystallin
b = beta crystallins
i = intermediate crystallin
o = origin well

All plates to same scale

a)

ADULT

b

a

+

+

+

b)

ADULT

b

a

c)

NORMAL ST. 59

i

b

a

d)

NORMAL ST. 57

i

b

a

e)

NORMAL ST. 54

i

b

a
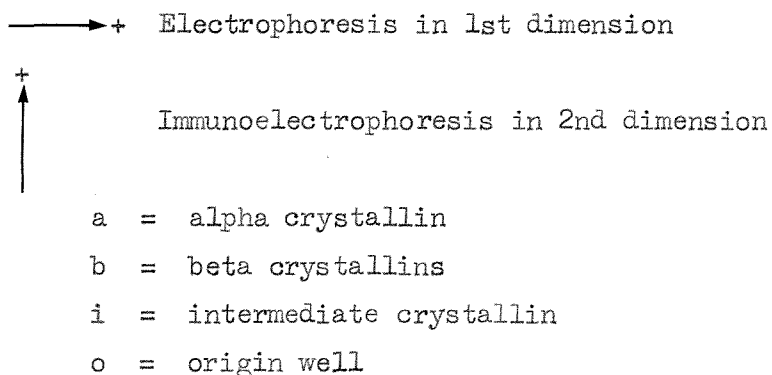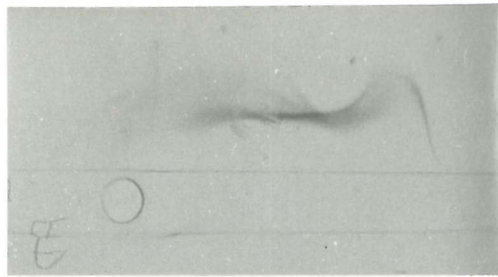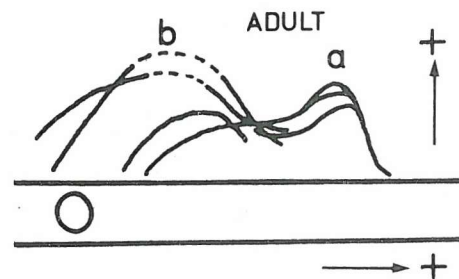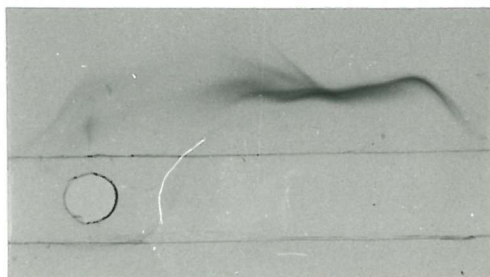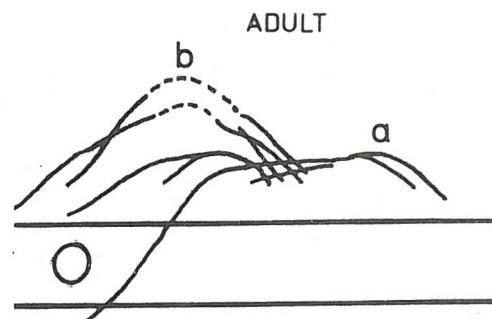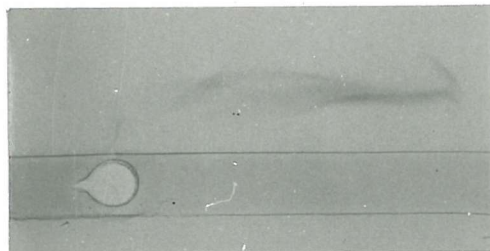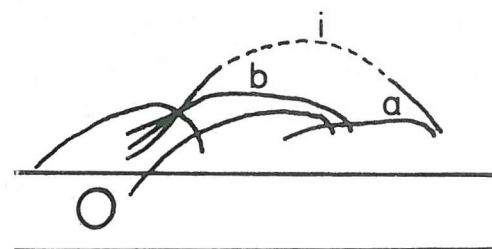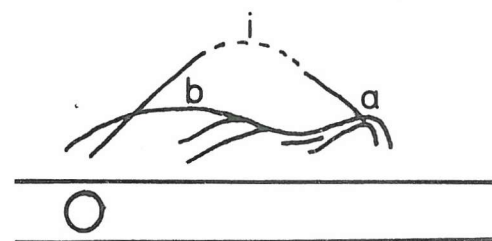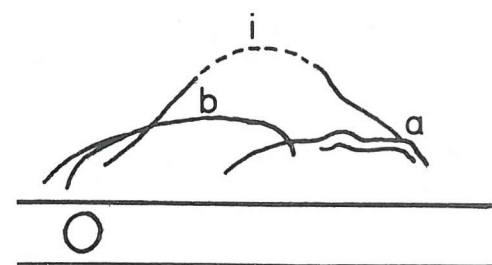
FIGURE 6/12

Two dimensional immunoelectrophoresis comparing the lens
crystallins of large and stunted propylthiouracil treated
Xenopus larvae with those of normal pre- and postmetamorphic
animals.

    a)   Large treated tadpole lens crystallins

    b)   Stunted treated tadpole lens crystallins

    c)   Adult female lens crystallins

    d)   Normal stage 54 lens crystallins.

Each photograph is accompanied by a drawing interpreting the IEP
data. Dotted lines were only faintly visible on the original
plates.

The area under each peak is directly proportional to the
concentration of crystallins precipitated.

⟶ +   Electrophoresis in 1st dimension

+
↑
| Immunoelectrophoresis in 2nd dimension
|
|

   a = alpha crystallin
   b = beta crystallins
   i = intermediate crystallin
   o = origin well

All plates to same scale

a)

LARGE TREATED

STUNTED TREATED

b)

ADULT

c)

NORMAL ST.54

d)
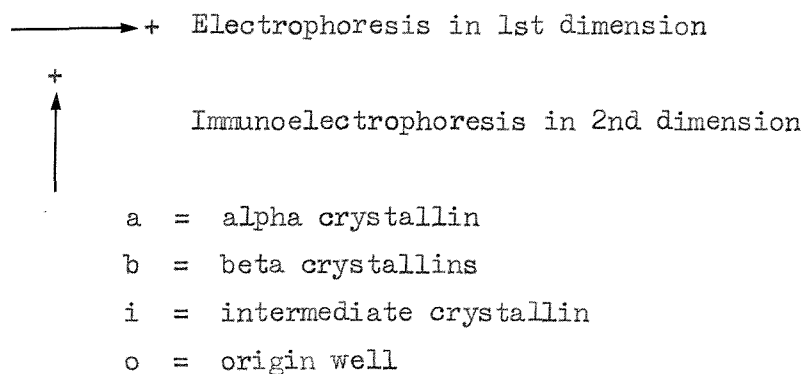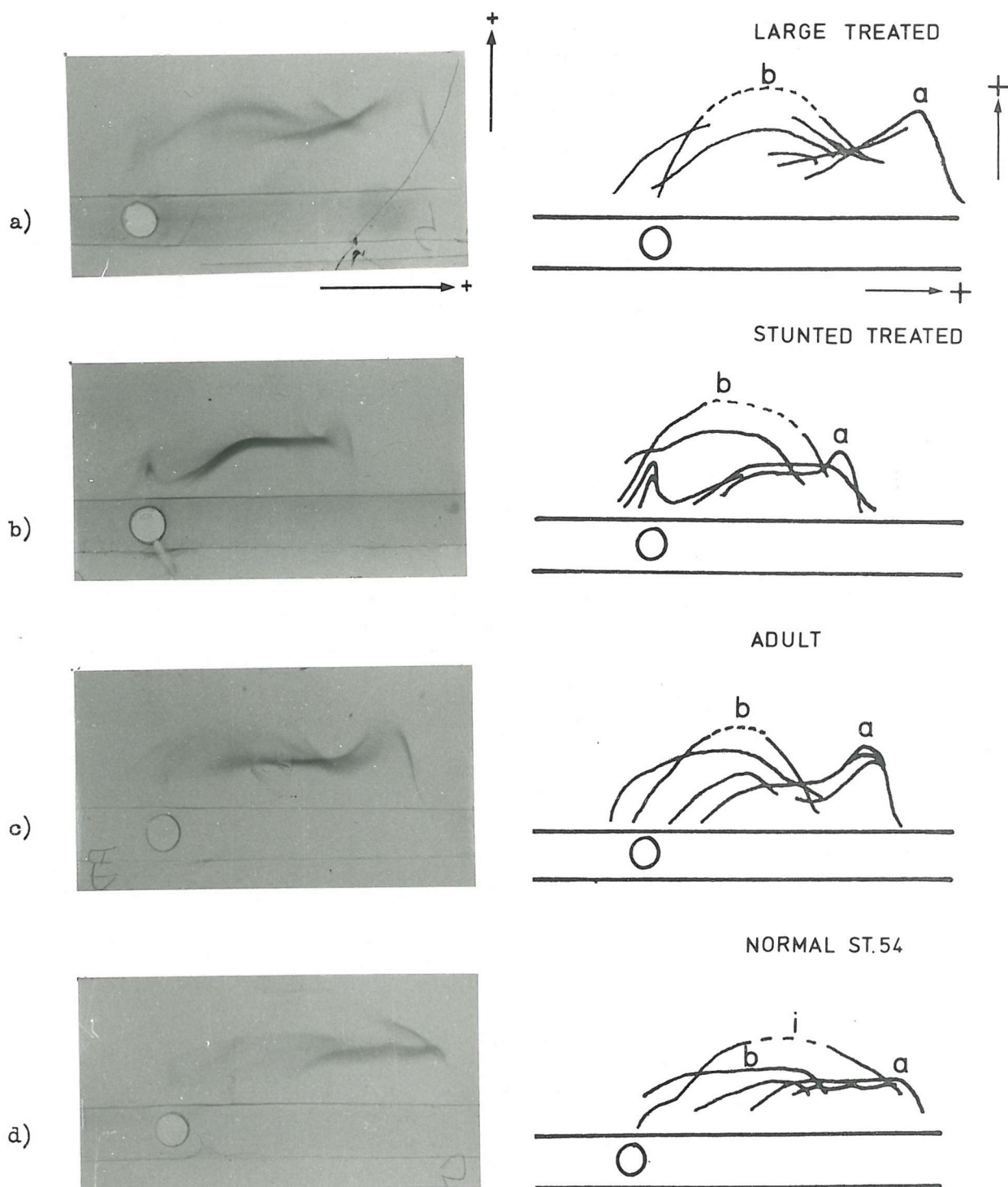
which differed only in body size and therefore in lens size. This conclusion supports Clayton's (1970) hypothesis that the difference between the crystallins of the inner and outer lens is a response to lens growth. This relationship between lens size and crystallin pattern explains the absence of variation in the crystallin patterns of giant tadpoles. It also suggests that the lens crystallin system is controlled in a different way from haemoglobin or coelomic fluid protein synthesis in which marked individual variation occurred amongst giant tadpoles (Chapters 3 and 4).

The results of this chapter provide no evidence that thyroxine is involved in the control process. Although Kaltenbach (1953) showed, by means of local implantations of thyroxine- containing pellets, that the hormone induces metamorphic changes in the extrinsic ocular muscles, bulging of the eye, formation of the upper eyelid, fusion of the two larval corneae and formation of the nictitating membrane, there has been only one study suggesting thyroid control of lens development. Polansky and Bennett (1973) studied changes in lens diameter in bullfrog larvae and separated crystallins by cellulose acetate electrophoresis. Both normal and T4 - induced metamorphoses were accompanied by lens growth and by an increase in the proportion of alpha- and beta- crystallins and a decrease in the proportion of gamma- crystallins. The authors admitted that the crystallin changes could be the result of lens growth rather than a direct response to T4. The possible nature of crystallin transition control is discussed in Chapter 8.

That lens growth is related to whole body growth is suggested by several papers. De Jongh (1967) showed, in Rana temporaria, that during larval development, the whole eye grows with a positive allometric relationship to body size. At metamorphosis this relationship changes to negative allometry. It is well known that the size of the lens is related to the size of the whole orbit (Harrison, 1929; Coulombre and Coulombre, 1969). Thus it may only be necessary for the crystallin shifts to be controlled directly or indirectly by lens size for a relationship with whole body size also to be apparent.

Several of the treated larvae used to provide crystallins had also previously been used as haemoglobin and/or LDH donors (Table 6/1). There was no correlation between possession of adult - or tadpole - like

patterns within a single animal. Thus one stage 54 giant tadpole pool possessed adult crystallins and LDH but tadpole-like haemoglobins. A stage 56 tadpole arrested for 41 weeks also still had a tadpole haemoglobin pattern but adult-like crystallins. These data support the above statement that the control of the crystallin transition may be quite different from the control of the haemoglobin transition.

CHAPTER 7

Assay of circulating thyroxine and triiodothyronine in normal
and propylthiouracil - treated Xenopus laevis larvae

7.1 INTRODUCTION

The preceding chapters have shown that "biochemical metamorphosis"
can occur in X. laevis larvae severely retarded by propylthiouracil
treatment. The efficacity of the goitrogen was indicated by the long-
term developmental effects observed. However one explanation for the
observed continuation of biochemical transitions is that, with time,
treated larvae are able to synthesise elevated levels of thyroid hormone
perhaps by a proliferation of extrathyroidal tissue. The total hormone
levels in circulation would still be low, to account for continued
developmental arrest, but might be sufficient to initiate some of the
biochemical events. Copenhaver (1955) noted that colloid - containing
thyroid tissue developed in the gills of Ambystoma punctatum reared in
0.01 to 0.005% (0.59 - 0.30 mM) propylthiouracil. Turner (1973) found
extrathyroidal tissue in giant X. laevis tadpoles treated with 0.01 to
0.015% (0.59 - 0.89 mM) propylthiouracil.

The aim of the work in this chapter was to measure circulating T4
and T3 titres separately in normal and treated pre- and prometamorphic
tadpoles. If treated tadpoles are unable to produce significantly more
T4 or T3, than premetamorphic controls, then the above explanation will
have been eliminated.

In spite of intensive research on amphibian metamorphosis for over
60 years there have been no published direct measurements of circulating
T4 and T3 titres in tadpoles. However a number of studies have identified
the hormones produced by the amphibian thyroid and various, more or less
indirect, estimates of glandular activity or hormone titre have been made.
The methods employed by previous workers were reviewed before selecting
the methods used here.

Most earlier workers used histological criteria such as height of
follicular epithelium and amount of follicular colloid to estimate the
synthetic activity of the thyroid gland (review by Lynn and Wachowski,

1951). However it has generally been accepted that a histologically "active" thyroid gland does not necessarily mean that circulating TH levels are high.

More recently the uptake of labeled TH precursors such as ^{131}I or ^{125}I have been used. Gorbman and Evans (1941) did not quantify their autoradiographs of Hyla regilla labeled tadpole thyroids but merely noted that the grain density increased as the gland grew larger and more colloid was visible. Saxen et al (1957) obtained relative measurements of thyroidal ^{131}I and protein-bound ^{131}I during X. laevis metamorphosis. The radioactivity was determined by scintillation counting of homogenised thyroid glands and whole larvae or by a microdensitometry method based upon autoradiographs (Saxen et al, 1956a). Similar whole gland or whole body counts have been used by Dodd (1955) and by Nataf et al (1975) with X. laevis; D'Angelo (1956) with Rana clamitans; Donoso and Trivelloni (1958) with Bufo arenarium; Bowers et al (1959) with R. catesbeiana and Kaye (1961) with R. pipiens. The isotopic material was administered either by injection or immersion of the whole animal. This has led to differences in the developmental stage at which peak uptake was obtained (Kaye, 1961; Neuenschwander, 1972; Dodd and Dodd, 1976). These studies have been mainly concerned with detailing the thyroid's ability to concentrate iodine at different stages of development. The method may be extended by homogenising labeled thyroid glands, followed by the chromatographic separation, identification and quantification of the labeled components.

Shellabarger and Brown (1959) used paper chromatography to separate tryptic hydrolysates of ^{131}I-labeled thyroids from X. laevis larvae and adults. Their data were not quantified however. Flickinger (1964) also used qualitative chromatography to show that T4 is first present in 16 mm long Rana pipiens tadpoles. Race and Cameron (1966) employed the thin-layer chromatographic technique with metamorphosing R. clamitans. Provisional semiquantitative results were obtained by densitometry of the stained separations. Hanaoka et al (1973) working with Bufo bufo japonicus and X. laevis employed thin-layer chromatography and detected T3 before stage 32 (external gills). In B. b. japonicus T4 became the main thyroid hormone after stage 34 (early premetamorphosis). In

Xenopus the reversal of values for T3:T4 took place between stages 40 and 46.

The only worker to express his data in absolute values was Thornburn (1967) working with relatively large volumes of adult Bufo bufo blood. Butanol extracts of serum were used with paper chromatography against known quantities of standards. Quantitation was achieved by estimating the catalytic effect of throidal iodo-compounds on the reduction of the ceric ion by arsenite.

Dodd and Dodd (1976) have recently estimated the rates of T4 synthesis during prometamorphosis and climax in Xenopus. Larvae were injected with a constant dose of ^{131}I and digests of the labeled thyroid glands separated by chromatography. The ^{131}I-T4 component was then measured.

An unusual method of estimating thyroid activity was used by Gorbman and Ueda (1963). Physiological activity was correlated with the electrical properties of individual thyroid follicles. Electrical measurements were made through microelectrodes.

The above methods give only an indirect estimate of circulating TH level. Even if precursors are taken up and incorporated into hormone the latter may be degraded to inactive molecules before or after release.

The range of methods currently used clinically to assay circulating TH has been reviewed by Varley, Gownlock and Bell (1976). Some methods are not suitable for use with the small samples which are obligatory in metamorphosis research. The protein-bound iodine (PBI) method is widely used for the routine investigation of thyroid disorders. The serum proteins are precipitated and the iodine in the iodine - containing compounds carried down with them is converted to inorganic iodine by digestion or incineration. Iodide is measured by its catalytic action on the oxidation of arsenite by ceric sulphate in which the yellow Ce^{4+} ion is converted to the colourless Ce^{3+} ion. PBI values include both T4 and T3, which in humans are almost entirely protein-bound. Also, depending upon the protein precipitant used, variable amounts of MIT and

DIT are measured. When present in large amounts inorganic iodide may interfere. The most serious source of difficulty arises when large differences in titres of thyronine-binding proteins occur between the samples under comparison. It was shown in Chapter 4 that total serum protein and the percentages of constituent components change during metamorphosis. Little is known about thyronine-binding proteins in amphibian body fluids but it may be that the levels of any which occur change greatly during larval life. A method which relies upon approximately constant TBG levels in human sera would not seem to be suitable for use in metamorphosing tadpoles.

The PBI method has the advantage of being usable on a micro scale (Malkin, 1965). It was therefore used by Just (1972) to assay changes in TH level in metamorphosing Rana pipiens sera. The author claimed that changes in serum total protein did not coincide with the observed changes in PBI values. It was argued that in practice the PBI method gave a reasonable estimate of total circulating TH.

The evidence which has accumulated to indicate that T3 may be the most biologically important thyroid hormone will be discussed in Chapter 8. As T3 is present in much lower concentrations than T4 in pre- and prometamorphic Xenopus (Hanaoka et al 1973) quite large fluctuations in T3 titre might be masked by T4 in PBI measurements.

For these reasons modern clinical assays measure the two hormones separately. The methods used are more direct than any of those attempted in amphibian metamorphosis research to date. The methods are the competitive protein-binding technique (Murphy and Jachan, 1965; Seligson and Seligson, 1972) and radio-immunoassay (Hollander and Shenkman, 1974).

These methods have been used to assay circulating TH in fishes. Competitive protein-binding was first employed by Refetoff et al (1970). Since then several other studies have made use of the technique in fishes (e.g. Higgs and Eales, 1973; Henderson and Lorscheider, 1975; Henderson, 1976). Packard, Packard and Gorbman (1976) determined serum T4 in adult R. pipiens in this way. The methods used by these workers all employed assay kits originally intended for human serum measurements.

A similar adaptation of human radioimmunoassay (RIA) kits had not been reported at the time when the present work was carried out. More recently however, Brown and Eales (1977) have reported that clinical T4 RIA's were insufficiently sensitive to measure plasma T4 in freshwater fishes. A more sensitive RIA technique, described in section 7.4, has been developed by these authors.

Special RIA's have not yet been developed for amphibian thyroid measurements but a study by Clemons (1976) used a homologous technique for growth hormone.

In the present work both competitive protein-binding and RIA methods intended for clinical use were adapted for the assay of Xenopus coelomic fluid hormones. The reason and rationale for the use of coelomic fluid, rather than serum, was given in Chapter 4. In this context it seems likely that both free- and bound-hormone are present in coelomic fluid in quanties directly proportional to those of the serum. The small size of TH molecules makes it likely that unbound hormone would move freely between the two fluids. The immunological demonstration, in Chapter 4, that identical proteins occur in serum and coelomic fluid makes it likely that specific TH transport proteins, if they exist in Xenopus, would also occur in both fluids. Thus coelomic fluid is a convenient medium with which to make the comparison of T3 and T4 titres between normal and propylthiouracil-treated larvae.

When using hormone assays designed for human serum assay with Xenopus coelomic fluid one is making certain assumptions, which are:

1. Xenopus T3 and T4 are chemically identical to the human hormones and will therefore be bound by antisera against the human hormones.

2. That non-specific hormone binding (i.e., binding of hormone to proteins other than thyronine binding globulins, TBG or thyronine binding prealbumin, TBPA) will not adversely affect the results.

3. That compounds used to prevent specific hormone binding to human TBG will also be effective with any amphibian TBG.

These points will be discussed in sections 7.2 and 7.3.

7.2 EXPERIMENTAL

For T4 a competitive protein-binding assay and a RIA were used. For T3 two different RIA methods were given preliminary tests but only one gave satisfactory calibration curves.

7.2.a. Competitive protein-binding with an anion exchange resin (Bauer et al, 1970)

The method of Bauer et al (1970) is closely followed in the 'Tetralute' kit (Ames Company, Slough, Bucks). The principle is that virtually all T4 is extracted from serum by placing it in a strongly alkaline (pH 13) Sephadex G-25 column. The 'Tetralute' column is similar to the barrel of a 5 ml plastic syringe. The extracted T4 along with the added radioactive ^{125}I-T4 tracer in the alkaline medium is bound by the Sephadex G-25. The column is washed with buffer to shift the pH to 8.6 and to wash out any free ^{125}I and the serum proteins which are free of T4. At pH 8.6 an equilibrium is established between the Sephadex column (secondary binding) and the eluting reagent which is then added. The eluting reagent is human serum diluted in barbital buffer providing a limited number of TBG sites. T4 bound to the eluting reagent is removed from the column by washing at pH 8.6.

The ratio of T4 bound to Sephadex after elution to that bound initially is established by counting the radioactivity before and after elution. This ratio ("percentage retention") is the percentage of radioactivity retained by the column and is an index to the amount of T4 in the sample serum. The T4 content of the sample is determined by relating the percentage retention to a calibration line prepared with standard sera of known T4 concentrations.

Details of Xenopus used to provide test samples are given in the legend to Fig. 7/1. Throughout this chapter the fluids assayed were coelomic fluids from normal and treated tadpoles. However it was useful also to assay Xenopus fluids which could be obtained in larger

quantities. Thus toadlet coelomic fluid and adult toad serum were collected and used both in preliminary experiments when testing methodology and also for comparison with tadpole data.

All calibration lines appear in Fig. 7/1.

Experiment 1. Standards only were used to check methodology and kit performance. The assay material volume was 50 μl. Reproducibility of duplicated standards was high and a straight line of the type illustrated by Higgs and Eales (1973) was obtained indicating satisfactory performance.

Experiment 2. A calibration line was again produced and in addition samples of adult sera A and B, goitrogen-treated tadpole, stage 56/59 and stage 48/50 coelomic fluids were assayed. The sample volume was 50 μl.

Experiment 3. Because of the extremely low level of T4 found in the previous experiment an attempt was made to extend the calibration line into the human hypothyroid region (less than 3.8 ug T4/100 ml). Samples with 0.44, 0.88 and 1.76 ug T4/100 ml were used with a sample volume of 20 μl.

Additionally the modifications recommended by Higgs and Eales (1973) for use with low T4 titres were incorporated. These workers were able to steepen the standard line by using a 1:30 dilution of human serum with barbital buffer as an eluting agent rather than the 1:16 dilution suggested by Bauer et al (1970). Assuming the 'Tetralute' eluting reagent dilution to be 1:16 (not stated by the manufacturers) a 1:1 redilution with buffer was made. Higgs and Eales also found the time after drainage of the eluting reagent (TBG) before elution with buffer to be critical. Thus a minimum of 5 minutes was allowed rather than the 2 minutes stated by the manufacturers.

The calibration line obtained is shown in Fig. 7/1 in which duplicability was again excellent but the line was still too flat.

Experiment 4. The calibration line of Experiment 3 was repeated together with samples of adult toad serum and coelomic fluid from goitrogen-treated tadpoles, normal toadlets, stage 52 tadpoles and stage 48/50 tadpoles. The sample volume was 20 µl.

7.2.b. Measurement of non-specific hormone binding

The availability of RIA materials made it possible to investigate the most serious potential source of error in this work, namely binding of TH to any Xenopus protein not effectively blocked by thiomersalate (referred to here as "non-specific hormone binding"). It is also convenient to discuss the other potential sources of error mentioned in section 7.1.

The simple nature of the molecule of either T3 or T4 removes the uncertainty about their similar structures in mammals and amphibians. This uncertainty is a problem when working with polypeptide hormones. In fact the standard T3 and T4 solutions supplied by RIA kit manufacturers for use with human samples are prepared with synthetic hormones. It was found that the antibodies of the anti-T4 and anti-T3 sera supplied were not of the precipitating sort as they would not precipitate synthetic T4 or T3 from human serum. Hence it was not possible to demonstrate the cross-reactivity of the antisera with Xenopus hormones by direct precipitation.

The compound used here to block specific hormone binding was thiomersalate (section 7.2.c). To demonstrate its efficacity with Xenopus material and to prove that a serious level of non-specific binding was not occurring a preliminary experiment was performed.

The non-specific T4 binding properties of treated-tadpole coelomic fluid, adult toad coelomic fluid and adult toad serum were compared with those of water. The methodology was similar to that of normal assays (Section 2.30 and 7.2.c) except that specific T4 antiserum was not used. To determine the total radioactivity the adsorbent powder was omitted and 1000 µl of water added instead. The results appear in Table 7/1.

Table 7/1. Percentages of non-specific T4 binding caused by Xenopus coelomic fluid and serum proteins.

Sample	^{125}I-T4 cpm (mean of 3 replicates)	Percentage of total ^{125}I-T4 retained. $\left(\dfrac{\text{cpm sample}}{\text{total activity}} \times 100\right)$	Percentage non-specific T4 binding (in excess of water)
Water	1,227	9.3	-
Goitrogen-treated tadpole coelomic fluid	1,683	12.7	3.4
Adult female coelomic fluid	1,463	11.0	1.7
Adult female serum	1,707	12.9	3.6
Total ^{125}I-T4 activity	13,255	-	-

The percentages of non-specific T4 binding were well within the range of that of human serum, namely less than about 5% (Mardell, 1976). Thus thiomersalate effectively blocks any specific binding which may occur with Xenopus body fluid proteins and non-specific binding is too weak to resist the adsorbent powder (anion exchange resin) added in these radioimmunoassays.

7.2.c. Principles of radioimmunoassay (RIA)

RIA is based upon the competition between labeled and unlabeled antigen for specific antibody sites, forming antigen-antibody complexes. This reaction is described by the expression (Goldsmith 1975)

antigen   +   ⟷ antigen-antibody complex

            specific antibody

radioactive +   ⟷ radioactive antigen -

antigen   antibody complex
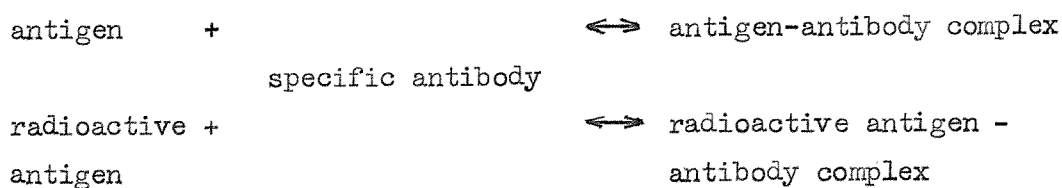
At equilibrium, the radioactive complex (bound - B) is separated from the radioactive antigen (free - F). The B/F ratio is dependent upon the amount of nonradioactive antigen. Antigen concentration in unknown samples is determined by comparing the B/F ratio to the B/F ratios obtained

by incubating varying amounts of known nonradioactive antigen with the
same   amount of antibody as in the unknown sample under similar assay
conditions.   Sensitivity of the order of 10^{-12} moles/litre may be
achieved.

Specificity is dependent upon the ability of the antiserum to
recognise subtle structural features of the antigen molecules.   The
measurement of T3 and T4 has lagged behind the development of RIA's
for other hormones by approximately a decade (Hollander & Shenkman, 1974).
Presumably the small size of the thyroxine molecule dissuaded investigators
from considering it a suitable antigen, but as has been subsequently shown
for T3, T4 and other small antigens as well, small size does not preclude
antigenicity if appropriate methods are employed.   After the demonstration
by Brown et al (1970) that antibodies to T3 could be raised in animals
injected with a poly-L-lysine-T3 conjugate, other investigators were
able to develop serum T3 and T4 RIA's suitable for routine laboratory
use.

Some further special problems are encountered with TH radioimmunoassays.
In the case of those for T3 the antiserum must be highly specific as, at
least in humans, it must normally be measured in the presence of 50-100
fold greater concentrations of T4 from which it differs by only one
iodine atom.   Preliminary separation of T3 and T4 may be hazardous since
it may prove incomplete and may induce conversion of T4 to T3 (Taurog,
1963).

Second, a direct serum assay is subject to interference by the
presence of thyronine-binding proteins in the serum in amphibia as well
as in humans (Farer et al, 1962).   These proteins interfere with both
the antibody— hormone reaction and with the separation of free hormone
by adsorption on a solid matrix.   Since the level of thyronine-binding
protein may vary from serum to serum this interference must either be
eliminated or corrected for by an additional 'blank' non-specific binding
measurement for each sample in the absence of antiserum.

Third, the presence of serum, even in the absence of thyroid
hormones, interferes slightly with the binding of TH to antiserum.   It
is necessary that the calibration curve should be constructed using serum

rather than aqueous standards.

Two types of RIA method were tried. The first was the "Phadebas" RIA for T3 (Pharmacia Diagnostics A B, Uppsala, Sweden) which employs T3 antibodies bound to Sephadex particles as a solid phase (Wide & Porath, 1966). This method did not give satisfactory calibration curves with standard T3 samples in human serum. In addition, the mean gamma count rate of Ong T3/ml samples should be around 10% of the mean count-rates of total activity added. In fact they lay between 19 and 27%. It was concluded that the reagents supplied were unreliable great care having been taken with the methodology.

The second RIA method is that used by the Radiochemical Centre, Amersham, Bucks. This, in both the T3 and T4 forms, gave excellent standard curves.

The method depends upon competition for binding sites on hormone specific antibody between the hormone in serum and ^{125}I-labeled hormone. The proportion of ^{125}I-labeled hormone bound to antibody is inversely related to the concentration of the unlabeled hormone present. This proportion is measured by separating the free hormone by adsorption on a solid matrix and estimating the radioactivity of the bound fraction remaining in solution.

The manufacturers claim that cross-reactivity of the anti-T4 serum with L-triiodothyronine is less than 5% and with DIT and MIT is less than 0.5%. With the anti-T3 serum cross-reactivity with L-thyroxine is less than 0.2% and with DIT and MIT less than 0.1%.

The problem of non-specific binding is circumvented in this method by the use of a TBG-blocking agent, thiomersalate and the appropriate sodium barbitone buffer. Huefner and Hesch (1973) found that, of several TBG-inhibitors tried (anilino-naphthalene-sulphonic acid, sodium salicylate, diphenyl-hydantoin and tetrachloro-thyronine) thiomersalate (1:1000 w/v) was the most satisfactory as it did not also inhibit the specific binding of the labeled hormone to the antibody.

Standards of T4 and T3 were used diluted in human serum.

7.2.d. T4 Radioimmunoassay

Details of animals used as coelomic fluid and serum donors are given in the legends to Figs. 7/2, 7/3 and 7/4.

Experiment 1. Although the manufacturers recommended 50 µl aliquots of standards and samples, 25 µl aliquots were used throughout to conserve the biological material.

In this experiment 0.15, 4.10, 10.8, and 19.2 µg T4/100 ml standards in human serum were used to construct a calibration curve. If the curve was satisfactory it was assumed that the methodology was correct. In addition a sample of adult Xenopus serum was assayed.

Experiment 2. As with the T3 RIA the advice of Dr. Smith (Radiochemical Centre) and Mr. Mardell (Southampton General Hospital) was followed, that the reconstituted reagents (antiserum, labeled T4 and adsorbent) should be further diluted 1:2 with distilled water. This was intended to make a more economical use of reagents with the low hormone titres of Xenopus fluids.

25 µl aliquots of 0.15, 4.10, 10.8 and 19.2 µg T4/100 ml standards and a sample of Xenopus adult serum were used with the rediluted reagents.

Experiment 3. Following the unsatisfactory result of the previous experiment unrediluted reagents were used to produce a calibration curve and to assay 25 µl samples of adult toad serum and coelomic fluids from toadlet, goitrogen-treated (non-giant) normal stage 56/59 and stage 49/50 tadpoles.

Experiment 4. Shortage of assay reagents again made it necessary to attempt their redilution. The results of Experiment 2 suggested that the unsatisfactory counts obtained with the 10.8 and 19.2 µg T4/100 ml standards was due to there being insufficient adsorbent. In the present experiment the reconstituted absorbent was not diluted while the antiserum

and labeled thyroxine were again rediluted 1:2 with distilled water.
Standards only were employed to produce a trial calibration curve.

Experiment 5.   The satisfactory results of the previous experiment
confirmed that rediluted antiserum and labeled thyroxine would perform
satisfactorily.   The reconstituted adsorbent was now rediluted 1:0.5
with distilled water and another calibration curve produced.   By this
means it was hoped to be able to assay a large number of Xenopus fluids
in the next experiment.   The calibration curve was extended downwards
by the use of a 0.06 µg T4/100 ml sample obtained by using 10 µl rather
than 25 µl of the 0.15 µg T4/100 ml standard.

Experiment 6.   1:2 rediluted antiserum and labeled thyroxine were
used with 1:0.5 rediluted adsorbent to assay 25 µl aliquots of standards
and samples.   The samples were adult toad serum and coleomic fluid
from toadlet, three separate giant tadpoles and normal stage 57/59, 54/56,
52/54 and 49/51 tadpoles.

7.2.e.   T3 Radioimmunoassay

Details of animals used as coelomic fluid and serum donors are
given in the legends to Figs. 7/5 and 7/6.

Experiment 1.   25 µl aliquots of both standards and samples were
used.   As the previous T4-assays had given satisfactory calibration
curves it was not necessary to run a preliminary experiment with
standards alone.   Two replicates of each standard and sample were run.

Experiment 2.   In the previous experiment it was apparent that
circulating levels of T3 are too low to be extrapolated from a standard
curve based on the human T3 range.   The present experiment and
experiment 3 compared two ways of obtaining lower points on the curve.
The method used here was to use smaller volumes of the 0.10 ng T3/ml
standard than the usual 25 µl in order to lower the T3 concentration.   5,
10 and 25 µl of this standard were used being equivalent to 0.02, 0.04
and 0.10 ng T3 ng T3/ml respectively.

FIGURE 7/1

Competitive protein binding ("Tetralute") assay of <u>Xenopus laevis</u> coelomic fluid and serum thyroxine

Experiment 1. Preliminary experiment to check
assay performance.
▲-▲ Calibration line with 1.76
3.52, 8.8 and 17.6 μg T4/100 ml
standards

Experiment 2.

■-■ Calibration line with 1.76,
3.52, 8.8 and 17.6 μg T4/100 ml
standards. The following
<u>Xenopus</u> samples were assayed:

Sample	Source	% Retention (& means)	μg T4/100 ml (extrapolated from line)
Adult A Serum	Single female	10.6 13.3 (12.0)	<1.76
Adult B Serum	Single female	12.9 13.3 (13.1)	<1.76
Goitrogen-treated tadpole coelomic fluid	24 St. 53/54 crowded & treated 26 weeks (pooled)	11.9 13.2 (12.6)	<1.76
St. 56/59 coelomic fluid	8 tadpoles (pooled)	14.4 14.7 (14.6)	<1.76
St. 48/50 coelomic fluid	42 tadpoles (pooled)	13.6 13.1 (13.4)	<1.76

Experiment 3. Preliminary experiment to check
assay performance with dilute
standards.
O-O Calibration line with 0.44
0.88, 1.76 and 4.4 μg T4/100 ml
standards.

Experiment 4.

□-□ Calibration line with 0.44,
0.88, 1.76 and 4.4 μg T4/100 ml
standards. The following
<u>Xenopus</u> samples were assayed:

Sample	Source	% Retention	μg T4/100 ml (extrapolated from line)
Adult C Serum	Single female	33.6	0.8
Toadlets coelomic fluid	4x2.0/3.0cm specimens (pooled)	33.5	0.75
Goitrogen-treated tadpole coelomic fluid	As in Expt. 2	33.9	0.85
St. 52 coelomic fluid	13 tadpoles (pooled)	34.1	1.00
St. 48/50 coelomic fluid	As in Expt. 2	32.4	0.50
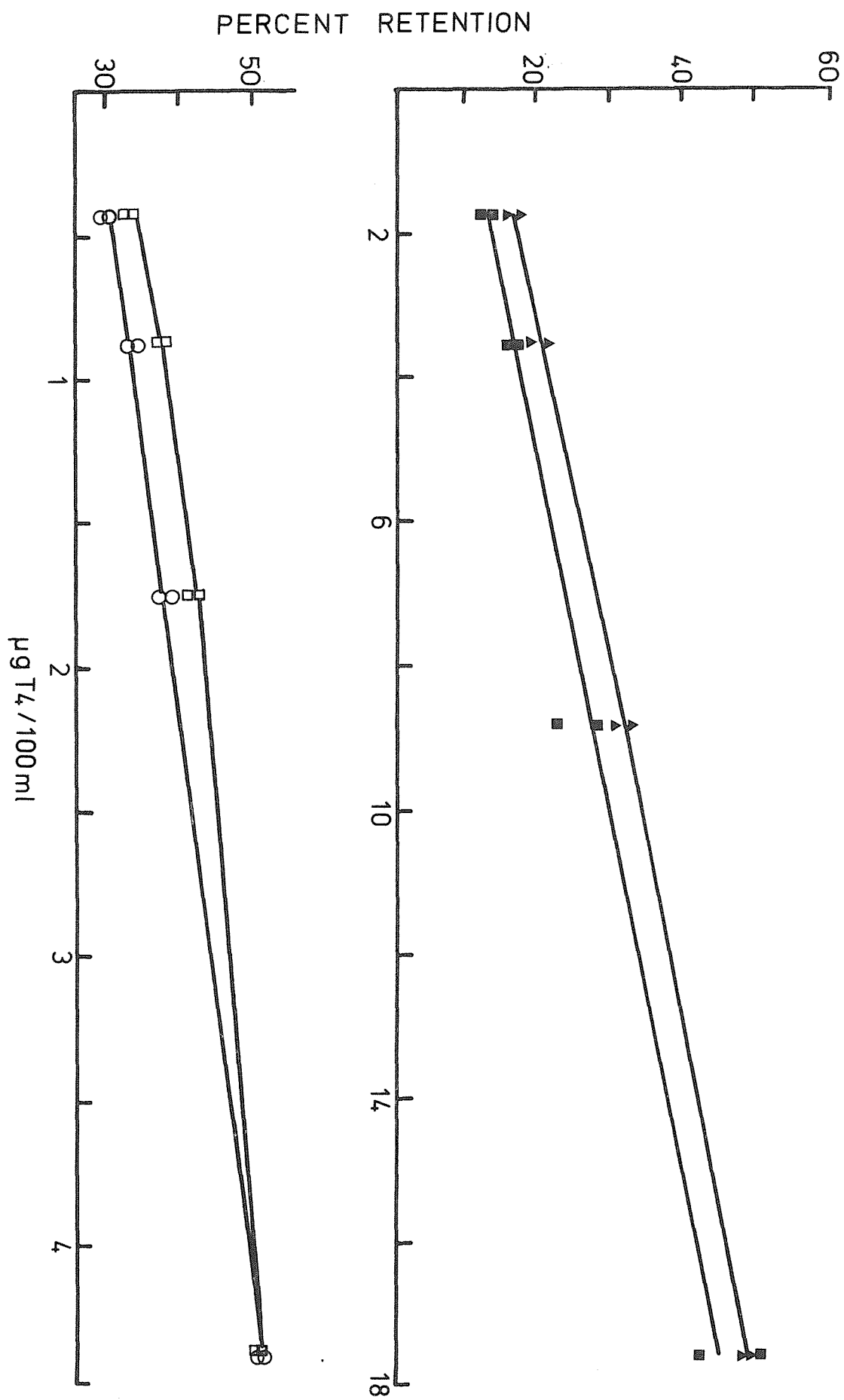
Experiment 3.   The alternative method was to dilute the 0.10 ng T3/ ml sample.   Dilution with buffer would alter the serum protein concentration also and so ideally T3-free serum ought to be used as a diluent .   Mr. R. Mardell of Southampton General Hospital generously gave me some vials of lyophilised T3-free human serum which were reconstituted with distilled water.   One vial was used to give points for 0 and 0.025 ng T3/ml.

In this experiment (as in the later T4-RIA) the reconstituted reagents (antiserum, labeled-T3 and adsorbent) were rediluted.   A 1:1 dilution was made with antiserum and labeled-T3 while a 1:0.5 dilution was made with the adsorbent.   To further conserve material 10 ul aliquots of both standards and samples were employed.

Experiment 4.   10 µl aliquots of standards were used to produce a calibration curve from which were extrapolated T3 titres for adult serum and coelomic fluids from three separate giant tadpoles.   Two further vials of T3-free serum were also assayed for comparison with that used in experiment 3.

Experiment 5.   The previous experiment was repeated to obtain T3 values for adult serum and coelomic fluid from normal stage 57/59, stage 56, stage 52/54, stage 52 and stage 49/51 tadpoles.   A fourth T3-free standard was tested.

7.3 RESULTS

In expressing T3 and T4 titres the units used by many clinical workers and by the RIA kit manufacturers have been followed.   Thus T3 is expressed as ng T3/ml and T4 as ug T4/100 ml.   To convert ng/ml to µg/100 ml the first value is divided by 10.

7.3.a.   Competitive protein-binding assay for circulating T4

The calibration lines are drawn in Fig. 7/1 and extrapolated T4 values for Xenopus body fluids appear in the legend to that figure.

FIGURE 7/2.   Thyroxine radioimmunoassay – preliminary experiments
(Experiments 1 and 2)

a)   Experiment 1
●-● Calibration curve using 0.15, 4.10, 10.8 and
19.2 μg T4/100 ml standards.   The reconstituted re-
agents (labeled T4, antiserum and adsorbent) were
used in accordance with the Radiochemical Centre's
recommendations and were not rediluted.   The follow-
ing Xenopus sample was assayed:

Sample	Source	Net ^{125}I counts per 2 min (& mean)	μg T4/100ml. (extrapolated from curve)
Adult serum	Single female	16,600 17,100 (16,850)	<0.15

b)   Experiment 2
O-O Calibration curve using 0.15, 4.10, 10.8 and
19.2 μg T4/100 ml standards.   In this experiment the
reagents (labeled T4, antiserum and adsorbent) were
rediluted 1 : 2.

147
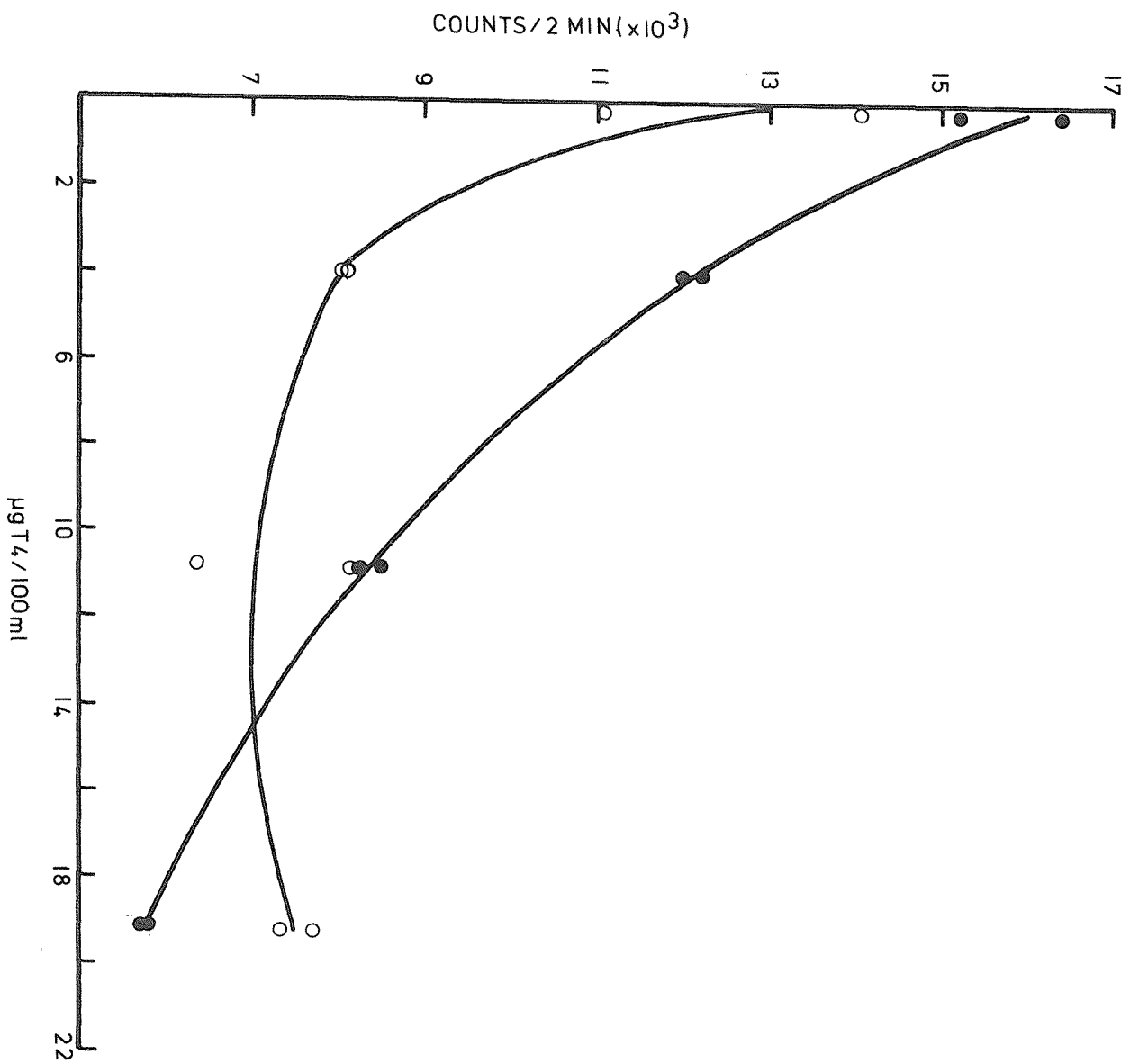
A summary of the thyroxine concentrations found in Xenopus serum, and coelomic fluids from treated and normal tadpoles, by the competitive protein-binding method is given in Table 7/2.

Table 7/2. Summary of Xenopus serum - and coelomic fluid - T4 titres obtained by the competitive protein - binding technique

Sample	µg T4/100 ml
Adult ♀ A serum	< 1.76
Adult ♀ B serum	< 1.76
Adult ♀ C serum	0.8
Goitrogen-treated tadpoles (stunted giants) coelomic fluid	0.85
Normal toadlet coelomic fluid	0.75
Normal stage 56/59 coelomic fluid	< 1.76
Normal stage 52 coelomic fluid	1.00
Normal stage 48/50 coelomic fluid	0.50

If one assumes an approximately straight line relationship to hold between percentage retention and µg T4/100 ml, as was found by Higgs and Eales, from 0 to 10 µg/100 ml then one can use the results of Experiment 2 to predict a scale of magnitude of T4 titres from the percentages retention. In that experiment an ascending order of T4 titres was as follows: goitrogen-treated tadpoles < stage 48/50 < stage 56/59.

This agrees with Experiment 4 in that goitrogen-treated tadpoles are lower than stage 56/59 but unlike Experiment 4 goitrogen-treated are also lower than the pre-metamorphic stage 48/50 group. The goitrogen-treated larvae used here were stunted by crowding.

7.3.b. T4 Radioimmunoassay

The duplicability and shape of the calibration curve obtained in Experiment 1 (Fig. 7/2a) were good. This suggested that the reagents and methodology were satisfactory.

However when all the reconstituted reagents (antiserum, labeled T4 and adsorbent) were rediluted 1:2, duplicability and curve shape declined in quality (Fig. 7/2b).

FIGURE 7/3.   Thyroxine radioimmunoassay (Experiments 3, 4 and 5).

a)   Experiment 3

●-●Calibration curve using 0.15, 4.10, 10.8 and 19.2 µg T4/ 100 ml standards.   The reconstituted reagents (labeled T4, antiserum and adsorbent) were used in accordance with the Radiochemical Centre's recommendations and were not re-diluted.

The following Xenopus samples were assayed:

Sample	Source	Net ^{125}I counts per 2 min (& mean)	µg T4/100 ml (extrapolated from curve)
Adult serum	Single female	16,500	<0.15
Toadlet coelomic fluid	Single 3cm specimen	16,000	<0.15
Goitrogen - treated tadpole coelomic fluid	22 St. 53/54 crowded & treated 28 weeks (pooled)	13,800 11,800 (12,800)	2.4
St. 56/59 coelomic fluid	16 tadpoles (pooled)	9,500 9,100 (9,300)	7.7
St. 49/50 coelomic fluid	15 tadpoles (pooled)	11,500 9,700 (10,600)	5.2

b)   Experiment 4

□-□ Calibration curve using   - 0.15, 4.10, 10.8 and 19.2 µg T4/100 ml standards.   The reconstituted labeled T4 and antiserum were rediluted 1 : 2.   The reconstituted adsorbent was not rediluted.

c)   Experiment 5

▲-▲Calibration curve using 0.06, 0.15, 4.10, 10.8 and 19.2 µg T4/100 ml standards.   The reconstituted labeled T4 and antiserum were rediluted 1 : 2.   The reconstituted adsorbent was rediluted 1 : 0.5.
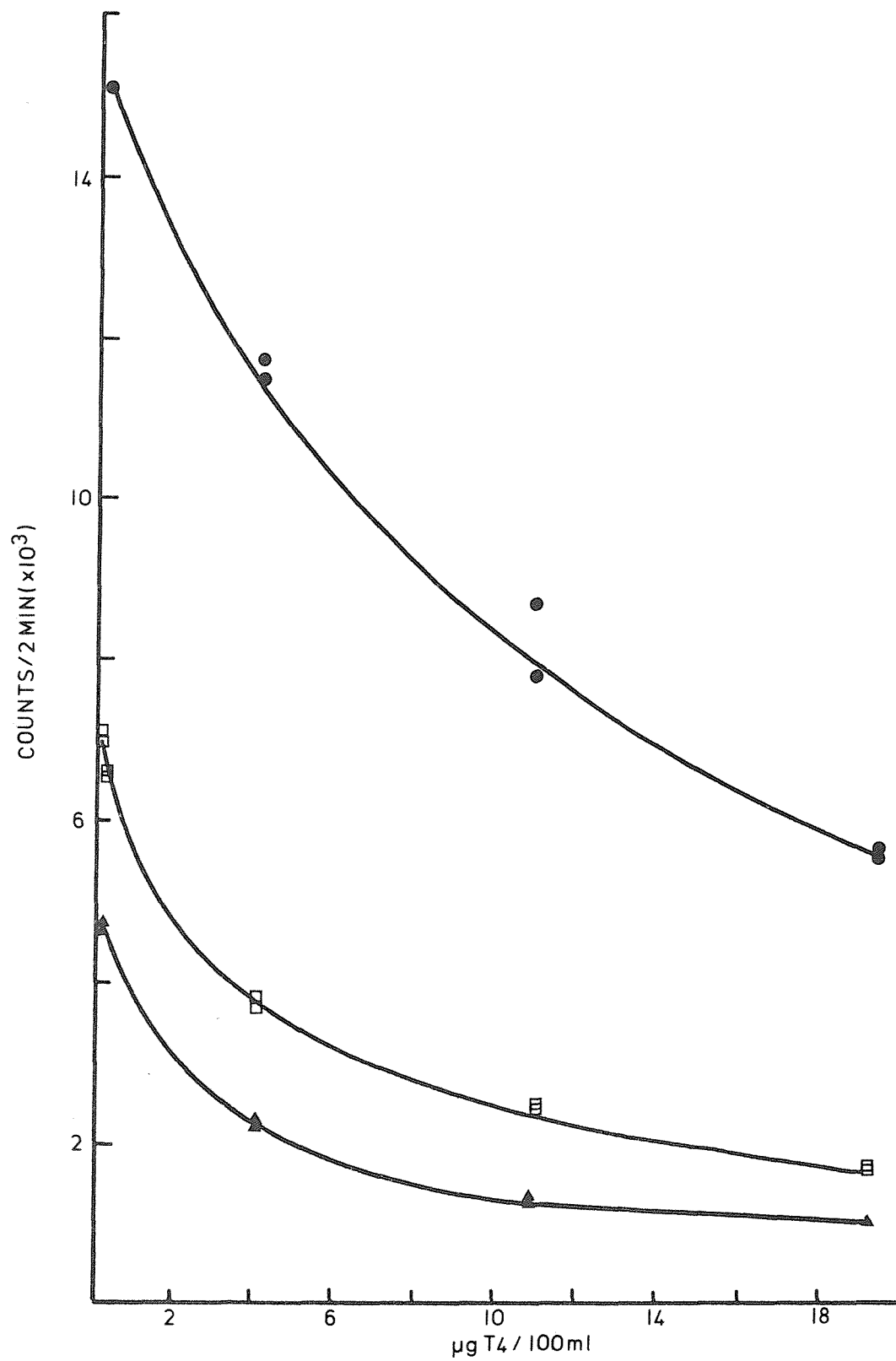
FIGURE 7/4.   Thyroxine radioimmunoassay (Experiment 6)

Experiment 6

Calibration curve using 0.06, 0.15, 4.10 and 10.8 μg T4/100ml standards.   The reconstituted labeled T4 and antiserum were rediluted 1:2.   The reconstituted adsorbent was rediluted 1 : 05.   The following Xenopus samples were assayed:

Sample	Source	Net ^{125}I counts per 2 min (& mean)	μg T4/100 ml (extrapolated from curve)
Adult serum	Single female	8,080 7,900 (7,990)	<0.06
Giant treated tadpole coelomic fluid (A)	1 X St. 58 tadpole. Uncrowded & treated 36 weeks. 1.38g.	7,640	<0.06
Giant treated tadpole coelomic fluid (B)	2 X St. 58 tadpoles. Uncrowded & treated 36 weeks. 1.39 & 1.80g. (pooled)	7,700	<0.06
Giant treated tadpole coelomic fluid (C)	1 X St. 56 tadpole. Uncrowded & treated 36 weeks. 1.85g.	7,760	<0.06
St. 57/59 coelomic fluid	14 tadpoles (pooled)	6,780 5,840 (6,310)	0.39
St. 54/56 coelomic fluid	38 tadpoles (pooled)	6,160 6,300 (6,230)	0.40
St. 52/54 coelomic fluid	29 tadpoles (pooled)	6,080 6,820 (6,450)	0.32
St. 49/51 coelomic fluid	47 tadpoles (pooled)	6,400	0.32

COUNTS / 2 MIN ($\times 10^3$)

µg T4 / 100 ml

150
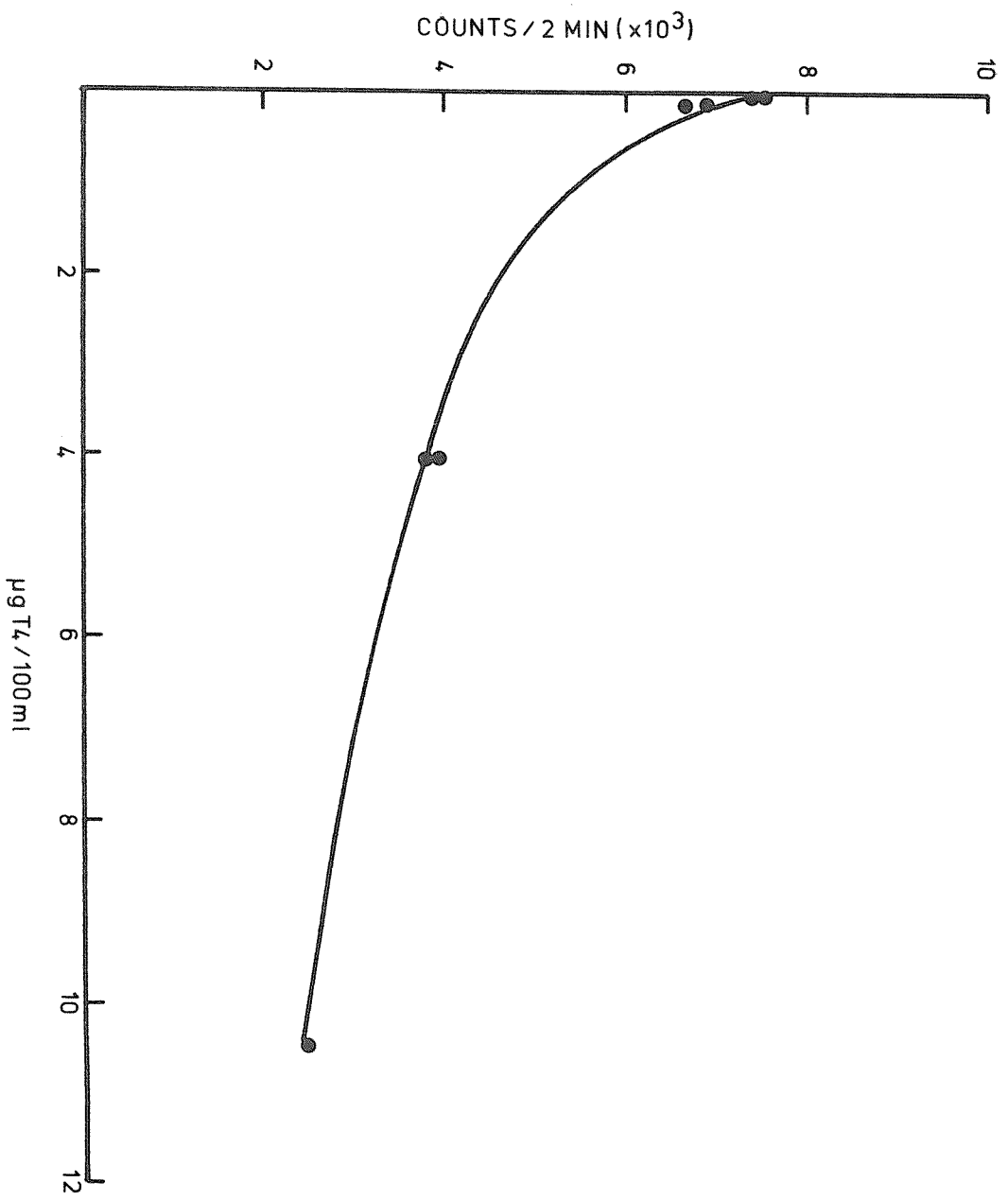
Thus in the first assay of _Xenopus_ fluids attempted (Experiment 3), non-rediluted reconstituted reagents were used. The results appear in Fig. 7/3a. The calibration curve was of a reasonable shape but the duplicability of the counts obtained with goitrogen-treated tadpole and normal stage 49/50 tadpole coelomic fluid was poorer. As the results obtained with stage 56/59 tadpoles were reasonable there was no reason to suspect some deleterious influence of coelomic fluid proteins.

Experiments 4 and 5 (Fig. 7/3b) proved that redilution of antiserum and labeled thyroxine (1:2) did not adversely affect the assay providing that the reconstituted adsorbent was not rediluted less than 1:0.5.

The latter dilution was used in Experiment 6 in which further _Xenopus_ fluids were assayed. Duplicability was more satisfactory than in Experiment 3 but was still poorer than that obtained with standards of T4 diluted in human serum (Fig. 7/4).

The T4 titres obtained in Experiments 1, 3 and 6 are compared graphically in Fig. 7/5 in which the results of the competitive protein-binding assay for T4 also appear.

There is agreement that T4 levels in 3 adult females' serum and one toadlet's coelomic fluid were very low - probably less than 0.06 µg T4/100 ml in each case. There is also agreement within Experiment 3 and within Experiment 6 that propylthiouracil treated tadpoles, whether large or stunted giants, possessed depressed T4 levels by comparison with normal tadpoles of even stage 49/50. However the agreement in absolute values between the latter two experiments is very poor. In both cases the calibration curve was satisfactory indicating that methodology was correct. Duplicability of sample counts was poor in Experiment 3 but this would not account for the differences in T4 values encountered.

Also in Experiment 3 the titre of stage 49/50 tadpoles was appreciably lower than that of stage 56/59 (5.2 to 7.7 µg T4/100 ml) whereas in Experiment 6 they were almost identical.

T4 titres obtained by the competitive protein-binding method were intermediate between those of Experiments 3 and 6 (T4-RIA). The T4 titre of goitrogen treated tadpoles was intermediate between normal stage 48/50 and stage 52 values.

7.3.c.   T3 Radioimmunoassay

The manufacturers state that, in addition to the shape of the calibration curve, a good performance check is to calculate the ratio of counts obtained with tubes 1 and 2 to tubes 3 and 4; between tubes 3 and 4 to 5 and 6; and finally between tubes 5 and 6 to 7 and 8. Tubes 1 and 2 are duplicates of the lowest standard and tubes 7 and 8 are duplicates of the highest standard.   This check was used with the 4 standards supplied but not with the extra low-titre standards obtained by dilution.

The calibration curve and performance check obtained in experiment 1 (Fig. 7/6a) suggested that the general methodology was satisfactory. The extrapolated T3 titres with _Xenopus_ fluids were all lower than the lowest standard titre of 0.10 ng T3/ml.   If one assumes the calibration curve to continue towards 0 ng T3/ml with the same slope one can suggest an _ascending_ order of titres from the order of counts thus: stage 49/50 < toadlet < stage 56/59 < treated tadpoles < adult It is unlikely that the differences between stage 49/50, toadlet, stage 56/59 and goitrogen-treated tadpole T3 titres are significant.   The difference between these and the adult may be attributable to differences between coelomic fluid and serum T4.

In experiment 2 the use of 5, 10 and 25 ul of the 0.01 ng T3/ml standard did not give good results.   Fig. 7/6b shows that the curve fit is poor in the lower region.   The fit in experiment 3 (Fig. 7/6b) was somewhat better when dilution rather than diminution of volume was used to obtain the low titre points.   Duplicability was poor however and most puzzling was the fact that the 0-T3 serum used as a diluent gave lower counts than did not solution it had diluted.   Thus a satisfactory 0-T3 point was not obtained.   The difference in the range of counts observed between experiments 2 and 3 results from the fact that 10 ul rather than 25 ul of ^{125}I— T3 were used in experiment 3.

FIGURE 7/5

Histograms summarising T4 titres obtained by radioimmunoassay
(Experiments 1, 3 and 6) and by competitive protein binding
assay ('Tetralute').

      T4 RIA (Experiment 1)

          1. Adult serum

      T4 RIA (Experiment 3)

          2. Adult serum

          3. Toadlet coelomic fluid

          4. Treated tadpole coelomic fluid

          5. Normal st. 56/59 coelomic fluid

          6. Normal st. 49/50 coelomic fluid

      T4 RIA (Experiment 6)

          7. Adult serum

          8. Treated tadpole A coelomic fluid

          9. Treated tadpole B coelomic fluid

         10. Treated tadpole C coelomic fluid

         11. Normal st 57/59 coelomic fluid

         12. Normal st 54/56 coelomic fluid

         13. Normal st 52/54 coelomic fluid

         14. Normal st 49/51 coelomic fluid

    Competitive protein binding assay (Experiment 4)

         15. Adult serum

         16. Toadlet coelomic fluid

         17. Treated tadpole coelomic fluid

         18. Normal st. 52 coelomic fluid

         19. Normal st. 48/50 coelomic fluid

FIGURE 7/6.   Triiodothyronine radioimmunoassay (Experiments 1, 2 and 3)

   a)   Experiment 1

   ●-●Calibration curve using 0.10, 0.69, 2.01 and 5.11 ng T3/
       ml standards.   The reconstituted reagents (labeled T3,
       antiserum and adsorbent) were used in accordance with the
       Radiochemical Centre's recommendations and were not
       rediluted.   The following Xenopus samples were assayed:

Sample	Source	Net ^{125}I counts per 2 min (& means)	ng T3/ml (extrapolated from curve)
Adult serum	As in Fig 7/3 legend	6,960 6,960 (6,960)	<0.10
Toadlet coelomic fluid	"	7,600 7,600 (7,600)	<0.10
Goitrogen – treated tadpole coelomic fluid	"	7,140 7,880 (7,510)	<0.10
St. 56/59 coelomic fluid	"	7,360 7,760 (7,560)	<0.10
St. 49/50 tadpole coelomic fluid	"	7,620 7,600 (7,610)	<0.10

   b)   Experiment 2.   Preliminary experiment to test assay perfor-
       mance with low T3 titre standards.   The standard titres were
       reduced by using smaller volumes of the normal standards.
       The standards used were, 0.02 (5 μl of 0.10), 0.04 (10μl of
       0.10), 0.10 (25μl of 0.10), 0.28 (10μl of 0.69), 2.01 (25μl of
       2.01) ng T3/ml.
       The reconstituted reagentswere not rediluted.
       ▲-▲Calibration curve.

       Experiment 3.   Preliminary experiment to test assay perfor-
       mance with low T3 titre standards.   The standard titres were
       reduced by dilution with a "0 ng T3/ml" sample.   The stand-
       ards used were 0, 0.025, 0.10, 0.69, 2.01 and 5.11 ng T3/ml.
       The reconstituted labeled T3 and antiserum were rediluted 1:1
       while the adsorbent was rediluted 1 : 0.5.
       O-OCalibration curve.
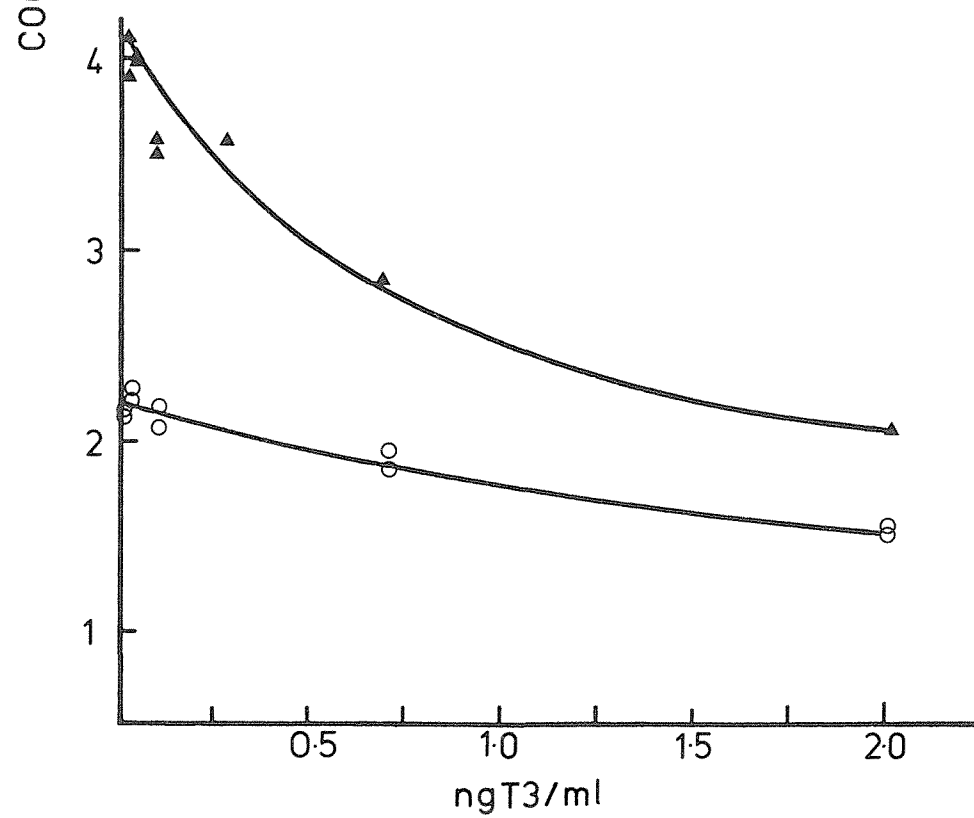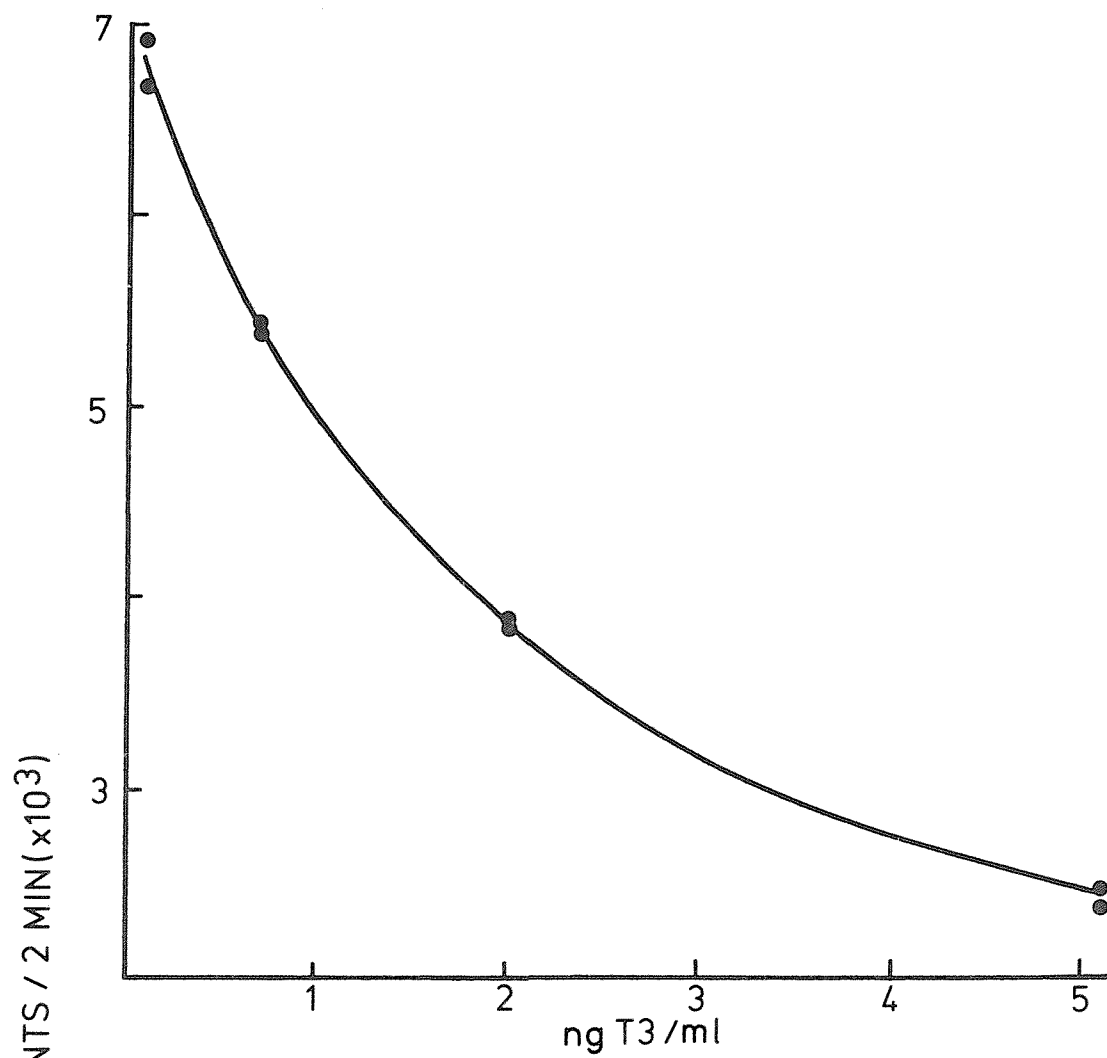
The fact that circulating T3 titres in _Xenopus_ had been shown to be very low in experiment 1 made it essential to attempt calibration below 0.10 ng T3/ml and the dilution method was chosen for experiments 4 and 5.

In experiment 4 further batches of O-T3 serum were tried (Fig. 7/7a). The results obtained with vial C shows that duplicability can be good whereas vial B which was assayed in triplicate suggests that low repeatability may be due to minor methodological variation. The repeated pipetting of 5 μl aliquots is suspected as the source of occasional error.

Again the counts of O ng-T3/ml standards were lower than those of the 0.025 ng T3/ml standard.

The values for 0.025 ng T3/ml standards were satisfactory and this adds to the acceptability of the extrapolated values for adult serum. The flatness of the curve means that relatively small differences in counts lead to large differences in T3 titres extrapolated from the abscissa.

The order of counts confirms the conclusion of experiment 1 that T3 titres are lower in goitrogen-treated tadpole coelomic fluid than in adult serum.   This does not, of course, imply a metamorphic difference. The difference may be attributable to the difference in body fluid type.

In experiment 5 vial D of O ng T3/ml serum provided more satisfactory results both as a standard and as a diluent.   Nevertheless the counts of most of the normal _Xenopus_ tadpoles samples were higher than those with T3-free serum.

Clearly in _Xenopus_ body fluids one is encountering T3 titres which are less than one hundredth of those in normal human serum. Even with diluted standards the reliability of differences between titres in the O to 0.02 ng T3/ml is not expected to be great.   Experiments 4 and 5 were carried out sequentially and both used serum from the same female toad to provide a criterion for comparison.   The count rate for adult serum was 5% higher in Experiment 4 than in Experiment 5.   If the count rates for the 3 giant tadpole titres of Experiment 4 are reduced by 5% and
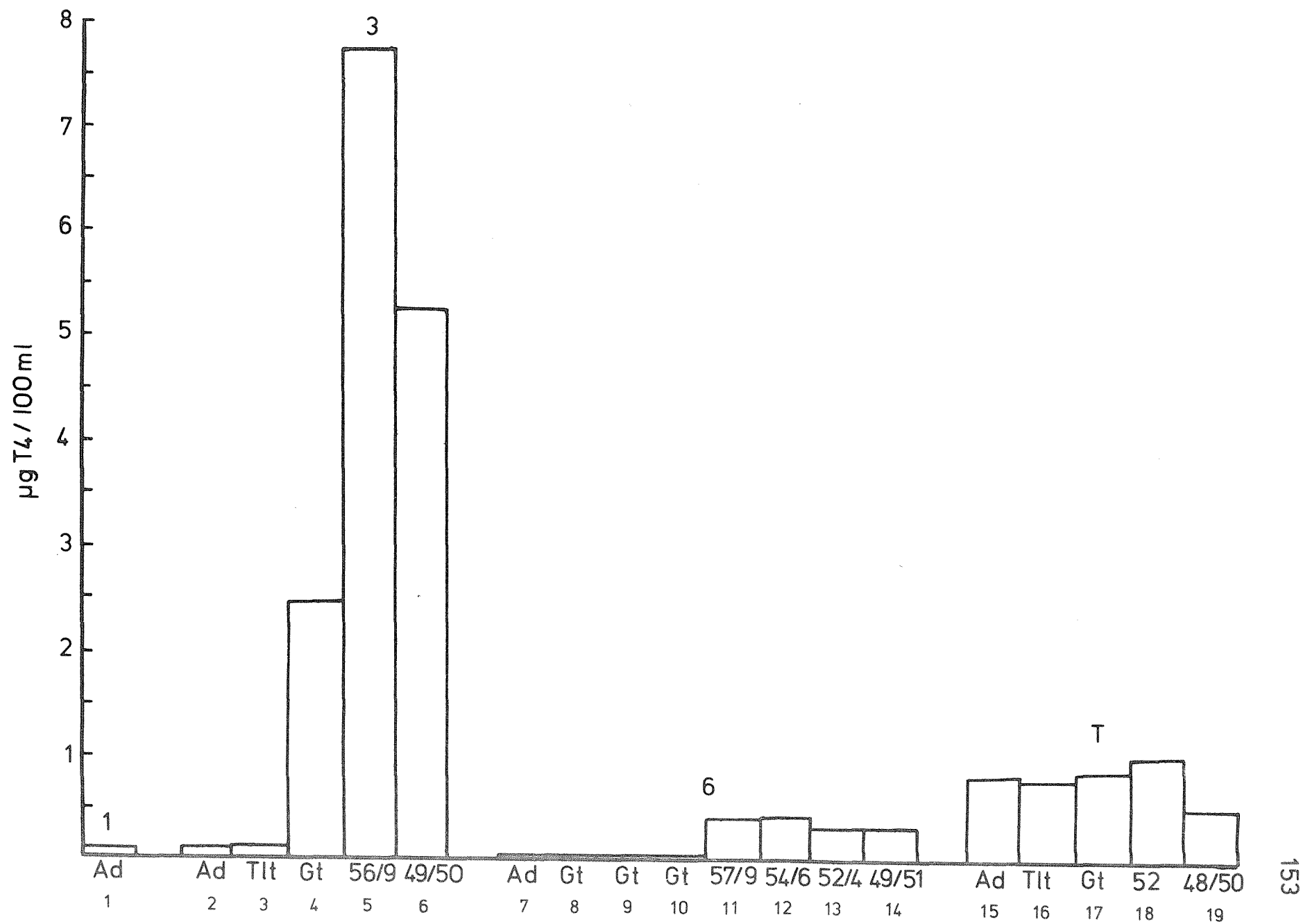
FIGURE 7/7   Triiodothyronine radioimmunoassay (Experiments 4 and 5)

Experiment 4.

● – ● Calibration curve using 0, 0.025 0.10, 0.69, 2.01 and 5.11 ng T3/ml standards. The reconstituted labeled T3 and antiserum were rediluted 1:1 while the adsorbent was rediluted 1:05. The following _Xenopus_ samples were assayed.

Sample	Source	Net ^{125}I counts per 2 min (& means)	ng T3/ml (extrapolated from curve)
Adult serum	As in Fig 7/4 legend	1,580 1,560 (1,570)	0.05
Giant treated tadpole coelomic fluid (A)	"	1,600	<0.025
Giant treated tadpole coelomic fluid (B)	"	1,560	0.06
Giant treated tadpole coelomic fluid (C)	"	1,660	<0.025

Experiment 5

▲ – ▲ Calibration curve using 0, 0.025, 0.10, 0.69, 2.01 and 5.11 ng T3/ml standards. The reconstituted labeled T3 and antiserum were rediluted 1:1 while the adsorbent was rediluted 1:05. The following _Xenopus_ samples were assayed.

Sample	Source	Net ^{125}I counts per 2 min (& means)	ng T3/ml (extrapolated from curve)
Adult serum	Single female	1,480 1,500 (1,490)	<0.025
St. 49/51 coelomic fluid	As in Fig 7/4 legend	1,400 1,380 (1,390)	<0.025
St. 52 coelomic fluid	13 tadpoles (pooled)	1,360 1,420 (1,390)	<0.025
St. 52/54 coelomic fluid	As in Fig 7/4 legend	1,380 1,500 (1,440)	<0.025
St. 56 coelomic fluid	As in Fig 7/6 legend	1,320 1,360 (1,340)	0.10
St. 57/59 coelomic fluid	As in Fig 7/4 legend	1,420 1,380 (1,400)	<0.025
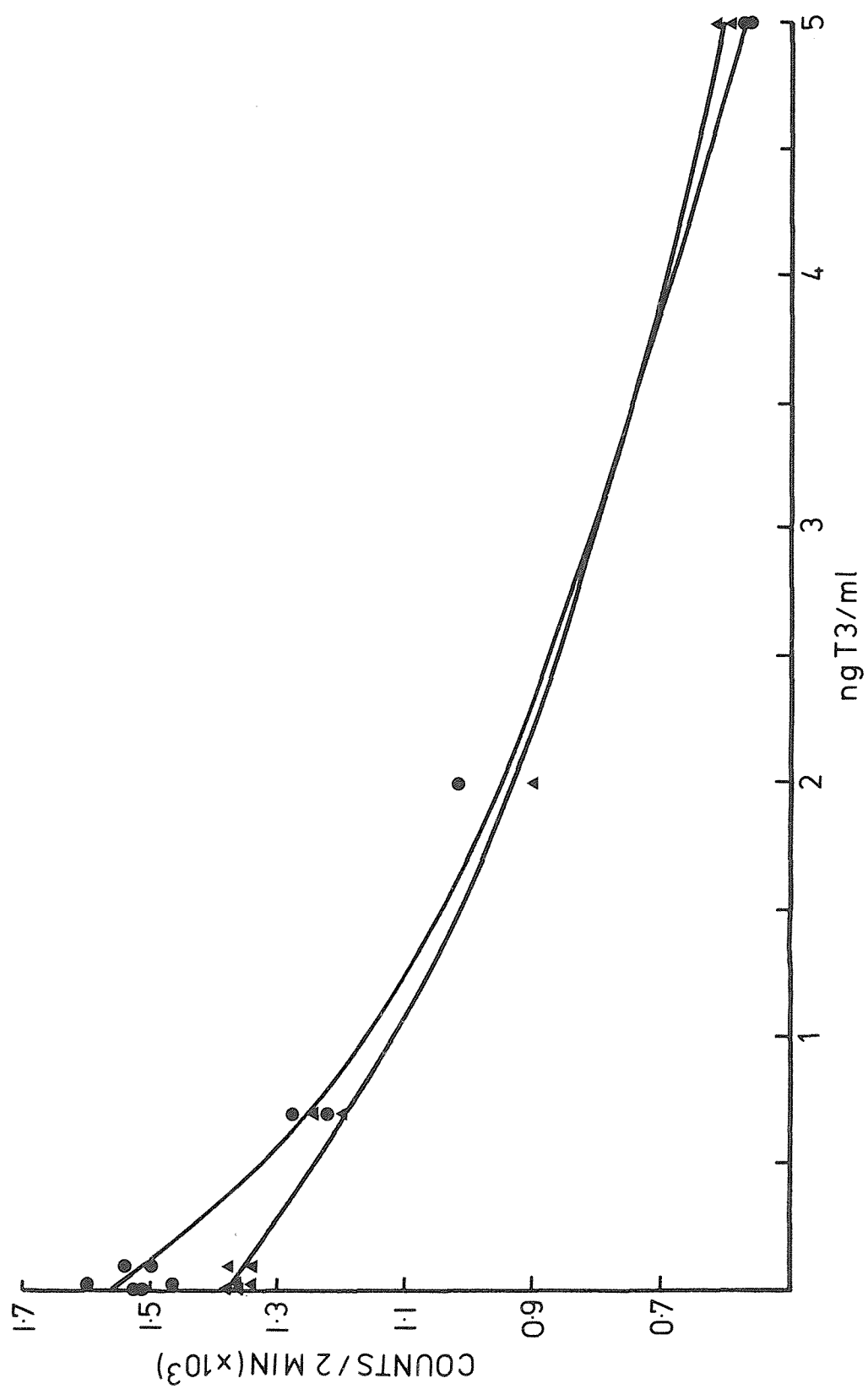
averaged the value for comparison is 1,526 counts/2 min. If the hormone titres suggested by the count rates are arranged in an _ascending_ order:

Goitrogen-treated $<$ stage 52/54 $<$ stage 57/59 $<$ stage 49/51 & 52 $<$ stage 56

Again it is doubtful whether these values are significantly different from one another in view of the flatness of the calibration curve in the low titre region.

7.4 CONCLUSIONS

The assays of coelomic fluid T3 and T4 were performed to discover whether propylthiouracil treatment for prolonged periods did depress hormone titres to, or below, normal premetamorphic levels. The most sophisticated methods available were chosen but it is clear that they do not give repeatable results with the extremely low hormone levels encountered in _Xenopus_ fluids. Nevertheless provisional conclusions can be drawn from the rank order of ^{125}I-count rates obtained with different samples. These results suggest that propylthiouracil does indeed depress T4 levels to below those of premetamorphic normal tadpoles.

T4 titres on tadpoles and adults are not comparable here because coelomic fluid and serum respectively were used in the assays. However the hormone titres obtained with adult serum may be compared with the scanty published data. When these experiments were performed the best comparison with other lower vertebrates was with the competitive protein-binding studies of Canadian workers. Packard _et al_ (1976) assayed 1.7 µg/100 ml of thyroxine in Mexican _Rana pipiens_ adult serum. In the present work adult titres determined both by competitive protein-binding and RIA were slightly lower than this but the _Rana_ data support the conclusion that amphibian circulating total T4 values are much lower than those of humans. Seligson and Seligson (1972) reported 3.0-6.0 µg T4/100 ml in normal human sera by the "Tetralute" method.

The other comparison in amphibia was with the work of Thorburn (1967) who measured 10 to 22.6 µg T4/100 ml in adult _Bufo bufo_. It seems certain that the discrepancy is due to his relatively insensitive method of quantitative paper chromatography.

In other cold-blooded vertebrates Higgs and Eales (1973) assayed less than 0.5 μg T4/100 ml in most individuals of 10 species of freshwater fishes. In the Pacific Hagfish, Eptatretus stouti Henderson and Lorscheider (1975) found 2 to 3 μg T4/100 ml of plasma.

Recently RIA data on fish T4 levels have been reported. Brown and Eales (1977) and White and Henderson (1977) used a new technique, which is described below, with salmonid plasma. Brown and Eales (1977) assayed 0.1 to 0.61 μg T4/100 ml in different pooled samples of Salmo gairdneri plasma. White and Henderson (1977) found from 0.08 to 0.37 μg T4/100 ml in Salvelinus fontinalis plasma. These figures confirm that the T4 titres of lower vertebrates are so low that they require a more sensitive assay than any available for clinical use.

However the general agreement between the adult serum T4 levels reported here and those found by Packard et al (1976) in Rana pipiens suggests that the preliminary conclusion drawn above, that Xenopus larvae are unable to raise their circulating T4 titres, when treated for prolonged periods with propylthiouracil, is valid. It seems probable that had T4 titres been elevated markedly, as predicted for normal metamorphosis by Etkin (1964a), this would have been detected by the RIA. The question of whether large increases in T4 titre do occur at climax is discussed in Chapter 8. Shortage of larvae prevented study of this aspect. Except in Experiment 3, of the T4 RIA's, there was no marked elevation during prometamorphosis.

The PBI measurements of Just (1972) have cast doubts on the hypothesis that a large (up to 100-fold) increase in T4 titre does occur at climax. Just was only able to demonstrate a 10-fold increase in a proportion of climax larvae. Even this value may be too high as Henderson and Lorscheider (1975) were able to compare PBI and competitive protein-binding assay data with divided samples of hagfish plasma. They found that thyroxine iodine comprised less than one-third of plasma organic iodide as measured by the PBI method.

The T3 titres assayed by RIA were even lower when compared with normal human levels. In Xenopus they were below 0.025 ng T3/ml whereas

Chopra et al (1971) reported 1.0 to 1.7 ng T3/ml in normal human sera.
Thus it is unlikely that significant Xenopus fluid results can be
obtained with human RIA's.   Thornburn (1967) claimed 8.2 to 139 ng
T3/ml in adult Bufo bufo.   The insensitivity of his method makes these
figures suspect.   In the present work there was certainly no evidence
for a large rise in T3 titre in treated tadpoles and in Experiment 5 of
the T3 RIA's the count rates suggested treated-tadpole coelomic fluid
to have the lowest hormone titre.

Brown and Eales (1977) reported 0.5 to 6.3 ng T3/ml of Salmo
gairdneri plasma while White and Henderson (1977) assayed 2.4 to 8.6 ng
T3/ml in Salvelinus fontinalis plasma.   In humans the normal T4:T3
ratio is about 30:1 on a molar basis.   The recent RIA results suggest
that in salmonids the T4:T3 molar ratio may be less than 1:1. The
situation in adult Xenopus seems   similar to that in humans with
a considerable excess of T4 over T3.

Radioimmunoassay was chosen for this work because it was likely
to be the most sensitive and specific technique available for T4 and T3
assays.   With Xenopus fluids it seems that the human assay kits were
at or beyond, their lower limits of accuracy.   Particularly worrying
was inconsistency between experiments.   That general methodology was
satisfactory was indicated by good duplicability with standards and in
the forms of calibration curves.

A priori the most likely cause of spurious results was thought to
be differences in hormone binding properties between human and Xenopus
body fluid proteins.   Good evidence was obtained here that no adverse
affect was occurring.

Very little information is available on thyronone-binding proteins
in amphibian body fluids.   Farer et al (1962) found labeled thyroxine
bound to a fast-moving albumin-like fraction of Rana catesbeiana serum.
Most of the hormone migrated in the human beta-globulin position though,
on paper chromatograms.   However Dowling et al (1964) studied temperature-
dependent turnover rates of T4 and T3 in adult R. pipiens and Bufo
marinus.   They investigated plasma hormone binding by equilibrium dialysis

and suggested that T4-binding acitivity in the adult anuran plasma is very low. The turnover of these hormones was slowed by injection of human plasma. Medda and Premachandra (1968) found that normal rabbit serum (and apparently any good T4 binding protein) was a potent inhibitor of bullfrog metamorphosis. This was interpreted as evidence that mammalian serum proteins can affectively bind TH against competition from endogenous serum proteins. The bound hormone was assumed to be inactive as a morphogenetic compound. In fishes Refetoff et al (1970) found that a large proportion of total thyroxine was free (unbound) and so it may be that specific TH transport proteins are not as important in lower vertebrates as they are in humans.

A further possible source of variation was deterioration of samples when deep frozen. This seems unlikely as Postmes et al (1974) stored human serum at $-20^{\circ}C$ for 2 years without any RIA-detectable deterioration. Even at $5^{\circ}C$ storage for 7 days did not cause the deterioration of human material (Lieblich and Utiger, 1972).

Just (1972) found great variation in individual levels of PBI in Rana pipiens tadpoles. It seems unlikely that the variation was entirely genuine here as all samples were pools of coelomic fluid from a number of individuals. However the RIA data of Brown and Eales (1977) and White and Henderson (1977) cited above showed considerable variation in TH titres amongst pooled plasma samples from salmonids. In Salmo gairdneri a four-fold T4 variation and a twelve-fold T3 variation were noted (Brown and Eales, 1977). In Salvelinus fontinalis the four-fold variation in both T3 and T4 was correlated with season. Minimal values occurred in November at the time of spawning and maximal values were in mid-spring. It may be that some of the variation between Xenopus larval TH assays was genuine.

Until more satisfactory TH assays have been developed it will be impossible to obtain accurate data on circulating TH levels in normal or goitrogen-treated tadpoles. Human patients receiving anti-thyroid drugs are not normally allowed to become goitrous so that RIA data showing the effect of treatment on the more readily assayed human thyroid hormones are not available. However T4 was certainly reduced to below 0.7 μg T4/

100 ml in one mildly goitrous patient compared to normal levels of 3.0 to 6.0 μg T4/100 ml (Mardell, 1976)

A number of technical difficulties would attend the development of a RIA technique to measure the extremely low amphibian TH titres. Supposedly zero T3 or T4 serum is produced by mixing labeled hormone with normal serum and then repeatedly passing the mixture through anion exchange columns until the count rate of the eluate approaches zero. In practice small levels of hormone remain and so it is possible to get higher count rates with samples than with "zero TH serum". A further problem is that even highly purified T4 or T3 contains impurities which may become radioactively labeled. With the very steep shape of calibration curves, as they approach zero TH, small differences in count rates due to such contaminants or due to technical errors will produce large differences in extrapolated hormone titres.

The TH assay developed by Brown and Eales (1977) combined the "Tetralute" competitive protein-binding technique with that of RIA. The assays were performed on miniature Sephadex columns but the specific hormone binding agent was an anti-T3 or anti-T4 serum rather than human TBG. The authors claimed a great increase in sensitivity over normal clinical methods. Mean detection limits were 0.012 μg T4/100 ml and 0.095 ng T3/ml. Although the T4 assay would have been adequate for Xenopus work the T3 assay still lacks the necessary sensitivity. Kistler et al (1977) have suggested that the great preference for T3 shown by cytosol receptors from bullfrog tadpole tail (discussed in Chapter 8) might be usable as a radio-receptor assay more sensitive than conventional RIA's.

CHAPTER 8

GENERAL DISCUSSION

8.1 Outline of Discussion

In each of the five preceding experimental chapters only the
literature directly relevant to the biochemical metamorphic marker, or
hormone, concerned was reviewed. Similarly, the conclusions drawn
from my results were confined to the immediate context of each system.
In this final chapter these main conclusions will be interpreted in
the wider context of the hormonal control of amphibian metamorphosis.

Firstly the principal conclusions which were made in Chapters 3 to
7 are summarised. Two important overall points emerge. Four biochemical
transitions, normally temporally correlated with morphological
metamorphosis, can occur in **Xenopus** larvae immersed continuously in
propylthiouracil solutions. The observation that morphological
development was severely retarded and the results of hormone assays both
support the contention that propylthiouracil is a potent goitrogen (Section
8.2).

The interpretation of these overall points is the primary aim of
the present chapter. Three of the protein systems (haemoglobin,
albumin and crystallins) have previously been studied during spontaneous
and induced metamorphosis. In Section 8.3 the evidence which has
suggested a controlling role for thyroid hormones is considered.

The simplest interpretation of my data is made in terms of the
Etkin (1964a) hypothesis for the hormonal control of amphibian
metamorphosis (Section 8.4). However the hypothesis was originally
propounded on the basis of fragmentary evidence and has received both
support and criticism since 1964.

One alternative explanation is that residual levels of circulating
thyroid hormone might have a progressive ("stoichiometric") effect on
certain tissues especially if the latter have low thresholds of response.

Thus it might be argued that haemopoietic tissues, hepatocytes and lens epithelium have lower thresholds of response than the developing limbs or regressing tail (Section 8.5).

Although thyroid hormone control of several biochemical transitions has been suggested by the studies reviewed in Section 8.3 it has become increasingly apparent that other hormones, and especially the growth promoting hormone "prolactin", have a role in the control of morphogenesis (Section 8.6). In view of the relationship between crystallin pattern and lens diameter irrespective of morphological stage, demonstrated in Chapter 6, growth regulating morphogenetic hormones are especially relevant.

Thyroid hormones have been observed to produce long-term or "triggering" effects on amphibian metamorphosis. The importance of this phenomenon is not yet known and it has not been demonstrated with haemopoietic, liver or lens tissues in Xenopus. However it could be argued that a brief exposure to premetamorphic circulating thyroid hormone levels was sufficient to begin the biochemical differentiative process which continued in some animals under goitrogen treatment (Section 8.7).

Until recently stoichiometric hormone action, threshold levels and long-term hormonal effects have lacked any basis at the molecular level with thyroid hormones. Several researchers have sought a mechanism for thyroid hormone action in terms of receptor proteins and direct interaction of hormone-receptor complexes with chromatin. As these studies may eventually provide an explanation for the metamorphic phenomena discussed in Sections 8.4, 8.5 and 8.7 they are reviewed in detail in Section 8.8.

The Etkin model (Etkin 1964a; 1968) for the hormonal control of amphibian metamorphosis predicted that the normal orderly sequence of morphogenetic (and presumably biochemical) events was coordinated by a sharply rising circulating thyroid hormone level. As published data had not supported this contention Dodd and Dodd (1976) produced a revised metamorphosis hypothesis. This proposed that, although thyroidal output of hormone increases markedly at climax, circulating levels do not rise because of increasing intracellular hormone binding and utilisation.

This hypothesis together with the evidence for rising thyroid hormone levels is discussed in Section 8.9.

Propylthiouracil treatment produced an apparent dissociation of development and growth. A possible interpretation of my results is that "biochemical metamorphosis" is correlated with body growth rather than with gross morphology. Evidence that this is so was obtained in the case of the lens proteins and this system is discussed further in Section 8.10.

The Etkin hypothesis remains the best substantiated explanation of metamorphosis control. Thus one is forced to conclude that continued "biochemical metamorphosis" in the presence of propylthiouracil suggests that haemoglobin, albumin, LDH isozyme and crystallin shifts are not under primary thyroid control. The final Section (8.11) compares the biochemical transitions of anurans such as Xenopus with those of salamanders and other vertebrates. It is suggested that thyroid hormone control of metamorphosis may only have evolved in those systems (such as tail regression) which are unique to metamorphosing Amphibia.

8.2. Summary of principal conclusions from Chapters 3 to 7.

Four protein systems were chosen which were expected to change markedly during the morphological metamorphosis of Xenopus laevis. It has generally been assumed by previous writers that these biochemical changes, like the changes in morphology of organs such as the legs and tail, are directly controlled by the thyroid hormones, thyroxine and triiodothyronine. In addition, it has been argued that the different properties of the metamorphosed protein systems confer physiological advantages on the post-metamorphic anuran which might be expected to be necessary for survival.

The thyroid hormone synthesis of premetamorphic X. laevis tadpoles was blocked by immersion, for up to 14 months, in 0.59 mM aqueous solutions of the goitrogen, propylthiouracil. The effect of the absence of TH on the protein systems was assessed to determine whether the presence of hormone is necessary for continued biochemical metamorphosis. Treated tadpoles were morphologically severely retarded but continued to grow to gigantic proportions.

Data presented in Chapters 3 to 6 inclusive confirmed that during
normal larval development ontogenetic changes occur in the four
protein systems.   In each case the changes were quantitative rather
than qualitative.   No new proteins were observed but there was an
increase in the synthesis or assembly of certain proteins, perhaps
accompanied by changes in degradation.   Thus, although the proteins
remained more or less the same, some fractions were more prominent in
the adult than in the larva and vice versa.

In the case of haemoglobins, the Hb A_1 fraction became the main
fraction of the adult whereas it had been a minor one, in terms of
relative concentration, in the tadpole.   The tadpole haemoglobins Hb F_1,
F_2 and F_3 were not detectable in adult haemolysates.   These results are
in accord with those of Maclean and Jurd (1971a).

Of 24 goitrogen-treated animals examined 42% had haemoglobin patterns
characteristic of toadlets or adults rather than of larvae, of similar
morphology.   The remaining 58% possessed 'tadpole' haemoglobins.   That
the synthesis of adult globins was actually being enhanced was shown by
studies on the incorporation of the amino acid, leucine into Hb A_1
haemoglobin.

In the second system the total protein concentrations of coelomic
fluid increased from about 2 mg/ml in stage 54 to about 8 mg/ml in toadlets.
The relative concentration of albumin increased by a factor of 10 during
the same developmental period.   As with haemoglobins, goitrogen-treated
larvae were variable both in terms of total protein and relative albumin
concentrations.   The animals examined exhibited every stage of change
from that of normal premetamorphic tadpoles to that of normal adults.
Like haemoglobin, the remarkable discovery was that some treated larvae
had transformed in this respect in the apparent absence of thyroid
hormones.

Neither the variation in haemoglobin patterns nor coelomic fluid
albumin concentration was clearly correlated with the body weight, stage
of arrest or duration of goitrogen treatment which also varied.

Changes in the heart LDH isozymes proved to be less amenable to research than the other systems because of the need to pool a number of hearts from even giant tadpoles. The results obtained during normal development showed that the relative concentrations of the more cathodal isozymes increased markedly through metamorphosis. A series of heart homogenates from different adult toads proved that this was not attributable to genetic polymorphism. When this marker was examined in giant tadpoles they were found to be 'adult' in their LDH isozymes.

In the final system, lens crystallins, the markers used were the increase in relative concentrations of alpha and fast-moving beta components as shown by polyacrylamide gel electrophoresis and two-dimensional immunoelectrophoresis. Qualitative IEP showed that alpha crystallin antigens only reached detectable levels in adult lenses and a number of 'new' antigens also were first detected after metamorphosis. In all the propylthiouracil retarded larvae examined, which had been treated for more than 8.5 weeks and which had reached abnormally large sizes, the 'adult' crystallin markers were found. A study of crystallins in normal and treated tadpoles showed a significant correlation between lens diameter and antigenic pattern.

In the last experimental chapter the best available methods, competitive protein binding and radioimmunoassay, were used to determine the long term effect of the goitrogen on thyroid hormone levels in the coelomic fluid.

In premetamorphic coelomic fluid there was about 0.5 μg T4/100 ml (6.0×10^{-6}M) and less than 0.025 ng T3/ml (3.8×10^{-8}M). In prometamorphic tadpoles there was no consistent detectable increase. Due to shortage of material climax coelomic fluid could not be assayed but in adults the titres of both hormones, in serum, were even lower than in tadpoles and scarcely distinguishable from zero.

Propylthiouracil depressed T4 titres to below the premetamorphic level mentioned above in giant tadpoles which had been treated for 40 weeks, with T3 the titres of all animals, treated or untreated, were very close to zero. There was certainly no evidence for a rise in circulating T3 under prolonged goitrogen blockage.

8.3.   The mechanisms of haemoglobin, albumin and crystallin transitions
       and the effect of exogenous thyroid hormone

To understand the role which morphogenetic hormones might play in
the control of protein transitions it is first necessary to study the
transition itself at the cellular level.   Following this the current
evidence that exogenous thyroid hormones do exert an effect will be
reviewed.   The control of haemoglobin transitions has received by far
the most attention from researchers but unfortunately there are a number
of conflicting reports.

One possibility is that the Hb transition is brought about by the
production of a new population of cells at metamorphosis which contain
only adult Hb.   The tadpole erythrocytes, containing only tadpole Hb,
would be removed from circulation.   An additional possibility which has
been included in this argument is that tadpole Hb and adult Hb cells are
produced at different erythropoietic sites.   Metamorphosis could then
include a shift from tadpole to adult stem cell site.

The evidence supporting these ideas is as follows, in which the
key experiment must be attempts to show that individual cells contain
either tadpole or adult Hb but not both.   Rosenberg (1970) used a micro-
PAGE technique to separate Hbs from individual bullfrog tadpole and adult
red cells.   She found adult cells to contain only adult Hb and tadpole
cells to contain only tadpole Hb.   Maniatis and Ingram (1971c), also
working with R. catesbeiana, raised specific antisera to tadpole and
frog Hbs separately.   Using immunofluorescence they were unable to
detect both Hb types in single peripheral red cells.   That a number of
erythropoietic sites are involved has been shown by several authors.
Hollyfield (1966) performed erythropoietic tissue transplants between R.
pipiens embryos of different ploidy.   It was claimed that grafts of the
pronephric or mesonephric kidney anlagen became the erythrocyte stem cell
sites in the recipients.   However Maniatis and Ingram (1971a) found that
the liver was the primary site of red cell maturation in bullfrog tadpoles,
the kidney being of lesser importance.   In the adult frog bone marrow
was the principal site.   Further work by the same authors (Maniatis and
Ingram 1971b) with fluorescent antisera showed that frog Hb could first
be detected in the liver indicating that this was the site of red cell
maturation during the transition.   Broyles and Frieden (1973) found both

kidney and liver to be sites in bullfrog tadpoles but the kidney was of greater importance.

The connection between the different sites and the idea that even different tadpole red cells may contain different tadpole Hbs was also made by Broyles and Frieden (1973). Electrophoresis of haemoglobins synthesised in vitro in tissue slices from the two sites showed kidney red cells to contain mainly Hb-Td3 whereas liver cells synthesised Hb Td-1 and Td-2. Thus the tadpole multiple haemoglobin pattern, obtained by electrophoresing haemolysates of circulating red cells, was produced by a mixture of Hbs from both sites. Deutsch and Broyles (1975) made bullfrog tadpoles anaemic with phenylhydrazine injections. Upon recovery it was found the slower Hb components, shown to originate in the kidney, predominated. This was taken to indicate two cellular populations in tadpoles with different sensitivities to phenylhydrazine.

The main criticism of the above argument has come from conflicting results with the key experiment on whether only a single class of Hb, tadpole or frog, occurs in single circulating red cells. Jurd and Maclean (1970) had performed an essentially similar experiment with immunofluorescent labeling, as that later reported by Maniatis and Ingram (1971c). The former authors used X. laevis and found that up to 25% of red cells from metamorphosing animals contained both adult and tadpole Hbs. The proportion of cells showing double labeling declined as metamorphosis proceeded. Similarly, during recovery from phenylhydrazine anaemia when adult Xenopus toads resynthesise "tadpole" Hb, this resynthesis was not confined to a small population of cells but occurred in most of them (Maclean and Jurd, 1971a). That this result was not due to species difference was indicated by Benbassat (1974b). He found that 98% of circulating red cells of climax bullfrog tadpoles stained with anti-tadpole Hb serum while 16% also stained with anti-adult Hb serum. However the acceptance of these percentages is complicated by the finding (Benbassat 1974c) that although the antisera do not cross-react with isolated tadpole or adult Hb they are unable to distinguish between them in mixtures.

Perhaps the simplest explanation of this difference is that immunochemical detection of a haemoglobin type is likely to be more

sensitive than PAGE. This could explain the discrepancy between the data of Rosenberg (1970) and Benbassat (1974b). The difference between the latter author's results and those of Maniatis and Ingram (1971c) might be explicable if the antisera of Benbassat were more potent than those of the American authors. The positive results obtained by Benbassat (1974b) and Jurd and Maclean (1970) are more convincing than negative results. This evidence does not negate the conclusion that erythropoietic sites change during metamorphosis and that this change may be implicated in the control of the Hb transition. However it shows that the transition is not produced simply by a switch in erythropoietic cell sites each of which synthesises only either tadpole or adult Hb.

In looking for differentiative processes which might be controlled by thyroid hormones the evidence that a new red cell population does indeed appear at metamorphosis will now be given. Hollyfield (1967) reported that during the metamorphosis of R. pipiens a morphologically distinct population of erythrocytes appear which gradually entirely replaces the former population. A similar claim was made by Moss and Ingram (1968b) in the bullfrog. It was claimed that the new population of cells synthesised adult Hb in vitro. De Witt (1968) showed that these morphologically distinct bullfrog cells are different in morphology from the immature erythrocytes of normal tadpoles suggesting that they are not merely released at an early stage of maturation. Later work by the same author (De Witt et al, 1972) also showed that cells containing adult Hb are not produced as a response to the sudden removal of tadpole cells from circulation by phenylhydrazine induced anaemia. The replacement cells also contained tadpole Hb. In the axolotl a morphologically distinct population of red cells appears at the time of induced metamorphosis. Whereas haemolysates of pre-metamorphic and post-metamorphic blood contained only larval and adult Hb respectively, haemolysates of the intermediate cells contained a mixture of haemoglobins (Ducibella, 1974b).

A turnover of circulating red cells appears to occur at metamorphosis therefore. Forman and Just (1976) estimated the normal lifespan of circulating larval bullfrog red cells at about 100 days. Just and Atkinson (1972) found the haemoglobin transition to be nearly complete

in 1 week old froglets.   Although Benbassat (1970) found this transition
to be protracted for from 4 to 10 weeks in some larvae, it is obvious
that there must be a premature selective removal of the tadpole cells
at metamorphosis.

Another possibility which has received little attention is that
the circulating red cells could be switched to synthesise adult
haemoglobin and that tadpole haemoglobin might be metabolised.
Although mature erythrocytes are considered to be terminally
differentiated cells Hilder and Maclean (1974) have shown that
isolated <u>Xenopus</u> red cell nuclei can be induced to synthetic activity
by manipulation of the ionic concentration of the culture medium.
Rosenberg (1972) had earlier produced dikaryons of frog-frog and tadpole-
tadpole erythrocytes.   The nature of the Hb synthesised by these
dikaryons was compared to that synthesised by frog-tadpole heterokaryons.
The differences obtained were interpreted as due to changes in
cytoplasmic repression of the genome following cell fusion.   These
papers make it conceivable that a hormone could cause a resynthesis of
haemoglobin, but of a new type, in circulating red cells.

The above synopsis suggests that if thyroid hormones are directly
involved as triggers in the haemoglobin transitions they might be
expected to act either by changing the Hb synthesis of maturing cells,
by causing a switch in site, or by reactivating the synthesis of Hb
in circulating cells.   While there is no evidence to support the last
possibility, most of the above differentiative processes which have
been observed in the Hb transition have been produced precociously by
TH treatment.

Following T4 treatment of bullfrog tadpoles Moss and Ingram (1968b)
found an increased <u>in vitro</u> synthesis of adult Hb to be preceded by a
decline in tadpole Hb synthesis. Hirayama (1967) demonstrated changes in
amino acid incorporation by red cells <u>in vivo</u> during normal metamorphosis
such that incorporation increased during the climax.   Premetamorphic
tadpoles treated with T4 also showed enhanced amino acid incorporation
into haemoglobin.   Similar work by McMahon and Dewitt (1968) indicated
that after T4 treatment incorporation of uridine dropped for 11 days but
between 14 and 22 days there was another increase accompanied by the

reappearance of ribosomal RNA peaks.   This was interpreted to mean
that synthesis in old erythrocytes producing tadpole Hb was repressed
to be followed by a new cell line synthesising frog Hb.   Just and
Atkinson (1972) have suggested that some of these effects may be the
result of altered amino acid pool sizes following hormone treatment.
They found that although bullfrog larvae induced to metamorphose with
T3 began to synthesise adult haemoglobin larval haemoglobin still persisted
in circulation quite unlike the situation in spontaneous metamorphosis.
They suggested that although TH may be involved in the transition other
factors must also operate.   One of the most interesting experiments
followed from the work of DeWitt et al (1972) previously discussed.
Maniatis and Ingram (1972) found that although tadpole haemoglobin was
contained in new red cells produced as a response to phenylhydrazine
injection, the new cells would contain adult Hb if thyroxine was
administered to recovering larvae.   Certainly there is evidence that
the appearance of the morphologically distinct red cell populations
previously mentioned can be induced precociously by TH treatment of
larvae (Hollyfield, 1967; DeWitt 1968; Ducibella, 1974b).

Thus there is a considerable body of evidence to suggest that
thyroid hormone administration can partly, but not wholly, mimic the
haemoglobin transition at metamorphosis.   The interpretation of the
apparent conflict between this conclusion and the conclusion of this
thesis will be made when the other system for which some evidence of
TH control exists, namely serum proteins, has been discussed.

Studies on the mechanism of hormone action in the tadpole liver
(discussed in Section 8.8 in connection with increases in urea cycle
enzymes) have also noted the appearance of new serum albumin in the
blood following TH administration (Tata,1967).   From the work of Thornburg
et al (1975) and from Chapter 4 of this thesis it follows that the liver
is the source of coelomic fluid albumin.   However a variety of hormones
have been shown to have a similar effect in the mammalian liver.
Atkinson et al (1972) have shown that during spontaneous metamorphosis
there is a burst of DNA synthesis preceding the appearance of new tissue
specific proteins.   This was also produced by in vivo T3 injection.
Tata (1971) has pointed out that there may be restricted DNA synthesis as

a prerequisite for differentiation in tadpole liver unconnected with growth of the cell population. In this connection McGarry and Vanable (1969) found cell division to be a necessary prerequisite for T4 induced changes in <u>Xenopus</u> skin glands. Several other authors have found the normal serum and coelomic fluid, total protein and albumin, concentrations to increase when premetamorphic tadpoles were induced to metamorphose with exogenous TH (Herner and Frieden, 1960; Tata, 1967; Chen, 1970; Ledford and Frieden, 1973).

Thus in both the haemoglobin and coelomic fluid protein systems there is evidence apparently in conflict with that adduced in Chapters 3 and 4 of this thesis. It seems clear that the complexity of the systems has been overlooked by some of those administering exogenous thyroid hormones. There is a constant problem in experimental endocrinology that when one administers a hormone to an animal the response observed may be pharmacological rather than endocrinological. If treatment leads to abnormally high circulating hormone titres the response observed may not be the normal one produced by physiological titres. This is especially true in the present case in which so little is known of circulating physiological hormone levels in normal and treated larvae. A classic solution to this problem is to apply the opposite strategy - to surgically or chemically ablate the endocrine organ. When results are in conflict it is perhaps safer to rely more on the latter approach which does not involve the complication of pharmacological responses to exogenous hormone. In addition many other factors may be involved in the transitions studied. The role of prolactin as an antimetamorphic compound has already been discussed but its involvement in the control of haemoglobin and coelomic fluid protein shifts is not known. In the case of haemoglobin, erythropoietin may also exert control. Maclean and Jurd (1972) have pointed out that the reported effects of thyroxine on the erythroid system of amphibians are remarkably similar to those of erythropoietin in mammals. The increase in coelomic fluid and serum total protein concentration at metamorphosis could partly be due to a loss of body water. Fletcher and Myant (1960) showed the dry weight:wet weight ratio of <u>Xenopus</u> larvae to rise during metamorphosis and prolactin has been implicated in this process (Schultheiss <u>et al</u>, 1972; Goldenburg & Warburg, 1977). The normal transition from a pronephric to mesonephric kidney is only retarded by

the propylthiouracil treatment of _Xenopus_ larvae, true giant tadpoles
having a well-developed mesonephros (Oates, 1977) which may contribute
to the loss of body water.   This is also an example of a morphological
change normally associated with metamorphosis but not prevented by
goitrogen treatment.   However, unlike many features of amphibian larval
development (such as leg development in the swimming larva and tail
regression) a transition from pro- to mesonephros is a vertebrate
characteristic which may not be controlled by thyroid hormones.   This
point is discussed further in Section 8.11.

Two interpretations seem possible in this conflicting situation.
Either thyroid hormone is not the primary cellular signal or it is the
primary signal exerting its normal effect on certain biochemical transitions
through the stoichrometric attainment of low hormone requirements or by
long-lived effects following signals received before the onset of
goitrogen treatment.   That TH had some effect on haemoglobin and
coelomic fluid shifts is clear both by the protracted nature of the
transition in some animals and by its absence in others which were still
biochemically tadpole-like.   However, the presence of all intermediate
stages between 'tadpole' and 'adult' makes it likely the transition
would have occurred in most animals given sufficient time.   That tissue
response to TH is not prevented by aging has been shown by Hsü _et al_
(1971) who kept surgically thyroidectomised bullfrog larvae alive for
4 years.   The animals were capable of metamorphosis at any time that T4
was administered.   The evidence for the stoichiometric attainment of
competence and for long-lived hormonal effects is reviewed in Sections
8.5 and 8.7 respectively.

In the other two protein systems, LDH and lens crystallins, it was
shown in Chapters 5 and 6 that the appearance of adult markers occurred
in all propylthiouracil-treated larvae which had been arrested for over
8.5 weeks and which had grown abnormally large.   In the absence of
published data to the contrary it is assumed that thyroid hormones are
not centrally involved in these transitions.   The adaptive significance
and control of ontogenic changes in LDH isozymes in amphibians are
obscure and will not be discussed further here.   In Chapter 6 not only
were adult crystallin markers shown to be present in giant tadpoles but
also their appearance and relative concentration was found to be strongly

correlated with lens diameter. It was argued that as lens diameter is related to orbit size and whole body size the crystallin shift is a result of larval growth. This was the first experimental support of Clayton's (1970) hypothesis that crystallin shifts are necessitated by lens growth, possibly to maintain its refractive properties. It also raises the interesting question of how crystallin gene control and body size might be coordinated. This point is discussed in detail in Section 8.10.

8.4. Interpretation under the Etkin hypothesis

Perhaps the simplest explanation of the data summarised in Section 8.2 can be based on the Etkin model for the control of amphibian metamorphosis (Etkin 1964a and 1968). In essence, Etkin stated that larval growth and metamorphosis are controlled by the direct tissue actions of thyroid hormones (mainly T4) and by prolactin. During premetamorphosis the thyroid gland releases only low levels of T4 and the pars distalis thyrotrophs are sensitive to the negative feedback of the hormone so that TSH is not released. The thyroxine levels therefore remain low. Growth, in larvae, is controlled by prolactin which is released in large quantities by the pars distalis during premetamorphosis. The balance of these two hormones explains the observation that pre-metamorphosis is characterised by rapid growth but slow development.

During early prometamorphosis the median eminence of the hypothalamus becomes competent to respond to TH. Its differentiation is completed and a capillary plexus develops which can then transport the neurohormones, thyrotropin releasing hormone (TRH) and prolactin inhibiting hormone (PIH) from the preoptic nucleus to the pars distalis. TRH is supposed to desensitise the thyrotrophs to TH negative feedback, thus enhancing thyroxine release, while PIH is supposed to have the reverse effect on prolactin release. Throughout the remainder of prometamorphosis an escalation of TH release occurs because of positive feedback on TRH synthesis and release. Thus it is predicted that TSH titres will also rise while those of prolactin continue to decline. The changed balance between T4 and prolactin accounts for the observation that, at climax, morphological development is precipitous whereas growth is retarded.

One can assume that propylthiouracil prevents circulating T4 levels ever rising to even normal premetamorphic levels. Under Etkin's hypothesis the pars distalis thyrotrophs respond by secreting more TSH leading to the observed goitrous thyroid gland which is still unable to synthesize TH because of the continuous presence of the goitrogen. The low circulating T4 levels prevent final differentiation of the median eminence so that the effects of TRH and PIH are not produced. Thus the premetamorphic low levels of T4 and high titres of prolactin persist indefinitely producing negligible morphological change but steady growth.

As biochemical changes normally associated with metamorphosis occurred in a majority of goitrogen-retarded tadpoles one can conclude that thyroid hormones are not directly responsible for the control of the protein shifts studied.

This argument is subject to many criticisms which involve the most basic concepts of the roles of morphogenetic hormones in amphibian development. They will therefore occupy much of the remainder of this chapter.

Etkin's model itself has received a number of criticisms which were reviewed by Just (1968), Frieden and Just (1970) and Dodd and Dodd (1976).

The experimental data upon which Etkin's hypothesis is based are as follows. It is well-established that surgical thyroidectomy arrests anuran metamorphosis (e.g. Allen, 1918; Hoskins and Hoskins, 1919). Treatment with thyroxine restored this developmental capacity (Kendall, 1919). Hypophysectomy and, in fact loss of the pars distalis alone, also prevents metamorphosis (Adler, 1914) and eventually it was shown that mammalian TSH would induce climax in pituitary-ablated larvae providing the thyroid gland was present (Uhlenhuth et al, 1945). The activity of the thyroid gland, as judged by histological criteria (review by Lynn and Wachowski, 1951) and by ^{131}I uptake (Kaye, 1961), increased during prometamorphosis and especially at climax. Etkin (1935) had shown that, to obtain a normal sequence of changes in induced metamorphosis of thyroidectomised Rana sylvatica tadpoles, it was necessary to treat with gradually increasing concentrations of thyroxine. Chang (1957) and

Rémy and Bounhiol (1965) proved that surgical removal of the median
eminence prevented metamorphic climax although the larvae still fed
and grew well.   Etkin (1964b) claimed that growth was restored by
injection of mammalian prolactin into hypophysectomised tadpoles.

In spite of these fundamental observations Dodd and Dodd (1976) have
pointed out that much of the important detail of the hypothesis was
speculative.   For example, there was no knowledge of circulating levels
of TSH, T3, T4 or prolactin.   In fact, it was not known whether TSH or
a growth promoting hormone (GPH) like mammalian prolactin were present in
larval pituitaries.   If a GPH did exist its supposed anti-metamorphic
properties had not been demonstrated.   There were no data in support of
the contention that circulating T4 has a positive feedback effect on
hypothalamic TRH secretion or that TRH desensitises the pars distalis
thyrotrophs to T4 negative feedback.   Finally, the nature of the control
exerted by the hypothalamus on GPH secretion was unknown.

Since the hypothesis was produced a number of highly relevant papers
have been published but, in general, there have been few critical
attempts to directly test Etkin's assertions.

Dodd and Dodd (1976) have reported that chromatographic studies have
confirmed that thyroid hormone synthesis increases during prometamorphosis
to reach a peak between climax stages 60-62 in X. laevis.   Synthetic rates
were then at least 40 times those during early prometamorphosis.   A rapid
synthetic decline followed climax.   The same authors used a bioassay to
determine the pituitary TSH content.   Although this work did not directly
show how much TSH was released, taken in conjunction with the chromato-
graphic data, a case was made that the TSH synthesised actually entered
into circulation.   Another approach was used by Eddy and Lipner (1976) who
injected Rana catesbeiana tadpoles with a specific antiserum against
mammalian TSH, known to cross-react with larval pituitary extract.   This
work shows a TSH to be present in the tadpole pituitary gland.   Compared
to control animals injected with normal rabbit serum, TSH antiserum
depressed the rate of hind limb development during T4-induced metamorphosis
to about 50% of the response to T4 alone and completely blocked hind limb
development during spontaneous metamorphosis.   Thus the thyroid gland was
unable to release TH in the absence of TSH and consequently metamorphosis
was prevented.

The development and role of the median eminence has received more attention since Etkin's hypothesis was first promulgated. Etkin (1965) showed that the development of the capillary plexus, in Rana pipiens, thought to transport TRH to the pars distalis, did not develop until prometamorphosis, the process being completed by the beginning of climax. Further work (Etkin, 1966) showed that development of the median eminence does not occur in thyroidectomised tadpoles but that when such operated larvae are raised in T4 solutions the median eminence reached a thickness typical of normal climax specimens. This sensitivity to T4 stimulation did not develop until the end of premetamorphosis. In spite of a single publication by Guardabassi (1961), which claimed that a proportion of hypothalamectomised X. laevis larvae were able to metamorphose, all other reports have stated that this was not the case (Chang, 1957; Voitkevitch, 1962; Hanaoka, 1967). There is always the possibility of incomplete ablation in some animals.

Norris and Gern (1976) studied the question of whether positive feedback of hypothalamic TRH release can occur in neotenic Ambystoma tigrinum larvae. The Colorado population used do not normally metamorphose but their peripheral tissues respond to exogenous TH and their thyroid glands to exogenous TSH. The authors injected a small amount of T4 directly into the region of the hypothalamus and observed a high incidence of metamorphosis. Similar injections into the peritoneal cavity did not induce metamorphosis. Hypothalamus-injected larvae were able to take up significantly more ^{131}I into their thyroid glands than sham injected controls. Because previous work (Norris, Jones and Criley, 1973; Platt, 1976) had shown pituitary prolactin to decline during spontaneous or induced metamorphosis it was speculated that prolactin acts as a blocking agent to hypothalamic production of TRH which can only be countered by high, local T4 concentrations.

In adult Bufo bufo the two types of pars distalis acidophil cells have been positively identified with anti-prolactin and anti-growth hormone fluorescent sera (Hansen and Hansen, 1976). Nicoll and Licht (1971 a & b) separated the two hormones by PAGE and assayed their activities. Clemons (1971 - cited by Dodd and Dodd, 1976) has claimed that cells secreting both hormones are present in the pituitaries of

bullfrog larvae but this work has not been published. Only one type of acidophil cell is present until metamorphosis however. The most convincing evidence for an antimetamorphic role of larval prolactin has come recently from Eddy and Lipner (1975). Using a highly specific antiserum raised against ovine prolactin the metamorphosis of injected tadpoles was studied. Tadpoles treated with prolactin antiserum or normal rabbit serum remained unchanged. However when T4 was injected 1 day after the prolactin antiserum metamorphosis was accelerated compared to injection of T4 alone. It was concluded that the antiserum blocked the peripheral antimetamorphic effect of prolactin allowing a more rapid response to exogenous T4. However an Ouchterlony test of tadpole pituitary homogenates against the prolactin antiserum failed to produce visible precipitation of antigens. The accelerating effect of the antiserum was noticeable only during pre- and pro-metamorphosis as would be predicted by Etkin's hypothesis.

All of the above observations have tended to support the Etkin model but other recent data have not. One of the first pieces of contradictory evidence was the failure of injected ovine TRH to elicit precocious metamorphosis in bullfrog tadpoles (Etkin and Gona, 1968). However it was quite possible that the mammalian and amphibian hormones differed chemically. McKeown (1972) working with adult Bufo bufo claimed that prolactin secretion was controlled by an inhibitory factor in agreement with Etkin. A radioimmunoassay was used to measure relative levels of circulating hormones after ectopic transplantation of the pars distalis. However Dodd and Lacharojana (1977) obtained different results with X. laevis larvae. Hypophysectomy and reimplantation of the pituitary into an ectopic site always resulted in a depressed growth rate compared to controls even when endogenous TH secretion had been inhibited by a goitrogen. This suggests that, contrary to Etkin's hypothesis, the secretion of prolactin is not under hypothalamic inhibitory control.

The control of TH release by TSH has also received some criticism. Guardabassi (1961) found that the thyrotrophs are partly independent of TRH control. In X. laevis tadpoles in which the hypothalamus had been ablated a goitrous response was still produced by thiouracil treatment. Again Guardabassi's results are at variance with other authors'.

Hanaoka (1967) in R. pipiens and Goos (1969) with X. laevis observed
thyroid hypertrophy in similar experiments. Guastalla et al (1972)
studied the effect of thyroidectomy on pars distalis thyrotrophs in
Bufo bufo larvae which had also been hypothalamectomised. There was
no hypertrophy of the thyrotrophs suggesting that TH negative feedback
acts mainly on the hypothalamus rather than on the pars distalis as
hypothesised by Etkin.

Dodd and Dodd (1976) have reviewed the evidence that negative
feedback by T4 on the hypothalamus-pituitary axis operates throughout
larval life and not, as predicted by the hypothesis solely during
premetamorphosis. Goos (1968) treated premetamorphic X. laevis with
propylthiouracil and found a depletion of neurosecretory material in the
dorsal preoptic nucleus. When the goitrogen solution was replaced with
water the neurosecretory material reaccumulated. Later work by Goos'
group (van Oordt et al, 1972) showed that this material contained TRH
since its release was correlated with histological and physiological
evidence of enhanced TSH titres.

Just (1968), using data on the half-lives of mammalian TSH and TH
questioned whether a spiralling escalation of TH titre could be
produced by positive feedback on the hypothalamus. The half-life of
TSH in mammalian plasma is under 2 hours whereas that of TH is
approximately 3 days. He suggested that there would not be a greatly
elevated TSH titre and therefore the initial thyroxine levels, after
first being raised indirectly by TRH release, would tend to level off.
This argument was used by Just to explain the most serious criticism of
the Etkin hypothesis, namely that there is little evidence to support his
contention based on immersion studies, that circulating TH levels would
increase during premetamorphosis by 5 to 25 times. Further, at the time
of foreleg emergence he predicted a 200-250 fold increase over premetamorphic
levels and 10-fold over prometamorphic titres. Just's (1972) PBI data,
although subject to criticism, are the only fully published data on
circulating TH levels and they showed, at most only a 10 to 20 fold
increase over premetamorphic and prometamorphic levels in Rana pipiens.
Some individuals showed little climactic increase at all. Recently
the unpublished data of Miyauchi et al (1977) have suggested that serum
T4 levels changed little during bullfrog development whereas a

substantial rise in serum T3 was detected. The involvement of T3 in
metamorphosis is discussed in more detail below. In the PBI method
used by Just (1972) both thyroid hormones would be measured but there
would be a large excess of T4 (about 200:1 of T4:T3 in Chapter 7 of
this thesis) such that even marked changes in T3 might not have been
detected.

As recently as 1974, Etkin and Gona have reiterated their belief
that the integration of metamorphic events stems from rising hormone
titres, rather than from say, sequential changes of tissue receptors.
Thus although there is now evidence in support of much of Etkin's
hypothesis the failure, to date, to detect large increases in circulating
TH is a serious criticism. In addition the mechanism of the feedback
loops still remains to be elucidated.

The work of Goos et al (1968) and of Goos (1969) has shown that
treatment of premetamorphic X. laevis tadpoles with 0.01% (0.59 mM)
propylthiouracil prevented development of the capillary plexus in the
median eminence. Also cells of the preoptic nucleus, thought to be
concerned with TRH synthesis, were inhibited. This information, in
conjunction with the well-known goitrogenic effects of propylthiouracil,
can be used to explain the metamorphic arrest but continued growth
observed in the present thesis.

Etkin's hypothesis predicts that metamorphic changes are produced
by rising TH levels. The continued protein transitions demonstrated
here suggest that these biochemical changes are not under primary
thyroid control. However several other concepts, frequently discussed
in the literature on hormonal control of amphibian morphogenesis, need
to be considered before final conclusions are drawn.

8.5. Tissue competence and threshold levels of response to thyroid
      hormones

Two concepts which have some experimental support and which could
affect the interpretation of my data are tissue competence to respond
to TH, and threshold levels required for a response to occur. In

addition evidence is presented in Section 8.7 that a brief exposure to thyroid hormone may have a "triggering" effect by which transitions, once initiated, can continue in the absence of the hormone. These topics will lead naturally to a consideration of recent work on cytoplasmic and nuclear binding of hormone molecules which is the basis for a modified model for the control of amphibian metamorphosis introduced by Dodd and Dodd (1976) and discussed in Section 8.9.

It is generally agreed that some tisses become competent to respond visibly to exogenous TH at about the time of opercular membrane formation (Moser, 1950 with Rana temporaria; Etkin, 1950 with R. pipiens). There has been dispute, however, over whether all tissues which subsequently metamorphose acquire this competence at the same time. This point is clearly relevant to the interpretation of my data because it might be claimed that the protein shifts were initiated before later metamorphic events such as forelimb development.

Etkin (1950) claimed that there was an all-or-nothing acquisition of competence but detailed supporting data were not provided. Subsequent work has shown that, in some tissues at least, competence is not acquired until prometamorphosis. The case of the median eminence has already been discussed. Heady and Kollros (1964) found that, in the plical glands of R. pipiens, the response elicited by T4 treatment depended upon the stage of the skin donor. Responsiveness was found to develop gradually rather than in an all-or-nothing fashion. Similarly, Kollros and McMurray (1956) showed that the growth response of mesencephalic V nucleus cells is not present before stage III in R. pipiens larvae and not fully developed until stage V.

Even if it is accepted that not all tissues acquire competence simultaneously the mechanism of the process after acquisition is highly controversial. Etkin's (1935) work with thyroidectomised R. sylvatica tadpoles was mentioned above. He found that the sequential order of events was not affected by the concentration of T4 used but that development was telescoped as concentration increased. Using low thyroxine concentrations of 0.01 to 0.0001 μg/100 ml(1.3×10^{-9} to 1.3×10^{-7}M) he found that early metamorphic events occurred at normal time

intervals but later events were much delayed. Nevertheless it was claimed that even the lowest concentrations would produce all metamorphic events if sufficient time was allowed. Etkin (1964a) used these data as the basis for his 'stoichiometric model' of competence. This states that even very low levels of TH will produce all metamorphic changes in a progressive manner. Low doses will take a long time to produce full metamorphosis but as dosage rises the duration of metamorphosis shortens. There is no specific threshold level, of exogenous or circulating hormone, necessary to produce a given change; rather each tissue has its own total thyroxine requirement which it can gradually acquire. On the other hand Kollros (1956, 1958 and 1961) has argued that each tissue has a definite threshold requirement for circulating hormone. He has produced data which appear to refute the stoichiometric model. Using hypophysectomised R. pipiens tadpoles and low immersion dosages of T4 (0.0002 to 0.06 µg/100 ml; 2.6 x 10^{-9} to 7.7 x 10^{-7} M) he found that early metamorphic events would be stimulated to a certain point and would then enter an apparently indefinite stasis period unless the hormone level was raised. For example, the threshold for the onset of the corneal reflex was found to be lower than that for perforation of the operculum (Kollros, 1958) and the latter threshold was lower than that for gill resorption (Kollros, 1961). Thus Etkin (1935), Kollros (1961), and previously Allen (1932) with Bufo boreas larvae, found it necessary to increase thyroxine concentration to obtain an approximately normal spacing of events, but Kollros (1961) claimed that within the period of study (up to 61 weeks) metamorphic arrest would persist at low hormone levels whereas Etkin believes metamorphosis to be merely retarded. Direct comparison of the data produced is difficult because in one case tadpoles were thyroidectomised whereas in the other they were hypophysectomised. Also, for most of his work Etkin used much higher thyroxine concentrations than Kollros and studied his animals for only short periods.

The importance of this argument to the present thesis is made clear by the interpretation which Ducibella (1974a and b) put on his experimental data obtained with Ambystoma mexicanum. Neotenic animals were found to complete the transition of haemoglobins and serum protein in a similar way to other specimens which had been induced

to metamorphose by immersion in T4 solutions (4.5 to 9.0 x 10^{-8} M).
Ducibella argued that the untreated animals have low levels of TH
release which were nevertheless sufficient to provide enough circulating
hormone to meet the low threshold requirements of the tissues responsible
for haemoglobin and serum protein transitions. It was assumed that the
thresholds for overt morphological changes were higher and could not be
attained naturally. Ducibella produced no specific evidence to support
this interpretation.

A number of authors have interpreted their data in terms of one or
other of the above competence hypotheses but the evidence has not been
unambiguous. For example, Verma (1965) continued Kollros' work on skin
gland development. He showed that high levels of exogenous T4 altered
the order of glandular development to produce an abnormal sequence.
Verma suggested that the threshold levels of most glandular cells was
being met simultaneously rather than sequentially as in normal
metamorphosis when, it was assumed, circulating TH levels were increasing
gradually. Asynchronous development was also reported by Kaltenbach
(1968) who stated that at high T4 concentrations severe abnormalities,
such as tail regression before the completion of limb development, could
produce inviable larvae with no satisfactory means of locomotion.

The interpretation of many experiments is complicated by the well-
established "lag periods" which differ from tissue to tissue and which
are intercalated between hormone treatment and the appearance of a
detectable response. These lag periods were studied by Derby (1968).
Using discs of R. pipiens tadpole tail fin, cultured in Hank's medium,
he studied the effect of different T4 concentrations on the lag period
and on the rate of regressive response in tissue taken from donors of
differing metamorphic stages. Increasing the T4 concentration did not
alter the duration of regression (3 to 4 days). Rather, the lag period
before the beginning of visible response, shortened. Discs from donors
in late prometamorphosis-climax required lower concentrations of exogenous
T4 to regress than did discs from early prometamorphic tails. If doses
were the same, with tissues from both classes of donor, then the lag
period of the more advanced stages was shorter. Derby suggested that
discs from climax tadpoles had already been exposed to higher levels of

endogenous T4 and for longer periods before excision than had those from early stages. Thus in terms of the stoichiometric model the more advanced tissues had already acquired a large part of the total TH requirement. In a recent paper Kim et al (1976) have shown that when tail fin discs, treated in vitro with T4 for several days were washed thoroughly and grafted to previously untreated discs, the latter could then be induced to regress with much shorter lag periods than ungrafted discs. It was assumed that no unbound T4 was transferred directly but that an intermediate, produced by TH action during the graft's lag period, had been carried over. Of course, it is also possible that the substances transferred were hydrolytic enzymes.

Chou and Kollros (1974) performed somewhat similar experiments to study lag periods in R. pipiens. In this case embryos from a single batch of eggs were reared at different temperatures to give a range of developmental stages at the same time. The lag period and time of tail shortening response in whole larvae were studied under T4 treatment. Again lag periods were shortest for developmentally more advanced larvae while the duration of response was similar in all stages. However these results were interpreted to support the absolute threshold, rather than the stoichiometric, hypothesis. It was argued simply that the threshold became lower as the larvae progressed through metamorphosis.

The stoichiometric model has the advantage that it suggests an outline mechanism by which TH is bound or involved in progressive processes thus shortening lag periods. Kollros has suggested no way in which thresholds change. However the most direct evidence was that of Kollros (1961) discussed above. Unfortunately this work was never published fully and has been criticised by Etkin on the grounds of its statistical validity bearing in mind the well known metamorphic variability of anuran tadpoles.

The stoichiometric model suggests that tissues responsible for changes in haemoglobins, albumin and crystallin shifts might progressively utilise the remaining low thyroid hormone levels present under goitrogen blockage. However the assumption must be made that the threshold of response is lower in these tissues than in the limbs and tail. There is

no direct evidence in support of this supposition.   Until further
evidence appears stoichiometric binding must remain a possible
alternative interpretation of protein shifts under goitrogen blockage.

8.6.   Growth promoting hormones in metamorphic control

Interpretation of the experiments discussed in Section 8.5 and
indeed of all metamorphosis experiments has been complicated by the
discovery that a prolactin-like GPH exerts antimetamorphic effects on the
peripheral tissues.   Gona (1967) found that simultaneous injection of
T4 and mammalian prolactin into premetamorphic bullfrog tadpoles
resulted in an induced metamorphosis similar to that produced by T4
injection alone.   When TSH and prolactin were injected together
metamorphic stasis resulted.   Prolactin therefore appeared to be acting
as a goitrogen by blocking glandular TH synthesis or release.   Norris
and Platt (1973) showed that treatment of larval Ambystoma tigrinum with
prolactin had no effect on ^{131}I uptake by the thyroid gland.   However
Regard and Mauchamp (1973) supported Gona's data in experiments with
prolactin and TSH.

Much more evidence that prolactin has peripheral effects has
accrued.   Some of this was discussed in connection with the Etkin
hypothesis for metamorphic control.   The most interesting experiments
in the context of tissue competence and lag periods are those of Derby
and Etkin (1968) and Derby (1970).   These authors have again used the
regression of cultured tail fin discs under T4 influence to study the
additional effect of grafted larval pituitaries or treatment with
mammalian prolactin and growth hormone.   Derby and Etkin (1968) claimed
that both treatments blocked or delayed the regression of discs but the
pituitary grafts were found to be more effective.   Derby (1970) extended
the data by grafting pituitaries taken from donors in different
metamorphic stages.   Early prometamorphic pituitaries showed the greatest
antimetamorphic activity followed by glands from climax larvae.   Derby
(1975) showed that whereas exogenous T4 increased the activity of the
hydrolytic enzymes acid phosphatase and B-N-acetylglucosaminidase in vivo,
prolactin as one of its effects reduces the level of activity of these
enzymes.   In addition to these effects on morphology and its underlying

mechanism prolactin has also been shown to exert antimetamorphic effects on certain biochemical transitions. Medda and Frieden (1972) showed prolactin treatment to produce opposite effects to T3 injection on the ammonia excretion of bullfrog tadpoles. However Crim (1975) found that prolactin did not antagonise thyroxine-induced rhodopsin synthesis in the retina of R. catesbeiana tadpoles.

This discussion of antimetamorphic action by prolactin emphasises the danger of interpreting metamorphosis experiments solely in terms of TH action and shows that such concepts as competence and threshold of response may be even more complicated than indicated above.

8.7. Long-term effects of thyroid hormone treatment

It might be predicted from Kollros' (1961) hypothesis on response thresholds that the role of TH is merely to act as a trigger to some metamorphic process which, once initiated, could continue in the absence of the hormone. If this were the case then it might be that a protein shift initiated by endogenous TH before goitrogen treatment could continue, in at least some animals, in the eventual absence of hormone. There is good reason to believe that the protein transitions studied occur gradually over the whole larval period and not solely during climax. Jurd (1972) found the relative concentrations of tadpole and adult haemoglobins to change steadily from NF stage 43 (the earliest studied) through to adulthood. However Kollros (quoted in Etkin, 1964a) has denied this prediction and believes that the continued presence of TH is necessary for metamorphosis. Other authors have produced evidence for long term effects by pulse treatments of thyroid hormones. Two types of experiment have been performed. Frieden et al (1965) administered T3 to bullfrog tadpoles with a single injection. At $25^{\circ}C$ precocious metamorphosis proceeded swiftly but at $5^{\circ}C$ the process was still in an early stage after 80 days. When tadpoles were returned to $25^{\circ}C$ after stasis at the lower temperature metamorphosis proceeded on the same time scale as in the normal T3-induced experiment. Induced metamorphosis could be severely retarded at any stage by lowering the temperature to $5^{\circ}C$. The authors considered these results could be explicable either because an appreciable fraction of T3 survived the

prolonged $5^{\circ}C$ period and became active again at $25^{\circ}C$, or that some long-lasting "imprint" had been made on the responding tissues. The biological half-life of ^{131}I-T3 was found to be 12 days at $5^{\circ}C$. Thus after 80 days less than 1% of the original dose would be expected to remain. They concluded that come "imprint", produced by T3 but more stable than the hormone itself, must be made.

Another approach was that of Prahlad and DeLanney (1965). 10µg of T3 were injected into the perivitelline space of 6 day old axolotl embryos which were subsequently released from the jelly into fresh water 9 days later. After 2 days tissue changes were observed in sequence until the induced metamorphosis was complete within 25 to 35 days. These results appear to refute the assumption of Etkin's hypothesis, that the continuous presence of circulating hormone is necessary for metamorphosis to occur. An earlier paper by Paik and Cohen (1961) is also relevant. While studying the effect of goitrogen on thyroxine-stimulated protein synthesis in tadpole liver it was discovered that thiouracil acts, not only on the thyroid gland, but is also incorporated into RNA as a uracil analogue. The most disruptive effect on the T4-induced stimulation of carbamyl phosphate synthetase synthesis occurred when thiouracil was administered during the lag period suggesting that TH stimulates RNA synthesis some of which may have long-lived effects.

Although the stoichiometric competence model and long-lived effects of TH treatment could account for the continuation of biochemical metamorphosis in goitrogen treated Xenopus larvae they lacked any firm mechanism until recently. Because the mechanism of thyroid action is so fundamental to understanding its morphogenetic role the recent work will be discussed in some detail.

8.8. Thyroid hormone action in amphibians at the molecular level

Unfortunately the study of TH action even in mammals, has lagged behind that of other morphogenetic hormones such as the steroids. As our knowledge of steroid hormone action appears to have served as a model to those investigators who have studied TH it is in order to review briefly how the steroids are thought to control gene action.

The much more fragmentary evidence available on amphibian TH will then be more comprehensible. Reference will not be made to original sources but to the reviews of Lewin (1974) and Rosen and O'Malley (1975).

The system employed has been the hen oviduct in which 17-beta-oestradiol induces the specific synthesis of the proteins, ovalbumin and lysozyme. Progesterone, on the other hand, induces avidin synthesis. These hormones are thought to cause changes in gene expression at the transcriptional level partly because actinomycin, puromycin and cycloheximide all block the hormonal response. In addition the morphological response of oviduct cells to hormone is followed by an increase in the numbers of RNA sequences hybridising with repeated DNA. By isolating the RNA released from oviduct polysomes after hormone treatment it has been shown that messengers appear which can be translated to give ovalbumin in vitro.

The steroids appear to enter the cells by passive diffusion and to bind with high affinity and specificity to 9S cytoplasmic receptor proteins which can reversibly dissociate to 4S or 5S size. This ionic and temperature dependent activation of the receptor sites occurs coincident with translocation into the nucleus. The activated nuclear receptor can then be shown to undergo a hormone-dependent high affinity binding reaction with chromatin. The receptor binds to chromatin acceptor sites which appear to be primarily composed of the DNA backbone although certain chromosomal nonhistone (acidic) proteins seem to quantitatively modify this DNA binding.

In the case of the larval amphibian it was first shown by Tata (1970) that X. laevis tadpoles suddenly acquire the ability to bind T3 some 36-60h after fertilisation (NF stages 34-41). This coincided with the first detection of tissue competence judged in terms of changes in the rates of synthesis of nucleic acids, proteins and phospholipids plus changes in PO_4^{3-} uptake, hydrolase induction or, after a longer lag period, in gross morphology. The binding was shown to be temperature dependent being much greater at $25^{\circ}C$ than at $5^{\circ}C$. This, and other, properties suggested that binding was specific. The work was performed with homogenates of whole larvae and can be criticised on

the grounds that circulating thyronine binding proteins, if present in
Xenopus, could have increased a binding which would not be
intracellular.

Later work was more sophisticated. Griswold et al (1972) injected
bullfrog tadpoles with ^{14}C-thyroxine and determined the subcellular
distribution of the labeled hormone. At $5^{o}C$ and $25^{o}C$ T4 accumulated in
the liver cytoplasm but only at $25^{o}C$ did a large percentage of hormone
enter the nucleus where it was tightly bound to chromatin in a
chemically unmodified form. Yoshizato et al (1975a) studied the binding
of T3 by bullfrog tadpole tail fin, tail muscle, kidney and liver
cytosol homogenates. All tissues studied bound the hormone the binding
being metal ion dependent. The degree of metal ion dependency varied
but was highest in the tail fin which also had the largest maximal
binding capacity (10.4 picomoles per mg of crude cytosol protein as
opposed to 0.04 picomoles in the liver). The cytosol hormone receptors
had properties of an acidic protein.

Nuclear binding of T3 and T4 were also studied by the same group.
In the case of in vitro cultures of tail fin Yoshizato et al (1975b)
found evidence for high affinity, limited capacity binding sites in the
nucleus only. The numbers of sites were estimated at 1500 (T3) and
800 (T4) but both had similar dissociation constants of about 10^{-10}M.
Both binding and competition studies supported the conclusion that the
nucleus had more sites for T3 than for T4. Kistler et al (1975) found
similar evidence for T3 and T4 receptors in the nuclei of tadpole liver
cells to which binding was temperature dependent. The high affinity
saturable sites could not be detected in the cytoplasm and in this
tissue receptors for the two hormones differed both in number and dissocia-
tion constant. There were 12,300 sites for T3 ($K_d = 6.8 \times 10^{-10}$M) and
2,300 for T4 ($K_d = 4.6 \times 10^{-10}$M). In both tail fin and liver nuclei
hormones were bound in a chemically unmodified form.

The relevance of such research to metamorphic control is indicated
by two other recent papers. Yoshizato and Frieden (1975) claimed that
the binding capacity for T3 in the tail nuclei increased at metamorphosis
in Rana catesbeiana. Thus at stage X there were 1,330 sites, at stage XV

there were 1580 and at stage XVII-XIX there were 2830. The ratios of
bound to unbound hormone remained approximately the same for the
extranuclear portion of the cell. It was suggested that the increase
in hormone sensitivity of tadpole tail during metamorphosis might
result from this increase in nuclear binding capacity for T3 rather than
from increases in affinity as the affinity constants appeared to decrease
during larval development. Durban and Paik (1976) investigated the
binding properties of the whole cell sap in the tail and liver of
metamorphosing bullfrogs. They claimed that specific T4-binding
proteins were absent in premetamorphic tails but appeared at the
beginning of tail resorption. T4 binding in the liver was present in
premetamorphic cells but increased during the transition. In the tail
these authors claimed that the dissociation constant for thyroxine
increased four-fold whereas the total capacity decreased by one-third.
Thus there appears to be a difference between metamorphic changes in
the cellular binding of T3 and T4.

The above comparison has shown that with both steroid and thyroid
hormones, signalling activity may be mediated by receptor proteins.
Differences between the two systems are apparent however. It seems
that with TH a cytoplasmic binding step is not obligatory as cytoplasmic
receptors have not definitely been identified and, in mammals, isolated
nuclei will bind the hormone to the nuclear receptors.

A number of questions arise in both the steroid and amphibian systems
(Lewin, 1974). Are the cytoplasmic receptor proteins present only in
target tissues so that other types of cell are unable to respond to the
hormone? And do the receptor proteins alone mediate the action of the
hormones, or are the nuclei of target tissues differentiated, perhaps
in the properties of chromatin, to possess the ability to respond?

In the case of steroids it seems that hormone action is mediated
only through the interaction with cytoplasmic receptors because the
affinities of receptors for oestradiol analogues corresponds well with
their overall biological activity. However, recent work by Kistler
et al (1977) indicates that this is not so with tri-substituted thyronine
analogues of T3 in bullfrog tadpole tail fin cytosol. The relative

strengths of binding of analogues were compared to T3 by competition experiments. These results were compared to previous data from Frieden and Yoshizato (1974) on the thyromimetic activity of the analogues using tail regression as a bioassay. A correlation was observed in only three cases. Because of the uncertain occurrence of extra-nuclear cytoplasmic binding these experiments might have been more informative if carried out with nuclear receptors alone.

All of the tadpole tissues so far examined have contained TH receptors whereas in the case of steroids they are present in the oviduct but absent from spleen and lung which do not respond to steroid hormone treatment. Most, if not all, tissues of the larval amphibian are involved in metamorphosis so that this difference is not surprising.

It has been argued in the case of the chick oviduct that the key factor in the differentiative response is the proteinaceous receptor rather than the hormone molecule which may only serve to move the preformed receptor into the nucleus. It appears that the receptor-hormone complex as a whole binds to the chromatin. The level of binding of receptor-hormone complexes to chromatin appeared due to the nature of non-histone (acidic) proteins present. Fractionation of acid proteins showed a single fraction to promote binding and it has been hypothesised that some acidic protein, present in oviduct chromatin but absent from other chromatins, may specifically bind the hormone-receptor complex. This binding presumably results in changes of gene expression. O'Malley's group have produced a number of hypotheses to explain the mechanism by which binding of the complex allows polymerase to transcribe the structural gene element. In Amphibia work has yet to progress to this level of analysis but a highly speculative model for gene control by mammalian TH suggested by Oppenheimer et al (1976) is mentioned below.

This summary of our scanty knowledge of amphibian TH action at the gene level nevertheless suggests ways in which competence, thresholds and metamorphic triggering could be produced by changes in receptor numbers and affinity as well as by tissue-specific differentiation of chromatin permitting hormone-receptor complexes to bind to only certain regions of the genome.

In addition, as was mentioned previously, Paik and Cohen (1961) and Frieden et al (1965) had suggested that TH treatment might lead to the production of long-lived RNA molecules which might also partly explain the above phenomena. Although a number of researchers have attempted to study TH-induced transcriptional and translational events and relate them to the eventual appearance of metamorphosis-specific proteins this field is still in its infancy and has yet to suggest substantial models. Some of the most relevant data obtained in the induction of liver enzymes will be discussed briefly before proceeding to consider how the Dodd and Dodd (1976) hypothesis could affect the interpretation of my data.

Following exogenous TH administration there is a 6 day lag period before new proteins, characteristic of normal metamorphosis, can be detected in the bullfrog larval liver (Tata, 1967). The increase in enzyme is preceded, by about 2 days, by an abrupt increase in the rate of amino acid incorporation into protein in vivo. At least part of the lag time is assumed necessary for additional RNA to be synthesised and processed in the nucleus. That a proportion of this RNA is important for the de novo synthesis of hepatic metamorphic proteins is indicated by the fact that actinomycin D, administered with or soon after thyroxine, will prevent a rise in carbamyl phosphate synthetase (Kim and Cohen, 1968). However the latter authors concluded that control was not entirely transcriptional. Part of the hormonal induction is due to control of transcription which involves all species of RNA but simultaneously the hormone controls the activation of an inactive form of the enzyme. The evidence was a discrepancy between the immunological detection of the enzyme and measurement of enzymic activity. Part of the inactive enzyme was preformed and part was synthesised following hormone administration. Tata (1967) and Nakagawa and Cohen (1967) found that within the 6 day lag period there was an acceleration of both nuclear and cytoplasmic RNA synthesis in tadpole liver following T3 treatment. The interpretation is complicated by changes in RNA precursor pool sizes also caused by the hormone, but Kim and Cohen (1966) claimed to have detected an alteration in template activity of liver chromatin from thyroxine-treated tadpoles. However Wyatt and Tata (1968) were unable to demonstrate that a significant part of the additional nuclear RNA

synthesised _in vivo_ was messenger - or even DNA-like. They were inclined to attribute this failure to the difficulty of detecting a small change in a wide spectrum of nuclear RNA molecules.

More recently Griswold and Cohen (1972) separated the activities of tadpole liver RNA polymerase I and II with alpha-amanitin and showed that the activities of both enzymes increased after T4 treatment. Also observed was an increase in the activity of the mitochondrial enzyme carbamyl phosphate synthetase. In further work Griswold and Cohen (1973) developed techniques for the solubilisation and separation of RNA polymerase I and II and studied their reaction characteristics using exogenous DNA template. The T4-induced increase in polymerase activity observed with endogenous template was also seen when the separated enzymes were assayed with the exogenous system. Thus the increase in RNA polymerase activity after T4 treatment represents either an activation or increased synthesis of the enzyme itself. By suppressing protein synthesis 70-80%, with cycloheximide, it was shown that T4 still induced an increase in polymerase activity. The tentative conclusion is therefore that the hormone merely leads to enzyme activation.

A highly speculative model for genetic regulation in mammals by thyroid hormone, mentioned previously, has also resulted from studies involving alpha-amanitin (Oppenheimer _et al_ 1976). This compound is usually regarded as a specific inhibitor of RNA polymerase II which is responsible for the assembly of heterogeneous RNA. When the drug was administered concomitantly with T3, and 8h afterwards, there was almost complete inhibition after 66h of T3-induced malic enzyme formation (Dillman _et al_, 1977). However after cessation of alpha-amanitin inhibition(72h) a dramatic increase in the rate of enzyme synthesis occurred, so that the level of malic enzyme activity was identical to that observed in animals treated with T3 alone. The possibility of stimulation by residual unbound T3 was considered unlikely and Oppenheimer _et al_ (1976) have suggested that a "long-lived imprint" was formed prior to assembly of heterogeneous RNA. This is reminiscent of the long-lived effects of T3 in tadpoles previously noted. Oppenheimer _et al_ suggested that T3 interacts with nuclear receptors reversibly

to lead to a slow accumulation of the "long-lived imprint" which was thought to be responsible for stimulating RNA polymerase. Alpha-amanitin was assumed to block RNA polymerase but not to affect the hormone-receptor complex. After T3 and alpha-amanitin have been metabolised the "long-lived imprint" allowed renewed stimulation of RNA polymerase and formation of new malic enzyme. Although the time period indicated here is much shorter than those observed in larval metamorphosis, models such as this help to bridge the gap which exists between whole-animal metamorphosis experiments and experiments on the transcriptional - translational activities of the hormones.

Following the burst of nuclear RNA synthesis described by Tata (1967) in tadpole liver cells additional cytoplasmic RNA appeared mainly in heavier polyribosomal aggregates. This was accompanied by a decrease in the relative amount of monomeric ribosomes and their subunits. The polyribosomes increased just before the appearance of new specific proteins but there was no detectable total increase in ribosome number suggesting a turnover. A redistribution of ribosomes attached to endoplasmic reticulum membranes was also observed by Tata (1967). Ribosome synthesis occurred almost simultaneously but no evidence is yet available to prove that these new ribosomes are involved in hormone-induced de novo protein synthesis.

Somewhat similar conclusions have been drawn from studies on tail regression (review by Tata, 1971; Ryffel and Weber, 1973) which indicates that thyroid hormone treatment probably exerts both transcriptional and translational effects.

8.9. Interpretation under the Dodd and Dodd hypothesis

Taken with the failure to demonstrate the marked increases in circulating thyroxine required by the Etkin (1964a) model the work discussed in Section 8.8 has lead to a revised hypothesis for metamorphic control by Dodd & Dodd (1976). They accepted that the thyroid gland was synthesising and releasing more hormone during metamorphosis and also that circulating TH levels did not rise markedly at this time. It was therefore suggested that the hormones were being utilised peripherally at

an ever increasing rate to account for the difference. This hypothesis removes the need for the complicated feedback changes, in the presence of high circulating hormone titres, necessitated by the Etkin model. In the Dodd hypothesis the well-established negative feedback system would ensure increased thyroidal output as tissue utilisation increased. In support of their hypothesis Dodd and Dodd cited the work of Ashley and Frieden (1972) who found that in premetamorphic bullfrog tadpoles plasma levels of labeled T3 and T4 were maximal 45 minutes after an intraperitoneal injection and thereafter dropped rapidly. Amongst the various routes for removal of TH from the plasma which includes excretion, Dodd and Dodd suggest that hormone is utilised by the tissues.

This modified hypothesis states only that TH is being used peripherally at an increased rate but the work of Yoshizato and Frieden (1975) on increases in receptors at metamorphosis was cited by Dodd and Dodd (1976) so that a large part of the usage was likely to be due to increased specific nuclear binding. If this were so one would expect that levels of hormone in the peripheral tissues of climax larvae would be substantially higher than those in circulation. The work of Reynolds (1971) does not support this contention however. Reynolds showed that the rate of uptake of ^{125}I-T4 by live or dead R. pipiens tadpoles differed little but that subsequent loss of hormone was much higher in live animals suggesting an active process. Individual tissue determinations of radioactivity as well as autoradiographic observations suggested that, at most, concentrations in but a few tissues exceeded circulating levels by any magnitude. Further, once the supply of hormone diminished tissue levels fell precipitously along with total body levels. In Reynolds' work tadpoles from TK stages IV to XV were apparently pooled so that the method may not have been sensitive enough to show the increase in tissue hormone predicted by the Dodd model even if it occurred in these pre-climax stages. Data are not yet available on the stability or turnover of receptor-bound hormones but if they are rapidly turned over it must be assumed that they are released as deiodinated molecules which cannot subsequently be reused as hormone and which will not affect the feedback mechanism.

Another possibility is that metamorphosis requires tissue metabolism of hormone at an ever increasing rate in such a way that high levels are never built up intracellularly. Yamamoto (1964) studied changes in thyroxine deiodination activity in the whole body, viscera, tail and liver of metamorphosing X. laevis larvae. Changes in the tail were slight but in the liver there was a marked increase in enzymatic activity during the transition, followed by a decline. A series of studies by Galton and coworkers (1961, 1962 and 1976) have elucidated this mechanism further. Galton and Ingbar (1961) showed that larval R. pipiens liver, which is sensitive to TH signals, possesses a mechanism to deiodinate T4 whereas adult frog liver which is insensitive, does not. The same authors (Galton and Ingbar, 1962) showed that the neotenous Necturus maculosa which is insensitive to T4 also lacked a peripheral deiodinating system. However a recent paper by Robinson and Galton (1976) has shown that the deiodinating activity can be separated from metamorphic events. Xenopus tail tips regressing spontaneously in vitro did not exhibit a deiodination ability. Also the enzymatic reaction, when present, possessed properties characteristic of peroxidase activity. It did not appear to achieve monodeiodination and hence cannot be considered a mechanism for the peripheral conversion of T4 to T3.

Thus it seems that the peripheral utilisation of T4 does increase at metamorphosis as required by the Dodd hypothesis but that much of the hormone is deiodinated to di- and monoiodotyrosine which have low thyromimetic activities.

Reynolds' (1971) work involved only the use of labeled thyroxine. The data previously reviewed showed that many more nuclear sites for T3 are available in the liver and tail. Kistler et al (1977) have stated that in tadpole tail fin cytosol they found about 250 times greater affinity for T3 than for T4. They consider that this finding makes it unlikely that the T3 cytosol binding sites in tadpoles, which also possess the potential to bind T4, do so under physiological conditions. In exogenous hormone treatment experiments T4 may be relatively inactive as the presence of less than 0.4% T3 in purified T4 preparations is difficult to achieve. A number of authors (e.g. Roth, 1953; Frieden and Westmark, 1961) have found T3 at least ten times more active than T4, when injected, as judged by changes in morphology. A recent paper by

Yoshizato et al (1976) has also disputed the results of Durban and Paik (1976) by stating that no high-affinity, saturable sites for T4 were detectable in bullfrog tail. The unpublished data of Miyauchi et al (1977) mentioned previously, suggests that there may be a substantial rise in serum T3 at climax.

There has been considerable controversy amongst mammalian TH workers as to whether T3 is the active hormone while T4 is merely a prohormone (e.g. Chopra et al, 1973). Also, Oppenheimer et al (1972) has shown that, in addition to its goitrogenic properties, propylthiouracil stimulates the peripheral monodeiodination of T4 to T3 in mammals when injected at the same time as T4. These facts, together with the amphibian binding data suggest that more attention should be paid to T3 in the control of amphibian metamorphosis. In the context of this thesis, even if T3 were the active hormone and propylthiouracil has the above affect on amphibians, the almost total arrest of morphological metamorphosis indicates that this goitrogen is also a highly potent inhibitor of glandular T3 synthesis or release.

In summary, whether the Etkin (1964a) or Dodd and Dodd (1976) hypothesis is correct, all the evidence points to a need for active glandular release of TH for amphibian metamorphosis to proceed. The empirical phenomena of lag periods and stoichiometric competence may be partly explicable in terms of the accumulation of hormone-receptor complexes if these can be shown to be stable for prolonged periods.

However should Dodd and Dodd (1976) prove to be correct attempts to demonstrate the efficacity of propylthiouracil treatment by comparing hormone levels in normal premetamorphic and treated tadpole coelomic fluid might be futile if circulating titres do not rise in spontaneous metamorphosis. The data of Just (1972) and unpublished T3 RIA results of Miyauchi et al (1977) both suggest that a detectable rise does occur although not on the scale predicted by Etkin (1964a). Thus the provisional results of Chapter 7 are likely to be meaningful.

8.10. The role of body growth in biochemical metamorphosis

Maclean and Turner (1976) suggested that the haemoglobin transition

might be a response to body growth rather than to morphological metamorphosis. The technical difficulty of rearing sufficient goitrogen treated larvae to investigate this problem systematically with each protein system prevented other than provisional conclusions with haemoglobin and albumin. However data presented in Chapters 3 and 4 made it clear that if the biochemical transition is a response to growth then the relationship much be a complicated one as some very large larvae possessed tadpole-like proteins and _vice versa_.

However in the case of lens crystallins good evidence was obtained here for a direct relationship between lens diameter and protein pattern category. The question was raised in Section 8.3 of how crystallin gene control and body size might be coordinated.

I should now like to speculate on the nature of this coordination. The most obvious adult marker was the presence of detectable alpha-crystallins and so the present argument, which could easily be extended to beta- and gamma-crystallins, will be confined to the alpha-group. Several workers (e.g. McDevitt, 1968; Polansky and Bennett, 1970 & 1973) have claimed that alpha crystallins are not detectable in the embryonic lens but appear later in the secondary fibres. As the lens grows the proportion of tissue containing alpha-crystallins to that which does not contain alpha-crystallins will rise. Consequently the concentration of alpha crystallins will rise in whole lens homogenates of lenses of increasing diameter. Until a certain point is reached, determined by the sensitivity of the technique employed, alpha crystallins will be undetectable and after that point their relative concentration on electropherograms will rise. Therefore it is only necessary that a gene switch occurs at the beginning of secondary fibre differentiation for the observed results to be produced. The connection between crystallin electropherogram and lens diameter will then be the connection between whole body growth and lens growth. De Jongh (1967) has shown the relationship between body size and lens diameter to be constant throughout a given phase of amphibian larval development.

Polansky and Bennett (1973) working with _Rana catesbeiana_ tadpoles separated electrophoretically the crystallins from whole lenses and also

from specific layers within lenses. Studies of whole lenses showed that the lens proteins did not change rapidly, one to another, prior to or during metamorphosis. However, changes became apparent during postmetamorphic development. These changes included an increase in the relative concentration and mobility of alpha crystallin, a decrease in the relative concentration of gamma crystallin and an increase in the relative concentration of beta crystallin. Examination of specified layers within tadpole and frog lenses demonstrated that changes in the patterns of lens protein synthesis and modification may occur during development. For example, alpha crystallin from tadpole and frog lenses is concentrated in the exterior cell layers but the tadpole alpha crystallin has a faster electrophoretic mobility than frog alpha crystallin. Another example is the presence of a new beta crystallin in the adult frog lens periphery and not in the tadpole lens periphery. This also suggests change in protein synthesis during anuran lens development. Thus although the hypothesis of a single switch at the beginning of secondary fibre differentiation is adequate for the data obtained with Xenopus lenses the situation seems more complex in the bullfrog. Nevertheless in general the findings of Polansky and Bennett (1973) agree well with the growth hypothesis.

Polansky and Bennett (1973) also found that the changes in lens physical parameters (including growth) and crystallin patterns occurred precociously during induced metamorphosis. They admitted, however, that the effect could be indirect and be related to lens diameter rather than morphological stage. This has been confirmed in the present thesis.

In adult amphibians both "growth hormone" and "prolactin" appear to have GPH activity (Nicoll and Licht, 1971a and b). These hormones have been implicated in the control of mitosis in the lens epithelium of adult Rana pipiens. Normally epithelial mitosis has a cyclic nature (Rothstein et al, 1975) which is abolished by hypophysectomy. Although a number of exogenous mammalian hormones could temporarily replace the stimulating effect of the pituitary only mammalian growth hormone did so permanently (Van Buskirk et al, 1975). Recent work (Wainwright et al, 1976) has shown that partially purified preparations of R. pipiens "growth hormone" and "prolactin" have the same effect. As the equatorial region of the lens epithelium is the germinative region

producing new lens fibres this control may relate seasonal lens growth
to seasonal body growth. In amphibian larvae the GPH is prolactin and
an interesting line of further research would be to investigate its
involvement in lens epithelium mitosis and the differentiation of
secondary lens fibres.

The results of this thesis have shown that developmentally
retarded larvae are highly variable with respect to two "metamorphic"
protein shifts and that individual specimens can be a mosaic of "tadpole"
and "adult" proteins. The point was raised in Chapter 1 that animals
selected for biochemical study are usually grouped in stages based upon
external morphology. In the light of this thesis, which shows that
morphological and biochemical metamorphoses can be dissociated, it might
be predicted that a given stage might include larvae with heterogeneous
metamorphosing proteins. However the thesis has also shown that under
normal circumstances a predictable protein transition will occur which is
correlated with gross morphology and which served as the control
situation in my work. Heterogeneity of samples might only arise if
experimental animals were raised under conditions of, for example, low
light intensity (Saxén et al, 1956b) when developmental arrest and
gigantism can occur.

8.11. Protein transitions during the development of non-metamorphosing vertebrates

Until further experimental work produces a more substantial
foundation for the concepts of stoichiometric hormone action, differential
thresholds of response and long-term triggering effects the main overall
conclusions of this thesis are best interpreted in terms of the Etkin
(1964a) hypothesis. To date there is little experimental support for
the modified hypothesis of Dodd and Dodd (1976) and it seems likely that
the sequence of metamorphic events is produced by at least a slight rise
in the circulating levels of T3.

In spite of the published evidence which has claimed a central role
for thyroid hormones in the haemoglobin, albumin and lens crystallin
transitions the results of this thesis show that the change in these

proteins and in LDH isozymes can occur under thyroid blockage. Before assuming that this conclusion is surprising I should like to speculate that there is no a priori case for believing that TH control, of all changes observed during overt metamorphosis is inevitable. Solomon (1965) has reviewed the development of a number of non-enzymic proteins in vertebrates. It is apparent that ontogenetic shifts, in haemoglobins, serum proteins and lens crystallins, occur in all vertebrate classes. The situation with LDH isozymes is similar (Manwell and Baker, 1970). Although there is evidence, discussed in previous chapters, that the protein shifts may be adaptive in those post-metamorphic anurans which leave the water, the fact that the shifts also occur in non-metamorphosing vertebrates makes it likely that they are a common property of the vertebrate subphylum which has been exploited by some amphibians as a preadaptation. There is variation even within the amphibia however as was shown in the axolotl Ambystoma mexicanum by Ducibella (1974a). Neotenic individuals completed a haemoglobin and red cell type transition when 3 to 4 months old. A serum protein transition occurred between 5 and 9 months of age. Experimental induction of morphological metamorphosis could be induced by thyroxine treatment at any time. In the closely related tiger salamander Ambystoma tigrinum which metamorphoses spontaneously at about 5 months of age the haemoglobin and red cell changes occur at the same time.

Barrington (1968) and Etkin and Gona (1974) have reviewed the hypotheses concerning the origin of the thyroid gland and its hormones in the lower chordates. It was argued that the hormones arose as an accident of the chemical affinity of tyrosine for iodine which was a common element in the marine environment of the vertebrate ancestors. Monoiodotyrosine and diiodotyrosine were probably common compounds, which in living lower chordates have a physiological role, but there is no evidence for their possessing a hormonal i.e., signalling, function. In protochordates, proteins in the blood and tunic couple with the iodinated tyrosines facilitating the synthesis of T3 and T4. The universal presence of these compounds in vertebrates suggests a continuing role for them, but at present no overall functional activity, can be ascribed to them (Etkin and Gona, 1974). In general, they do not appear to play a central role in the processes in which they have been implicated.

A definite signalling role appears to have evolved only in amphibian metamorphosis and in the metabolic regulation of homeotherms (Frieden and Kent, 1976). Only in the amphibians is there definite evidence that thyroid hormones serve as the primary signal that permitted the evolution of a specialised larval period and associated metamorphosis.

It is therefore possible that although the unique amphibian morphological metamorphic events are directly controlled by the thyroid hormones, those biochemical changes which occur in all vertebrate embryos and may be only temporally correlated with metamorphosis, are controlled by quite different signals. Like many of the processes in other vertebrates, in which thyroid hormones have been implicated, they may only be involved in the metabolism of the system. Until we have a deeper understanding of the control of such processes as haemoglobin and serum protein transitions it appears that they are not the model systems in which to study metamorphic gene control by thyroid hormones that they have often been considered to be.

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