The role of steroid hormones in the differentiation of the gonads of the clawed toad, Xenopus laevis Daudin

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The role of steroid hormones in the differentiation of the gonads of the clawed toad, *Xenopus laevis* Daudin.

by

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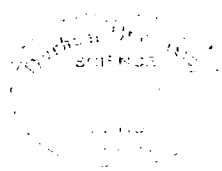

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Πρῶτον μέν γάρ τρία ἦν τά γένη τά τῶν ἀνθρώπων,
οὐχ ὥσπερ νῦν δύο, ἄρρεν καί θῆλυ, ἀλλά καί τρίτον
προσῆν κοινόν ὄν ἀμφοτέρων τούτων, οὗ νῦν
ὄνομα λοιπόν, αὐτό δέ ἠφάνισται· ἀνδρόγυνον γάρ
ἓν τότε μέν ἦν καί εἶδος καί ὄνομα ἐξ ἀμφοτέρων
κοινόν τοῦ τέ ἄρρενος καί θήλεος, νῦν δ' οὐκ ἔστιν
ἀλλ' ἤ ἐν ὀνείδει ὄνομα κείμενον.

Plato, 'Symposium',
189D-E, page 138.

'In the first place, there were three kinds of human beings, not merely the two sexes, male and female, as at present: there was a third kind as well, which had equal shares of the other two and whose name survives though the thing itself has vanished. For "man-woman" was then a unity in form no less than name, composed of both sexes and sharing equally in male and female; whereas now it has come to be merely a name of reproach'.

ABSTRACT

Past experiments had demonstrated feminization of male larvae of Xenopus by treatment with oestrogens but the androgens available had had only slight effects on gonadal development in this species. The author's experiments confirmed the former effect with oestrogens at concentrations of 25µg/l to 500µg/l. Feminization was however, prevented by simultaneous administration of actinomycin D or puromycin. At the high concentration of 2mg/l, oestradiol caused paradoxical masculinization. The effect of progesterone was likewise dosage-dependent. At low concentrations it had only slight effects while at 500µg/l it accelerated ovarian development. It was again confirmed that testosterone had only slight effects on gonadal development, but it was discovered that 5α-dihydrotestosterone caused a considerable degree of gonadal masculinization in genetic female larvae. It is suggested that the inactivity of testosterone is a consequence of the absence or at least inactivation, in the larva, of the enzyme required to metabolise the androgen into the 5α-form. Cortisone at low concentrations had little effect on gonadal development while the 2mg/l dosage caused partial intersexuality in females. Oestrogens and androgens in addition led to hyperplasia of the interrenal. Furthermore oestrogens, androgens and cortisone induced precocious development of the vasa differentia in genetic males.

Differentiated gonads of larvae continued to develop well in vitro and they were not affected by oestradiol treatment. Adult ovaries also survived well in vitro but undifferentiated gonads and adult testes disintegrated.

Δ^5-3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase were present in the interrenals from stages 49 onwards. In the gonads they first appeared at stage 56, shortly after sexual differentiation became apparent. Treatment with 2mg/l oestradiol led to failure of these enzymes to appear in the gonads but not in the interrenals.

Injections of tritiated oestradiol or progesterone into larvae led to accumulation of radioactivity initially in the liver and the mucosa of the duodenum, later confined to the latter. Individuals so treated seven months after metamorphosis accumulated radioactivity mainly in the kidney tubules, blood and choroid plexus and only slightly in the duodenal mucosa, indicating that by this stage the kidney had developed its adult function with respect to steroid metabolism and the brain its regulatory function over sexual development. In the larvae pretreatment with cold oestradiol prior to the injection with tritiated oestradiol, reduced the accumulation of radioactivity in the duodenal mucosa but there was no effect when this pretreatment was followed by injection of tritiated uridine, suggesting that oestradiol manifests its feminizing action by inducing production of neoprotein.

The role of administered steroid hormones in modifying differentiation of the gonads is discussed. It is concluded that they can act as sexual inductors (the medullarins and cortecins of Witschi) via mediating protein synthesis.

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CHAPTER 1

BACKGROUND AND AIMS

1.1 Review of Literature

Vertebrate embryos are inherently susceptible to undergoing sex reversal spontaneously or experimentally, since all individuals regardless of genetic sex, pass in early development through a sexually undifferentiated phase in which the primordial structures of both sexes are present. The ease with which inversion occurs varies with species since the degree to which the structures of the recessive sex developed varies with systematic group. Sex reversal is easier when the organs of the recessive sex persist for a long time in the embryo or when they are still present in the adult. The bipotentiality of the embryonic sex can be demonstrated experimentally using sex steroids in vivo or in vitro, gonadal grafting, parabiotic experiments, or altering environmental parameters such as temperature, ionic concentration, illumination and diet. Early attempts to influence embryonic sex differentiation using crude hormone preparations were unsuccessful, but with the purification, isolation and eventual synthesis of sex steroids, and achievement of direct control over dosage and timing of hormonal administration, sex reversal was achieved. The first successful experiments with pure hormones were done on chick embryos in the early 1930's by workers such as Wolff and Ginglinger 1935; Willier, Gallagher and Koch, 1935, 1937. By injecting a sex hormone solution into the egg they achieved structural transformations of the gonads and accessory organs and eventually sex reversal. A summary of such experiments in vertebrates is given in Table 1(p 14).

Of all vertebrates, fishes show the most divergent expressions of sex, ranging from synchronous hermaphroditism, protandrous and protogynous hermaphroditism to gonochorism both differentiated and undifferentiated. Therefore these vertebrates provide excellent material to approach the problems of sex differentiation and of evolution of sex among animals. The reproductive organs of teleosts and holosteans differ fundamentally from those of other fish and from the amphibians, birds and mammals. According to D'Ancona (1943, 1950 and 1955); Ashby (1952) and later Dodd (1960) and Forbes (1961), there is no medullary contribution to the gonad but only the cortical region is present from which both testis and ovary develop.

Thiebold (1954) injected oestradiol into embryos of the small shark Scylliorhinus caniculus within the egg. Ovotestes developed in males. Likewise the ovaries remained unaffected by testosterone injections while the testes were again intersexualised. Oestradiol affected the development of the gonoducts of both sexes. It stimulated persistence of the Mullerian duct in males and hypertrophy of the anterior parts of the ducts in both male and female sexes. Likewise testosterone caused hypertrophy of the Wolffian ducts in both embryonic sexes, in addition to causing hypertrophy of the anterior part of their Mullerian ducts. Chieffi (1959) using oestradiol brought about hypertrophy of the Mullerian ducts but observed no effect on the Wolffian ducts. Testosterone on the other hand caused marked hypertrophy of the Mullerian and Wolffian ducts in both sexes.

Experiments investigating the modification of sex differentiation using steroids on juvenile teleosts, have been largely restricted to

cyprinodonts and the results less easy to interpret than those in amphibians and birds. In the rainbow trout, Salmo gairdneri irideus, Padoa (1937a, 1939) reported that testosterone treatment induced testicular tissue growth in the ovaries. Ashby (1952, 1956, 1959, 1965, pers. commun.) reported paradoxical similarity of action of oestradiol and testosterone in the brown trout, Salmo trutta. At medium and high concentrations both hormones retarded gonadal development but also produced hypertrophy of the somatopleure to form a columnar ciliated epithelium. At low concentrations there was evidence of acceleration of gametogenesis but none of sexual reversal. Progesterone speeded up spermatogenesis in older animals. A number of attempts have been made to modify sex differentiation in viviparous toothcarps by administration of androgens in young newly born broods or adults. Androgens mainly affected the secondary sexual characters, the action on the gonads being incomplete, pathological or negative.

In the guppy, Poecilia reticulata, the effects of sex hormones have been studied by Witschi and Crown (1937); Berkowitz (1937, 1938, 1941); Regnier (1938); Eversole (1939, 1941); Gallien (1946, 1948); Hildemann (1954); Querner (1956) and Miyamori (1961). Berkowitz and Querner produced ovotestes by administering oestrogens to young male guppies, and Miyamori (1961) produced ovotestes by giving androgen to young females. In the top minnow, Gambusia holbrookii, Lepori (1942a,b, 1948) produced ovotestes in females by administration of an androgen and in males by administration of an oestrogen. Similiar results were obtained by Okada (1944) in Gambusia affinis. The same author (Okada 1943, 1949, 1952) using

Oryzias latipes, claimed that the formation of ovotestes in adult males could be induced by either oestrogens or androgens. In the swordtail Xiphophorus hellerii, Baldwin and Goldin (1940) reported that androgen treatment of young females, stimulated the male secondary characters and also induced masculinization of the gonad after degeneration of the oocytes. Querner (1956) showed that androgen treatment produced ovotestes in genetic females. Corticoids on the other hand, contrary to results in some amphibians, had no effect on sex differentiation.

Experimental procedures have been diverse and sometimes not completely documented. This makes the results discussed above difficult to compare. Failure to obtain complete sex reversal in those studies could have been due to the hormonal treatment starting after the onset of gonadal sex differentiation. Complete sex reversal of bony fishes was in fact achieved subsequently using mammalian or synthetic sex steroids. Yamamoto (1953, 1958, 1959a,b, 1961) was able to reverse both sexes of the egg-laying cyprinodont, Oryzias latipes. Sex-linked pigmentary patterns were used to indicate the genotypic sex of the treated fish and the results were confirmed by the abnormal sex ratios of their offspring. Males were fed oestrone or stilbestrol and females methyltestosterone, from the day after hatching (before sex differentiation) for 8-10 weeks. Thereafter they were raised on a normal diet until they reached maturity. When mated with normal females (XX), the sex reversed females (XX) gave an all female population. When the sex reversed males (XY) were mated with normal males (XY), they produced a sex population ratio of approximately one female to three males. One third of the male offspring had a genetic constitution of YY, which when mated with normal females gave an all-male population.

Yamamoto (1963) sex reversed these YY males, which subsequently laid eggs that all developed into males. When sex reversed males were mated with sex reversed females, a normal 1:1 sex ratio of offspring was produced (Yamamoto, 1961). Oestradiol and stilbestrol were about three times more potent than oestrone in reversing the sex of young male medakas (Yamamoto, 1962). Reversal of young females in the opposite direction by methyltestosterone was also achieved by Yamamoto (1958). The sex reversed females when mated with normal females gave an all female population. Other androgens such as androsterone and testosterone propionate had also a male inducing action (Yamamoto, Takenchi & Takai, 1968). On the other hand progesterone, deoxycorticosterone acetate (DOCA) and cortisone acetate were ineffective in both young males and females.

All attempts to control sex by hormone treatment after birth in the guppy, by which time gonadal sex differentiation was already established (Goodrich, Dee, Flynn & Mercer, 1934) failed until Dzwillo (1962) succeeded in getting functional XX males by injecting methyltestosterone into gravid females of Poecilia reticulata containing embryos having different gonads. Clemens (1965) was able to obtain androgen induced XX males in Tilapia mossambica by administration starting with newly hatched fry having indifferent gonads. In the goldfish Carasius auratus, Yamamoto and Kajishima (1969) obtained functional reversals in both directions by giving heterologous sex hormones to newly hatched fry with indifferent gonads for two months. By test crosses of the hormone treated fish evidence of male heterogamety (XY) in goldfish was obtained.

The above sex reversals in fish suggest that by finding the correct dose of a suitable sex hormone to treat the embryos or larvae at the indifferent gonad stage, it is possible to convert genetic females into functional males and vice versa.

The process of sexual development and the factors influencing it, have been studied extensively in amphibians. These lower vertebrates are excellent forms for studies of sex because of the manner in which the gonads develop embryologically and their responses to the many factors modifying sexual development such as temperature and diet. Many experimental studies have been made to determine the role of gonadal secretions or synthetic hormonal substances in the process of sex differentiation. In early investigations adult or juvenile animals were partially or completely castrated and grafts of gonads were transplanted from an individual of one sex to one of the opposite sex (Witschi 1927b). Later, as techniques became more refined much younger animals were used and larvae in tail-bud stages were placed in parabiotic pairs (placed side by side and allowed to grow together) or grafts of the presumptive reproductive system were transferred from one embryo to another. When it became possible to obtain synthetic hormonal substances, larvae and embryos were subjected to a variety of treatments.

Witschi (1942) reviewed the effects of temperature on sexual development in amphibians. Temperatures of 15-20^{o}C produced normal individuals. When larvae of Rana temporaria were kept in water at 10^{o}C there was retardation of the medulla and stimulation of the cortical gonadal region. The ovaries of the genetic females were larger than normal and in the genetic males there was dominance of

cortex over medulla. At 27°C in contrast sex differentiation started normally but as metamorphosis approached, the ovarian cortex of females begun to degenerate while the medulla grew bigger till eventually all females transformed into phenotypic males. Piquet (1930) had found that tadpoles of this species reared at 18-21°C developed into 50% males and 50% females. At 15°C hermaphroditic individuals were produced, at 10°C 100% were females at the time of metamorphosis and at 27°C all larvae had testes. Uchida (1973b) reported that high temperature favoured masculinization of the urodele Hynopius retardatus gonads. Heat-induced sex transformation from ovaries to testes in frog tadpoles was verified subsequently. Iwasawa and Oyanagi (1971) showed that higher than environmental temperatures encouraged testicular development in Rana togoi larvae. Hsu, Yu and Liang (1971) and Hsu, Chiang and Liang (1973) demonstrated that heat-induced partial or complete transformation of ovaries into testes, still occurred in tadpoles of Rana catesbeiana reared at 30°C if these had previously been hypophysectomised. When older, sexually differentiated frog larvae were submitted to such high temperatures, cortical regression accompanied by compensatory medullary enlargement and testicular organization in the female ovaries was induced (Witschi 1929).

In some instances the topography of the gonads permitted surgical extirpation of the dominant inductor system. Thus feminization of the male Bufo vulgaris by surgical ablation of the medulla, was reported by Ponse (1925). The testes of normal toads are capped by purely cortical lobes. After removal of the testicular parts the ovarian rudiments enlarged in the course of two years and eventually produced mature eggs. Thus the destruction of the dominant inductor system achieved sex reversal, indicating an antagonistic

relationship between cortex and medulla at larval and adult stages.

For the investigation of the possible nature of the inductor system inducing sex differentiation, parabiotic and implantation experiments were employed. In the parabiotic studies with frogs, toads and salamanders, the results obtained varied according to the group tested. Sex differentiation between parabiotic males or females proceeded normally whereas in heterosexual pairs modification of one or the other sex could take place. With Rana and Hyla frogs, Witschi (1927a, 1931) and Montalenti and Calisti (1936), found that in general the male partner gonads dominated and suppressed the development of the ovaries transforming them eventually into testes. The influence of the testes on the ovaries in the above species was found to be proportional to the distance separating them. Thus the areas of the ovaries nearest to the testes were completely transformed whereas those furthest away were intersexual. In contrast parabiotic pairs of the toad larvae allowed to develop to metamorphosis showed no modification of either testes or ovaries. Witschi (1931) suggested that in the latter case the masculinizing substance from the testes was inactivated before reaching the ovaries, while Ponse (1949) suggested that time was the important factor and that the masculinizing substance of the toad testis was not produced until after metamorphosis. Ponse (1949) verified her suggestion by finding an inhibitory effect of the testis on the Bidder's organ two months after metamorphosis. Chang (1953) using heterosexual parabiotic pairs of Xenopus laevis larvae, showed that testes inhibited the development of ovaries. The masculinizing substance from the testes had maximum potency some time after metamorphosis but prior to sexual maturity. However the ovaries were maximally susceptible during the early larval period. There

was agreement by Foote and Probstmeyer (1959) who cultured together testes and ovaries of Xenopus larvae in vitro. Mikamo and Witschi (1963) placed testicular grafts of adult Xenopus in the body cavity of larvae and found inhibition of both male and female gonads. Removal of the grafts after a year was followed by recovery of the remaining rudiments of the host gonads. Recovery was sometimes incomplete and in some genetic females the medullary elements led to testicular development.

In parabiotic studies of urodeles such as Triturus (Witschi and McCurdy 1929, 1943) and Ambystoma (Burns 1925, 1930, 1935a,b; Humphrey 1932, 1936a,b; Witschi 1936, 1937), it was confirmed that normally the testes of the male partner predominate and the ovaries of the female partner could be suppressed or even reversed to testes. Reversal of the female salamanders was obtained by parabiosis with males or by implantation of testes or testicular primordia (Humphrey 1945). The ovary of the parabiotic pairs could become the dominant organ in some instances, particularly if the male partner was small, weak or retarded in development (Burns 1935a,b; Witschi 1937). It has been suggested that the freemartin is an example of natural mammal parabiosis resulting in masculinization of the female twin (Lillie 1916, 1917, 1923; Witschi 1965).

The terms cortecin and medullarin were proposed by Witschi (1931, 1934, 1957) for the antagonistic inductor substances of sex differentiation these being protein in nature arising from the cortex and medulla of the embryonic gonad respectively. Other investigators however, proposed sex steroids of the embryonic or adult gonad to be the inductors of sex differentiation (Willier 1939; Vannini 1946; Wolff

1950; Burns 1955, 1961; Haffen 1977). Experiments with the foetal stages of birds (Wolff 1947; Mintz and Wolff 1954; Wolff and Haffen 1962) and rats (MacIntyre, Baker and Wykoff 1959) were claimed to support the steroid theory of sex differentiation.

Humphrey (1928a,b) studied the phenomenon of sex modification in amphibians using the technique of orthotopic transplantation in Ambystoma embryos. A graft of ectoderm and underlying mesoderm was removed from an embryo of the tail-bud stage and exchanged with a similar graft from another embryo of the same developmental stage. The ectoderm and mesoderm of the graft contained the primordia of the kidneys and gonads which developed and differentiated in the new host. He was thus able to obtain larvae having the anlage of an ovary on one side of the body and of a testis on the other. Humphrey (1929) also found that if the ovary and testis of the same animal were of equal size, it was the development of the former that was modified. There was an inhibitory action of the testis on the ovarian cortex which induced masculinization of the ovary. Where the testis was smaller than the ovary, the former became intersexual or was reversed by the action of the ovary. Humphrey (1931a,b) also showed that modified ovaries would return to their original state if the testis was removed before the inverted ovaries developed testicular lobules, but that if the ovary developed such lobules, its transformation into a testis would continue. Usually after reversal of an ovary to a testis by grafts in the axolotl, Ambystoma mexicanum, the animals were unable to reproduce because of the failure of the duct system to develop between the gonad and the kidney (Humphrey 1942). However Humphrey (1945) later obtained a sex reversed female of this species with a functional testis

from which offspring were obtained. Burns (1928) removed gonads of young Ambystoma larvae and placed them in the body cavity of older larvae. The grafts became intersexual if the host animal was of the opposite sex. In some cases, the host gonads were modified if grafts were large, well differentiated and near the host gonad.

Adams (1930) transplanted ovaries from adult females of Triturus viridescens into the body cavity of adult castrated males of the same species. He observed that after 7-10 months the ovarian grafts usually contained large yolked oocytes, as well as medium and small sized ones. The rudimentary oviducts of the castrated males hypertrophied under the influence of ovarian grafts, the cloacal glands were repressed and Wolffian ducts and collecting ducts of the kidneys were more dilated than in typical castrates. Uchida (1937a) found that adult testis-grafts placed in larvae of Hynopius retardatus caused reversal of the ovaries. Witschi (1927b) found that when grafts of parts of testes of adult Rana temporaria were transplanted into the body cavity of tadpoles of the same species all constituents of testis-grafts developed normally but that development of the host's gonads was unaffected by the grafts.

Therefore the above studies of gonadal grafts in larvae would suggest a masculinizing substance produced by the testis causing in most instances the ovaries of females to be reduced in size and in some cases to be reversed. Castration effects showed that the somatic sex characters particularly those of postpuberal animal were at least in part conditioned by hormonal substances from the gonads. The effects of castration and grafting in metamorphosed

anurans and urodeles were reviewed by Gallien (1955a).

Chromatographic and spectrophotometric methods showed sex steroids to be present in extracts of embryonic gonads of amphibians and in the water where tadpoles were kept. In Xenopus laevis, oestrogens were identified in extracts of young ovaries just after metamorphosis (Gallien and Chalumeau-Le Foulgoc 1960) and later investigations showed that Xenopus male and female larvae of a very early age (9-17 days old) were capable of oxidizing testosterone and 17β-oestradiol. The males oxidized and thus inactivated oestradiol more quickly than testosterone whereas the females did the reverse (Breuer, Dahm, Mikamo and Witschi 1966; Breuer 1969). Rao, Breuer and Witschi (1969) showed that in vitro incubation of larval Xenopus ovaries or testes with 17α-hydroprogesterone produced androst-4-ene-3,17-dione as the only C_{19} steroid metabolite. Rao, Breuer and Witschi (1968) found that male and female larvae of Xenopus (17 days old) were capable of converting 17β-oestradiol not only to oestrone but also to oestriol, 6α-hydroxyoestrone and 15α-hydroxy-oestrone the last of which was demonstrated in mammals. These results would suggest that Xenopus larvae possess the enzymes necessary for the metabolism of steroid hormones (17β-hydroxysteroid oxidoreductases, hydroxylases) during gonadal development. Dale (1962) found small amounts of androsterone and 17β-oestradiol in the water where tadpoles of Rana pipiens were raised. Furthermore, by marking incubating tissue with labelled precursors and chromatographing the metabolites, Ozon (1963, 1967) identified the enzyme systems controlling steroid synthesis in differentiating gonads of larvae of Pleurodeles waltlii. Using biochemical techniques, Collenot (1964, 1965b) showed that Δ^5-3β-hydroxysteroid

dehydrogenase enzyme system was present in the middle of larval life in Pleurodeles waltlii, before the start of gonadal differentiation. The results obtained in amphibians, reviewed by Ozon (1969), were consistent with observations in birds and mammalian embryos and probably indicate that the amphibian gonads elaborate sex hormones during sexual differentiation using hydroxysteroid dehydrogenase enzyme systems. Thus the embryonic mediators of gonadal sex differentiation would be expected to be closely associated with steroidogenic enzymes and sex steroids.

Synthetic steroid hormones have been used extensively for studies of their effects on sexual development in amphibians. However, because of diverse methods of administration, varying times of treatment and of concentrations of steroids employed, and the variety of amphibians treated, results obtained have often appeared to be contradictory or at least difficult to interpret. Gallien (1959b) and Foote (1964) summarised the effects of actions of sex steroids on the differentiation of sex in amphibians (see Table 1, p.14). Histologically complete and in some cases functional transformations of the gonads were produced in a number of species using sex hormones of adult type such as testosterone and oestradiol. However, in other species only partial or temporary reversals were obtained and negative or paradoxical results came from using the same hormones. The sex genotype was also involved in this variety of response, because in some species reversal proceeded easily in one direction whereas in the others it was difficult or impossible to produce. In general, androgenic hormones have a masculinizing action on the gonads and genital tracts of Ranidae and Hylidae. Sex reversed females of the Ranidae were mated to normal females resulting in an all female offspring, thus proving the homogamety of the female

TABLE 1 Effect of steroid hormones on sex differentiation in vertebrates

Species	Investigators	Method of administration	Oestrogens	Androgens	Progestins	Corticosteroids
CHONDRICHTHYES						
Scylliorhinus caniculus	Thiébold (1954), Chieffi (1959)	Inj.in egg embryos	oed.→♀ in males	test.→♂ in males		
TELEOSTEI						
Oryzias latipes	Yamamoto (1953,1958,1959a,b,1961) Yamamoto,Takenchi & Takai (1968)	Fed or alcoh.soln. in the medium	oed., oene, stilb. →♀	mtest.→♂	prog.→no effect	DOCA→no effect
Poecilia reticulata	Dzwillo (1962)	Inj.in gravid females		mtest→♂		
Tilapia mossambica	Clemens (1965)	Alcoh.soln. in medium		test→♂		
Carasius auratus	Yamamoto & Kajishima (1969)	"	oed.→♀	test→♂		
AMPHIBIA*						
Urodeles						
Hynobius retardatus	Hanaoka (1941a,b)	"		mtest.,test. pr.→gon.inhib.		
Hynobius nebulosus	Asayama & Yamamoto (1951), Asayama & Miyamori (1957), Asayama & Amanuma (1957)	"	oed.→♀			DOC→♀
Pleurodeles waltlii	Gallien (1950b, 1954a,c)	"		test.→inhib.→♀	pregn.→gon. inhib.	
Ambystoma tigrinum	Ackart & Leavy (1939), Foote (1940a)	"	oed.,oene→♀	mtest., test→♀		
Ambystoma opacum	Foote (1940b,1941), Mintz (1947),Bruner (1952), Bruner & Witschi (1954)	"	oed.,oene→♀	test.pr.→♀		
Triturus pyrrhogaster	Asayama (1953), Asayama & Matzuzaki (1958)	"		test→♀		DOC→partial ♂
Triturus cristatus	Chieffi (1958, 1962)	"	oed.→♀	test.→♀		
Triturus helviticus	Gallien (1959b), Gallien & Collenot (1960)	"	oed.,oene→♀			
Triturus alpestris	Gallien (1959b), Gallien & Collenot (1960)	"	oed.,oene→♀			
ANURANS						
Xenopus laevis	Witschi & Allison (1950), Gallien (1953,1954d, 1956), Foote & Foote (1959)	Inj.in larvae or dissolved in medium	oed.→♀	test.→gon. inhib.→♀ at high concn.		
Discoglossus pictus	Gallien (1945, 1948, 1949, 1950c,d)	"	oed.→partial & temp.♀		pregn.→ partial ♀	
Bombina bombina	Gallien (1959b)	"	oed.→no effect	mtest., or test.→ → no effect		
Alytes obstetricans	Witschi & Allison (1950), Witschi (1951b)	"	oed.→partial & temp.♀			
Pelobates cultripes	Gallien & Collenot (1958), Collenot (1965a)	"	oed.,oene→♀			
Pelodytes punctutus	Gallien & Collenot (1958)	"	oed.→partial ♀ & ♂			
Rana temporaria	Gallien (1937, 1938,1941,1944,1950d), Jost (1943), Uchida & Takahashi (1959)	"	oed or oene →♀ low conc. →♂ high conc.	mtest. or test. →♂	pregn.→♂	DOCA→inhib. of oogenesis
Rana dalmatina	Vannini (1941,1944,1945b,1948,1950b),Padoa (1947)	"				DOC→♀

Rana esculenta	Padoa (1936,1938,1942b,1950).Quattrini (1960)	"			prog.→♀ at low conc.→♂ at medium & high conc.	DOC ♀
Rana sylvatica	Mintz & Witschi (1946), Mintz (1948) Witschi & Chang (1950)	"	oed. or →♀ low conc.	mtest. or test →♂	prog.→ partial ♂ at low conc. pregn.→♂ at high conc.	DOCA→♂ at high conc. cortisone ♂ at high conc.
Rana pipiens	Witschi & Crown (1937), Foote (1938), D'Angele (1943), Chang & Witschi (1955b)	"	oene → ♂ high conc.			
Rana clamitans	Foote & Witschi (1939), Mintz, Foote & Witschi (1945), Foote & Witschi (1950,1953)	"			pregn.→♂	
Rana catesbeiana	Puckett (1939,1940),Vannini & Stagni (1966, 1967).	"				
Rana japonica	Kawamura & Yokota (1959)	"				
Rhacophorus schlegelii	Iwasawa (1958), Amanuma (1963)	"	oed.→partial ♀	test.→ partial ♂	prog.→toxic ovarian retard.	DOCA→toxic→ ovarian inhib.
Pseudacris nigrita	Witschi, Foote & Chang (1958)	"	oed.→partial ♀	test.→♂		
Bufo bufo	Padoa & Picchi (1946), Gallien (1947) Talluri & Padoa (1953),Chang (1954),Lugli (1956)	"	oed.→partial & temp. ♀	test.→no effect	pregn.→gon. inhib.	
Bufo americanus	Chang (1955)	"	oed.→partial & temp. ♀	test.→no effect		
Hyla arborea japonica	Takahashi (1958,1959),Uchida & Takahashi (1959)	"	oed.→partial ♀	test→partial ♂		DOCA→no effect
REPTILIA						
Lacertitia						
Anolis carolinensis	Gorbman (1939), Evans & Clapp (1940), Forbes (1941)	Inj.into larvae	oed.→inhib.of testes→♀ of repr. tracts			
Lacerta vivipara	Gorbman (1939), Kehl (1938, 1944)	"		test.→partly ♂ & ♀ stiml.repr. tracts no effect on ovaries		
AVES						
Gallus bankiva	Review by Willier (1939), Wolff & Wolff (1948), Vannini (1950a)	Inj.into egg	oed.,oene→♀	test.→slight ♀ or ♂		DOCA→♀
MAMMALIA						
Metatheria						
Didelphys virginiana	Burns (1939a,b, 1942a,b, 1955)	Inj.into mother	oed.→♀	mtest.→little effect		

*Classification of Amphibia according to Coin, Coin & Zug (1978) pp. 221-250.
oed., oestradiol; oene, oestrone; stilb., stilbestrol; test., testosterone; mtest., methyltestosterone; test.pr., testosterone propionate; prog., progesterone; pregn., pregnenolone; DOCA, deoxycorticosterone acetate; gon.inhib., gonadal inhibition.

♂, masculinization; ♀, feminization; ⚥, intersexuality

in the group. Oestrogens at low concentrations produce feminization whereas high doses cause paradoxical masculinization of female larvae.

Oestrogens also feminize male gonads in lower anurans Xenopus and Pelobates, producing functional sex reversal. In bufonids oestrogen treatment of tadpoles causes partial feminization and intersexuality as showed in Bufo bufo by Padoa and Picchi (1946) and in Bufo americanus by Chang (1955) while androgen treatment has no effect on sex differentiation. Adrenocortical steroids appear to feminize gonads of Ranids and Urodeles. Progesterone masculinizes some species but in Discoglossus it produces partial feminization. In the urodele Pleurodeles it only depresses gonadal development. In Triturus, Ambystoma and Hynobius, oestrogens cause feminization but androgens produce an inhibitory effect followed by a paradoxical feminization. The sex reversed animals when mated to normal males produce 100% males, indicating the homogamety of the males in the group. Androgens suppress testicular development in Xenopus but no sex reversal has been achieved, and in Pelobates there was no observable effect.

The slow differentiation of the reproductive system in most reptiles enables the persistence of considerable degrees of bisexuality after hatching or birth and even into the adult stage. The retention of potential or actual heterosexual structures is a prerequisite for experimental sex reversal. Both gonadal elements and accessory sex structures may be modified in a heterosexual direction by administration of sufficient amounts of hormone appropriate to the opposite sex.

There was a tendency towards feminization (especially of the reproductive tructs) after treatment of post-hatching or adult males of Anolis and Sceloporus with oestrogen. This hormone induced atrophy of the testicular medulla and sometimes hypertrophy of its cortex and of the remnants of the vas deferens, Mullerian duct and sexual segment of the kidney (Gorbman 1939; Evans & Clapp 1940; Forbes 1941). In contrast androgen administration, whether by testis grafting or by testosterone injection into the female Uromastix, Anolis and Lacerta, had generally no effect on the ovary although there was hypertrophy of the vas deferens, Mullerian ducts and sexual segment of the kidney (Gorbman 1939; Kehl 1938, 1944; Noble & Greenberg 1941). Thus androgen seemed to have a partly masculinizing and partly feminizing effect on the reproductive tructs. Administration of testosterone propionate or testosterone to female snakes of Thamnophis r. radix, was followed by masculine development of the sexual segment of the renal collecting tubule, whereas dehydroandrosterone and progesterone were less effective (Waters, 1940). Injection of oestradiol dipropionate into the amniotic sac of Thamnophis sirtalis caused a decrease in size of the seminiferous tubules (Hartley 1945).

Treatment of early embryos of the turtle Chrysemys marginata bellii with testosterone propionate resulted, in the case of females, in ovaries in which the medullary cords were modified to resemble seminiferous tubules (Risley 1940). Administration of the same hormone to the juvenile terrapin, Malachemys centrata resulted in ovarian and follicular size reduction, conspicuous development of the phallus and preputial glands in both sexes and of Mullerian

ducts in females (Risley 1941b). Treatment of the same species with oestradiol dipropionate, resulted in general testicular atrophy, hypertrophy of the cortical remnant of the testis, and hypertrophy of the Mullerian and Wolffian ducts. Intraperitoneal injection of oestrone into immature male Alligator mississipiensis produced hypertrophy of Mullerian duct segments (Forbes 1938a), while injection of testosterone into young females induced hypertrophy of the oviducts (Forbes 1938b, 1939).

While in birds, a specific morphologic basis for sex reversal exists during early development in the form of male (medullary) and female (cortical) components, the situation is complicated by the lateral assymetry in females which affects in some degree the entire genital system. The left embryonic ovary has a preponderance of cortical tissue, whereas in the rudimentary right ovary the cortex is almost absent, represented by a transient germinal epithelium which disappears even earlier than that of the testis (Wolff 1948). In the male there is a slight degree of such assymetry expressed in the better development and longer survival of the germinal epithelium which is the potential cortex on the left testis.

Early attempts to produce experimental hermaphrodites in fowl involved grafting ovaries, testes or both into normal males and females, into capons and into castrates of both sexes (Domm 1939). Testicular grafts masculinized the combs of female hosts, whereas ovarian grafts feminized the plumage of the male hosts. In some cases both the gonad of the host and the grafted gonad matured, so that both eggs and sperm were produced. In other cases the ovarian graft became an ovotestis, thus having both a feminizing and a

masculinizing effect on the capon. In addition, hormone preparations were injected into chick egg prior to the fifth day of incubation and thus before the onset of sex differentiation (see review by Willier 1939). After hatching there was a tendency for the experimentally modified gonads to revert toward the original sex (Wolff 1938). In general male hormones were less effective than female hormones in transforming the embryonic ovaries of birds. Extracts from bull testes or testosterone propionate had little effect on male embryos, depressing testicular size slightly and stimulating the Wolffian ducts. Paradoxical action of hormones appeared again in the case of avian gonads. Certain male hormones of urinary origin, androsterone and dehydroandrosterone, had a weak masculinizing effect on the female embryos but like oestrogens a strong feminizing effect on the males (Wolff 1938; Willier 1939). In large doses both substances induced cortical differentiation in the testes especially the left which could be transformed into an ovotestis. The Wolffian ducts hypertrophied in both sexes (Kozelka & Gallagher 1934; Willier, Gallagher and Koch 1935; Wolff 1935, 1936). Lewis (1946) reported a feminizing action of oestradiol benzoate and other oestrogens on the male duck embryo. However complete sex reversal was never observed. In general since testicular extracts and testosterone have little or no effect on ovarian differentiation in chick or duck embryos, it seems most unlikely that androgen plays an important role in producing spontaneous intersexes in the avian embryos.

Thus female hormones do not significantly affect the differentiation of chick embryonic ovaries but the testes are highly

transformed. The left testis is more easily reversed than the right one, because of it having a germinal epithelium. Low hormone doses convert it into an ovotestis (Willier et al. 1935) but with higher doses a complete sex reversal is achieved. After treatment the left gonad resembles a normal ovary and the right gonad is small resembling the right gonad of the female. There is no effect on the Wolffian ducts and the left oviduct usually persists as in a normal female.

The demonstration that oestrogens induced sex reversal, led to the reinvestigation of grafting embryonic gonads into the coelom of the host embryos. Wolff (1946) grafted gonads of chick embryos (6-11 days old) into hosts of about 50 hours incubation. The grafts differentiated according to their genetic sex. A testicular graft had little or no effect on the gonads of female host, but Mullerian ducts regressed. The ovarian graft however, caused the persistence and differentiation of a cortex on the left testis, resulting in an ovotestis. The degree of modification was greater when the graft and host gonads were closer together. Surprisingly, grafts of the right gonad from the female as well as the left gonad, feminized the host testis. Subsequently, it was shown that the medulla of the left ovary, free from all cortical tissue, could also feminize the host testis (Mintz & Wolff 1954). Also, in cultures in vitro of embryonic avian gonads, the ovary proves to be the dominant gonad (Wolff & Haffen 1952). It readily induces cortical differentiation in the testis which becomes an ovotestis. The same type of transformation occurs in testes after introduction of oestradiol benzoate into the culture medium. The above result provides evidence that embryonic gonads produce substances which can alter sex differentiation.

A marked contrast with the striking effects of steroid sex hormones on the differentiation of the gonads, of fish, amphibia, reptiles and birds, has been the failure so far to obtain similar effects in mammalian embryos with the exception of a single species, the north american opossum, Didelphys virginiana, a marsupial. The formation of intersexual gonads in the male as a result of oestrogen treatment in this species is a unique occurrence. A similar formation of intersexual gonads in eutherian mammals has never been obtained with steroid hormones. In the opossum, hormones may be administered directly to the pouch young before the completion of sex differentiation, which takes place almost entirely postnatally. In the male opossum, oestrogens feminize the urogenital sinus of pouch young, by inducing its division to a ventral urethra and a dorsal vaginal rudiment (Burns 1942a,b), and prostate glands fail to form if oestrogen is administered before their differentiation has begun (Moore 1947). A paradoxical effect of oestrogen in the male opossum is seen in some cases where the mesonephric ducts are stimulated (Moore 1941). Oestrogen treatment of the female opossum stimulates extensive development of the Mullerian ducts and the vas deferens. The epithelium of the urogenital sinus proliferates and keratinizes so that complete closure of the sinus and urethra may take place (Burns 1939a,b), the external genitalia retaining their female characteristics. Negative results have been reported for a number of species of placental mammals in which pregnant females were treated with relatively large doses of sex hormones during the period of sex differentiation. Experiments of this kind were carried out in the rat by Greene, Burrill and Ivy (see review by Greene 1942), the

guinea pig by Dantchakoff (1936, 1937), the mouse by Turner (1939, 1940) and Raynaud (1942), the rabbit by Jost (1947a), the hamster by Brunner and Wh.fschi (1946) and White (1949) and the monkey by Wells and van Wagenen (1954). The modifications induced in these eutherian mammals were minor in character. Both oestrogens and androgens caused a general retardation of growth and development of gonads but no sex reversal. There was a variable degree of hypertrophy of the medullary elements in ovaries after treatment with male hormone reported only in a few cases (Dantchakoff 1939; Jost 1947a; Wells & Wagenen 1954).

In mammals, the male secondary sex characters are incapable of developing after castration whereas morphogenesis of the female form is not significantly affected after ovariectomy (Jost 1947b; Raynaud & Friley 1947; Wells 1950). In castrated males, prostatic differentiation was prevented and development of a vagina resulted. Male and female castrates were morphologically very similar and both closely resembled the normal female. It seems that the embryonic testis is the essential factor in male development, whilst the female pattern is independent of hormonal conditioning and of sex constitution, since in the absence of gonads, it develops spontaneously in castrates of either sex. It could be concluded that the embryonic testis has a twofold function. First, it has a stimulatory effect leading to the induction of male type differentiation of ducts accessories and external genitalia, and secondly an inhibitory effect leading to the regression of the Mullerian ducts (Jost 1955). Therefore in the absence of foetal testis secretion an intersex condition may arise in the male individual.

Modification of gonadal differentiation in the mammalian embryo has been most successfully achieved through the technique of gonad transplant (MacIntyre 1956; MacIntyre, Baker & Wykoff 1959; MacIntyre, Hunter & Morgan 1960). The inhibitory action of testis secretion on ovarian development has been demonstrated, and also evidence of ovarian inhibition of testicular differentiation has been obtained. These results indicate that the mechanism of gonadal sex differentiation in mammals is very similar to that originally proposed by Witschi(1931, 1957) for amphibians: namely that the medullary or cortical area of the gonad eventually achieves dominance and the secretion of the dominant part then suppresses the development of the opposite gonadal component.

1.2 The Present Study

Initially the body growth, gonadal development and differentiation of Xenopus was closely followed, so that a standard table of normal body and sexual development was established. This formed the basis upon which constructive comparison of further experiments with larvae could be made.

The effects of sex steroids on the gonadal differentiation was carried out employing alcoholic solutions of the hormones and adding them to the aquarium water where the larvae were kept. Culturing Xenopus gonads in vitro and treating them with male and female hormones respectively, was also carried out in order to investigate the sexual development and the effects of the sex hormones in vitro. The aim of the in vivo and in vitro hormonal treatment was both to confirm certain of the results of others and to extend observations to the use of sex hormones not previously tested and link these techniques with histochemical and auto-radiographic studies.

To elucidate further the mechanism of hormone-induced sex change in Xenopus, two antibiotics inhibiting protein synthesis were employed, actinomycin D which inhibits protein synthesis at the nuclear level and puromycin at the cytoplasmic level respectively. These substances were administered simultaneously with oestradiol so as to establish whether feminization induced by oestrogen would still take place in the presence of these antibiotics. If hormone-induced sex reversal is linked with hormone-induced protein synthesis, then actinomycin D and/or puromycin should prevent any sex change manifestation.

Histochemical work was also carried out to investigate the presence and activity of Δ^5-3β-hydroxysteroid dehydrogenase and glucose-6-phosphate dehydrogenase. The first enzyme complex is essential for biosynthesis of sex steroids, and the second dehydrogenase is a provider of the coenzyme NADH which is required for steroid hydroxylations essential in steroid synthesis. Using established histochemical reactions, localization of both enzymes in tissues was made possible with the formation of coloured formazan deposits, the enzyme activity being linked to the colour intensity of these deposits. Various stages of larval development before and after sex differentiation were used together with adults to test for initial appearance and progressive activity (if any) of the enzymes in their gonads and interrenals so as to establish the exact time of enzyme appearance in the larval life and to construct an enzyme activity table of the various tissues. Presence of these enzymes would indicate an ability by the particular tissue of steroid synthesis. A further investigation to establish if the oestradiol-induced sex change affects the enzyme activity in the larval gonads and interrenals, was also carried out. Thus steroid enzyme involvement could

also be involved in the mechanism of hormone-induced sex reversal.

Finally to investigate the fate of exogenous steroid hormones in the body of larvae and juvenile Xenopus, tritiated steroids and uridine were injected into the animals. The aim of this experiment is to see if steroid hormones manifest their sex changes either directly on the gonads by stimulating protein neosynthesis or indirectly by involving the brain and/or any other tissue in the body.

CHAPTER 2

PRELIMINARY INVESTIGATION

2.1 Introduction

Initially a study of the normal development was done to determine the relation between increase in length and weight and the appearance of important features of the external anatomy and histological characteristics of the gonads and interrenals. The cytological aspects of gonads at certain stages of development were studied with the electron microscope at various magnifications. The larval stages were numbered according to the system of Nieuwkoop and Faber (1975). The materials and methods section for this investigation applies generally to the rest of the thesis.

2.2 Materials and Methods

A continuous supply of tadpoles was obtained by breeding from three pairs of adult Xenopus. These were about 2 years old, laboratory bred and bought from Xenopus Ltd. They were kept in stainless steel tanks with about 20 cm of water at 18°C, thermostatically controlled. The water was changed twice a week so as to get rid of the excretory products, faeces and detritus. The adults were fed on steak and kidney baby food, tubifex or small pieces of beef-heart and liver. The tanks were kept near the windows and exposed to daylight.

Spawning was induced by injecting the adults with chorionic gonadotrophin (hCG, Xenopus Ltd.) into their dorsal lymph sac. Two days before eggs were required, both male and female were given a priming injection of 100 i.u of hormone in 0.5ml of distilled water. Later, the male by then showing nuptial pads, was injected with 250 i.u of the hormone and the female with 500 i.u and both animals were put in a plastic tank containing water at 23°C and some pieces of

spawning mesh and were left undisturbed overnight. Next day, the adults were separated from the eggs. These hatched in four days. After hatching the larvae were distributed between several tanks at a density of 10 per 8 litres of water. This was dechlorinated, well aerated and changed three times a week. Both eggs and larvae were maintained at 23°C. The latter were fed with nettle powder following change of water. This was thoroughly mixed with water and the suspension was passed through a fine net; the residue was thrown away so as to avoid clogging their gills with large particles of the powder.

For the histological study, twenty tadpoles of each stage were anaesthetized in MSS 222 1% solution, and their body length and weight recorded. Then their tails were cut off just behind the anus and the animals were fixed in Bouin for 24 hours. After fixation they were processed for wax embedding. Serial sections of 8μ were cut from anus to head throughout the mesonephros and stained in haemalum and eosin (H & E) as follows for microscopical examination of the gonads:-

Dewax in Xylene for 15 minutes.

Sections to water through a graded series of alcohols.

Haemalum 5 minutes.

Wash in distilled water.

Differentiate in alkaline alcohol, about 10 seconds to blue.

Pass through 70% and 95% alcohols.

Counterstain in Eosin for 25 seconds.

Wash in 95% (two changes) and absolute alochol (two changes).

Clear in Xylene and mount.

With H & E, the nuclei stained blue, collagenous material and cartilage pale blue-grey, erythrocytes red and cytoplasm of cells shades of pink. Photographs were taken to support observations. These are of the anterior gonadal region, unless otherwise stated in the explanation of figures. Data concerning weight, length and sex are given in Table 2 (p30). The stages significant in the present connection are from stage (st.) 48 onwards. Metamorphosis starts with the eruption of the forelimbs out of the atrium at st.58 and concludes at st.66. Representative stages based on hind-limb development are illustrated in plates I and II. Details of germ cell development between st.48 and st.66 are shown in Table 3 (p33).

For the cytological study, larval gonads attached to their mesonephroi, at respectively st.49 (before sex differentiation), st. 56 (female only) and male juvenile (4 months old) were processed for electron microscope examination using the method shown below.

The excised gonads attached to their mesonephroi were fixed overnight in 5% buffered gluteraldehyde with sodium cacodylate made up from:-

(a) 10% gluteraldehyde, 40ml 25% gluteraldehyde plus 60ml of water giving 100ml of 10% solution.

(b) 0.2M sodium cacodylate adjusted to pH 7.3 by adding 0.1N hydrochloric acid.

These were mixed in equal quantities to give 5% gluteraldehyde in 0.1M sodium cacodylate. Following fixation they were treated for 2 hours with 1% osmium tetroxide in 0.1M sodium cacodylate (50ml 2% OsO_4 in 50ml 0.2M sodium cacodylate) and then:-

Washed in 0.1M buffer for half an hour.

Dehydrated in alcohols: 10 minutes 70% alcohol

10 minutes 95%

2 x 30 minutes absolute alcohol.

Impregnated with propylene oxide 2 x 10 minutes.

Infiltrated in 50/50 Epon/Epoxypropane overnight.

Infiltrated in Epon for 24 hours.

Embedded at 60°C for 48 hours to polymerise the resin.

Serial thick (1µm) and ultrathin sections were cut through the gonads from the same blocks, on LKB Ultratome using glass knives. The 1µm sections were mounted on glass slides, stained in 1% toluidine blue in 1% borax, dried on a hot plate at 60°C for about 2 minutes and used for light microscope examination. The ultrathin sections were mounted on grids, stained in 2% aquous uranyl acetate and lead citrate and examined in a Philips 300 Electron Microscope at 60kV. Photographs of the various organelles of gonadal cells were taken.

Treatment of tadpoles with hormones followed the investigation of the normal larval development, in order to check whether sex reversal would occur. Cultures of 10 larvae kept in tanks containing 8 litres of dechlorinated and well aerated water maintained at 23°C (in a water bath), were used. The larvae were supplied with fresh water and fed with nettle powder every second day. The hormone to be added was first dissolved in 0.8ml of absolute alcohol and then this solution was added to the tadpole water. The control cultures were given 0.8 ml of absolute alcohol. This concentration of alcohol had no detectable effects on body growth and development or on gonadal development. Pairs of cultures (total of 20 larvae) were treated with given amounts of each particular hormone. The concentrations used were as follows: 25µg, 100µg, 500µg and 2mg/l

of water. Seven synthetic hormones were employed (Sigma Chemicals).

17β-oestradiol ($\Delta^{1,3,5(10)}$-oestratien-3,17β-diol).

β-oestradiol-3-benzoate.

hexestrol (3,4-bis- [4-hydroxyphenyl] -hexane).

progesterone (Δ^{4}-pregnen-3,20-dione).

testosterone acetate.

5α-dihydrotestosterone (5α-androstan-17β-ol-3-one).

cortisone (Δ^{4}-pregnen-17α,21-diol-3,11,20-trione).

2.3 Results

The only difference noted in the development of the tadpoles from the description given by Nieuwkoop and Faber (1975), was their smaller size near the end of premetamorphosis and beginning of metamorphic climax (st.56,57,58). However they were much bigger than in Rapola's (1962) description; apparently in his case there was overcrowding since he raised 30 larvae per 8 litres of water. Premetamorphosis (st.48-57) lasted about 33 days and metamorphosis (st.58-66) about 16 days. The overall course of development, from fertilized egg to the young frog, lasted about 2 months. St.52 marks the beginning of sex differentiation, st.58 the beginning of metamorphosis and st.66 the end of metamorphosis (see Table 2 p30) In experiments with hormones, treatment started about st.50-51, that is before there was any visible sign of sex differentiation.

2.3.a. Development of Interrenal (adrenal cortex) and Gonads.

2.3.a(i) Interrenal. According to Nieuwkoop and Faber (1975) the interrenal tissue appears at st.42-43 (3 days old) as a cell proliferation from the apex of the dorsal mesentery extending from slightly anterior

TABLE 2. Normal developmental table of *Xenopus laevis*.
(mean values of 20 tadpoles)

Age (days)	Stage	Mean length mm	Mean weight mg	Phynotypic sex of gonads	External criteria of development
9	48	15.8±0.3[a]	42.5±1.1[a]	Indifferent	Hindlimb bud visible, semicircular in lateral aspect.
13	49	21.3±0.3	72.9±2.3	Indifferent	Hindlimb bud more distinct with distal outline circular. No constriction at its base.
15	50	25.1±0.3	95.5±1.8	Indifferent	Hindlimb bud longer, constricted at its base.
17	51	29.8±0.3	147.0±3.3	Indifferent	Hindlimb bud conical, melanophores on it.
21	52	34.1±0.3	182.0±3.1	Beginning of sex differentiation	Hindlimb bud showing ankle constriction and flattening of foot.
25	53	38.2±0.3	250.0±7.0	6 ♂ 6 ♂♀ 8 ♀	4th & 5th toes indicated in the paddle-shaped hindlimb
27	54	40.0±0.2	318.0±8.9	8 ♂ 12 ♀	All toes and fingers evident
34	55	43.4±0.3	420.0±5.0	11 ♂ 9 ♀	Hindlimb longer, 4th & 5th toes longer
39	56	50.0±0.4	514.5±7.5	13 ♂ 7 ♀	Elbow and wrist shown
42	57	57.4±0.6	828.5±21.3	12 ♂ 8 ♀	Fingers stretched out into forelimb atrium.
44	58	62.8±0.4	1032.5±18.0	9 ♂ 11 ♀	Forelimbs broken through to outside
52	63	49.8±0.3	434.0±3.9	12 ♂ 8 ♀	Tail still longer than body.
60	66	14.5±0.2	323.0±2.8	11 ♂ 9 ♀	Tail completely disappeared.

[a] mean ± s.e. (sample size always 20).

to the coeliaco-mesenteric artery to the posterior ends of the mesonephric blastemata. The cells of this interrenal primordium migrate towards the ventral wall of the dorsal aorta and form a solid cell mass in the space between the two postcardinal veins. At st. 44-45 (4 days old) these two veins unite forming the interrenal or caval vein while at the same time the interrenal blastema divides to form two blastemata lying side by side and placed between the dorsal wall of the caval vein and the ventral wall of the dorsal aorta. They are circular in cross section. During st.46-50 (5-15 days) the interrenal cell masses increase in size and move slightly ventrally (Figs. 6 & 9). From st.51-56 (17-39 days) the interrenal islets (IR) become more elongated (club-shaped) and also continue to move ventrally down the lateral walls of the caval vein (Figs. 15,17,19,23). The interrenal cells are large with abundant eosinophilic cytoplasm and a large nucleus. Between st.57 and st.66 the interrenal islets proceed to take their final position medio-ventrally with respect to each lobe of the mesonephric kidney (Figs. 30,32,38).

2.3.a(ii) Gonads. Serial sections of st.47-58, 62, 63 and 66 were examined and diameters of sex cells measured. Table 3 (p.33) shows the progress of sex cell development. For making diameter measurements of different type of gonadal cells, 8 tadpoles of the particular stage were used and 10 cells from serial gonadal sections (every two sections) of each larva were measured, using a micrometer eyepiece and starting at the anterior part of the gonad. Measurement of the nuclear diameter of 10 primary germ cells, spermatogonia and oogonia was not attempted owing to their irregular shape; only cells with clear and regular outlines were considered. The longest and shortest diameters of each cell,

were measured and the average of the two was taken as the true diameter. The mean diameters of the same type of cells (10 cells) of each tadpole were added together and the mean average diameter of the particular cell for the eight tadpoles was calculated, (see Table 3).

(p33)

Table 3 shows that between st.47 and 52, primordial germ cells (pgs) were present and that the sex of the gonads could not be determined. These pgs were surrounded by follicle cells. During this period their mean diameter increased from about 12μ at st.47 to 20μ at st.52. At st.53 most of the gonads showed definite signs of feminity or masculinity. Male gonads were compact structures lacking a distinct cortical region. Spermatogonia similar to but smaller than pgs were present likewise surrounded by somatic cells. The female gonads were compact structures with a well defined cortical region containing oogonia, which were also smaller than pgs. Spermatogonia and oogonia had similar diameters and were similar in appearance under the microscope. At st.54 the ovary was easily recognised since an ovarian cavity was evident with some medullary cells lining it. The testes at this stage were still compact structures packed with somatic cells and spermatogonia nests. Occasional small circular cavities within the testes suggests the appearance of primordia of seminiferous tubules. At st.55, nests of oocytes in preleptotene (early oocytes) appeared in the ovarian cortex. They were distinguished from oogonia by their round nuclei and clear cytoplasm. They were about 9μ in diameter. In contrast the testes were still solid structures full of somatic cells and nests of spermatogonia. The latter had by this stage diminished in size, probably due to repeated mitotic divisions. At st.56 the ovarian cavity of the female gonad had become more pronounced and the cortical region was full of nests of oogonia and oocytes at various stages of meiotic prophase and some somatic

TABLE 3. Gonadal development in Xenopus.
(mean diameters from 8 larvae per stage)

Stage	Phenotypic sex of gonads	Observations	Sex cell type and diameter (μ).
47	Indifferent	Primordial germ cells (pgs), 1-2 per section, large with highly lobed nucleus and large compact nucleoli, enveloped by much smaller follicle cells (Fig. 6).	pgs 12.3±0.0[a]
48	Indifferent	Gonads larger, containing pgs (1-2 per section) and follicle cells (Fig. 7).	pgs 15.4±0.6
49	Indifferent	Gonads containing pgs (1-3 per section) and follicle cells (Figs. 8 & 9).	pgs 16.6±0.6
50	Indifferent	A complete gonadal rudiment formed with pgs (2-4 per section) located around the periphery (Fig. 10).	pgs 18.0±0.7
51	Indifferent	Gonads showing cortex and medulla. Pgs (2-5 per section) in cortical region. (Fig. 11).	pgs 18.6±3.2
52	Beginning of sex differentiation.	Gonads showing distinct cortex and medulla. Size of pg (2-5 per section) at maximum. (Fig. 12).	pgs 20.3±1.0
53	Male	Testes compact structures having no clear remnants of cortical region. Spermatogonia similar to pgs, showing a highly lobed nucleus, surrounded by somatic cells. Occasional appearance of small circular cavities possibly the primordia of seminiferous tubules. (Figs. 17 & 18).	spermatogonia 18.0±0.6
	Female	Ovaries solid structures with a medulla and a well defined peripheral cortical area, containing groups of oogonia which are similar to pgs; somatic cells present but fewer than in testes (Figs. 13-16).	oogonia
54	Male	Testes containing more nests of spermatogonia and masses of somatic cells; some spermatogonia are dividing. Small circular cavities are seen more often (Figs. 19 & 20).	spermatogonia 18.9±0.9
	Female	Appearance of ovarian cavity lined with medullary cells. Nests of oogonia in cortex, some undergoing mitosis. Oogonia large with highly lobed nucleus and with nucleoli. Number of somatic cells in cortex much smaller than in testes (Fig. 21).	oogonia 19.4±1.1
55	Male	Testes containing an increased number of nests of spermatogonia which are diminished in size due to repetitive mitotic divisions, and surrounded by numerous somatic cells. Circular cavities also present (Fig. 24).	spermatogonia 12.9±0.5
	Female	Ovarian cavity larger and early oocytes appear in cortex. These have clear cytoplasm, large round nucleus and vesiculated nucleoli. Some oocytes are in leptotene (fibrillar chromosomes) and zygotene (Bouquette) stages. (Figs.22 & 23).	early oocytes 9.1±0.4 (N 5.7± 0.0)
56	Male	Testes possessing nests of spermatogonia and somatic cells. More small circular cavities in the soma (Fig. 25).	spermatogonia 12.9±0.8
	Female	Ovarian cavity more pronounced and lined with medullary cells. A few somatic cells appear in the cortex. Nests of oogonia and oocytes seen. Larger oocytes in early meiotic prophase (leptotene and zygotene) present in the cortex. First appearance of some early pachytene oocytes in the cortex (Figs. 27 & 28).	early oocytes 9.4±0.4 (N 5.7±0.0 zygotene oocytes 12.6±0.6 (N 7.4±0.5)
57	Male	More nests of growing spermatogonia in the testicular soma. Small circular cavities present (Fig. 26).	spermatogonia 13.1±0.5
	Female	Ovarian cavity very large (measuring as much as 51 x 28μ). Nests of oogonia early oocytes, and leptotene, zygotene and pachytene oocytes present in the thick cortical region (Fig. 29)	early oocytes 9.7±0.5 zygotene oocytes 12.9±0.5 (N 7.4±0.5)
58	Male	Nests of spermatogonia throughout the testicular soma, surrounded by masses of somatic cells. Small circular cavities present (Fig. 30).	spermatogonia 14.8±0.4
	Female	Prominent ovarian cavity, thick cortex nests of oogonia and growing oocytes evident. Early oocytes, leptotene, zygotene and many pachytene oocytes are present (Fig. 31).	early oocytes 11.7±0.5 (N 6.0±0.3) zygotene oocytes 13.1±0.5 (N 6.0±0.4)
62	Male	Numerous nests of spermatogonia in the testes; a few early synaptic spermatocytes are evident. Small circular cavities present (Fig.32)	spermatogonia 14.6±0.5 early synaptic spermatocytes 9.1±0.4 (N 7.7 ± 0.4)
	Female	Nests of oogonia and oocytes at various developmental stages present in the thick cortex; some somatic cells evident. Many pachytene oocytes present but also some in diplotene (Figs.33 & 34).	pachytene oocytes 14.3±0.4 (N 7.7±0.4)
63	Male	Numerous nests of spermatogonia in testes. Nests of early synaptic spermatocytes are also evident. Many somatic cells and some small circular cavities present (Fig. 35).	spermatogonia 14.6±0.7 early synaptic spermatocytes 9.4±0.7 (N 6.3±0.4)
	Female	Large ovarian cavity and very thick cortical region (in places 100μ), containing some nests of oogonia but mainly oocytes at various stages of meiotic prophase and a few in diplotene (Fig. 36).	late pachytene oocytes 18.3±0.5 (N 12.0±0.6) diplotene oocytes 36.6±1.7 (N 20.0±0.9)
66	Male	Numerous nests of spermatogonia and somatic cells in the testes. Nests of late synaptic spermatocytes and small circular cavities present in the soma (Fig. 37).	spermatogonia 14.9±0.6 late synaptic spermatocytes 10.3±0.6 (N 6.3±0.4)
	Female	Cortical region very thick containing some nests of oogonia and masses of oocytes at various meiotic stages; very few somatic cells present. Many oocytes have entered diplotene (Figs.38 & 39).	late pachytene oocytes 18.0±0.4 (N 11.7±0.7) diplotene oocytes 36.6±1.5 (N 20.0±0.9)

[a] mean ± s.e. (sample size 80)
N, nucleus

cells. In addition the oocytes were starting to enlarge (see Table 3). In the testes the spermatogonia had increased in mean size. Between st.57 and 66 the cortical region of the ovary continued to thicken and the ovarian cavity to get larger. Likewise the oocytes continued their growth and development, and oocytes in diplotene first appeared at st.62. The spermatogonia in the testes attained a large size by st.58 (about 14μ) and by st.62 spermatocytes at early meiotic prophase were already present. It is seen that spermatocytes first appeared after metamorphosis has started whereas early oocytes appeared much earlier, at st.55. By st.66 early synaptic spermatocytes were encountered with diameter of about 10μ. This size is markedly smaller than that of oocytes in early pachytene at st.62, which were almost 14μ in diameter. For comparison the histological structure of testis and ovary of 4 month old juvenile Xenopus, is shown in Figs. 40-42.

2.3a(iii) Ultrastructure of gonads. Primordial germ cells at st.49, oogonia and oocytes at st.56 and spermatocytes from a 4 month juvenile male are shown in Figs. 43-49. The pgs have the following striking characteristics (Figs. 43-45).

I. Very large cell size, up to 20μ in diameter.

II. A multi-lobed nucleus with a very large surface area which is surrounded by a double-layered nuclear membrane, containing electron-dense round nucleioli (1-3 per nucleus) and relatively diffuse chromatin. The nuclear membrane is associated at places with granular material as seen in Figs. 44 & 45.

III. Aggregation of mitochondrial masses on one or two sides outside the nucleus. The mitochondria are large, electron dense filamentous structures containing many transverse cristae and dense granules (see Figs. 44 & 45).

IV. Aggregation of granular material sometimes associated with mitochondria in the cytoplasm (see Figs. 44 & 45). Vesicles and tubules also evident in the cytoplasm. An electron-dense opaque body (NOB in Figs. 43 & 44), similar in appearance to the nucleolus was close to the nucleus.

A follicular cell (FC) adjacent to the pg (see Figs. 43 & 44) had a very large nucleus more densely stained than the pg nucleus, and containing condensations of chromatin particularly at the nuclear membrane. Most of the above characteristics of the pg and the follicle cell are in agreement with descriptions of other workers in the past (Al-Muktar Kawakib & Webb, 1971; Coggins, 1973; Wyllie & Heasman, 1976).

The oogonia and spermatogonia like pgs have multilobed nuclei, each surrounded by a double layered nuclear membrane. As seen in Fig. 46 the oogonium nucleus contains three compact, spherical and electron-dense nucleoli and also diffuse chromatin material which at places becomes dense, forming micronucleoli-like structures possibly resulting from fragmentation of the main nucleoli. An aggregation of mitochondria is present on one side of the nucleus. Some of the mitochondria are dumbell-shaped and possibly in process of division. Dense granular material is associated with the mitochondria and lipid bodies and microvesicles are evident in the cytoplasm.

The first sign of oocyte formation was the appearance in st.55 tadpole ovaries of groups of cells with large round nuclei and vesiculated nucleoli. The highly lobed nucleus, so characteristic of the pg, oogonium and spermatogonium, had been lost, this being the first visible transition to oocyte formation. These oocytes in

preleptotene (early oocytes) or early leptotene stage, were present both in later larval stages and in the adult ovary. The early oocyte possesses a very large and approximately spherical nucleus filling the greatest part of the cell, surrounded by an undulating nuclear membrane. The chromatin in the oocyte nucleus was denser than that in pg or oogonium and in the early leptotene stage the chromosomes had contracted and thickened just sufficiently to be resolved. Axial elements surrounded by a fibrillar network (see Figs. 47 & 48), were evident in the nucleus of these early leptotene oocytes and the dark granules possibly represented axial elements in cross section. The mitochondria, which were much fewer in number than in pg or oogonium, were scattered throughout the cytoplasm, suggesting that these cells represent a post-mitotic stage.

A nest of spermatocytes in early synaptene stage is shown in Fig. 49. Characteristic synapsis of the chromosomes had been completed and there is no polarization (as in zygotene) but dispersal throughout the nucleus of the synaptonemal complexes which are more or less interconnected (Fig. 49). A [illegible] is evident between spermatocytes in the juvenile testis as shown in Fig. 50.

CHAPTER 3

TREATMENT OF TADPOLES WITH OESTROGENS

(17β-oestradiol, β-oestradiol-3-benzoate & hexestrol)

3.1 Introduction

The effects of oestrogens on sex differentiation in amphibians vary according to the taxonomic group, dosage, timing and duration of treatment. In some species total and functional sex reversals have been achieved whilst in others feminization was partial and temporary and in some cases negative or paradoxical results were obtained.

In higher anurans, Ranidae, Hylidae and Bufonidae, the action of female hormones has been highly variable. Padoa (1936, 1938) using low doses of oestrogens, feminized larvae of Rana esculenta whereas with high doses he produced paradoxical masculinization. Similar results were obtained with Rana temporaria (Gallien, 1941) and Rana pipiens (Chang and Witschi 1955b). Takahashi (1957) suggested that the paradoxical effect in Rana temporaria was due to inhibition of the cortical gonadal region enabling development of the medulla and subsequent testicular formation.

Total, permanent and eventually functional reversal was obtained with oestrone or oestradiol in a number of lower anuran and urodele species. Xenopus laevis (Witschi and Allison 1950; Gallien 1953, 1956) and Pelobates cultripes (Gallien and Collenot 1958; Collenot 1965a) showed complete feminization after oestrogenic treatment. Cross-breeding of Xenopus neofemales (genotypic males) with normal males gave all male offspring, indicating feminization of the males and homogamety (ZZ) of the male sex (Gallien 1955b, 1956; Chang & Witschi

1955a, 1956). Treatment of male Xenopus with oestrogens following gonadal differentiation, induced development of oogonia in the testes of young animals just after metamorphosis but no cortical proliferation, whereas in older animals (juvenile) there was cortical proliferation and formation of oocytes (Chang 1958). It was suggested that there was a bipotential action first directly on the medulla which responded just after metamorphosis and later on the cortex. However no complete feminization of male larvae was achieved in Pelodytes punctatus (Gallien & Collenot 1958), in the discoglossides Bombina bombina (Gallien 1959b), Alytes obstetricans (Witschi & Allison 1950) and Discoglossus pictus (Gallien 1950d) or in the bufonids Bufo Bufo (Padoa & Picchi 1946) and Bufo americanus (Chang 1955). Instead partial feminization and intersexuality existed in the treated animals. Asayama (1959) following treatment of larvae of Bufo vulgaris formosus with oestrone, observed accelerated metamorphosis but no sex transformation, though there was inhibition of the medullary mesynchyme. Padoa (1937b) treating toad larvae with high doses of oestrogenic steroids observed suppression of Bidder's organ development.

Sex inversions induced by oestrogenic substances were demonstrated in the urodeles Pleurodeles waltlii (Gallien 1950a, 1951b, 1954c), Ambystoma opacum (Foote 1941), Ambystoma tigrium (Ackart & Leavy 1939), Ambystoma punctatum (Burns 1938a), Hynobius nebulosus (Asayama & Miyamori 1957a), Triturus helveticus and Triturus alpestris (Gallien & Collenot 1960). Gallien (1950a, 1951b, 1954b) found that sex reversed Pleurodeles waltlii neofemales (genotypic males) bred successfully with normal males producing an all male population but they stopped breeding after about five years and started to show male sexual behaviour (Gallien 1954a, 1961). When histologically examined the animals showed testicular elements

in the ovaries.

An investigation of the effects of three oestrogens on the gonadal differentiation of Xenopus was carried out, mainly to check previous observations.

3.2 Materials and Methods

Hormonal treatment was carried out for 26 days, starting just before sex differentiation at st.50-51 and ending near the beginning of metamorphosis at st.56-57. Each hormone was first dissolved in 0.8ml of absolute ethanol and then it was added in 8 litres of water containing the larvae. Twenty tadpoles were used for each treatment.

The three synthetic oestrogens used were 17β-oestradiol, β-oestradiol-3-benzoate and hexestrol. Four different concentrations of each hormone were used 25µg/l, 100µg/l, 500µg/l and 2mg/l, and an investigation of the progress of the effect of oestradiol was carried out by treating batches of larvae for respectively 11, 21, 26 and 33 days, starting at st.51. The final stage of tadpole development was assessed from the external body criteria wherever possible. At the end of each experiment, the tadpole body length and weight were recorded, then they were paraffin embedded and serial sections cut at 8µ and stained in haemalum and eosin. Photographs were taken to support observations.

For further details look at general methods section (pages 25-28).

3.3 Results

3.3a Treatment with 25, 100 and 500µg/l oestradiol for 26 days (from st.50 to 56).

All hormone-treated cultures produced only phynotypic female individuals at various levels of female development (see Table 4).

These were divisible into three discrete groups with differing histological appearance of gonads; individuals respectively with normal ovaries (♀ N) as in controls (Fig. 51), with sex reversed testes resembling inhibited ovaries (♀↓) and with intersexual gonads (⚥). Each normal ovary had an obvious ovarian cavity and thick distinct cortex containing oogonia, early oocytes, oocytes in leptotene and zygotene and some in early pachytene stage (Figs. 53, 55 & 57). The inhibited ovaries had no obvious ovarian cavity, some medullary cells were present and the cortex contained oogonia and early oocytes but no later meiotic stages (Figs. 54, 56 & 58). The intersexual gonads showed distinct cortex and medulla with masculine organization more evident at the anterior region of the gonad (Fig. 59). The first mentioned individuals could be genetic females, the second could be genetic males feminized by the gynagogen and the third group (found in 500 µg/l culture) could be genetic males in the process of feminization due to the high dose of oestradiol. Looking at Table 4, hormonal treatment produced 4 ⚥, 32 ♀↓(presumably genetic males) and 24 ♀ N (24 genetic females). Assuming the population ratio of genetic males to females is 1:1 then feminization of the male larvae must have taken place. The 100 and 500µg/l hormone treatment produced some interrenal hyperplasia.

3.3b Treatment with 2mg/l oestradiol for 26 days (from st.51 to 57).

Histological examination of the gonads at the end of treatment revealed four discrete groups of individuals as shown at the bottom of Table 4. The individuals with testicular structures mostly resembling controls (as in Fig. 61) showing precocious Wolffian duct development at the hilum of each testis and some additional cavities in the soma at the frontal testicular region (♂ Abn in Figs. 64-66). The Wolffian duct itself was lined with a single

TABLE 4. Effect of 17β-oestradiol on gonadal differentiation

(mean values of 20 tadpoles)

Treatment	Final stage	Mean length mm	Mean weight mg	Phenotypic Sex ♂ N	♂ Abn	♀-♂	♀-♂	♀S	♀+	♀N	Interrenal hyperplasia
(a) Controls*	56	52.3±0.7[a]	504.5±8.9[a]	9	0	0	0	0	0	11	-
25µg/l	56	53.2±0.7	516.0±14.1	0	0	0	0	0	12	8	-
100µg/l	56	53.9±0.7	533.0±13.0	0	0	0	0	0	13	7	+
500µg/l	56	54.7±0.6	546.5±12.1	0	0	0	4	0	7	9	+
(b) Controls*	57	55.9±0.5	770.5±16.5	12	0	0	0	0	0	8	-
2mg/l	57	49.1±1.0	510.5±20.1	0	7	4	0	2	7	0	++++

*All controls were alcohol treated

[a] mean ± s.e. (sample size always 20).

Sex type signs:

(i)	♂N	Males with normal testes.
(ii)	♂Abn	Males possessing testes resembling controls but with precocious Wolffian duct development at the hilum of each testis in the frontal gonadal region.
(iii)	♀♂	Intersexualised males. High masculinized frontal gonadal region possessing cavities and partially feminized or intersexual caudal gonadal region (about two thirds of gonadal length) showing distinct cortex and medulla.
(iv)	♀	Individuals with intersexual gonads; cortex and medulla distinct but masculine tendency evident at the frontal region of gonad.
(v)	♀S	Females possessing sterile ovaries. Ovarian structures small with practically no gonia in the cortex but only somatic cells.
(vi)	♀↓	Females with suppressed ovaries or sex reversed testes, both showing similar appearance. Cortex losing its distinct outline, no clear ovarian cavity but some medullary cells present. The cortical region contains nests of oogonia and early oocytes but no pachytene stage oocytes.
(vii)	♀ N	Females with normal ovaries.

layer of cuboidal epithelium. The individuals with partially intersexualised testes had intersexual caudal gonadal region (about two thirds of the whole gonad) showing distinct cortex and medulla and a highly masculinefrontal region (about a third of the gonad showing some cavities in the testicular soma (♀-♂ in Figs. 71-73). One individual had a left intersexual gonad and a right testicular structure (Figs. 62 & 63). Another individual showed precocious Wolffian duct development at the anterior of the testis whereas the caudal part of the gonad showed zygotene and pachytene oocytes but no obvious ovarian cavity (Figs. 67-70). The individuals classified as sterile females (♀ S) had vestigial and almost sterile ovarian structures after hormone treatment. They possessed very small (in comparison to controls) ovaries with cavity present and in the thin cortical region there was predominance of somatic cells; only a couple of gonia were evident in Fig. 75 and no gonia at all in Fig. 74. Finally individuals were found to possess inhibited (or suppressed) ovaries. Most of them had no obvious ovarian cavity and some medullary cells were present. The cortex had no zygotene or pachytene stage oocytes and some individuals showed cavities in the cortical region possibly indicating destruction by the hormone (Figs. 76 & 77 and compare with control ovary in Fig. 60). Therefore the high dose of oestradiol treatment produced paradoxical results by masculinizing rather than feminizing the larvae. It stimulated Wolffian duct development in the testes of genetic males (♂Abn.), produced partial intersexuality in the testes of other genetic males (♀-♂) and suppressed the ovaries of genetic females (♀ S and ♀ ↓). The presumed genetic ratio here is 11 ♂and 9 ♀, very close to the 1:1 male: female normal ratio.

Apart from the paradoxical sex effects the high hormone dose stimulated marked interrenal hyperplasia in all tadpoles (Figs. 62, 71 & 76). The hormone also had teratogenic effects. Most of the treated larvae developed branched tentacles (Fig. 79 and compare with control in Fig. 78) and limb-bud development was generally inhibited. Twelve tadpoles had no fingers or toes after treatment but only limb-stumps. Four larvae had two toes and no fingers, three had three toes on each limb and no fingers and one had four toes on each hind-limb and no fingers (see Figs. 81-84 for some of the defects and compare with control in Fig. 80). There was complete inhibition of front limb growth. Also all treated larvae developed curling of the tip of the tail towards the abdomen and their body colouration was darker than the control animals. The anatomical defects caused by the hormone made it impossible to assess the stage of the larvae at the end of the experiment, using the external body criteria. However agewise the tadpoles were at st.57 since they were kept alive for the same period of time as the controls. Furthermore, treated larvae were hyperactive in the water and aggressive behaviour was observed (tadpoles attacking each other).

3.3c Progressive effect of 500µg/l oestradiol treatment on gonadal differentiation (11,21,26 and 33 days respectively, starting at st.51).

As shown in Table 5 (p45), the hormone induced feminization confirmed the previous results when using various concentrations of oestradiol as indicated in Table 4. It was found that the longer the treatment period the more complete was the feminization. The results in Table 5 showed the gradual disappearance of intersexes in the cultures the longer the time period of treatment, until eventually no intersexes were found. Another chronic effect of oestradiol was the great increase

TABLE 5 Progressive effect of 17β-oestradiol (500µg/l) on gonadal differentiation, starting from st. 51.

(mean values of 20 tadpoles)

Treatment	Days	Final stage	Mean length mm	Mean weight mg	Phenotypic Sex* ♂ N	♂♀	♀♂	♀ N	Interrenal hyperplasia
Controls**	11	54	43.9±0.3	346.0±10.8	8	0	0	12	-
Oestradiol	11	54	43.5±0.4	317.0±11.5	0	4	9	7	-
Controls**	21	56	51.0±0.6	492.5±7.7	11	0	0	9	-
Oestradiol	21	56	54.0±0.4	537.5±8.3	0	4	7	9	+
Controls**	26	57	55.2±0.4	768.0±17.5	12	0	0	8	-
Oestradiol	26	57	58.5±0.5	892.5±29.3	0	2	9	9	+
Controls**	33	62	52.6±0.3	555.5±9.5	9	0	0	11	-
Oestradiol	33	62	71.1±0.2	1421.0±14.3	0	0	8	12	+

* Sex type signs as before, explained on page 42, of subsequent developmental stage.

** All controls were alcohol treated.

in tadpole body size and weight after continuous hormone treatment for 33 days. This effect was also seen to some extent in the 500µg/l hormone treatment in Table 4.

3.3d Treatment with 25, 100, 500µg/l and 2mg/l β-oestradiol-3-benzoate for 26 days (from st.51 to 57).

In each of the treated cultures in Table 6, there was again a clear distinction between two groups of approximately equal numbers of individuals which were respectively on the one hand indistinguishable from or similar to females in the controls (Figs 89, 100 and 101 and compare with controls in Fig. 85) and on the other hand intersexual with varying degrees of masculine tendency. It may be concluded that these are genetic females and males respectively. In the case of the former presumably genetic females, modification of gonadal development was confined to the culture treated with 2mg/l and even here was limited to the inhibition of the ovaries; these showed no clear ovarian cavity and no pachytene oocytes in the cortical region, and the size of the ovaries was slightly reduced as compared to those of control females (Figs. 109-111 and compare with control in Fig. 85). The genetic males had largely intersexual gonads, the anterior region (one third of the whole gonad) was highly testicular with cavities present (Figs. 90,95-96,102), whilst the posterior region (two thirds of the gonad) was intersexual showing distinct cortex and medulla (Figs. 91,94,97-99 and 103); one individual showed precocious Wolffian duct development (Figs. 92-93). The genetic males in the highest dose treatment had mostly normal testes with only part of the caudal region somewhat inhibited (Figs. 104-108 and compare with control in Fig. 86). As indicated in Table 6 there was a tendency for feminization though not as strong as in the case of oestradiol treatment. An indication of that was an individual

TABLE 6 Effect of 26 day β-oestradiol-3-benzoate treatment on gonadal differentiation, starting at st.51 and finishing at st.57

(mean value of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic sex ♂ N	♂>♂	♀>♂	♀	♀ ♀	♀ N	Interrenal hyperplasia
Controls*	57.1±0.6	861.0±20.5	9	0	0	0	0	12	-
25µg/l	57.2±0.6	813.5±20.5	0	0	12	1	0	7	-
100µg/l	56.9±0.5	802.0±24.7	0	0	9	0	0	11	-
500µg/l	55.5±0.4	728.5±18.0	0	0	11	0	0	9	-
2mg/l	49.8±0.8	502.5±12.6	0	12	0	0	8	0	++

*Controls were alcohol treated.

Sex type signs:

(i) ♂N — Males with normal testes.

(ii) ♂↓-♂ — Individuals with masculine gonads. The caudal gonadal region (about a third of the gonad) showing small suppressed testes with few nests of spermatogonia and the frontal gonadal region (about two thirds of the gonad) resembling normal control testes).

(iii) ♀-♂ — Intersexualised males. The frontal gonadal region (about a third of the gonad) resembling control testes but with some cavities present in the soma and the caudal (about two thirds of the gonad) intersexual.

(iv) ♀↓ — Individuals with suppressed ovaries. In most specimens no clear ovarian cavity and some medullary cells were present. No pachytene stage oocytes in the cortical region, and the size of the ovaries was generally smaller than controls.

(v) ⚥ — One intersex. One gonad was an inhibited ovary and the other a testicular structure.

(vi) ♀ N — Females with normal ovaries.

in the 25µg/l culture showing a left testis and a right ovary, the former being larger than the latter (Figs. 87-88).

As shown in Table 6, treatment with the three relatively low doses of female hormone, produced 32 partially intersexualised males (32 genetic males), 1 intersex (1 genetic male), and 27 normal females (27 genetic females), a good approximation to the 1 male: 1 female normal sex ratio.

3.3e Treatment with 25, 100, 500µg/l and 2mg/l hexestrol for 26 days(from st.51 to 57).

It is seen in Table 7, that as in the case of oestradiol-benzoate there were two distinct groups of individuals of approximately equal numbers in each treated culture, those similar to female controls and those showing intersexual gonads with varying degrees of masculine tendency. It may be assumed that these are genetic females and genetic males respectively. Treatment of genetic females with hexestrol resulted in a limited inhibition of the ovaries, some of them showing no obvious cavity and all of them having no pachytene oocytes in the cortex (Figs. 114 for 25µg/l; 122 for 100µg/l; 126 for 500µg/l and compare with the control in Fig. 113). Also similarly to oestradiol benzoate, the genetic male showed less deviation from the normal male orientation with the high 500µg/l hormone dose than the lower ones. However, the 2mg/l dose was deleterious and the larvae died very quickly. After hormonal treatment the surviving genetic males showed highly masculine anterior gonadal regions (Figs. 115, 117 for 25µg/l; 119,123 for 100µg/l; and compare with control in Fig. 112), and intersexual caudal regions (Figs. 116, 118 for 25µg/l; 120-121, 124-125 for 100µg/l). At the high 500µg/l dose, the males had testes similar to controls but with large cavities present in the testicular soma (Figs. 127, 128). Furthermore the 25 and 100µg/l treatments stimulated body growth (see Table 7).

TABLE 7 Effect of 26 day hexestrol treatment on gonadal differentiation starting at st.51 and finishing at st.57.

(mean values of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic sex ♂N	♂cav	♂♀	♂♀	♀♀	♀N	Interrenal hyperplasia
Controls*	55.2±0.4	683.0±13.9	13	0	0	0	0	7	-
25µg/l	57.4±0.4	810.5±13.5	0	0	13	0	7	0	-
100µg/l	58.4±0.5	910.0±26.0	0	0	11	0	9	0	-
500µg/l	54.9±0.5	660.0±19.5	0	8	0	0	12	0	-
2mg/l	Tadpoles died within half an hour								

*Controls were alcohol treated.

Sex type signs

(i) ♂N Males possessing normal testes.

(ii) ♂cav. Individuals possessing almost normal testes which have cavities in the testicular soma.

(iii) ♀-♂ Intersexualised males. The caudal gonadal region (about one third of the gonad) intersexual with more female-like gonads and the frontal gonadal region (about two thirds of the gonad) highly masculinized with very large size testes.

(iv) ⚥ Intersexes showing distinct cortex and medulla.

(v) ♀↓ Individuals possessing suppressed ovaries. Ovarian cavity not always clear, some medullary cells present and cortical region thin with no pachytene stage oocytes.

(vi) ♀ N Females with normal ovaries.

3.4 Discussion

Treatment of tadpoles with unesterified oestradiol had strongly affected the male genetic sex since all larvae examined after hormonal treatment had either normal control-like ovaries or gonadal structures resembling ovaries. The result was in agreement with oestrogenic feminization of Xenopus obtained by others (Witschi & Allison 1950; Chang & Witschi 1955a, 1956; Gallien 1956).

The sex reversed genetic males possessed ovary-like structures apparently suppressed, generally smaller than normal control ovaries. Since sex development at these larval stages was not under pituitary control (Hintz & Foote 1947; Witschi 1953; Chang & Witschi 1955b; Iwasawa 1962; Hsu, Chiang & Liang 1973; Hsu, Huang, Chiang & Liang 1974), the hormone might have caused feminization by direct action on the gonads. The gynagogen could have acted by inhibiting directly and/or indirectly the medullary component in the embryonic gonad, at the same time allowing cortical proliferation or even stimulating cortical differentiation and thus eventually completely suppressing testicular development by allowing ovarian development to proceed (Witschi 1931, 1934 & 1957). Implications of steroidogenic enzyme systems being involved in the mechanism of oestradiol induced sex reversal, are discussed in a later section of the thesis.

There was also a dosage effect. Treatment with the high dose 500µg/l, caused intersexuality, with individuals possessing mostly intersexual gonads but with the frontal part showing a high degree of masculinization. Therefore, it seems that the higher dose of oestradiol started to induce masculinization in genetic females. The effect was observed more clearly with the pharmacologically high dose of 2mg/l, when no individuals with normal ovaries were found after treatment.

The gonadal structures of the genetic females appeared to be either inhibited ovaries or vestigial ovarian structures (probably sterile since almost no germ cells were present), indicating a trend towards masculinization. On the other hand the genetic males showed either precocious Wolffian duct development in their testes or some degree of intersexuality at their caudal gonadal region. The paradoxical masculinization induced by the high hormone dose, was in agreement with similar results obtained by others in ranid larvae (Padoa 1938, 1942; Witschi 1951, 1953; Chang & Witschi 1955b). The paradoxical nature of the hormone action is difficult to explain. Takahashi (1957) has suggested an indirect effect by cortical inhibition and subsequently medullary proliferation.

There was an interrenal hyperplasia caused by the two highest hormone dosages especially the 2mg/l dose which induced marked interrenal hypertrophy in all larvae. This was caused likewise by the high dose androgenic treatment found later (see pages 67 & 70). It was observed by other workers for example Chang & Witschi (1955b) and Hsu et al. (1973, 1974). This androgenital syndrome caused interrenal hyperplasia in intact Rana tadpoles but not in hypophysectomized ones, indicating involvement of the pituitary. The hormone-induced sex transformation in contrast occurred in both hypophysectomized and intact animals indicating no pituitary involvement.

In the progressive treatment with 500µg/l oestradiol the degree of feminization increased with time, and in the longest treatment of 33 days no intersexes were found, indicating that all males were reversed. There was also a tremendous increase in larval body

length and weight due to the prolonged hormone treatment. The anabolic effect of oestradiol observed could be regarded as similar to the growth promoting hormone effects seen in body organs such as uterus by Emmens (1966) and Talwar (1970).

Oestradiol benzoate had much less feminizing action and body growth promoting effect than oestradiol. After hormonal treatment there was a weak feminizing effect on the male genetic sex but not strong enough to cause sex reversal. Instead, the lower hormonal dosages, produced partially intersexualized males and one intersex whilst females remained unaffected. In contrast, Gallien (1953) obtained complete feminization in Xenopus using the same hormone. However, he treated the larvae for a much longer period of time and he also employed a concentration of lmg/l hormone, a dosage that I did not use. Thus the weaker feminizing effect of oestradiol benzoate that I observed could be accounted for. The longer period of treatment that he used is the more likely of these factors to have been responsible. The lack of stimulation of body growth by oestradiol benzoate and the toxic effect at the highest concentration in contrast with the effects of the unesterified hormone was probably due to the difference in the stereospecificity of the former that failed to activate the necessary metabolic processes which promoted body growth in oestradiol's case. The pharmacological dose inhibited body growth but caused no anatomical malformations or behaviour changes similar to those produced by oestradiol. Again the stimulation of the interrenal gland was much less intense than that produced by oestradiol. The explanation for the absence of observation of paradoxical masculinization by Gallien (1953) probably lies in the lower concentrations (0.6 & 0.5mg/l) which he used.

Hexestrol treatment inhibited ovarian development in the genetic females. Also at the lower doses the genetic males showed intersexuality whilst the higher dose produced some larvae with almost normal testes and others with inhibited ovaries. Thus it could be assumed that hexestrol produced paradoxical effects even at low doses.

It could be concluded that oestradiol had a strong feminizing effect on tadpole gonads whilst oestradiol benzoate was a weaker feminizing agent. In contrast hexestrol produced paradoxical effects on the gonads. This difference may be due to the higher oestrogenic activity of hexestrol as in the case of Oryzias latipes found by Yamamoto (1969). Possibly therefore a lower dose of hormone than was tested could have achieved feminization. It could also be suggested that the feminizing activity would depend upon the specific stereostructure of the hormone interacting with the specific protein receptor molecule present inside the target cell, to form a protein-hormone complex which would ultimately control protein synthesis by acting on the genetic material of the cell (O'Malley & Schrader 1976). Thus it seems that in Xenopus oestradiol forms, more readily than the other two, the feminizing complex with the protein receptor, inducing thus sex reversal of the male larvae. The results suggested that further investigation into the hormone dosages and time periods of treatments might reveal an overall feminizing activity by all oestrogens.

Summary

At low dosages oestradiol treatment caused feminization while oestradiol benzoate had a weaker feminizing effect. Hexestrol stimulated intersexuality.

The 2mg/l oestradiol treatment was toxic and caused paradoxical masculinization, interrenal hyperplasia and had teratogenic effects on the tadpole body. The same oestradiol benzoate dosage had similar toxicity and sexual effects but caused only very little interrenal gland proliferation and no teratogenic effects. The 2mg/l hexestrol was poisonous.

CHAPTER 4

TREATMENT OF TADPOLES WITH PROGESTERONE

4.1 Introduction

Progestins have been used in the past for the investigation of sex differentiation in amphibians. Padoa (1942) obtained complete masculinization of larvae of Rana esculenta by treating them with an alcoholic solution of progesterone. Similar results were obtained by Vannini (1945, 1948) using larvae of Rana agilis. Witschi & Chang (1950) found that progesterone had toxic effects especially at early tadpole stages. They obtained partial masculinization of Rana sylvatica larvae using 250µg/l, and at 500µg/l which was a sublethal concentration, masculinization of all females was practically complete. Iwasawa (1958) observed similar progesterone toxicity in treating larvae of the frog Rhacophorus schlegelii, since a considerable number of animals died in the course of the experiment. After treatment, histological examination of the survivors, showed only slight progress of testicular development and some retardation of the ovaries. Also large ovarian cavities appeared in both sexes.

Pregneninolone or ethinyl-testosterone, may act as a gynagogen or an androgen. Eversole & D'Angelo (1943) obtained complete masculinization of larvae of Rana pipiens and hypertrophy of the Wolffian duct after pregneninolone treatment as did Jost (1943) with Rana temporaria. Witschi & Chang (1950) found that an alcoholic solution of pregneninolone, of 100 and 500µg/l, caused complete masculinization of all genetic females of Rana sylvatica, while 25µg/l concentration had no effect at all. Gallien (1945, 1948, 1949) obtained partial gonadal feminization and hypertrophy of the male sex characteristics in Discoglossus pictus, after pregneninolone

treatment. Also Gallien (1951a) showed that in Pleurodeles waltlii the same hormone inhibits gonadal development and brings about cloaca masculinization. Lugli (1956) treating Bufo bufo found no masculinization effects, moreover the gonads remain undifferentiated in this genus until after metamorphosis.

Treatment of Xenopus tadpoles with progesterone was carried out to see if any sex changes take place in this anuran.

4.2 Materials and Methods

Treatment of larvae with progesterone started at st.50 and was carried on for 26 days till st.56. After treatment all tadpoles were killed and histological examination of the gonads was carried out. For further details see section on general materials and methods on pages 25 - 28 .

4.3 Results

The hormone had no obvious effect on larval body growth and there was no indication of any sex reversal although there was an effect on the ovaries of the genetic females as shown in Table 8.

Hormonal treatment with the lowest dose had no effect on sexual differentiation and all individuals examined showed testes (Figs. 131) and ovaries (Fig. 132) similar to those of control animals (Figs. 129 & 130). The next two higher doses had no effect on genetic males (Figs. 137 & 139) but affected the female sex by inhibiting development of the ovaries of some individuals and stimulating that of others. The inhibited ovaries were small with obvious ovarian cavity but only oogonia and early oocytes present in the cortex and no pachytene stage oocytes (Figs. 135-136). The

TABLE 8 Effect of 26 day progesterone treatment on gonadal differentiation starting at st.50 and finishing at st.56

(mean values of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic sex ♂ N	♀↑	♀ N	♀↑	Interrenal hyperplasia
Controls*	49.9±0.4	561.5±9.8	11	0	9	0	-
25µg/l	51.6±0.5	607.0±13.0	9	0	11	0	-
100µg/l	50.8±0.5	575.5±10.6	8	4	0	8	-
500µg/l	51.2±0.5	580.5±12.3	12	0	0	8	-
2mg/l	Tadpoles died within an hour						

*All controls were alcohol treated.

Sex type signs:

(i)	♂N	Males possessing normal testes.
(ii)	♀↓	Females having ovarian inhibition; ovaries smaller than controls with thin cortex and no pachytene stage oocytes present giving a generally inhibited appearance.
(iii)	♀ N	Females possessing normal ovaries.
(iv)	♀↑	Females having ovarian stimulation. Ovaries resembling postmetamorphic state of development, possessing a very thick cortex with many nests of late pachytene stage oocytes and some diplotenic ones (stimulation of meiotic process); ovarian cavity very much reduced.

stimulated ovaries were large compact structures with reduced ovarian cavity and a very thick cortex containing many pachytene stage oocytes and some diplotenic ones (Figs. 133, 134 & 138).

The dose of 2mg/l killed all larvae within an hour of application.

4.4 Discussion

Progesterone had no effect on the genetic males, whilst it interfered with the female somatic sex by affecting ovarian and oocyte growth and development. The hormone acted in an ambiguous manner by stimulating the female gonads at the higher dose and stimulating or inhibiting the ovaries at the lower dose. The inhibited ovaries remained small, showing only oogonia and early oocytes in the cortex. In contrast ovarian stimulation by the hormone induced proliferation of the cortical region with subsequent reduction of the ovarian cavity and a great acceleration of the oocyte meiotic process. In fact the stimulated ovaries resembled histologically the ovaries of much older postmetamorphic animals.

The bipotentiality of progesterone, in slowing down or accelerating ovarian growth and development in Xenopus larvae, is difficult to explain. The stimulatory activity of this precursor of sex steroids, could involve release of LH and consequently ovarian stimulation. However, it was shown in the past (Witschi 1953; Chang & Witschi 1955; Hsu et al. 1973, 1974) that the pituitary was not involved in gonadal differentiation and development at early developmental tadpole stages. Therefore a direct effect of the hormone on the ovary and more specifically on the germ cells, whether stimulating or inhibiting could be the more appropriate hypothesis.

4.5 Summary

Progesterone had a dose dependent effect on ovarian and oocyte growth and development. There was no effect on testes. The pharmacologically high dose was poisonous to tadpoles.

CHAPTER 5

TREATMENT OF TADPOLES WITH ANDROGENS

(testosterone acetate and 5α-dihydrotestosterone)

5.1 Introduction

Androgenic steroids such as testosterone, testosterone acetate, testosterone propionate and methyltestosterone, injected into tadpoles or added to the aquarium water, have produced reversal of the female sex in Ranidae and Hylidae. Sex reversed females of Rana japonica mated to normal females produced all female offspring, confirming homogamety of the females (Kawamura & Yokota 1959). Hormone sex reversals, female to male, were obtained in Rana temporaria (Gallien 1937, 1938, 1944, 1950d), Rana pipiens (Witschi & Crown 1937; Foote 1938), Rana catesbeiana (Puckett 1940), Rana clamitans (Foote & Witschi 1939; Mintz, Foote & Witschi 1945), Rana sylvatica (Mintz & Witschi 1946; Mintz 1948), Rana dalmatina agilis (Vannini 1941; Padoa 1947), Pseudagris nigrita triseriata (Witschi, Foote & Chang 1958). Testosterone induced only partial masculinization in Hyla arborea japonica (Takahashi 1958a, 1959) and Rhacophorus schlegelii (Iwasawa 1958a; Amanuma 1963).

Some anurans did not respond to androgen treatment, their sex ratio and gonadal structure remaining normal. This was the case in Bombina bombina (Gallien 1959b), Alytes obstetricans (Witschi & Allison 1950), Discoglossus pictus (Gallien 1950c), Bufo bufo (Gallien 1947; Chang 1954; Talluri & Padoa 1953; Takahashi 1956). Gallien (1954d, 1956) and Foote & Foote (1959, 1960) treated Xenopus laevis with testosterone and found no sex reversal occurring although some gonadal inhibition was observed.

Treatment of urodeles with androgens had varied results but generally produced paradoxical feminization in males. Foote (1940, 1941), Mintz (1947) and Bruner (1952) treated several species of Ambystoma larvae with testosterone and found no masculinization; instead paradoxical feminization occurred. Burns (1939c) found that female larvae of Ambystoma punctatum injected with testosterone propionate developed intersexual gonads or testes whereas genetic males remained unaffected by the same treatment. Gallien (1950b, 1954a) treating larvae of Pleurodeles waltlii with testosterone obtained feminization of the males and he also observed acute mesonephric inhibition. Similar results were obtained with androgenic treatment in Hynobius retardatus (Hanaoka 1941b), Hynobius nebulosus (Asayama & Amanuma 1957a), Triturus cristatus (Chieffi 1958, 1962a), Triturus pyrrhogaster (Asayama & Matsuzaki 1958). Treating Triturus helveticus and Triturus alpestris with testosterone (Gallien 1959b), killed the larvae before sex differentiation has occurred, probably because of the high degree of mesonephric inhibition.

Past observers have reported that androgenic treatment causes no sex reversal of the female genotype in Xenopus. Therefore the female heterogametic sex was called "strong" whereas the male homogametic sex was the "weak" one since it was easily reversed by oestrogenic treatment. Xenopus laevis larvae were treated with testosterone acetate and 5α-dihydrotestosterone respectively. The aim of the androgenic treatment was to investigate the effects of the two hormones on gonadal differentiation, and also to see if the latter has higher androgenic activity in this species as in mammals (Mainwaring 1977). Possible higher activity could cause sex reversal in the female larvae.

5.2 Materials and Methods

Treatment of tadpoles with the androgens started at st.51, seventeen days after fertilization, and was carried on for 26 days up to st.57. The hormones used were testosterone acetate and 5α-dihydrotestesterone (5α-androstan-17β-ol-3-one). The same concentrations of hormones were employed as in the previous experiments already described. For further details on the methodology, see general materials and methods section on pages 25-28.

5.2a. Counting sex cells in tadpole gonads before and after the treatment with 5α-dihydrotestosterone

(i) The numbers of spermatogonia were counted in alcohol-controls and hormone treated males at st.57.

(ii) The numbers of oogonia and oocytes of various developmental stages were counted in ovary sections of control tadpoles at st.57.

(iii) The numbers of protogonia in sections of six tadpoles were counted at st.51, before treatment started.

(iv) The numbers of sex cells found in intersex sections of tadpoles st.57, after hormonal treatment were counted. The sex cells were in the cortical part of the gonad.

All hormone treated animals were used plus the controls plus six st.51 larvae. The total number of tadpoles used was 106. Every second serial section was used for cell counting in both left and right gonad to make up a total twenty sections per gonad per tadpole. The anterior third of the gonads was employed for cell counting since it had the largest diameter.

5.3 Results

5.3a Treatment with 25, 100, 500µg/l and 2mg/l testosterone acetate for 26 days (from st.51 to 57).

The three lower doses caused no sex reversal (see Table 9). Most of the genetic males after treatment, showed normal testes (♂N in Figs. 142, 145 & 150 and compare with control in Fig. 141). A further two individuals had testis-like gonads with additional cavities in the testicular soma (Fig. 145) and may be presumed to be genetic males. The genetic females showed inhibition of development of the ovarian structures (♀ in Figs. 144, 146-149, 151-152, and compare with control in Fig. 140). In addition there was an intersexual individual possessing on the left a large testis-like gonad and on the right a small ovarian structure (♂♀ in Fig. 143). Probably this represents an intersexualised male.

The treatment with 2mg/l inhibited development and growth. External criteria put these tadpoles at st.56 instead of st.57. A tremendous interrenal hyperplasia had taken place (Figs. 153, 156-157, 160), and the genetic females showed a trend toward intersexuality. Partially intersexualized females appeared showing highly feminized anterior gonadal regions and intersexual caudal regions (♂♀-♀ in Figs. 158-160, 161). There were partially intersexualized males with highly testicular anterior gonadal regions showing very large testicular structures and intersexual caudal regions (♂♀-♂ in Figs. 156-157). The genetic males showed abnormal testes by precocious Wolffian duct development at the frontal testicular region (♂Abn in Figs. 153-154, 155).

TABLE 9 Effect of 26 day testosterone acetate treatment on gonadal differentiation, starting at st.51 and finishing at st.57

(mean values of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic Sex ♂ N	♂ Abn.	♀-♂	♂	♂-♀	♀+	♀ N	Interrenal hyperplasia
Controls*	55.8±2.0	765.5±17.2	12	0	0	0	0	0	8	-
25µg/l	58.3±0.2	868.0±20.2	12	0	0	1	0	7	0	-
100µg/l	55.3±0.6	775.5±22.7	8	0	0	0	0	12	0	-
500µg/l	54.1±0.4	726.0±16.7	9	0	0	0	0	11	0	-
2mg/l	48.2±1.0	452.5±21.8	0	8	2	0	10	0	0	++++

*All controls were alcohol treated

Sex type signs:

(i) ♂N Individuals with normal testes.

(ii) ♂Abn. Individuals with testes showing precocious development of the Wolffian duct present at the hilum of each testis and cavities present in the soma, at the frontal region. The caudal region had normal control testicular appearance.

(iv) ♀♂ Intersexual larva, having the left gonad highly masculinized and the right one feminized.

(iii) ♀-♂ Partially intersexualized males having a frontal gonadal region (1/3) highly masculinized and an intersexual caudal region (2/3), with distinct cortex and medulla.

(v) ♀-♀ Partially intersexualized females. The frontal gonadal region possessed a thick cortex with nests of oogonia and zygotene and pachytene oocytes, no ovarian cavity but medulla present being small and insignificant. The caudal gonadal region intersexual and no pachytene stage oocytes present in the cortical area.

(vi) ♀+ Females with somewhat inhibited ovaries; cortical region distinct with no pachytene stage oocytes, no obvious ovarian cavity and some medullary cells present.

(vii) ♀ N Individuals with normal ovaries; cortical region containing pachytene stage oocytes and a clear ovarian cavity present.

5.3b Treatment with 25, 100, 500µg/l and 2mg/l 5_{α}-dihydrotestosterone for 26 days (from st.51 to 57).

Hormone treatment had no significant effect on body growth and development. The larvae survived well at all concentrations including the 2mg/l. As far as sex differentiation was concerned, the results in Table 10 show that there was incomplete sex reversal in a substantial proportion of the genetic females. The male somatic sex was also affected by the hormone treatment, the main effect being stimulation of development of the vasa differentia.

The three lower hormone doses (25, 100 and 500µg/l), produced six types of individuals as judged by the histological appearance of their gonads.

(i) Individuals possessing almost control-like testes (♂N).

(ii) Individuals with testes showing precocious development of the Wolffian duct at the testicular hilum (♂Abn.).

(iii) Individuals with testicular structures containing very few spermatogonia but packed with somatic cells (♂Sub ↑). The size of these structures was similar to or larger than control testes.

(iv) Individuals showing gonads similar to (iii) above but of very much smaller size (♂Sub.↓).

(v) Individuals with intersexual gonads showing a distinct cortical and medullary region respectively (♂♀). Generally all the gonia were present in the cortical region.

(vi) Individuals with partially intersexualized female gonads (♂-♀). Feminine tendency was prominent at the anterior gonadal region.

TABLE 10 Effect of 26 day treatment with 5α-dihydrotestosterone on gonadal differentiation, starting at st.51 and finishing at st.57

(mean values of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic sex ♂N	♂Abn.	♂Sub.↑	♂Sub.↓	♀	♀-♀	♀N	Interrrenal hyperplasia
Controls	52.6±0.5	704.5±14.3	11	0	0	0	0	0	9	-
25µg/l	53.2±0.5	703.5±23.8	7	0	8	3	2	0	0	-
100µg/l	54.9±0.4	810.0±19.7	6	2	5	2	5	0	0	++
500µg/l	55.4±0.8	848.0±27.0	3	7	4	2	3	1	0	++
2mg/l	52.9±0.4	698.5±1.6	0	9	0	0	0	11	0	++++

All controls were alcohol treated

$$\chi^2 = \frac{n(|ad-bc|-\frac{1}{2}n)^2}{(a+b)(c+d)(a+c)(b+d)}$$

For the three lower hormone concentrations:

H_1 49 genetic ♂ (♂N + ♂Abn. + ♂Sub↑ + ♂Sub↓) and 11 genetic females (♀N+♀-♀+♀) χ^2 = 12.0 P<0.001

H_2 25 genetic ♂ (♂N + ♂Abn) and 35 genetic ♀ (♀N+♀-♀+♀+♂Sub↓ + ♂Sub↑) χ^2 = 0.54 P≈0.4

H_3 42 genetic ♂ (♂N + ♂Abn + ♂Sub↑) and 18 genetic ♀ (♀N + ♀-♀+♀+♂Sub↓) χ^2 ≈ 4.2 P<0.05

H_4 32 genetic ♂ (♂N + ♂Abn + ♂Sub↓) and 28 genetic ♀ (♀N + ♀-♀+♀+♂Sub↑) χ^2 ≈ 0.03 P>0.8

Sex type signs:

(i) ♂N Individuals possessing normal testicular structures. Testes compact, with spermatogonia nests surrounded by masses of somatic cells.

(ii) ♂Sub↑ Individuals possessing subnormal testicular structures. Testes similar or slightly larger than control size, but very few spermatogonia in the soma (most sections had none at all) which was packed with somatic cells. The very small spermatogonia numbers present, probably due to losses or destruction by the hormone, could indicate degrees of sterility.

(iii) ♂Sub↓ Individuals possessing subnormal testes with very few spermatogonia but of smaller than control size, perhaps indicating growth inhibition.

(iv) ♂Abn Individuals with abnormal testicular structures. Testes of similar or larger than control size with many spermatogonia and somatic cells. An additional characteristic circular cavity lined with simple epithelium was present at the hilum of each testis, perhaps representing precocious development of Wolffian duct. Some of the testes had additional cavities in the soma.

(v) ⚥ Individuals possessing intersexual gonads showing distinct cortical and medullary regions, possibly indicating inhibition of sex differentiation by the hormone.

(vi) ⚥-♀ Individuals with partially intersexualized female gonads, showing a thick cortex with oogonia and early oocytes and zygotene and pachytene oocytes. There was no ovarian cavity present but also medulla was small and insignificant. Femini- the tendency was more prominent at the frontal region of

of the gonad.

(vii) ♀ N Individuals possessing normal ovaries.

The low 25µg/l dose produced 7 males with almost normal testes (♂N, see Fig. 164 and compare with controls in Fig. 163), showing a somewhat reduced number of spermatogonia present as compared to that of the controls (see Table 11 and in Appendix ii and iv). These individuals are most probably genetic males.

There were 11 individuals possessing subnormal testes (8 ♂Sub ↑ and 3 ♂Sub.↓, see Figs. 165 and 166 respectively) with very few spermatogonia. Indeed many of the sections examined had no spermatogonia at all (see Appendix Table v and vi), suggesting that hormone treatment induced a situation approaching sterility. It could be argued that these individuals were genetic females, the hormone having stimulated medullary cell proliferation and inhibited the cortical region. The two intersexes found in this culture (⚥, see Fig. 167) could also be genetic females in the process of being reversed.

The 100µg/l treatment produced 6 males with almost normal testes (♂N, see Fig. 168) and 2 with abnormal testes (♂Abn., see Figs. 169-171 containing many spermatogonia (see Table 11 and Appendix Table ix) and somatic cells and showing precocious Wolffian duct development. These two groups of individuals could be genetic males. The rest of the larvae found in this culture were 7 individuals with subnormal testes (5 ♂Sub.↑ and 2 ♂Sub.↓ , see Figs. 172 and 173) and 5 intersexes (⚥, see Figs. 174, 195). The individuals in these two groups were considered as before to be genetic females. Furthermore, most of the larvae showed variable stimulation of the interrenal glands (Fig. 175).

The 500µg/l treatment produced again a range of male-like and intersex individuals. There were 10 genetic males (3 ♂N, 7 ♂Abn., see Figs. 176, 177, 178-179, 180, 181) and at least 18 genetic

and probably 10 (including 2 ♂ sub ♀) —

females (4 ♂ Sub.♀ , [illegible] 3 ♀ and 1 ♀-♀), see Figs. 182,183, 184-185 and 186-187 . All individuals showed interrenal hyperplasia (see Figs. 177, 180, 183) with the interrenal islets much larger than in controls.

The highest 2mg/l dose produced prominent interrenal hyperplasia (see Figs. 188, 189, 191) and two histologically distinct types of individuals. There were 9 individuals showing precocious Wolffian duct development at the hilum of the testes, lined by a simple epithelium (♂ Abn., see Figs. 188-189, 190). In one larva the duct measured 87 x 188μ and in two cases additional cavities were found in the testicular soma (Fig. 190). These animals could be considered genetic males. There were also 11 partially intersexualized females (♀-♀, see Figs. 191, 192). Their gonads possessed a very thick cortex containing oogonia, early oocytes and many zygotene and pachytene oocytes especially at the anterior gonadal region (see counts of oocytes in Table 11 and Appendix Table ix). These individuals are probably genetic females in the process of being masculinized.

The hormone treatment altered the numbers of germ cells present in the gonadal tissue and this was substantiated by counts of the various germ cells (see methods for counts in page 65). Tables i to ix in the Appendix, show the numbers of germ cells in both gonads, in control and hormone treated animals. Table 11 shows the mean number of germ cells per gonadal section per tadpole. In general there was no significant difference* in numbers between left and right gonad of the same tadpole, or between tadpoles in a given culture of the same group type, and the difference was slight between the same group in different cultures. From Table 11 it can be seen

* indicated by Student's t test

TABLE 11 Mean numbers of germ cells per gonadal section per tadpole, in controls and in 26 day 5α-dihydrotestosterone treated larvae

Treatment and numbers of larvae	Primordial germ cells	Gonia in cortical region	Spermatogonia	Oogonia and Oocytes	Oogonia and early Oocytes	Zygotene and Pachytene Oocytes
Indifferent						
6 st.51	2.3					
Alcohol-controls						
11 st.57 ♂ N			19.1			
9 st.57 ♀ N				61.2	25.2	36.0
25µg/l						
7 ♂ N			11.9			
8 ♂ Sub.↑			1.1			
3 ♂ Sub.↓			0.9			
2 ♂♀		11.1		11.1	11.1	
100µg/l						
6 ♂ N			15.0			
2 ♂ Abn.			11.4			
5 ♂ Sub.↑			1.1			
2 ♂ Sub.↓			0.5			
5 ♂♀		11.9		11.9	11.9	
500µg/l						
3 ♂ N			21.4			
7 ♂ Abn.			11.9			
4 ♂ Sub.↑			1.2			
2 ♂ Sub.↓			1.0			
3 ♂♀		26.8		26.8	14.6	12.2
1 ♀-♀				41.1	17.5	23.6
2mg/l						
9 ♂ Abn.			15.8			
11 ♀-♀				56.9	18.4	38.4

that there was a tremendous increase of germ cell numbers from st. 51 to st.57 control gonads, this being about a ten times increase in the males and a thirty times increase in the females. There was a large decrease in all subnormal testes when compared to the st.57 control testes and also some decline in relation to the st.51 gonads, indicating that not only multiplication of germ cells did not take place after hormonal treatment but that even net losses might have occurred. All abnormal testes examined showed higher germ cell numbers than st.51 gonads and st.57 subnormal testes. The individuals found possessing normal testes after treatment with 25, 100µg/l and 500µg/l had similar numbers of germ cells per gonadal section to st.57 control testes. The intersexes in the 100µg/l treatment had fewer germ cell numbers per gonadal section than the st.57 female controls. The partially intersexualized females found mainly in the 2mg/l treatment, had similar germ cell numbers to the st.57 female controls.

5.4 Discussion

Treatment of tadpoles with testosterone acetate caused no sex reversal. There was however an inhibition of ovarian growth and oocyte development indicating that the hormone probably slowed down the oocyte meiotic process. The genetic males were not affected by the treatment although some testes showed cavities in the soma, probably indicating some inhibition of testicular development by the hormone. The 2mg/l dosage produced results similar to those of 5α-dihydrotestosterone high dosage. Some individuals showed precocious development of the Wolffian ducts at the hilus of the testes, and others partial intersexuality of

the gonads. The same high dosage produced interrenal hyperplasia and it was also toxic to the larvae.

In all the lower 5α-dihydrotestosterone treated cultures there was not one single unmodified female. The male somatic sex was also affected to a lesser extent. As seen in Table 10 using x^2 the sex ratio can be determined and two of the four hypotheses can be correct. First, all control-like and abnormal males (♂N and ♂Abn.) could be considered genetic males and all subnormal males, intersexes and partially intersexualized females (♂Sub ♀, ♂Sub ♀, ♀ and ♀-♀) could be considered genetic females. This would give 25 original genetic males and 35 genetic females, a ratio not significantly different from the 50% ♂:50% ♀ ratio as occurs naturally. Secondly, all ♂N, ♂Abn., and ♂Sub♀ could be genetic males and the ♂Sub ♀, ♀ and ♀-♀ genetic females, thus giving a ratio of 32 males to 28 females, very close to the natural sex ratio. Assuming one of hypotheses is correct, the hormone has modified the female genetic sex by producing subnormal males with variable degrees of sterility (testes practically devoid of spermatogonia) and intersexes. In the subnormal males 5α-dihydrotestosterone might have inhibited the cortex and the germ cells in it, allowing only a few spermatogonia to develop in the testicular structures. In the intersexes the hormone might have inhibited gonadal differentiation or most probably initiated masculinization in female genotypes. However it remains to be seen if animals treated continually with a low dosage for 2-3 months and raised to maturity are completely sex reversed (and/or sterile). Furthermore the particular size of the testes in subnormal males could suggest variable reactivity of the gonads toward the androgenic hormone.

Apart from female sex modification, 5α-dihydrotestosterone has interfered with the male somatic sex by affecting the mitotic process of spermatogonial multiplication and Wolffian duct development. As shown in Table 11 the number of spermatogonia in the subnormal testicular structures (♂ Sub† &†) was much lower than in the same stage control testes, and perhaps even represented a decline from the number of gonia present in the indifferent gonads. The precocious development of the Wolffian duct caused by the hormone treatment (100, 500μg/l and 2mg/l) seemed to be dose dependent given that the higher the dose the more tadpoles developed the duct; there was also interrenal hyperplasia due to hormone treatment, which was dose dependent with the highest dose showing maximum stimulation of the interrenal glands. In contrast, only the pharmacological dose of testosterone acetate stimulated interrenal hyperplasia and early development of the Wolffian duct. Gallien (1956) and Foote and Foote (1960) treating Xenopus larvae with testosterone saw no such stimulation of the Wolffian duct but only some inhibition of the embryonic gonad development.

Iwasawa and Kobayashi (1974) however achieved Wolffian duct stimulation in young frogs of Rana nigromaculata of both sexes by testosterone treatment. Oestradiol and testosterone administered together (20:1 ratio), induced a more striking response on the Wolffian duct and a remarkable hypertrophy of the Mullerian duct was caused in young female frogs by oestradiol treatment alone when applied just before the appearance of sexual characters. Tojio and Iwasawa (1977) obtained similar results with first year summer frogs of Rana japonica. In Xenopus and some urodeles it was noticed (Lofts 1974, p.539) that oestradiol treatment produced apart from

feminization of the gonad, hypertrophy of the Mullerian ducts when the hormone was applied after the ducts started their growth. Also, in urodeles if hormone treatment started earlier on, the Mullerian differentiation was inhibited as well as its extension towards the cloaca.

The 2mg/l treatment with 5α-dihydrotestosterone produced paradoxical results. There were individuals with partially intersexualized female gonads in which the frontal cortical region hypertrophied and contained large numbers of oogonia and advanced oocytes similar to control st.57 females, indicating no real affect on the female sex by the hormone. The caudal gonadal region was intersexual with distinct cortex and medulla without pachytene stage oocytes in the former. So in contrast to paradoxical masculinization obtained in ranid larvae with high doses of oestradiol (Padoa 1938; Gallien 1941; Chang & Witschi 1955b) and in Xenopus larvae with the pharmacological dose of oestradiol (see previous Table on page 41), the 5α-dihydrotestosterone caused only partial intersexuality in Xenopus female larvae when applied at the pharmacological level. Thus the high dosage of hormone did not have any affect on the phenotypic sex ratio.

In contrast to testosterone acetate, 5α-dihydrotestosterone did not retard body growth. All treated tadpoles survived well and their body lengths and weights were not significantly different from controls. Even the pharmacological treatment had no deleterious effect, suggesting no interference of the hormone with the normal metabolism of the animals. When, however, the same dose of oestradiol was administered (see page 44) there was an inhibition of body growth and development, so much so that it was impossible to assess the

developmental stage of the animals from the external characteristics; in some larvae, limb-buds did not develop at all and in others there was a variable number of digits present. It was also noticed that the oestradiol treated larvae showed aggressive behaviour. Neither testosterone acetate nor 5α-dihydrotestosterone pharmacological dose treated larvae showed similar anatomical defects or behaviour changes.

Treatment of tadpoles with 5α-dihydrotestosterone thus gave generally different results to testosterone acetate, the former modifying the female genetic sex by producing individuals in various degrees of maleness and intersexuality. The modification of the female genetic sex by 5α-dihydrotestosterone, its higher anabolic activity and its non toxicity in contrast to the comparatively low androgenic activity and higher toxicity of testosterone acetate, could suggest that the tadpole body at this stage does not possess the necessary enzyme system (or at least is not active) to convert testosterone into its more active 5α-dihydro form. Therefore female sex reversal in this species by hormone treatment in the past was impossible since the 5α-dihydro androgen was not used.

5.5 Summary

Testosterone acetate inhibited normal ovarian growth and development and some somatic inhibition was observed on some testes. The high dose treatment produced interrenal hyperplasia, some paradoxical sexual effects, precocious Wolffian duct development and it was toxic to the larvae.

The 5α-dihydrotestosterone treatment modified the female

genetic sex of Xenopus by producing individuals with varying degrees of maleness and intersexuality. It also affected the male somatic sex by interfering with the mitotic process of spermatogonial multiplication and by causing precocious development of the Wolffian duct. The high hormone dose was not toxic and it stimulated interrenal hyperplasia, partial intersexuality to females, and precocious Wolffian duct development.

CHAPTER 6

TREATMENT OF TADPOLES WITH CORTISONE

6.1 Introduction

Past research on the effect of mineralocorticoids in sexual differentiation has been limited in extent and given rather conflicting results, although there has been a general tendency towards feminization. Padoa (1942b) and Vannini (1944, 1945b, 1950b) reported DOCA to cause feminization in Rana esculenta and Rana dalmatina. Witschi and Chang (1950) using DOCA found a masculinizing effect on Rana sylvatica, at hormone concentrations approaching lethal level. Iwasawa (1958) found that DOCA had a toxic effect on Rhacophorus schlegelii larvae, and that the hormone (50µg/animal - 17 days) produced an inhibitory effect on ovarian development with differentiation of testes only slightly retarded. Uchida and Takahashi (1959) using the same hormone found no sex change in Rana temporaria and Hyla arborea japonica larvae, though they noticed inhibition of oogenesis in Rana. Asayama and Yamamoto (1951) obtained feminization of the gonads of the urodele Hynobius nebulosus with deoxycorticosterone treatment, but Asayama (1953) using the same hormone obtained no feminization with Triturus pyrrhogaster, though some of the animals became intersexual.

Research with glucocorticoids has been even more limited. Witschi and Chang (1950) treated Rana sylvatica with a high dose of cortisone (1mg/l) and found that the ovaries were transformed into testes. They reported initially oocyte degeneration and later compensatory development of the medullary component. Iwasawa (1958) after treating Rhacophorus larvae with the same hormone noticed a slight inhibition of the ovaries and a slight stimulation of the testes. Quattrini (1960) using hydrocortisone on Rana esculenta found no effect on sex differentiation.

Xenopus tadpoles were treated with cortisone to investigate its effects on sex development.

6.2 Materials and Methods

Cortisone treatment started at st.51 and it was carried on for 26 days intil st.57. Hormone doses of 25, 100, 500µg/l and 2mg/l were employed, as in the previous experiments.

For further details on methodology see general methods section on pages 25- 28.

6.3 Results

Cortisone treatment at the three lower concentrations caused no sex reversal (see Table 12). It had no effect on the male (see Figs. 195, 202 and compare with control in Fig. 194) or female (see Figs. 196, 203 and compare with control in Fig. 193) genetic sex. The hormone caused somatic ovarian inhibition in some female individuals at the lower doses (Figs. 197-198, 200-201, 204-205).

The 2mg/l treatment stimulated precocious Wolffian duct development in the testes of two individuals (Figs. 207-209) whilst the rest of the genetic males resembled control males (Fig. 206).

Individuals with normal ovaries were replaced by partially intersexualized females (Figs. 210-212). No stimulation of the interrenals was observed.

As shown in Table 12, the three lower dosages of cortisone stimulated body growth while the 2mg/l inhibited growth.

TABLE 12 Effect of 26 day cortisone treatment on gonadal differentiation, starting at st.51 and finishing at st.57

(mean values of 20 tadpoles)

Treatment	Mean length mm	Mean weight mg	Phenotypic Sex ♂ N	♂ Abn.	♀	♀-♀	♀+	♀ N	Interrenal Hyperplasia
Controls*	55.3±0.5	763.0±18.6	13	0	0	0	0	7	-
25µg/l	55.7±0.6	852.0±22.0	12	0	0	0	4	4	-
100µg/l	57.2±0.6	855.0±16.7	13	0	0	0	7	0	-
500µg/l	57.9±0.5	901.5±15.1	7	0	0	0	3	10	-
2mg/l	51.8±0.7	546.5±16.7	8	2	0	10	0	0	-

*All controls were alcohol treated

Sex type signs:

(i) ♂N Individuals possessing normal testes.

(ii) ♂Abn. Individuals showing abnormal testes. There was precocious development of Wolffian duct at the hilum of each testis and additional large cavities were present in the soma of the frontal testicular region.

(iii) ♂♀ Individuals with intersexual gonads showing distinct cortex and medulla.

(iv) ♂-♀ Partially intersexualized females. They possessed an ovarian anterior region (two thirds of the whole gonad) showing pachytene stage oocytes, whilst the caudal region (one third of the gonad) was intersexual. Cortical region getting gradually thicker towards the front of the gonad, reducing the ovarian cavity.

(v) ♀+ Individuals possessing suppressed ovaries, which were smaller than control ovaries, showing thin cortical region with no pachytene stage oocytes present and no clear ovarian cavity at places.

(vi) ♀ N Individuals possessing normal ovaries.

6.4 Discussion

Cortisone treatment had no effect on the larval sex ratio. There was, however, an inhibitory effect on the somatic female sex when using the lower doses, similar to that of progesterone (see previous Table on page 84). The hormone inhibited ovarian growth and development possibly by direct action on the ovary. Furthermore, there was no ovarian stimulation in contrast with progesterone, and no noticeable effect on the testes.

The pharmacological steroid dose caused precocious Wolffian duct development in very few males and partial intersexuality in all genetic females. Witschi and Chang (1950) inverted the ovaries of Rana sylvatica into testes by using a high dose (1mg/l) of cortisone. It remains to be seen if longer time treatment with the 2mg/l cortisone employed in this experiment could have caused complete intersexuality and possibly masculinization of the ovaries in Xenopus.

6.5 Summary

Cortisone treatment had no feminizing or masculinizing effect on Xenopus larval gonads, but some ovarian inhibition was observed.

CHAPTER 7

DEVELOPMENT OF GONADS *IN VITRO* AND THE EFFECT OF OESTRADIOL ON ORGAN CULTURES

7.1 Introduction

In the past a lot of work was done in an attempt to clarify some of the problems of sex differentiation and hormone-induced sex reversal, by using tissue and organ cultures. Preston (1949) observed stages of sperm formation in culture of *Rana temporaria* at various months of the year, and Gardenghi and Zaccanti (1968, 1969) cultured successfully genital ridges of *Bufo bufo* larvae, at various developmental stages for 5-20 days. Basu, Nandi and Nandi (1966) investigated the effects of various hormones on adult *Rana pipiens* using chemically defined media. Foote and Foote (1957, 1958a,b,c, 1959, 1960, 1963) cultured gonads from adult *Triturus cristatus* and *Xenopus laevis* and from larval *Rana catesbeiana*, *Xenopus laevis* and axolotl, in various natural media and monitored tissue maintenance and growth.

The effect of sex steroids on sex differentiation has also been investigated *in vitro*. Foote and Foote (1960) found that undifferentiated *Xenopus* gonads attached to their mesonephroi did not survive *in vitro*, whereas differentiated gonads of later stages survived well with some of them showing a degree of development similar to that *in vivo*. Oestradiol present in the culture medium caused no alteration of the sex ratio of either tadpole or adult gonads.

In mammalian gonad cultures, maintenance and initiation of spermatogenesis was reported in young rat testes by Steinberger, Steinberger and Perloff (1964a,b) and Steinberger and Steinberger

(1964, 1965). A study of sex differentiation of the Wolffian and Mullerian ducts of foetal rats *in vitro*, was made by Price and Pannabecker (1956) and of foetal guinea-pigs by Price, Ortiz and Zaaijer (1967). The morphogenetic changes of the ducts observed, were compared with those *in vivo*.

An attempt was made using a culture medium different to those used previously, to culture gonads of larval and adult *Xenopus* with and without oestradiol. This medium was used by the immunologists of the department to maintain *in vitro* thymus and spleens of *Xenopus*. The aim was to examine how well the gonads were maintained *in vitro* and to investigate whether this hormone will cause feminization *in vitro* as well as *in vivo*.

7.2 Materials and Methods

The culture medium employed had been used by the immunologists of the Department for culturing the thymus gland and spleen of *Xenopus*.

The medium was made as follows:

Leibovitz, L-15 medium (Flow Laboratories)	29.00 ml
Double distilled water	12.00 "
2mM Mercaptoethanol	1.65 "
Hepes Buffer (1.0M) pH 7.2	0.40 "
L-glutamine (2mM)	0.25 "
Fungizone (Gibco, Scotland)	0.40 "
Penicillin/Streptomycin (equal parts) (50 i.u/ml penicillin and 50µg/ml streptomycin)	0.40 "
10% Foetal Bovine Serum (FBS)	5.90 "
Total culture medium volume	50.00 "

Mercaptoethanol was added to stabilize the FBS. The medium was stored at $4^{o}C$ and used within a week.

The main problem encountered with the organ cultures was contamination, so that after initial trials Fungizone (antimycotic) was included as well as the usual antibiotics. The constituents of the medium were sterilized beforehand. The mixture was forced through a Millipore filter (size 0.22µm, Millex filter from Millipore Co., France) as an additional precaution for getting rid of bacterial spores. The whole procedure was carried out in a sterilizing chamber, possessing u.v. lamps and a ventilator. After the medium was made up, 5.0ml were added to a vented sterilized tissue culture petri-dish, 35mm in diameter and 10mm deep. Nucleopore polycarbonate membranes (0.4µm pore size and 13mm diameter) were boiled twice in distilled water and autoclaved before use. The membranes were floated on top of the culture medium ready to support the gonads.

Before dissecting the gonads out with their mesonephroi, the larvae were made bacteriologically sterile by placing them in a solution of 1% Sulphadiazine (Sigma Chemical Co.) to which a few drops of penicillin/streptomycin mixture were added. The mesonephroi and gonads were removed under these aseptic conditions from the tadpoles anaesthetized in 0.05% MS 222 Sandoz solution. The vented petri-dishes with their tops on, were put in sterilized stainless-steel boxes in the CO_2-Incubator containing 95% air and 5% carbon dioxide at 28°C and 100% relative humidity (all automatically controlled). The effective pH of the medium in the incubator varied between 7.0 and 7.2. It was changed every three days. The cultivation was carried on 12 days for tadpole gonads of stages 49, 52, 54, 55, 56 and 15 days for explants of adult gonads; 20 paired gonads with their mesonephroi, at each tadpole stage, were used. Gonadal pieces from five from each sex adult 12 month toads were cultured. The pieces of adult gonads cultured were completely

immersed in the medium. At first all gonads were cultivated in the medium to follow their maintenance and survival. Later, part of the distilled water was replaced with oestradiol solution giving concentrations of 1µg and 3µg per 5ml.

The media for the various treatments were made up as follows:

Group 1. Sham-ethanol controls. 1.5ml of distilled water was replaced with the same volume of o.1% ethanol in the 50.0ml culture medium.

Group II. Treatment with 1µg/5ml oestradiol. 1.5ml of distilled water was replaced with 0.5ml of oestradiol alcoholic solution (10µg/50ml) plus 1.0ml of 0.1% ethanolic solution, to give an effective hormone concentration of 1µg/5ml of medium.

Group III. Treatment with 3µg/5ml oestradiol. 1.5ml of distilled water was replaced with the same volume of oestradiol alcoholic solution (30µg/50ml) to give an effective hormone concentration of 3µg/5ml of medium.

The ethanolic solution was made up by adding 1.0ml of absolute ethanol in 100ml of distilled water. The oestradiol solution was made up by dissolving 2.0mg of 17β-oestradiol in 1.0ml of absolute ethanol and then added to 100ml of distilled water. Both solutions were autoclaved.

At the end of the culture period, the gonads were fixed in Bouin's solution for 24 hours and then processed for wax embedding. Serial sections of 5µ were cut, stained in haemalum and eosin and examined under the microscope. A comparison was made between the hormone treated and the sham-ethanol controls. Also a comparison of the histological appearance of gonads in vitro and gonads in vivo was made. Photographs were taken to support the observations.

7.3 Results

The effects are summarized in Tables 13 to 15 (p 92-94). The gonads from tadpole stages 49 and 52 disintegrated, whilst most of those from stages 54, 55 and 56 survived well, with some ovaries and testes showing development similar in degree to that *in vivo* (Figs. 213, 214, 215, 216-217, 218, 219-220). Out of a total of 60 pairs of cultured gonads from tadpole stages 54, 55 and 56, in cultures not treated with hormone only 11 pairs of gonads failed to differentiate and it was impossible to determine their sex due to bad maintenance. The oestradiol concentrations of 200µg/l and 600µg/l had no effect on either sex ratio or gonadal development (Figs. 221, 222, 223, 224, 225) and thus the sex ratio showed no sign of deviation from normal.

Pieces of adult testes cultured *in vitro* were not successful. Somatic deterioration and degeneration took place and necrotic areas could be seen in the central area of the testis (Fig. 234). This could be due to anoxia. Also the cystic appearance of most seminiferous tubules was lost as compared with the *in vivo* situation (Figs. 233-234 compared with the *in vivo* controls Figs. 231-232). However, some seminiferous tubules around the periphery of the testis, maintained their integrity (Fig. 233). As shown in Tables 14 and 15 no changes were detected in the oestradiol treated testicular cultures (Fig. 235).

Pieces of adult ovaries *in vitro*, survived well and in general the oocytes maintained their integrity (Table 13). A small degree of degeneration was apparent in some instances (Figs. 227-228 and compare with the *in vivo* control in Fig. 226). Tables 14 and 15 show that oestradiol treatment *in vitro* had no detectable effect on the ovarian structure (Figs. 229, 230).

TABLE 13. Development of gonads cultured *in vitro*

Size of sample	Days *in vitro*	Initial stage	Phenotypic Sex ♂	♀	Undetermined[a]	Comments about maintenance *in vitro*
Larvae						
20[b]	12	49	0	0	0	Disintegrated
20	12	52	0	0	0	ditto
20	12	54	9	7	4	Most maintained and survived well. Development slowed down. 2 ♂ progressed to st.55
20	12	55	7	8	5	Maintained well, development slowed down. 1 ♂ and 2 ♀ progressed to st.56
20	12	56	8	10	2	Maintained well, development slowed down. 1 ♀ progressed to st.57.
Adult						
5 ♂[c]	15	12 months old	5	0	0	Testes not successful. High degree of degeneration of seminiferous tubules.
5 ♀	15	"	0	5	0	Ovaries survived well, although slight degeneration of some large oocytes was observed.

[a] Undetermined sex

[b] 20 pairs of gonads attached to their mesonephroi

[c] Pieces of adult gonads.

TABLE 14. Effect of 200µg/l oestradiol on gonads cultured *in vitro*.

Size of sample	Days *in vitro*	Initial stage	Phenotypic Sex ♂	♀	Undetermined	Comments and comparison with untreated cultures
Larvae						
20	12	54	8	10	2	Survived well but no hormone effect. 2 ♂ and 1 ♀ progressed to st.55.
20	12	55	9	7	4	Survived well but no hormone effect. 3 ♂ and 1 ♀ progressed to st.56.
20	12	56	8	9	3	Survived well but no hormone effect. Development slowed down.
Adult						
5 ♂	15	12 months old	5	0	0	Testes degenerated; no detectable hormone effect.
5 ♀	15	"	0	5	0	Ovaries maintained well. No detectable hormone effect.

TABLE 15. Effect of 600µg/l oestradiol on gonads cultured *in vitro*.

Size of sample	Days *in vitro*	Initial stage	Phenotypic Sex ♂	♀	Undetermined	Comments and comparison with untreated cultures
Larvae						
20	12	54	8	6	6	Survived well but no hormone effect; no feminization. 3 ♂ and 2 ♀ progressed to st.55.
20	12	55	7	9	4	Survived well but no hormone effect. 1 ♂ and 2 ♀ progressed to st.56.
20	12	56	9	8	3	Survived well but no hormone effect. 1 ♂ and 1 ♀ progressed to st.57.
Adult						
5 ♂	15	12 months old	5	0	0	Testes degenerated; no hormone effect.
5 ♀	15	"	0	5	0	Ovaries survived well; no detectable hormone effect

7.4 Discussion

Gonads from tadpoles prior to and at the beginning of sex differentiation did not survive *in vitro*. At the end of the culture period, no gonads nor germ cells could be identified in the sectioned material. Inability of these early larval gonads to grow and differentiate *in vitro*, could be due to absence of an active principle necessary for growth stimulation of these early gonads which is present *in vivo*, and enables them to develop normal function. Larval gonads of later stages survived well, although the development was slowed down and at the end of the culture period ovaries and testes could be distinguished. This is in agreement with observations by Foote and Foote (1960). In some cases progressive development comparable to growth *in vivo* was observed. Adult ovaries survived well *in vitro*, whilst testes showed high degrees of degeneration. Large necrotic areas were found in the middle of the testicular structures, probably caused by lack of oxygen.

The inability of oestradiol treatment to feminize or change the sex ratio of the larval or adult gonads, was also observed by Foote and Foote (1960) when they showed that oestradiol had no effect *in vitro* on larval or adult *Xenopus* testes. This contrasts with the *in vivo* situation where complete feminization of larval testes took place (Gallien 1953; Chang & Witschi 1955a, 1956). Likewise Foote and Foote (1959) produced, using a water soluble testosterone compound, partial sex reversal of ovaries of *Rana catesbeiana* larvae *in vitro*, but the same compound had no similar effect on *Xenopus* tadpole ovaries. Again Basu, Nandi and Nandi (1966) cultured adult *Rana pipiens* testes and found that testosterone had no effect *in vitro*

whereas *in vivo* it inhibited spermatogenesis (Basu and Nandi 1965).

A reason for the diversity of results obtained by different workers *in vitro*, could be due to the varying nature of the culture media employed. Foote and Foote (1958c) concluded that growth and maintenance of amphibian gonads, were better with media containing components from natural sources. Amphibian tissue extracts, cock plasma, chick embryo extract were better than any of the defined media such as medium 199, medium 1066, White's nutrient solution etc. Similar media were used more recently by others such as Grasi-Milano, Di Castro and Manelli (1971). Nevertheless Basu and Nandi (1965), Basu et al (1966), Ball, Simnett and Arthur (1969), Simnett and Balls (1969) and Godsell and Balls (1976) successfully used defined complex media for amphibian organ culture (medium 199, Eagle's medium, medium CMRL-1415, Parker's medium, Trowell's medium, Wolf and Quimby's amphibian culture medium or Leibovitz L-15 medium). Details of these media were given by Paul (1975). Also an excellent review on amphibian organ culture was given by Monnickendam and Balls (1973).

Foote and Probstmeyer (1959) studied sex differentiation in *Xenopus* by maintaining heterosexual combinations of larval-ovaries and testes, of different stages together in the same culture. They found that in heterosexual pairs of early st.53 gonads, the testis predominated by depressing cortical development and causing intersexuality in some of the ovaries, In pairs where the male larvae were older, depression of the younger ovaries also occurred. When ovaries were older both ovaries and testes were in similar stages of development at the end of the experiment.

In ovary-testis pairs of gonads from metamorphosizing animals, testes were better differentiated and developed than ovaries. No marked changes occurred in ovaries or testes, in heterosexual pairs of two month old young Xenopus. The results of these authors substantiated the findings of Chang (1953) on parabiotic experiments with Xenopus laevis. All three authors suggested that the predominance of male gonads could be due to a morphogenic substance from the testis depressing ovarian development in heterosexual pairs. They concluded along with Witschi and Allison (1950), Chang and Witschi (1955a, 1956) and Gallien (1956), that the substance was not a sex steroid, since male and not female sex reversal was obtained in vivo in this particular species. However, my findings that 5α-dihydrotestosterone induced masculinization of the heterogametic females in vivo suggests the production of the steroid by larval testis and subsequently inhibition of the ovaries in the heterosexual parts in vitro.

In general, isolated gonads of Xenopus were maintained well in an artificial isolated environment, but they were not responsive to hormonal treatment. In assessing the oestradiol treatment in vitro, the stage of gonadal development should be considered. The hormone has its most pronounced effect, in vivo, when applied just before or at the beginning of sex differentiation, at st.50-52. Therefore it would be desirable to maintain the gonads in vitro, prior or during sex differentiation, to enable us to see if oestradiol treatment would cause feminization or not, something that I failed to do in the present experiment. Also, because of the very slow development of gonads in cultures, it would be advisable to maintain and treat them with oestradiol for longer periods of time. Furthermore, a possible change in the activity of oestradiol in vitro due to the different chemical environment,

could account for the non-feminizing effect. It remains to be seen whether it is possible to maintain and grow undifferentiated and differentiating Xenopus larval gonads in vitro, perhaps by modifying the cultivation method or the culture medium that I have used. Also, 5α-dihydrotestosterone treatment in vitro could give useful information in elucidating further, sex differentiation and hormone induced sex reversal in Xenopus.

7.5 Summary

Differentiated gonads of Xenopus larvae placed in organ cultures survived well, with some of them showing development similar in degree to the in vivo situation. In contrast undifferentiated or differentiating gonads did not survive in vitro. Oestradiol treatment had no detectable effect or larval gonads in vitro.

Adult ovaries were maintained in vitro whereas testes degenerated. Treatment of adult gonads with oestradiol caused no detectable effect.

CHAPTER 8

EFFECT OF COMBINING ANTIBIOTICS INHIBITING PROTEIN SYNTHESIS WITH OESTRADIOL TREATMENT

8.1 Introduction

Many workers have investigated the oestradiol effects on development, growth and protein synthesis in various tissues. A single physiological dose of oestrogen injected into the immature or ovariectomized female rat will convert the atrophic uterus into an actively growing organ, as shown by many workers such as Jensen and Jacobson 1962, Gorski 1964, Segal, Davidson and Wada 1965 and Emmens 1967. By employing radioactively labelled isotopes they showed retention and accumulation of oestradiol in the hormone treated uterus, with an increase in nuclear and later cytoplasmic RNA synthesis, this being stopped by the metabolic inhibitor actinomycin D (Talwar and Segal 1963; Hamilton 1971).

Since oestradiol administration increases protein synthesis, sex reversal by this hormone could induce production of specific feminizing protein agents, by the indifferent gonad or other gland in the larval body. In order to investigate the mechanism of hormone-induced sex reversal two antibiotics, actinomycin D which inhibits RNA synthesis at the nuclear level and puromycin which inhibits it at the cytoplasmic level, were administered separately and in conjunction with oestradiol to tadpoles prior to sex differentiation. If oestradiol-induced sex reversal involves new protein synthesis, then the antibiotics should stop it; as the production of RNA is an essential step in its genesis. Conversely if reversal is thus prevented this would suggest that oestradiol was at least imitating inductor-functioning if not a natural inductor.

8.2 Materials and Methods

Groups of 20 larvae at st.50 were kept in the following cultures for 33 days at 23°C in 200ml of aqueous medium and in complete darkness which was necessary on account of the photolability of the antibiotics. The larvae were crowded in this small volume of medium because of the high expense of the antibiotics. Treatment ended when the external criteria of the larvae were at st.56.

Group I. Ethanol treated controls.

Group II. Treatment with 100µg/l oestradiol.

Group III. Treatment with 2.5mg/l Actinomycin D

Group IV. Treatment with 5.0 mg/l Puromycin

Group V. Treatment with 100µg/l oestradiol plus 2.5mg/l Actinomycin D.

Group VI. Treatment with 100µ g/l oestradiol plus 5.0mg/l Puromycin.

The above media were kept in glass beakers, they were well aerated and changed every day. The antibiotic supply was discontinued for one day every four days to avoid accumulation to toxic levels in the tadpoles.

After treatment the body length and weight were recorded and larvae were successfully fixed in Bouin's for 24 hours and processed for wax embedding (for further details on methodology, see pages 25 - 28).

8.3 Results

The larvae showed a 9 day delay in development over the period of treatment and since this lasted 33 days starting at st.50 (15 days old), the tadpoles at the end of the experiment should have been at st.61 (48 days old) according to Nieuwkoop and Faber's

developmental table and not at st.56 as they were found to be. All larvae in the various cultures survived well and no deaths occurred.

Treatment with the antibiotics further inhibited body growth and development as shown in Table 16. Actinomycin D showed the strongest inhibitory effect and furthermore the tadpoles concerned, showed small and narrow heads as compared to the tadpoles in the rest of the cultures. Nevertheless, the testes and ovaries were well developed and differentiated (Figs. 243, 244). In the oestradiol culture some individuals showed suppressed ovaries most probably belonging to genetic males (sex reversal), as shown in Figs. 240-242, and some resembled female controls (Figs. 238-239). The suppressed ovaries had no obvious ovarian cavity or pachytene stage oocytes in the cortical region. In the alcohol treated controls only individuals with normal testes (Fig. 236) or ovaries (Fig. 237) were found. Also because of the prolonged treatment with oestradiol some interrenal hypertrophy was observed (Fig. 241).

Puromycin treatment also inhibited body growth, but again had no effect on sex development, histological examination revealing individuals with normal testes and ovaries respectively (Figs. 245 and 246).

When actinomycin D was administered simultaneously with oestradiol no feminization was observed and all individuals showed normal testes or ovaries (Figs. 247 and 248). Treatment with puromycin and oestradiol again produced no sex change and all individuals showed normal male or female gonads (Figs. 249, 250).

TABLE 16. The effect on gonadal differentiation of combining antibiotic and oestradiol treatment for 33 days, starting at st.50 and finishing at st.56.

Treatment	Mean length mm	Mean weight mg	Phenotypic Sex ♂ N	♀↓	♀ N	Interrenal Hyperplasia
Ethanol controls	45.5±0.7	418.5±15.2	12	0	8	-
100µg/l oestradiol	44.6±0.7	398.0±16.5	0	13	7	++
2.5mg/l actinomycin D	34.9±0.4 (23%)*	215.0±0.5 (50%)*	9	0	11	-
ACTINOMYCIN D + OESTRADIOL	34.0±0.4 (25%)	217.0±5.2 (48%)	12	0	8	-
5.0mg/l puromycin	41.5±0.8 (9%)	361.5±8.9 (14%)	11	0	9	-
PUROMYCIN + OESTRADIOL	42.4±0.8 (7%)	376.5±10.6 (10%)	9	0	11	-

* % decrease in body length and weight respectively, as compared to the ethanol treated controls.

8.4 Discussion

There was an inhibition of tadpole growth and development by the end of the treatment , due to combination of crowding , lack of daylight, and presence of antibiotics or a combination of the three. However, the tadpoles survived well and histological examination showed no gonadal malformation.

In earlier experiment it was found that oestradiol treatment induced feminization in Xenopus larvae. The sex hormone produced a sex reversal in the developing amphibian gonads achieving morphogenetic effects, normally expressed by sexual genetic factors. It was found in the present experiment that treatment with oestradiol induced feminization whereas administration of oestradiol simultaneously with actinomycin D or puromycin caused no such an effect. It remains to be seen if the antibiotics can stop the masculinizing action of 5α-dihydrotestosterone.

Witschi (1957) suggested that steroids or other external factors cause feminization or masculinization by suppressing the medullary or the cortical function respectively. In contrast Vannini and Stagni (1967, 1972) proposed that the administered steroids act as 'derepressors' of sex genes either causing masculinization of the genetic female by inducing the latent male sex genes to produce messenger-RNA (m-RNA), or alternatively causing feminization by activating the latent female sex genes in the male genotype. Vannini and Stagni (1967, 1968, 1971, 1972) elaborated their 'derepression' theory by experimenting with larvae of Rana dalmatina. In this species the gonads develop as ovaries till the end of metamorphosis in both sexes and later the ovaries invert into testes in the male genotype. Using testosterone,

they induced masculinization of the ovaries in the Rana larvae, whereas testosterone treatment combined with a sub-lethal dose of actinomycin D had no such effect. They claimed that both the antibiotics, actinomycin D and puromycin, suppressed the medullo-stimulating effect of testosterone but did not prevent inhibition and degeneration of the oocytes caused by testosterone. Likewise, antimedullary activity was not observed in my antibiotic experiment. They hypothesized that the medullo-stimulating activity of testosterone given alone, would depend on a 'derepression' or activation of the latent male sex genes, in the female genotype, to produce male m-RNA responsible for the translation of a male protein (medullarin ?). They also suggested that the antioogenic activity of testosterone was derived from a direct influence of the androgen on the growing oocytes. Vannini, Stagni and Zaccanti (1975) showed later by autoradiographic methods, a reduced level of RNA synthesis in the oocytes of testosterone treated Rana esculenta and Rana latastei tadpoles. In addition they found a stimulation of RNA synthesis in the hilum of the ovary after testosterone treatment, indicating proliferation of the medullary component.

Similar results on stimulating protein synthesis were observed using oestradiol. Gorski (1964) found that oestradiol injected into immature or castrated female rats caused uterine growth by stimulating the nuclear RNA polymerase activity to synthesize m-RNA, thus increasing cytoplasmic RNA and protein synthesis subsequently. Segal, Davidson and Wada (1965) showed that administration of RNA extracted from stimulated uteri, into the inhibited uteri of overiectomized rats, caused growth and

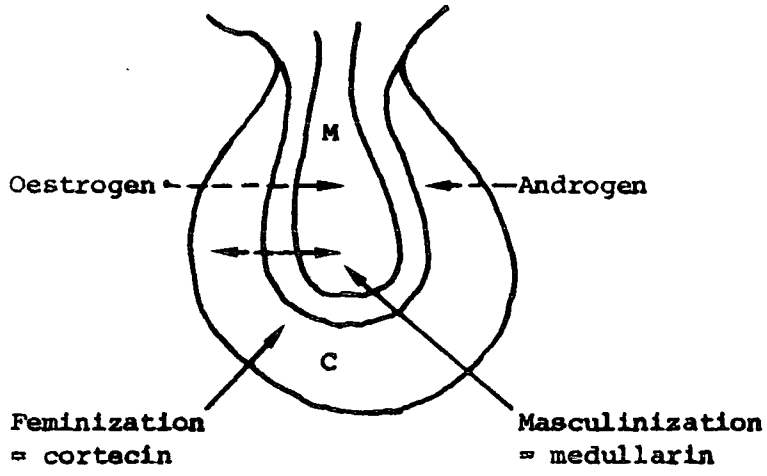
hyperplasia indistinguishable from that produced by oestradiol stimulation. Talwar and Segal (1963) prevented oestradiol action on the vagina of ovariectomized rats, by topical application of actinomycin D. It seems therefore, that oestrogens and androgens manifest their different sexual effects by influencing m-RNA in the nucleus.

The concept of 'derepression' proposed by Vannini and Stagni (1967) was in opposition to Witschi's (1931, 1957, 1967) inductor theory of sexual differentiation. He stated that the cortex produces cortecin, inducing the germ cells to differentiate into oocytes, and the medulla produces medullarin pushing the germ cells towards spermatogenesis. He suggested, without direct evidence, that both cortecin and medullarin were probably proteins, the former being produced by the follicle cells in the cortex and the latter by an interstitial-like cell type in the larval gonad. Furthermore, he said that an antagonism exists between the two inductor systems and differences in the range of inhibition, according to amphibian species, were demonstrated by parabiotic experiments. He also proposed that the sex steroids induce their effects by acting as inhibitors and not as inductors of sex differentiation, and therefore their effects are pharmacological.

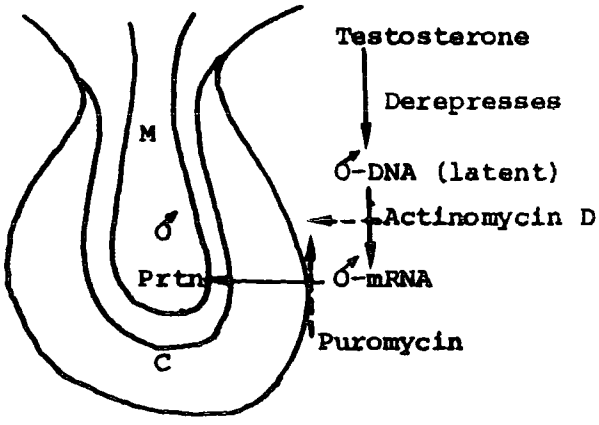
Diagrams i and ii in Text Fig. 1 (p106) compare Witschi's and Vannini and Stagni's ideas, in explaining sex differentiation. Diagram iii shows possible mechanisms of sex reversal in Xenopus using oestradiol or 5α-dihydrotestosterone by applying the ideas of these workers and also by introducing ideas from the steroid theory of sex differentiation. Thus we have a more complete picture, amalgamating all possible mechanisms in explaining the hormone-induced sex reversal in Xenopus, bringing us nearer to

Text Fig. 1. Possible mechanisms of sex differentiation and hormone-induced sex reversal in Amphibia.

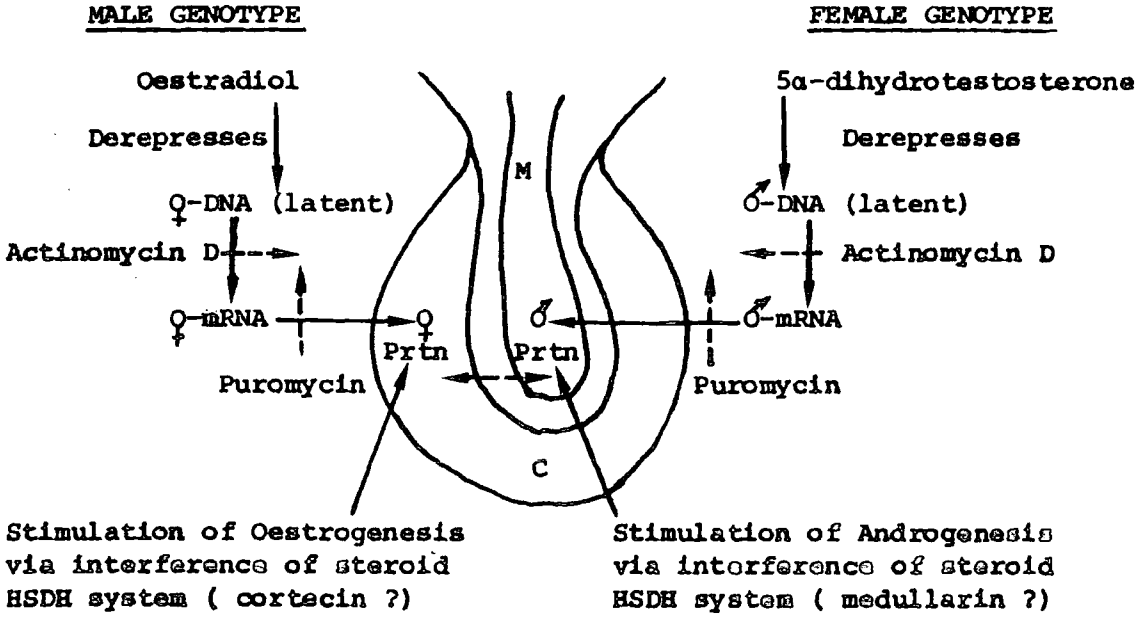
(i) Antagonism of cortex and medulla in Witschi's Inductor Theory (1931, 1934, 1957, 1967).



(ii) Vannini & Stagni's (1966, 1972) Derepression Hypothesis as applied to a male Rana tadpole.



(iii) Sex reversal effects of sex hormones in Xenopus



Continuous arrows indicate activation-stimulation.
Interrupted arrows indicate inhibition-derepression
HSDH: hydroxysteroid dehydrogenase enzyme
C: cortex
M: medulla
Prtn: protein

the solution of the problem of sex differentiation.

8.5 Summary

Oestradiol-induced sex reversal in *Xenopus*, was stopped by both actinomycin D and puromycin.

The possible mechanism of oestradiol action could have been activation of a protein-enzyme system, by direct stimulation of the cortex, indirect inhibition of the medulla or a combination of both or by interference with the normal steroid metabolism in the gonads.

CHAPTER 9

HISTOCHEMISTRY OF INTERRENALS AND GONADS DURING DEVELOPMENT (Δ^5-3β-HSDH, G-6 PO_4DH AND LIPIDS).

9.1 Introduction

Evolution of the steroidogenic cells has been closely correlated with that of the use of steroid molecules as hormones. Among vertebrates, both individual and species survivals have been dependent upon the activity of steroidogenic enzyme systems in the adrenal, gonads and in the placenta of mammals (see review by Bentley 1976). The multienzyme systems coupled with oxidation and reduction reactions which are essential in steroid metabolic pathways (see Talalay and Asham-Williams 1960), have enabled the glands to produce individual steroids recognisable by specific receptor sites.

An essential step in steroid hormone biosynthesis, is the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3β-ketones, involving the Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSDH) enzyme complex. The existence of this enzyme in mammalian tissues was first described by Samuels and Helmereich (1956). A short time later, Wattenberg (1958) established a histological technique for the demonstration of the enzyme by coupling oxidation of the hormone to the reduction of a tetrazolium salt (TS), thus forming coloured deposits and making histochemical localization possible in tissue sections. The degree of enzyme activity in various tissues was studied by Levy, Dean, and Rubin (1959a,b), by Hanke and Weber (1964) and Rubin and Strecker (1961). These latter authors found enzyme activities in the rat liver to differ in the two sexes. Furthermore Gallien, Certain and Ozon (1964) investigated Δ^5-3β-HSDH activity in the interrenal

of Pleurodeles waltlii at various developmental stages, and Baillie, Ferguson and McKHart (1966) demonstrated the presence of various steroid dehydrogenases including Δ^5-3β-, in the genital ridge of a 14mm human foetus. More recently Hay and Moor (1975) have investigated distribution of the enzyme in the graafian follicle of the sheep.

Glucose-6-phosphate dehydrogenase enzyme (G-6-PO_4DH) was shown biochemically to be a provider of NADH, a coenzyme required for steroid hydroxylations and cleavage of cholesterol and C_{17-20} side chains. Haynes and Berthet (1957) and Haynes (1958) showed evidence of G-6-PO_4DH involvement in adrenal steroid synthesis in the rats, and Goldman, Baker and Stanek (1972) investigated the development of the enzymes in the testes, ovaries and adrenals of rabbit foetuses. Since the enzyme system of G-6-PO_4DH is a potential NADH provider, a reductant important for steroid synthesis, its biochemical or histochemical demonstration could indicate active steroid synthesis in the particular tissue tested.

In the present instance histochemical work was carried out to investigate the occurrence and activity of the two important steroidogenic enzymes in the interrenals and gonads at various stages of development of Xenopus. A histochemical test for lipids was undertaken, using Sudan Black B, and the amount of lipid was compared with the activity of the two enzymes. An attempt was also made to establish the nature if any, of the relationship between high dosage treatment with oestradiol, interrenal hyperplasia, masculinization of the gonads and steroidogenic enzyme activity.

9.2 Materials and Methods

For the histochemical demonstration of Δ^5-3β-HSDH, Hsu's procedure and incubation medium were used (personal communication).

Tadpoles were instantly frozen in an isopentane-liquid nitrogen mixture, and cryostat sections of 12μ were cut at -25°C. Vertical sections through the tadpole's body were obtained, incorporating interrenal, gonadal, kidney, muscle and intestinal tissues. As soon as the mesonephric kidney was visible, in sectioning from the anal end of the tadpole, serial sections were taken for the following tests:

(i) Demonstration of Δ^5-3β-HSDH activity in tadpole sections, with control sections to test for any enzyme activity without substrate (dehydroepiandrosterone, DHEA).

(ii) Sections for G-6-PO_4DH activity demonstration, with control sections to test for enzyme activity without substrate (potassium salt of G-6-PO_4).

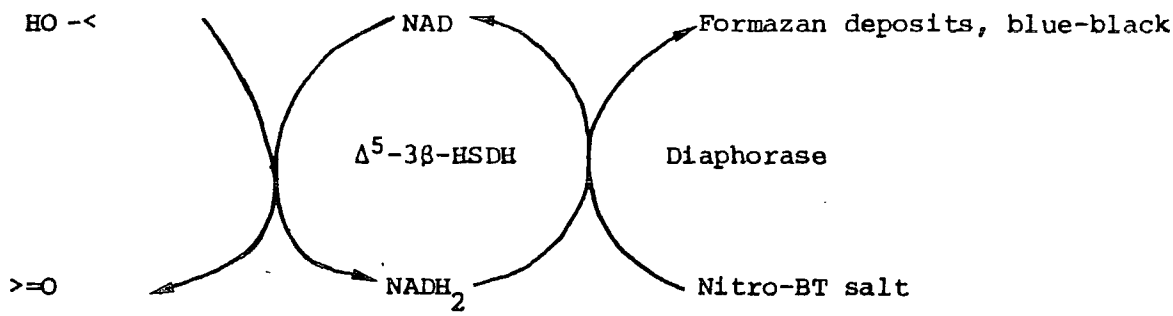
(iii) Sections for demonstration of lipids, with control sections where the lipid was extracted in acetone.

For each of the six tests one tadpole at a given developmental stage was used. The stages tested were 48 to 59 inclusive plus st.63 and 66. For comparison tests were carried out on a male and a female adult aged two years. In addition to the above tests, when the tadpoles were big enough (from st.54 onwards), three sections of 12μ each were cut out at the middle of the mesonephros of each individual, to check both enzyme activities and lipid presence in the interrenals and gonads of all tadpoles. These sections were kept in the cryostat chamber at -25°C until sectioning was complete and then enzyme demonstration and lipid staining were performed. Two late stage

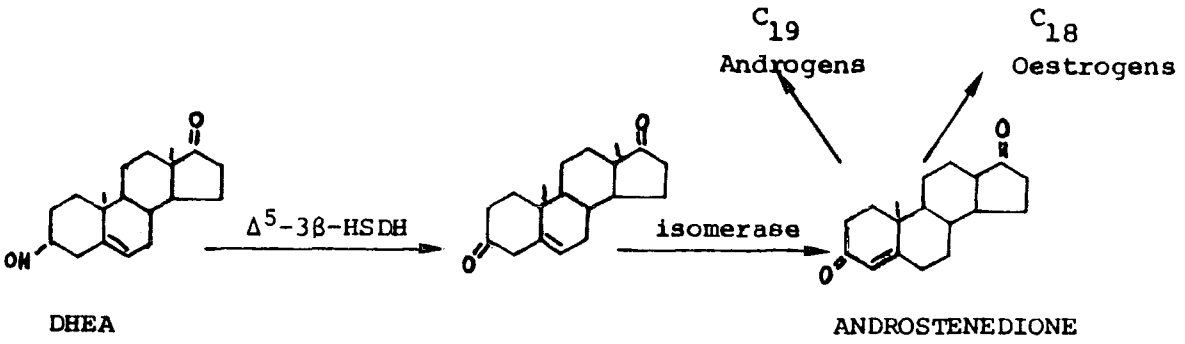
Rana catesbeiana tadpoles were also tested for Δ^5-3β-HSDH enzyme activity in the ovaries and interrenals (for species comparison).

Demonstration of Δ^5-3β-HSDH activity was carried out as follows. The sections on slides, were incubated at pH 7.6 and 37°C for 2 hours in the medium made up as follows. A few drops of dimethyl formamide were added to a small beaker containing 5mg of DHEA substrate, until the hormone was dissolved. Then 40mg of β-nicotinamide adenine dinucleotide (NAD) acting as the hydrogen-transferring agent between the hydroxysteroid and the Nitro-blue tetrazolium salt (Nitro-BT) and 10mg of Nitro-BT (to act as the electron acceptor forming the reduced, blue-black formazan deposits) were dissolved in 20ml of Tris-HCl buffer at pH 7.6 in another beaker. The pH of the buffer, Trizma base or Tris (hydroxmethyl)aminoethane, was adjusted by adding dropwise 1 N HCl acid. When the two were added together the incubation medium was ready for use. Control incubations without the substrate were carried out concurrently.

The mechanism of histochemical Δ^5-3β-HSDH demonstration and therefore formation of coloured formazan deposits is shown below:



The enzyme complex, Δ^5-3β-HSDH together with Δ^5-Δ^5-ketosteroid isomerase, converts Δ^5-3β-hydroxysteroids to Δ^4-3β-ketosteroids as shown below.



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After incubation, the tadpole sections were fixed in neutral formalin for ten minutes, washed several times in Tris-HCl buffer and mounted with gum-syrup. Microscopic examination of the temporary slides showed the distribution of formazan deposits and thus Δ^5-3β-HSDH activity in the tadpole tissues. Photographs were taken to give permanent records.

The histochemical demonstration of G-6-PO_4DH followed Cohen's (1959) method with some modifications. Tadpoles were frozen in the isopentane-liquid nitrogen mixture and then sections at 12 µ were cut using the cryostat as before. These were incubated for 20 minutes at 37°C in the following medium: 30mg of K_2G-6-PO_4 substrate (D-glucose-6-phosphate dipotassium salt), 5mg of TPN (triphosphopyridine nucleotide) and 5mg of Nitro-BT salt dissolved in 10ml of Sorensen's buffer to pH 7.5. The right pH was achieved by adding 0.1M KH_2PO_4 (potassium phosphate, monobasic) solution. 2ml of 0.1M KCN (potassium cyanide) was added to the above solution to inhibit the cytochrome system, function of which would confuse the results.

After incubation, the sections were washed several times in Sorensen's buffer, fixed in neutral formalin, rewashed with buffer and finally mounted with gum syrup. Controls were incubated without the substrate. Microscopic examination revealed any formazan deposits (blue-black) and thus the position and activity of G-6-PO_4DH in the tissues.

The preparations were photographed.

For the histochemical demonstration of lipids frozen sections at 12µwere stained with Sudan Black B. The mounted sections were successively fixed in formal-saline for 10 minutes, washed in water, stained in saturated Sudan Black B in propylene glycol, drained, agitated successively in 85% and 50% propylene glycol for 3 minutes each, washed in running tap water for 5 minutes, drained and mounted in glycerine. Microscopic examination of the temporary slides showed neutral lipids stained as black droplets in the tadpole tissues. Photographs were taken to give a permanent record.

The effect of high dose oestradiol treatment on Δ^5-3β-HSDH activity in interrenals and gonads was also investigated, as follows. Ten tadpoles of st.51 were treated with 2mg/l oestradiol for 26 days whilst another 10 tadpoles of the same stage were treated for the same period of time with 0.1 ml of absolute ethanol per litre of water, acting as controls. At the end of the treatment the controls were at st.57 but the stage of the oestradiol treated larvae was impossible to assess from the external criteria owing to the teratogenic effects of the hormone. It was therefore assumed to be 57 (as in previous experiment on treatment with 2mg/l oestradiol on page 41). To test the activity of the dehydrogenases in the interrenals and gonads, the same procedure was followed as described above. A comparison of the activity was made between controls and oestradiol treated larvae and photographs were taken.

9.3 Results

The extent of enzyme activity and lipid presence in interrenals and gonads at different stages of development is shown in Table 17 (p 115)

Both Δ^5-3β-HSDH and G-6-PO_4 DH enzymes first appeared in the interrenals, at st.49. At this stage there was only a very slight activity present (Figs. 251, 276). It increased progressively up to st.56 (Figs. 252, 253, 256, 277, 278) and then remained constant. The gonads first showed activity of both enzymes at st.56, when formazan deposits were distributed throughout the testis and ovary in the form of blue-black granules. The activity of Δ^5-3β-HSDH as indicated by the intensity of formazan deposits, was higher in the testis (Figs. 254, 255, 260, 262, 263, 264) than in the ovary (Figs. 256, 257, 259, 261) at all stages of development following its appearance. In addition much bigger granules, similar to those in the interrenals, were found in the testicular soma, whereas only fine granules were observed in the ovarian cortex. In the adults enzyme activity was found in the interstitial tissue of testis (Figs. 270, 271), in follicular membranes of the oocyte in the ovary (Fig. 273), and in the interrenals (Figs. 272, 274, 275). Presence of G-6-PO_4 DH activity paralleled that of Δ^5-3β-HSDH, but remained weak in the gonads of larvae (Fig. 279) at all stages and in the adult gonads.

There was likewise weak Δ^5-3β-HSDH activity from st.56 onwards in the wall of some proximal kidney tubules (Fig. 255) and in the fat body (Fig. 258). Both enzymes appeared to be present in the wall of the small intestine (duodenum) from st.56 onwards (Fig. 282, 283, 284), but not in adults. Also both enzymes were present in small quantities in the Mullerian ducts of adult females (Figs. 285, 286, 287) and in the livers of adults only of both sexes (Fig. 288). No sex difference in enzyme activity was observable. Fine lipid

TABLE 17 Distribution of Δ^5-3β-HSDH, G-6-PO_4 DH and lipids in the interrenals and gonads of *Xenopus*, at different stages of development.

Stage of develop.	Phenotypic Sex Ind[a]	♂	♀	♀	Mean length	Mean weight	Δ^5-3β-HSDH IR	T	OV.	G-6-PO_4 DH IR	T	OV.	Lipid IR.	T.	OV
48	6	0	0	0	15.8±0.6	38.3±5.4	-			-			-		
49	6	0	0	0	21.5±0.2	86.7±1.7	+[c]			+[c]			-		
50	6	0	0	0	25.2±0.5	110.0±7.3	+			+			-		
51	6	0	0	0	28.0±0.5	156.7±4.9	+			+			-		
52	6[b]	0	0	0	33.5±0.4	176.7±6.7	+			+			-		
53		2	2	2	37.7±0.5	263.3±8.5	++	-	-	++	-	-	-	-	-
54		3	0	3	39.3±0.5	295.0±7.6	++	-	-	++	-	-	++[d]	-	-
55		3	0	3	42.3±0.7	390.0±13.7	++	-	-	++	-	-	++	-	-
56		3	0	3	46.5±0.8	471.7±13.5	+++	++	+	+++	+	+	+++	-	-
57		3	0	3	56.3±0.8	816.7±25.5	+++	++	+	+++	+	+	+++	-	-
58		3	0	3	61.5±0.8	1001.7±31.4	+++	++	+	+++	+	+	+++	-	-
59		3	0	3	61.3±1.2	980.0±29.8	+++	++	+	+++	+	+	+++	-	-
63		3	0	3	50.5±0.4	536.7±10.5	+++	++	+	+++	+	+	+++	-	-
66		3	0	3	14.5±0.2	328.3±9.1	+++	++	+	+++	+	+	++	-	-
Adult[e]		1	0	1			+++	++	++	+++	+	+	++	++	++

[a]: larvae with indifferent gonads

[b]: six larvae at the beginning of sex differentiation

[c]: degree of formazan deposit intensity (relative enzyme activity). Maximum colour intensity as in adult interrenals (+++); - negative, + weak, ++ strong, +++ very strong.

[d]: relative numbers of lipid droplets

[e]: two year old adult female weight 80g, male 50g.

IR: interrenal; T, testis, OV, ovary.

grains of formazan deposits were seen mainly around the lipid vacuoles in the liver.

For comparison, Δ^5-3β-HSDH activity was also demonstrated in two late stage tadpoles of Rana catesbeiana. The enzyme was found between the oocytes and in the cytoplasm and membranes of the developing oocytes, and in large quantities in the interrenals (Figs. 265, 266).

Sudanophilic deposits first appeared in the interrenals at st.54 in the form of both large and small dark droplets scattered in the gland on either side of the caval vein (Figs. 289, 290, 291) and throughout the length of the mesonephros. At st.56 the numbers of sudanophilic droplets had increased (more closely packed together) in the interrenals (Fig. 291) and compared with Fig. 289), the glands being packed with dark mainly small droplets, giving the appearance of dark patches on either side of the caval vein. No sudanophilic deposits were found in the gonads of either st.56 or any later larval stages. Treatment with 2mg/l oestradiol had no effect on the interrenal sudanophilic droplets (Figs. 291, 292).

In the testis of the adult, very fine sudanophilic droplets were present in the interstitium, and to a lesser extent inside the seminiferous tubules containing the germinal cysts (Figs. 294, 295). In the adult ovary, the oocytes contained large droplets in the middle area of the cytoplasm and there was a zone of finer ones near the follicular membrane (Fig. 296b). Atretic ovarian follicles contained sudanophilic droplets of intermediate size, evenly distributed throughout the soma (Fig. 297). Large, widely spaced, sudanophilic droplets were present in adult liver (Fig. 298). Similar droplet size and

TABLE 18 Enzyme activity and interrenal cross-sectional area before and after treatment with 2mg/l oestradiol

(mean values of 10 tadpoles)

Treatment	Mean length mm	Mean weight mg	Δ^5-3β-HSDH IR.	Δ^5-3β-HSDH T.	Δ^5-3β-HSDH OV.	G-6-PO_4 DH IR.	G-6-PO_4 DH T.	G-6-PO_4 DH OV.	Δ^5-3β-HSDH Formazan deposit. Interrenal area
Ethanol-controls	56.9±0.5	836.0±20.0	+++	++	+	+++	+	+	normal
Oestradiol (2mg/l)	49.6±0.7	498.0±19.6	+++[a]	-*	-*	++	-**	-**	increased approximately tenfold

[a]. intensity of formazan deposits similar to controls but area of interrenals larger due to hormonal stimulation. Overall enzyme activity in the interrenals may be similar to or higher than controls.

*. the experimental larvae did not possess typical testes or ovaries (see previous experiment for sex types on page 41). Only two individuals (out of three) with inhibited ovaries showed very weak Δ^5-3β-HSDH activity in the cortical area.

**. the experimental larvae showed no G-6-PO_4 DH activity in their gonads. Treatment of larvae with oestradiol started at st.51 and was carried on for 26 days.

arrangement was found in the interrenals of both adult males and females (Fig. 296a), but of lower colour intensity.

As shown in Table 18, oestrogen treatment caused a change in enzyme activity and interrenal size. The hormone treated larvae showed paradoxically very strong formazan deposit intensity in the interrenal (Figs. 267, 268, 269), and on account of hormone-induced interrenal hyperplasia the area covered by the deposits was larger than that of the ethanol treated controls. Enzyme activity in the ethanol treated controls was similar to that in the stage 57 untreated controls (see Table 17). There was no enzyme activity in the gonads of most hormone treated larvae and only two individuals with inhibited ovaries (Fig. 269) showed any (very little) Δ^5-3β-HSDH activity in the cortical area of the gonad. In the oestradiol treated larvae, interrenal hyperplasia resulted in an approximately tenfold increase of the cross-sectional area, as shown by the Δ^5-3β-HSDH formazan deposits (Figs. 268, 269). In contrast G-6-PO_4DH formazan deposit intensity was somewhat lower than in controls (Figs. 280, 281 and compare with Fig. 279).

9.4 Discussion

Whereas in the present instance (see Table 17), Δ^5-3β-HSDH activity appeared first in st.49 tadpoles, and at this stage only present in the interrenal gland, Horn (1976) claimed that Pehlemann and Hanke (unpublished) had observed enzyme activity in st.40 tadpoles. However they gave no supporting evidence. In the present work enzyme activity was observed in the interrenals of older Xenopus larvae and in the adults. Again a cumulative increase in enzyme activity paralleled by an increase in interrenal sudanophilic droplets has been observed, whereas Rapola (1962) reported decrease

TABLE 19. Reports of Δ^5-3β-HSDH activity in the gonads of amphibia.

Species	Tissues with Δ^5-3β-HSDH activity	Investigators
URODELA		
Salamandra salamandra	Thecal and granulosa layers of oocytes and lutea cells	Joly and Picheral (1972)
Pleurodeles waltlii	Follicular cells of oocytes. Interstitial tissue of testis. Larval gonads.	Certain, Collenot, Collenot and Ozon (1964). Ozon (1967). Picheral (1970). Collenot (1965b).
Triturus cristatus	Interstitial tissue of testis. Thecal and granulosa layers of oocytes in the ovary.	Della Corte, Galgano and Cosenza (1962). Botte and Cottino (1964). Vellano (1969).
ANURA		
Xenopus laevis	Interstitium of adult tests and follicular cells of adult ovary.	Redshaw and Nicholls (1971)
Rana temporaria	Testicular interstitium of adult.	Oordt and Brands (1970). Lofts, Wellen and Benraad (1972).
Rana esculenta	Interstitium of adult testis	Chieffi and Botte (1963). Botte and Cottina (1964). Lofts (1964). Notte and Delrio (1967).
Rana catesbeiana	In indifferent larval gonad. In later tadpole stages present in developing oocytes and follicular cells in frogs.	Hsu, Hsu and Liang (1977).
Nectophryonoides occidentalis	Thecal and granulosa layers and corpora lutea cells.	Xavier, Zuber-Vogeli, Le Quang Troug (1970).

in Δ^5-3β-HSDH activity from st.55 to st.58 larvae.

The most important development at st.56 was the appearance of enzyme activity in the gonads. Several previous attempts (Gallien and Chalumeau-Le Foulgoc 1960; Pesonen and Rapola 1962; Rapola 1962; Redhsaw and Nicholls 1971) failed to show such enzyme activity in the larval gonads of Xenopus. Weak enzyme activity in juvenile and adult amphibian gonads has been found by several workers (see Table 19). Ozon (1967) reported Δ^5-3β-HSDH activity in the follicular cells of the newt Pleurodeles waltlii, confirming observations of Botte and Cottino (1964), of enzyme activity in both thecal and granulosa oocyte layers. Hsu, Chiang and Liang (1977) demonstrated Δ^5-3β-HSDH activity in the ovaries of late stage Rana catesbeiana larvae and also claimed enzyme presence in the indifferent gonads of the same species. I found no enzyme activity in the indifferent gonad of Xenopus.

Confirmation of some of Hsu's findings has been provided by the demonstration of high Δ^5-3β-HSDH activity in the interrenals and in the follicular cells as well as the cytoplasm of the oocytes in the ovaries of two late stage Rana catesbeiana tadpoles. The sexual specificity of enzyme activity found in the Xenopus gonads (it being higher in the testes than ovaries) has been observed in various animal tissues by other workers. Rubin and Strecker (1961) and Rubin, Strecker and Koff (1963) found biochemically that the liver homogenates of male rats had higher enzyme activity than the female homogenates. Baillie, Calman, Ferguson and McKHart (1966) obtained similar results histochemically. The higher level of enzyme activity in the larval testes could be due to the influence of male sex hormones probably present or produced in the young testis.

Rubin et al. (1963) showed that testosterone treatment of female rat raised the Δ^5-3β-HSDH activity in the liver to that of the normal male, whilst castration in the males reduced the level to that in the normal female.

In the adult Xenopus, the enzyme activity in the interrenals remained very high whilst the gonads continued to show only weak activity. Redshaw and Nicholls (1971), showed similar levels of enzyme activity and also the presence of 17α- and 3α-HSDH in the vitellogenic and mature oocytes of the adult Xenopus. A similar distribution of Δ^5-3β-HSDH within the ovary was found in the viviparous anuran Nectophryonoides occidentalis (Xavier et al. 1970) and in the ovoviparous urodele Salamandra salamandra (Joly and Picheral 1972). Redshaw and Nicholls (1971) also found that Δ^5-3β-HSDH activity in the follicular wall of the adult Xenopus ovary and the rate of oestrogenic synthesis in vitro, was increased after administration of pregnant mare serum gonadotrophin (PMSG).

Biswas (1970) observed increased enzyme activity in the testis of hypophysectomised Bufo melanostictus, after administration of ascorbic acid and follicle stimulating hormone (FSH). At the same time Wiebe (1970) investigated the mechanism of action of mammalian gonadotrophins on stimulating the enzyme activity in the testes of adult methallibure-injected Xenopus (the compound inhibits gonadotrophin secretion in a similar manner to hypophysectomy). He showed that injections of PMSG, human chorionic gonadotrophin (HCG), FSH or luteinizing hormone (LH), stimulated Δ^5-3β-HSDH activity, the PMSG eliciting the strongest effect.

The enzyme activity was inhibited by simultaneous administration of actinomycin D, suggesting increase in rate of synthesis of protein-enzyme as a possible mechanism of gonadotrophin action. Similarly in mammals, Samuels and Helmreich (1956) found that HCG increased the enzyme activity in the testes of hypophysectomised rats.

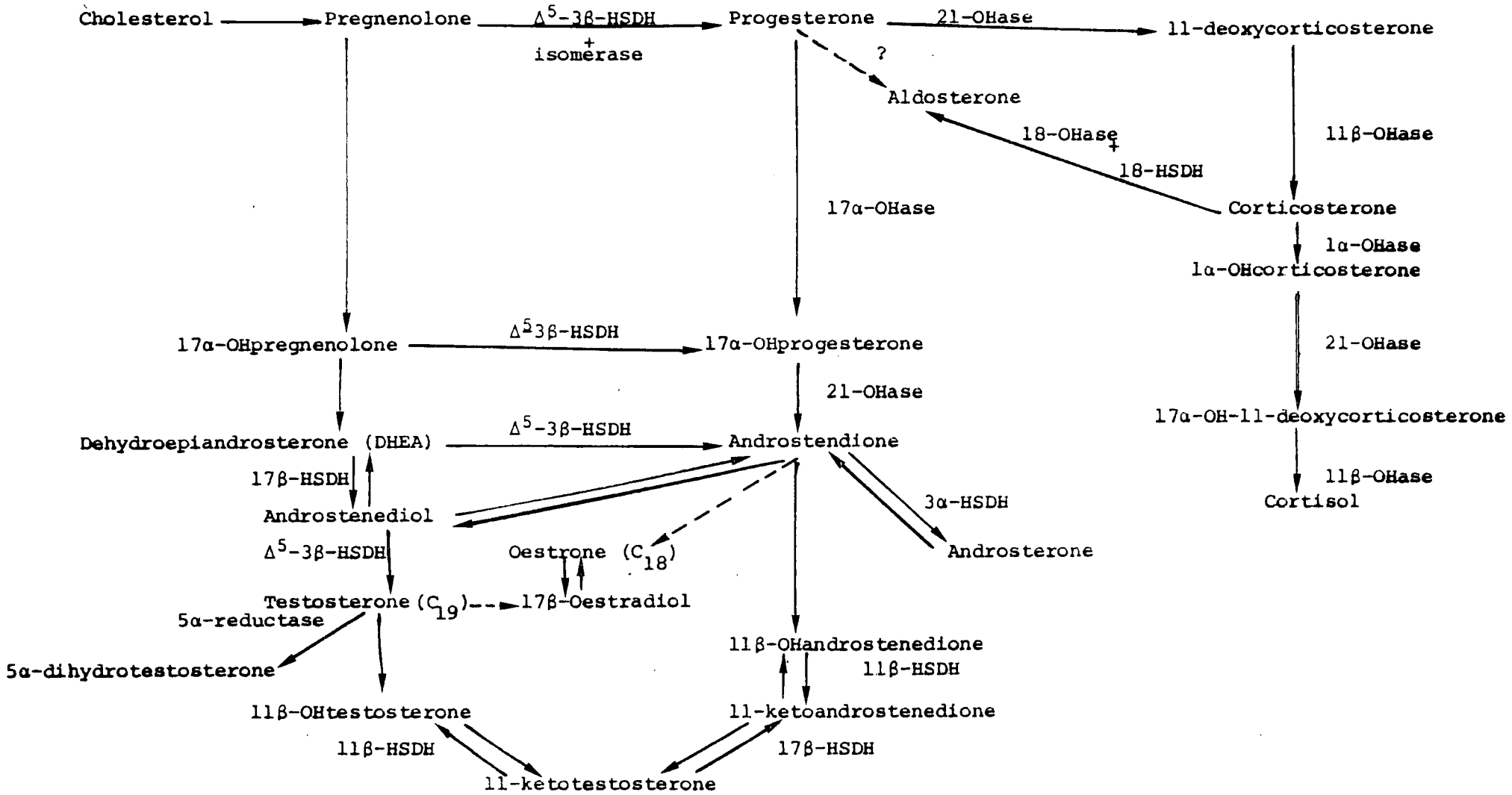
Studies of the gonadotrophic activity in stimulating synthesis of the steroidogenic enzymes by the above authors was based on research using heterologous hormones. Muller (1977) in contrast used homologous gonadotrophin in investigating gonadotrophic activity in Rana catesbeiana testicular steroidogenesis. He found that LH of Rana catesbeiana was more potent than FSH, in raising 5α-dihydrotestosterone concentration in the amphibian plasma. Therefore it could be said that exogenous gonadotrophins of all types have a stimulatory effect on the rate of steroid biosynthesis in Xenopus and other amphibians, indicating the involvement of the adenohypophysis in the gonadal function of adult animals. Furthermore, it would seem that gonadal steroidogenic activity in amphibians does not differ significantly from that in mammals. Both the metabolic pathways and enzymes involved in the sex steroid synthesis from labelled precursors in vitro, in gonads, show many similarities between amphibians and mammals (Ozon 1967).

Tadpole sex differentiation, prior to metamorphosis, was found in the past to be independent of hypophyseal control, being demonstrated in frogs by Chang and Witschi (1955a) and in salamanders by Mintz and Gallien (1954). In contrast interrenal growth and

development, and presumably Δ^5-3β-HSDH activity, was found to be under strict hypophyseal control (Witschi 1955). Hanke and Weber (1964) found that long day length conditions or ACTH injections stimulated the activity of Δ^5-3β-HSDH in the adrenal cortex of inactive winter adults of Rana temporaria. Recently Horn (1976) investigated the possible involvement of brain areas other than the pituitary, such as the hypothalamus or mesencephalon, in the interrenal control and Δ^5-3β-HSDH activity in Xenopus larvae. She transplanted sections of 3 month old Xenopus brain into st.48/49 hypophysectomised larvae and found that the juvenile hypothalamus had an ACTH-like effect on larval interrenals independent from the hypophysis, whereas the mesencephali ACTH-like activity was dependent on the adenohypophysis. It remains to be seen whether a similar, direct or indirect ACTH-like stimulation of the tadpole gonadal Δ^3-3β-HSDH activity could be obtained in Xenopus.

Whereas no enzyme activity was observed in the present work in the liver of Xenopus larvae, Hsu and Hsu (1976) demonstrated histochemically Δ^5-3β-HSDH activity in the liver of Rana catesbeiana tadpoles. There could be a species specificity of the enzyme. In the adult Xenopus the enzyme was present in the liver, perhaps indicating its involvement in bile acid production from cholesterol, as it was shown in rat liver by Bjorkhem (1969) and Bjorkhem, Gustafsson and Gustafsson (1970). The enzyme could be involved in the conversion of circulating pregnenolone, 17α-hydroxypregnenolone and DHEA, to their respective metabolites (see Text Fig. 2). Therefore the liver could be a site of Δ^5-3β-HSDH activity involved in Δ^5-3β-ol steroid metabolism, additional to the interrenals and gonads. The inability to show the enzyme in the liver of tadpoles

Text Fig. 2. Metabolic interrelationships of steroid hormones and function of Δ^5-3β-steroid dehydrogenase and hydroxylases.*



* compiled from mammalian investigations

histochemically, could mean either that the enzyme was present in insufficient quantities to evoke a histochemical reaction or was completely absent at that stage. The absence of the enzyme would probably indicate that not enough circulating Δ^5-3β-ol steroids were available in the larvae to require its presence in the blood-stream, the interrenals and gonads possessing sufficient enzyme to handle the steroids with these organs. The demonstration of the enzyme in the wall of the Mullerian ducts of the adult female, probably indicates active transduction of steroids by the oviducts in the adult Xenopus.

Small amounts of the same Δ^5-3β-HSDH enzyme was found in the wall of the duodenum of both male and female larvae at st.56 onwards, but , no enzyme activity was observed in the duodenum of the adult. Very few demonstrations of hydroxysteroid dehydrogenase activity have been reported in the intestinal wall of other vertebrates. Baillie, Calman and MacKay (1966) recorded presence of 17β-HSDH only in the wall of the small intestine in man, and no HSDH enzyme activity was observed in fish, amphibian or reptilian intestines (Baillie, Ferguson and McHart 1966). A possible function of the enzyme Δ^5-3β-HSDH in the tadpole intestine, could be involvement in steroid inactivation and excretion. The possibility of this was supported by autoradiography results (see page 139) where injected radioactively labelled oestradiol and progesterone respectively were found in the mucosa of the duodenum in larvae and juveniles. Diczfalusy, Franksson and Martinsen (1961) and Diczfalusy, Franksson, Lisboa and Martinsen (1962) showed that the human intestine can convert administered oestriol and 17β-oestradiol, into water soluble glucoronosides. Likewise Smith , Tapley and Ross (1963) using

everted gut sacs from rat small intestine demonstrated, *in vitro*, the formation of a glucoronoside from $|C^{14}|$-17β-oestradiol. They found that there was a net movement of radioactivity from the mucosa to the serosal side and that firstly oestradiol was oxidised to estrone and this in turn formed a water soluble glucoronide by conjugation. Therefore, the small intestine in *Xenopus*, could function as steroid metabolic organ too, converting steroids or steroid metabolites coming from the liver in the bile or the intestinal blood circulation, into their water soluble glucoronides which are reabsorbed and finally excreted by the kidney.

The interrenal hyperplasia and masculinization induced by the high dose oestradiol, confirmed the findings by Padoa (1938), Gallien (1941), Witschi (1951b, 1953) and Chang and Witschi (1955). The oestradiol treatment was carried out to establish whether there is any change in Δ^5-3β-HSDH activity in the gonads and interrenals thus examining the possibility of steroidogenic enzyme involvement in hormone induced sex change. It has been found that oestradiol inhibited stoichiometrically the activity of Δ^5-3β-HSDH in *Pseudomonas testosterone* (Goldman 1967) in bovine adrenal microsomes (Goldman 1968) in human placenta preparations (Schwarzel, Kruggel and Brodie 1973) and in gonadal preparations of *Pleurodeles waltlii* larvae (Collenot 1965). Furthermore Botte and Delrio (1967) showed histochemically that in adult *Rana esculenta* and *Lacerta sicula*, oestradiol administration diminished the activity of Δ^5-3β-HSDH in the testes of both species. Spiegel, Jones and Snyder (1978) suggested inhibition of the enzyme activity by oestradiol in *Rana pipiens* ovarian follicles and Hsu

and Hsu showed histochemically and biochemically similar inhibition of the same enzyme in the liver (1976) and in the interrenals and ovaries (Hsu et al. 1977; Hsu, Liang and Hsu 1978) of Rana catesbeiana tadpoles.

The pharmacological dose of oestradiol practically abolished enzyme activity in the gonads (see Table 18). There was similar-to-control formazan deposit intensity of Δ^5-3β-HSDH in the interrenals and an extensive increase in the area covered by them due to the hormone-induced hyperplasia. Thus it seems that there was, paradoxically, no visible decrease of enzyme activity in the interrenals. Furthermore, it could be possible that the induction of paradoxical masculinization caused by oestradiol, was due to the disturbance of steroidogenesis in the gonads of larvae by inhibition of Δ^5-3β-HSDH activity. Hsu and Liang (1978) and Hsu, Hsu and Liang (1979) reported that treatment with cyproterone acetate caused masculinization of the ovaries similar to that induced by oestradiol. Chronic treatment with cyproterone acetate was shown to inhibit enzyme activity of the gonads and the liver of the Rana catesbeiana larvae. They had already shown (Hsu and Hsu 1976) using the same species that treatment with metapirone, a competitive inhibitor in the biosynthesis of C_{11} and C_{19} steroids, causes degeneration of the larval ovaries and interrenal hypertrophy. All the above evidence points out to a direct relationship between disturbance of steroidogenesis and paradoxical masculinization. Hsu, Liang, Yu and Chiang (1978) suggested that inhibition of Δ^5-3β-HSDH activity would lead to blockage of oestrogen production and accumulation of DHEA (see Text Fig. 2), a weak androgen, which could be acting as a masculinizing agent. This of course could have been

the case if tadpole gonads were shown for certain to be able to synthesize sex steroids. The undifferentiated gonad of Rana catesbeiana was found to possess Δ^5-3β-HSDH activity (Hsu et al. 1977). Biochemical evidence of gonadal steroid biosynthesis at early stages of development in other amphibian species was given by Dale (1962), Ozon (1963), Breuer (1969) and Rao, Breuer and Witschi (1968, 1969). Traces of oestrone were demonstrated by chromatography in the ovaries of juvenile Xenopus by Gallien and Chalmerau-le Foulgoc (1960). However, for any final conclusions to be reached, a more direct demonstration of steroid biosynthesis by tadpole gonads and interrenals would be necessary.

The adrenogenital syndrome obtained by high dose oestradiol treatment was first reported by Witschi (1953) and confirmed by similar findings by others (Chang and Witschi 1955b; Hsu, Chiang and Liang 1973; Hsu, Huang, Chiang and Liang 1974). The interrenal hyperplasia obtained in intact tadpoles by oestradiol treatment was caused by the hormone reducing Δ^5-3β-HSDH activity in the interrenals and thus shortage of corticosteroids was created triggering the negative feed back mechanism and consequent hypertrophy. Likewise Bongiovanni, Eberlein, Goldman and New (1967) reported a genetic defect of Δ^5-3β-HSDH enzyme in the adrenogenital syndrome in man. Also Parks, Bermudex, Anast, Bongiovanni and New (1971) attributed the adrenogenital syndrome in a pubertal boy to Δ^5-3β-HSDH deficiency. Furthermore, Saidabur and Nadkarni (1975) showed that oestradiol acetate injection in adult Rana cyanophlyctis and Rana tigrina, caused regression of the testicular interstitial tissue cells, decreased Δ^5-3β-HSDH activity and increase in the amount of cholesterol

in the testes, possibly indicating reduction of testosterone formation in the frog testes. Since also Collenot (1964) found no enzyme activity in the gonads of Pleurodeles waltlii tadpoles, treated with oestradiol benzoate, the above evidence would suggest that gonadal masculinization and interrenal hyperplasia in Xenopus could be separate expressions of oestradiol action, direct or indirect on Δ^5-3β-HSDH activity, the former possibly being pituitary independent and the latter pituitary dependent.

The contrast between the hypothesis of Witschi (1931) of the gonadal inductors (cortecin and medullarin) being proteinaceous substances and unrelated to steroid hormones and that of Willier (1939) supported by Wolff (1950), Haffen (1977) and Woods and Erton (1978) claiming their steroid nature, may in fact prove to be a false antithesis and the two hypothesis be capable of amalgamation. Recently Chieffi, Iela and Rastogi (1974) caused masculinization of Rana esculenta tadpoles by cyproterone acetate treatment. They concluded that since cyproterone acetate in adult frogs was acting as an antiandrogen competing with testosterone and thus inhibiting the adult testes, masculinization of the larval ovaries provided further evidence that the inductors of gonadal differentiation were not sex steroids. However Hsu et al. (1979) found that cyproterone acetate apart from being an antiandrogen, clearly acted as an inhibitor of Δ^5-3β-HSDH activity in the liver and ovaries of intact and hypophysectomised larvae of Rana catesbeiana, causing ovarian masculinization. This close relationship between steroidogenic enzyme activity and ovarian masculinization after treatment with oestradiol (Hsu et al. 1978) or cyproterone acetate, led them to support the steroid theory of sex differentiation.

Therefore it could be suggested that oestradiol might have brought about gonadal changes in Xenopus larvae by interfering with steroidogenesis, and that cortecin and medullarin inductors could be enzyme systems in the steroid metabolic pathway, perhaps steroid dehydrogenases. Also as it was shown in earlier experiment (see page 70), 5α-dihydrotestosterone treatment caused modification of the heterogametic female sex, and a possible change of Δ^5-3β-HSDH activity in the larval gonads by androgen would give additional support to the steroid theory.

G-6-PO_4 DH was demonstrated in the interrenals and gonads of Xenopus larvae, its activity following a similar pattern to that of Δ^5-3β-HSDH (see Table 17). Since the enzyme was shown to be NADPH provider in carboxylic pentose phosphate pathway, which is an important coenzyme necessary for steroid synthesis, its presence in the gonads indicate a possible steroidogenic potential of the larval testes and ovaries. Cohen (1959) demonstrated high G-6-PO_4 DH activity in the fasciculata and reticularis of rat adrenal cortex, and Goldman, Baker and Stanek (1972) showed biochemically the existence of both G-6-PO_4 DH and Δ^5-3β-HSDH enzymes in the adrenals, testes and ovaries of rabbit foetuses. Thus areas of active steroid synthesis seem to possess G-6-PO_4 DH activity. Treatment of tadpoles with a high dose of oestradiol completely inhibited enzyme activity in the gonads but not in the interrenals, where paradoxically there was no observable inhibition. Therefore both dehydrogenases were affected in the gonads by oestradiol.

A positive lipid reaction appeared in the interrenals later than the enzyme activity, at st.54 (see Table 17). Perhaps a more

sensitive fluorescence method using 3:4-benzpyrene or phosphine 3R and other lipid stains such as Fettrot or Oil Red O, should have been used rather than Sudan Black B alone, in determining the exact tadpole stage of lipid appearance and the nature of lipids. No sudanophilic droplets were found in the larval gonads, indicating either absence or most probably insensitivity of the stain to pick out minute amounts of lipid material. In Xenopus interrenals, lipids appeared in the form of large and fine black droplets. The numbers of fine droplets and the intensity of colour increased at st.56 remaining constant till the end of metamorphosis at st.66 when the interrenals contained mainly large, low colour-intensity droplets; this probably indicates a decrease in the total amount of lipid (Rapola 1962). The gradual depletion of the lipid material could be due to the persistently high enzyme activity present, probably indicating active steroid synthesis.

After oestradiol treatment, the interrenals showed similar to control droplets present, possibly indicating an overall non-interference by the hormone in the amount of lipid material. In the adult interrenals, coarse, low colour-intensity sudanophilic droplets appeared, probably indicating active steroid synthesis and thus depletion of the precursor material. The adult ovary showed large lipid droplets in the cytoplasm of oocytes, whereas fine droplets only were found in the interstitial tissue of testes. Guraya (1972) suggested that the lipids in the interstitium could be the precursors of steroid hormones in the male. Lofts and Boswell (1960) and Lofts, Wellen and Benraad (1972) showed that cyclical events occurred in the interstitial tissue involving seasonal accumulation and depletion of the lipids and cholesterol

probably indicating the degree of androgenic activity in the tissue.

9.5 Summary

The two enzymes, Δ^5-3β-HSDH and G-6-PO_4 DH, first appeared in the interrenals at st.49 and lipids later on at st.54, giving these glands a potential power for steroid biosynthesis. There were no lipids present in the larval gonads indicating either presence of amounts too small to be picked up by the stain or total absence. Perhaps the interrenals which are very near to the gonads provide the necessary precursor material. Larval gonads first acquired enzyme activity and possibly steroidogenic ability at st.56. Weak enzyme activity was found in the mucosa of the duodenum of larvae.

Treatment with a high dose of oestradiol, induced interrenal hyperplasia and paradoxically caused no reduction in the enzyme activity in these glands. It also almost eliminated altogether the enzyme activity in the gonads, indicating a direct interference of the hormone in steroid metabolism perhaps causing the sex change.

CHAPTER 10

FATE OF RADIOACTIVELY LABELLED SEX HORMONES AND URIDINE IN THE BODY OF XENOPUS

10.1 Introduction

It has been noted that in teleosts and amphibia (see page 4) functional sex reversal has been achieved using sex steroids, while in lizards, in the chick and in the opossum although full inversion has not been achieved by this method, administration of small quantities of oestrogens produced ovotestes in the male embryos (Gorbman 1939; Willier 1939; Burns 1955, 1959). Furthermore, endocrine control of the development of the accessory sex structures involves the production, secretion and utilization of minute amounts of sex hormones. Following the fate of these sex hormones in the body of animals or tissues in vitro, was an important factor in the understanding of hormone-induced sex reversal. Detection and localization of physiological amounts of sex steroids in the living animals or in isolated organs has been made possible by auto-radiographic techniques. Initially Barry, Eidnoff, Dorbiner and Gallagher (1952) injected testosterone-4-C^{14} intraperitoneally into rats and mice respectively, but they failed to detect any significant accumulation of the labelled steroid in the sex organs of the animals. Later Greer (1958) employing the same radioactively labelled male hormone of higher specific activity, successfully demonstrated accumulation of radioactivity in the ventral prostates and seminal vesicles of pubescent rats. Hishida (1962, 1965) using radioactively labelled testosterone and oestrone respectively, demonstrated accumulation of radioactivity in the differentiating gonads of larvae

of the medaka, Oryzias latipes. By analysing all steroids present in the larval body he demonstrated that a conversion of oestrone to oestradiol has taken place in the larvae.

Autoradiographic experiments have also been carried out on reptiles and amphibians to investigate the uptake, retention and accumulation of steroid hormones in the particular target organs. Dufaure and Chambon (1978) injected tritiated testosterone into castrated male lizards of Lacerta vivipara and found selective accumulation of radioactivity in the epididymis only during the spring, the period of sexual activity. At the same time Martinez-Vegas, Keefer and Stumpf (1978) demonstrated selective concentration of tritiated oestradiol by various areas of the brain of another lizard, Anolis carolinensis. Morrell, Kelley and Pfaff (1975) using both male and female adult Xenopus, showed by autoradiography retention of tritiated oestradiol and testosterone respectively by various areas of the amphibian brain.

Radioactively labelled oestradiol, testosterone and progesterone hormones were also administered to intact or castrated mammals and the distribution and accumulation of these hormones or their metabolites in the various target organs was followed by autoradiography. Flesher (1965) demonstrated preferential uptake of radioactivity by the rat endometrium following the injection of tritiated oestradiol and King and Gordon (1966) showed accumulation of radioactivity in the uterus of ovariectomized female rats following the injection of the same tritiated sex steroid. Furthermore Stumpf (1969) using dry-mount high resolution autoradiography showed accumulation of radioactivity in the ovaries, vagina, testes, parts of the brain, liver and adrenal, after injection of tritiated oestradiol in intact immature and castrated mature rats. More recently Sar and Stumpf

(1973) demonstrated localization of radioactivity in the brain of intact immature and mature castrated male rats by dry-mount autoradiography following the injection of tritiated testosterone. They found radioactivity in the brain areas associated with the regulation of gonadotrophin secretion and male sex behaviour. Using a precursor of sex steroids, Taylor and Wright (1971) detected accumulation of radioactivity in the bile ducts in the liver of mice two minutes after the injection of tritiated progesterone.

Tritiated oestradiol was injected into larvae of Xenopus of various developmental stages and into 7 month old juveniles and autoradiographs were prepared in order to investigate the fate of the female hormone in the body of the animals. In addition the effect on the distribution of tritiated oestradiol and tritiated uridine respectively in the target tissues was investigated following pretreatment of larvae with cold oestradiol. By associating cold oestradiol treatment with the uptake of a radioactively labelled RNA precursor such as uridine, any hormone stimulation of RNA synthesis could be detected. Finally tritiated progesterone was injected into larvae and juveniles, the animals being killed at various time intervals and autoradiographs prepared in order to follow the fate of this hormone in the body of Xenopus.

10.2 Materials and Methods

For the preparation of autoradiographs, tadpoles and juveniles were fixed for 24 hours in Bouin dehydrated through a series of alcohols and chloroform and finally embedded in wax.* Serial sections of 8μm, from anus to the brain, were mounted on precleaned glass

* parallel trials using frozen sectioning gave same ultimate results. The method of processing described here was therefore adopted as it was much more convenient

slides coated with gelatin solution. A couple of drops of distilled water were put on to the slide surface so that the sections floated and thus could be manipulated easily. Then the slides were dried on a hot plate. Before applying the nuclear photographic emulsion on top of the sections, all the wax was removed by leaving the slides in xylene for 20 minutes.

The slides had been cleaned by allowing them to soak overnight in a mixture of potassium bichromate and concentrated sulphuric acid (100g of $K_2Cr_2O_7$ in 800ml of water plus 200ml of conc.H_2SO_4 which was added very slowly). After the acid bath the slides were washed in tap water, followed by two 15 minute changes of distilled water. They were then coated by dripping them in the gelatin solution made up as follows:- 2.5g of gelatin and 0.25g of chrome alum were added to 500ml of distilled water and the mixture was warmed up to dissolve the gelatin. Filtering the solution ensured elimination of any undissolved material. The final solution was allowed to cool before use. The coated slides were put in slide containers and in the oven at about 35°C to dry. The layer of dried gelatin on the slides provides good adhesion both for the sections and the emulsion.

The preparation of the autoradiographs was done in the dark room under safe light conditions. Using a plastic spoon, Ilford K_5 photographic emulsion was put in a clean measuring cylinder and was left for half an hour in a water bath at 43°C to melt. 25ml of this liquid emulsion was added to a dipping jar already containing 24.5ml of distilled water and 0.5ml of glycerol.

The mixture was stirred slowly with a glass rod and then left standing in the water bath at 43°C for about 10 minutes to get rid of any bubbles. Each slide was dipped twice vertically downwards in the emulsion, touching its corner on the side of the dipping jar to get rid of excess emulsion. Then each slide was allowed to drain for a couple of minutes and its back wiped clean with tissue paper. Afterwards all slides were placed on the precooled copper plate to gel with the emulsion side facing upwards (the crushed ice was left in the side arms of the cooling apparatus for an hour to ensure precooling of the copper plate). They were left on the plate for about 2 hours and then put on trays in slide boxes. Each box was tightly wrapped in a black polythene bag to protect slides from daylight. All boxes were put in a refrigerator where the slides were exposed for 21 days at 4°C. After exposure they were developed in total darkness for 3 minutes in D19 Kodak developer at room temperature, then quickly washed in distilled water and finally fixed in 1:5 dilution of kodafix for 4 minutes. After fixation the slides were washed in running water for 15 minutes, stained in haemalum for 5 minutes, washed quickly in distilled water, differentiated in alkaline alcohol and then taken through alochols to xylene. Coverslips were mounted with DPX and after drying on a hot plate, the slides were ready for observation.

Three radioactively labelled compounds were employed for autoradiography:-

(i) $|6,7\text{-}^{3}H|$-oestradiol in benzene/ethanol (9:1 v/v) solution, in borosilicate multidose vial sealed under nitrogen, of specific activity 42 Ci/mmol (154 mCi/mg), from Radiochemical Centre, Amersham.

(ii) 250µCi of |1,2,6,7-^{3}H|-progesterone in benzene solution, specific activity 81 Ci/mmol (0.5 Ci/l).

(iii) 250µCi of |5,6-^{3}H|-uridine in sterilized aqueous solution, activity 40-60 Ci/mmol.

The benzene and ethanol in the first two solutions were evaporated under a stream of nitrogen gas, the steroid being redissolved in two drops of absolute ethanol (analytical grade) and made up to the required volume with warm amphibian Ringer. The new radioactive steroid solution was then used for the injections.

Using the radioactive compounds mentioned, experiments were devised in order to investigate the fate of steroids in the larval and juvenile body of Xenopus and the effect, if any, of oestradiol on RNA synthesis. At first, 10 larvae were put in 400ml of tap water containing 15µCi of tritiated oestradiol. Five larvae were killed after 4½ hours, the remaining five after 24 hours of treatment and autoradiographs were prepared. Five extra control larvae were used for each period of treatment respectively, left in water containing alcoholic ringer but no tritiated steroid. Furthermore, groups of five larvae of various developmental stages (52, 54, 55, 56, 57, 58 and 62) were injected intraperitoneally with tritiated oestradiol (5µCi/10 l/tadpole), killed at various time intervals (after respectively 3, 15, 20, 30 minutes and 1, 5, 24 and 48 hours) and autoradiographs were prepared. An extra control tadpole per time interval was injected with alcoholic ringer only.

Another experiment was carried out to investigate the effect, if any, of pretreatment with cold oestradiol on the fate of injected tritiated oestradiol (5µCi/10µl/tadpole) in the larval body

(5 larvae treated with 100µg/l oestradiol for 26 days from st. 51 to st.57). Larvae were killed at ½hour, 5 hours and 24 hours respectively after injection of the tritiated steroid. There were five control tadpoles per time interval injected with tritiated oestradiol only. Also for comparison tritiated oestradiol was injected into 7 month old juvenile animals (5µCi/10µl/Xenopus). These were killed for autoradiography after 5, 15 minutes, 24 and 48 hours respectively.

To investigate the effect of oestradiol on RNA synthesis, tritiated uridine was employed. Two groups of five larvae each, were pretreated for 21 days with cold oestradiol (100µg/l from st. 51 to st.56), before they were injected with tritiated uridine and killed after 4½ and 24 hours respectively. The two groups of controls were only injected with tritiated uridine and killed at the same time intervals.

For comparison a precursor of sex steroids, tritiated progesterone was injected into larvae and juveniles (5µCi/10µl/animal) and then killed at various time intervals. There was a control animal per time interval devoid of labelled progesterone.

10.3 Results

When larvae were kept in water with tritiated oestradiol and killed after 4.5 and 24 hours respectively, the autoradiographs obtained showed masses of black silver (Ag) grains in the mucosa of the duodenum (Figs. 299, 300, 301) which were close together and practically impossible to dinstinguish from one another (+++++ in Table 20 p140) There was no detectable difference in numbers of

TABLE 20. Fate of |6,7-^{3}H|-oestradiol in the body of larvae

Time after commencement of treatment	Stage	Mean length mm	Mean weight mg	Phenotypic Sex ♂	♀	Ind.	Index of Ag grain numbers[a] Muc.	Gon.	Liv.
Treatment A[b]									
4.5 hours	56	46.2±0.7	460±11.0	3	2	0	+++++	-	-
24 hours	56	46.4±0.6	466±7.1	4	1	0	+++++	-	-
Treatment B[c]									
3 minutes	56	46.4±0.5	468±15.0	2	3	0	+++++	-	+++++
15 "	55	44.2±0.9	442±19.1	3	2	0	+++++	-	+
15 "	57	54.2±0.9	724±43.4	2	3	0	+++++	-	+
15 "	58	61.2±0.9	968±36.8	1	4	0	+++++	-	+
15 "	62	53.6±0.5	572±16.9	3	2	0	+++++	-	+
20 "	54	42.2±0.7	338±7.4	1	4	0	+++++	-	-
30 "	55	46.2±0.6	438±22.9	3	2	0	+++++	-	-
1 hour	56	46.4±0.5	454±13.3	4	1	0	+++++	-	-
5 hours	52	34.0±0.7	196±6.8	0	0	5	+++++	-	-
24 hours	56	46.6±0.6	460±9.5	4	1	0	+++++	-	-
48 hours	56	46.8±0.6	464±8.7	2	3	0	+++++	-	-

[a] Number of crosses represent the relative numbers of Ag grains. Five crosses indicate dense accumulation of grains (equivalent to very high radioactivity) practically impossible to separate, whereas one cross indicates very few Ag grains present. The negative sign indicates no grains present.

[b] larvae raised in 400ml of water with 15µCi of oestradiol present.

[c] larvae injected with 5µCi/10µl each.

Ind: indifferent gonadal sex
Muc: mucosa of the duodenum
Gon: gonads
Liv: liver

grains deposited after two lengths of period of treatment. It is certain that no Ag grains were deposited anywhere else in the tadpole body since autoradiographs throughout the body from the anus to the brain were carefully examined. The control larvae showed no Ag grains in their body.

The autoradiographs of larvae injected with tritiated oestradiol showed a similar picture. When tadpoles were killed 3 minutes after injection there was again a very dense accumulation of Ag grains in the mucosa of the duodenum and in the liver (Figs. 304-305, and +++++ in Table 20). After 15 minutes most of the radioactive steroid had left the liver (Fig. 306 and + in Table 20), but there was still very high numbers of Ag grains in the duodenal mucosa. From 20 minutes onwards only the intestinal mucosa showed Ag grains (Figs. 302, 303). All controls showed no radioactivity.

Pretreatment of larvae with cold oestradiol seemed to reduce the numbers of grains in the duodenal mucosa and the grains were also distributed throughout the wall (Fig. 308-309) as compared to the non-pretreated controls (Fig.307) probably indicating faster metabolism of the tritiated oestradiol due to cold oestradiol pretreatment (see Table 21 p 142). On the other hand such pretreatment with cold oestradiol had no effect on the distribution of tritiated uridine (at 4.5 and 24 hours) in the gonads or any other part of the larval body (see Table 22 p 143). Similar numbers of Ag grains appeared everywhere in both control and pretreated larvae (see Figs. 310-313 and compare with Figs. 314-316; Figs. 317-320 and compare with Figs. 321-323).

TABLE 21 Distribution of injected |6,7-^{3}H|-oestradiol (15µCi/10µl/st.57 larva) following 26 days pretreatment with cold oestradiol

Treatment	Time after injection (hours)	Mean length mm	Mean weight mg	Phenotypic Sex ♂	♀↓	♀	Index of Ag grain numbers Muc.	Gon.	Liv.
C + ^{3}H-E_2 *	0.5	54.2±0.9	698±30.7	1	0	4	+++++	-	-
E_2 + ^{3}H-E_2 **	0.5	55.2±1.1	720±35.6	0	3	2	++	-	-
C + ^{3}H-E_2	1.0	54.8±1.1	722±56.1	2	0	3	+++++	-	-
E_2 + ^{3}H-E_2	1.0	55.0±1.0	730±36.5	0	2	3	++	-	-
C + ^{3}H-E_2	5.0	54.2±1.7	704±57.8	4	0	1	+++++	-	-
E_2 + ^{3}H-E_2	5.0	54.0±0.7	706±29.3	0	1	4	++	-	-
C + ^{3}H-E_2	24.0	53.6±1.0	686±32.0	3	0	2	+++++	-	-
E_2 + ^{3}H-E_2	24.0	54.4±1.2	710±41.0	0	2	3	++	-	-

* control, alcohol-treated larvae, injected with tritiated oestradiol.

** larvae pretreated with cold oestradiol (100µg/l for 26 days) and then injected with tritiated oestradiol.

TABLE 22 Distribution of injected |5,6-^{3}H|-uridine (5µCi/10µl/st.56 larva) following 21 days pretreatment with cold oestradiol.

Treatment	Time after injection (hours)	Mean length mm	Mean weight mg	Phenotypic Sex ♂	♀↓	♀	Index of Ag grain numbers MB	Gon.	IR
C + ^{3}H-U*	4.5	51.0±0.6	530±14.1	2	0	3	+++++	++++	++++
E_2+^{3}H-U**	4.5	50.4±0.8	524±22.5	0	2	3	+++++	++++	++++
C +^{3}H-U	24.0	51.0±0.7	544±22.3	1	0	4	+++++	++++	++++
E_2+^{3}H-U	24.0	51.2±0.6	550±21.0	0	3	2	+++++	++++	++++

MB: mesonephric blastema

Gon: gonads

IR: interrenal gland

* : control, alcohol-treated larvae, injected with tritiated uridine

**: larvae pretreated with cold oestradiol (100µg/l for 21 days) and then injected with the tritiated uridine.

TABLE 23 Fate of injected |6,7-^{3}H|-oestradiol (5µCi/10µl/animal)

in juvenile, 7 months old, Xenopus.

(2 males and 2 females used in each case)

Time after injection	Mean length cm	Mean weight g	Index of Ag grain numbers KT	BV	DM	CP
5 minutes	3.7±0.6	3.5±0.5	+++++	++++	++	+++
15 minutes	3.3±0.1	3.3±0.5	+++++	++++	++	+++
1 hour	3.2±0.1	3.0±0.2	++++	++++	++	+++
5 hours	3.0±0.1	2.8±0.1	++++	++++	++	+++
24 hours	3.3±0.2	3.4±0.6	++	++++	+	+++
48 hours	3.3±0.1	3.6±0.4	++	++++	+	+

KT: wall of kidney tubules

BV: blood vessels, including hepatic and heart

DM: duodenal mucosa

CP: choroid plexus

TABLE 24 Fate of |1,2,6,7-^{3}H|-progesterone (5µCi/10µl/st.56 larva) injected into the larval coelom

Time after injection	Mean length mm	Mean weight mg	Phenotypic Sex ♂	♀	Index of Ag grain numbers Muc.	Gon.	Liv.
3 minutes	50.2±1.5	552±46.2	2	3	+++++	-	+
15 minutes	51.6±1.3	590±26.8	1	4	+++++	-	-
1 hour	48.0±0.7	510±26.8	3	2	+++++	-	-
5 hours	48.8±0.9	528±27.3	3	2	+++++	-	-
24 hours	49.6±1.3	524±31.9	2	3	+++++	-	-
48 hours	48.0±2.0	474±60.8	4	1	+++++	-	-

TABLE 25. Fate of |1,2,6,7-^{3}H|-progesterone (5µCi/10µl/animal) injected into the coelom of juvenile, 7 months old, Xenopus.

(2 males and 2 females used in each case)

Time after injection	Mean length cm	Mean weight g	Index of Ag grain numbers KT	BV	DM	CP
5 min	3.0±0.2	3.0±0.2	+++++	++++	++	+++
1 hour	3.1±0.1	3.2±0.2	++++	++++	++	+++
24 hours	3.2±0.1	3.1±0.1	++	++++	+	+++
48 hours	3.0±0.1	2.6±0.2	++	++++	+	+

Autoradiographs of juvenile Xenopus injected with tritiated oestradiol, showed varying numbers of Ag grains in the wall of the kidney tubules (Figs. 325, 326), the blood (Fig. 324), duodenal mucosa (Figs. 327, 328) and choroid plexus of the brain (Figs. 329, 330). No Ag grains were detectable in the gonads or anywhere else but those tissues already stated. With increasing time after ibjection the numbers of Ag grains in the wall of the kidney tubules and the duodenal mucosa were decreased probably indicating excretion of the steroid (see Table 23).

Tritiated progesterone injection of larvae, produced again high accumulation of grains in the duodenal mucosa (Figs. 332, 333). There was only a small number of grains in the liver at 3 minutes after injection (see Fig. 331 and Table 24), probably indicating faster metabolism of progesterone than of oestradiol. Autoradiographs of juvenile Xenopus injected with labelled progesterone showed a similar picture to the juveniles injected with tritiated oestradiol (see Figs. 334-339 and Table 25).

10.4 Discussion

The results obtained by autoradiography indicate that the duodenum and the liver play an important role in the metabolism of sex steroids in the larvae of Xenopus. Larvae kept in water containing tritiated oestradiol for 4.5 and 24 hours respectively showed intense accumulation of radioactivity as revealed by the Ag grains, in the mucosa of the duodenum. Autoradiographs following the injection of the radioactively labelled hormone into tadpoles of various developmental stages showed a similar picture.

It was also found that three minutes after the injection there was an intense accumulation of radioactivity in the liver. Most of it disappeared from the liver after 15 minutes but the duodenum still showed very large numbers of Ag grains 48 hours later. Thus the oestrogen was taken up by the liver in a matter of minutes probably metabolized and then excreted via the bile duct into the duodenum for further metabolism and final excretion. The absence of any radioactivity in the kidney suggests that the duodenum is the major metabolic-excretory organ during larval life. Thus the liver and duodenum of larvae seem to be closely associated with the metabolism of oestradiol from an early stage. Similar results have been obtained by others. Ryan and Engel (1953) demonstrated oxidation of oestradiol to oestrone and further degredation of both steroids to unrecognizable metabolites in vitro, by slices of human liver. The same workers found that the ileum converted part of oestradiol into oestrone. Likewise Engel, Bagget and Halla (1962) reported the conversion of oestradiol to oestrone and then by conjugation, to water soluble oestriol glucosiduronate, by human foetal liver in vitro. Diczfalusy, Franksson and Martinsen (1961) and Diczfalusy, Franksson, Lisboa and Martinsen (1962) demonstrated in vivo the human intestinal mucosa to be an important site of oestrogen conjugation, forming water soluble glucosiduronates. Similar conversion of oestradiol was shown to take place in vitro by pieces of the rat small intestine (Smith, Tapley and Ross 1963).

The persistence of radioactivity in the mucosa of the duodenum and absence in the mesonephros of larvae, even 48 hours following the injection of tritiated hormone probably indicates that the duodenum is the major metabolic excretory organ able to

take care of oestradiol and its metabolites during the larval life of Xenopus. The presence of Δ^5-3β-HSDH enzyme in the wall of the duodenum found earlier (see page114) could be an additional indication that the tadpole duodenum is involved in steroid inactivation and excretion. So it seems that oestradiol and its metabolites in the liver go to the duodenum through the bile, where they are further converted into the water soluble and thus more easily disposable glucurosiduronates, which in turn are secreted into the intestinal blood circulation or more probably are lost in the faeces.

The absence of radioactivity in the larval gonads at any time indicates that oestradiol causes sex reversal affecting the gonads either indirectly via a metabolite or by stimulating neosynthesis of protein in the gonads,

A direct effect of the hormone on the gonads is possible, although its action would have to be extremely rapid as soon as the hormone enters the body. The fact that actinomycin D and puromycin respectively inhibited the oestradiol-induced sex reversal (see page 102) indicates that oestradiol produces its feminizing effect by ultimately stimulating protein synthesis. Failure to observe any increase of radioactivity in the tissues especially the gonads of pretreated larvae that have been injected with tritiated uridine, would suggest that only small quantities of new RNA are involved, via which the hormone manifests its action. Zaccanti and Pasquini (1974a,b) claimed that testosterone induced sex reversal in Rana esculenta

and latastei was linked to RNA neosynthesis. After treating larvae with testosterone and then injecting them with tritiated uridine, they found accumulation of radioactivity in the medullary component of the sex reversed ovaries and in the extragonadal interrenal cells ('medulloblasts') which had descended towards the gonads. These extragonadal cells showed the highest accumulation of radioactivity. Based on these and the antibiotic results (Vannini and Stagni 1967, 1968, 1972), Vannini, Stagni and Zaccanti (1975) applied the derepression mechanism of sex differentiation to the hormone-induced sex reversal in the above two ranids.

Absence of radioactivity in the brain of larvae might indicate that this organ is not involved in early sexual development, whereas the accumulation of Ag grains in the choroid plexus of animals after metamorphosis points to an active involvement of the brain during later life. Also a high accumulation of Ag grains in the wall of the kidney tubules and a respectively low accumulation in the duodenal mucosa probably indicates that the kidney has by this time assumed its primary importance in the excretion of steroids, and that the intestine is no longer the major metabolic-excretory organ of sex hormones.

Pretreatment of larvae with cold oestradiol prior to the injection of tritiated oestradiol showed a markedly reduced radioactivity in the duodenal mucosa in contrast to the control animals which showed a very high accumulation. Thus it seems that the long oestradiol pretreatment has induced the larvae to acquire a much faster metabolism in respect to oestradiol.

The idea behind this pretreatment was that perhaps a basic level of oestradiol is required in the larval body to induce the production of a special oestradiol-carrier-protein, which would take the hormone to the gonads to induce sex reversal. In fact no radioactivity was found in the gonads of any pretreated larvae. Furthermore there was no observable difference between autoradiographs of oestradiol-pretreated larvae and control larvae injected only with the tritiated uridine. The radioactively labelled RNA precursor was found in most tissues of the larval body. High levels of radioactivity was observed in the gonads and interrenals, but the mesonephric blastemata showed the highest accumulation. However, there was no difference in the numbers of Ag grains present in these tissues between the hormone-pretreated and control larvae that had been injected with tritiated uridine.

As shown in the results, progesterone is metabolised very quickly by the liver of st.56 tadpoles. There was very little radioactivity in the liver three minutes after the injection of tritiated progesterone, as compared to the amount of radioactivity found at the same time interval after the injection of tritiated oestradiol. Perhaps the larval liver is more adapted to metabolise progesterone than oestradiol, which may not be present in Xenopus at this stage. Tritiated progesterone injected into juvenile toads presented an autoradiographic pattern similar to that of oestradiol referred to above, perhaps indicating that after metamorphosis all steroids are metabolized similarly in Xenopus.

10.5 Summary

An intense accumulation of radioactivity had appeared in the liver and in the mucosa of the duodenum of tadpoles within

three minutes of the injection of tritiated oestradiol. Radioactivity in the former had disappeared completely within 15 minutes whereas in the latter it persisted for 48 hours. Pretreatment with cold oestradiol prior to the injection of tritiated oestradiol markedly reduced the radioactivity which developed in the mucosa of the duodenum. Following the injection of tritiated progesterone little radioactivity was present after three minutes in the larval liver but the duodenal mucosa showed a very high level of accumulation.

Pretreatment of larvae with cold oestradiol prior to the injection of tritiated uridine had no effect on the distribution of radioactivity as compared to the non-pretreated controls.

Following injections of tritiated oestradiol and progesterone into juvenile Xenopus radioactivity appeared in the wall of the kidney tubules, the blood, the duodenal mucosa and the choroid plexus of the brain in both instances.

CHAPTER 11

CONCLUSIONS

This study has shown that oestradiol and oestradiol benzoate cause sex reversal in male larvae, with the former showing the more obvious feminizing effect. Although the concentrations of hexestrol tested produced no complete feminization, the results suggest that a more extensive investigation into hormone dosages and time periods of treatments would reveal an overall feminizing activity by all three oestrogens used. The 2mg/l (high) dosage of oestrogens showed varying degrees of toxicity, with oestradiol benzoate being the least toxic and hexestrol being highly poisonous to the larvae. Unesterified oestradiol inhibited body growth and development, causing anatomical defects in the larvae and inducing aggressive behaviour. It produced a trend toward intersexuality and it also caused interrenal hyperplasia in all larvae and precocious development of the Wolffian duct in some male individuals.

Progesterone treatment caused no masculinization of female larvae similar to that obtained in ranids by others, merely inhibiting or stimulating ovarian and oocyte growth and development. The high dosage was poisonous to the larvae.

In contrast to oestrogens, the androgen 5α-dihydrotestosterone was not toxic to the larvae even when the high dosage was employed, and testosterone acetate showed toxicity only at the high concentration. It was shown that unlike testosterone acetate, the 5α-form modified the female genetic sex, producing individuals with varying degrees of maleness. It is suggested that either the larval body does not contain the necessary enzyme system to convert

ordinary testosterone into the more active 5α-form or that if present the enzyme is not active. This would explain the inability of testosterone acetate to modify the female genetic sex. Paradoxically the 2mg/l high dosage of 5α-dihydrotestosterone left the normal male:female ratio unaffected. In addition both androgens caused interrenal hypertrophy in all larvae and precocious Wolffian duct development in some males.

The lower dosages of cortisone inhibited the ovaries of female individuals. The high dosage caused intersexuality of females and in a few males stimulated precocious Wolffian duct development. The high dosages of oestradiol, testosterone acetate, 5α-dihydrotestosterone and cortisone stimulated precocious Wolffian duct development in male individuals, suggesting similarity in the anabolic action of these hormones perhaps due to the common basic carbon ring (cycloperhydrophenanthrene nucleus) present in steroids. Progesterone which also has this nucleus was poisonous at high concentration.

Simultaneous administration of actinomycin D or puromycin with oestradiol prevented feminization suggesting that the hormone manifests its action by stimulating neosynthesis of RNA. From consideration of a possible mechanism of hormone-induced sex reversal in Xenopus, taking into account Witschi's inductor theory, Vannini's and Stagni's derepression theory and the steroid theory of sex differentiation, it can be concluded that the sex inductors may be steroids. The sex reversal of the male (homogametic) sex by oestradiol and the female (heterogametic) sex by 5α-dihydrotestosterone gives further support to the steroid theory. The discovery

and identification of such steroids in the larval body prior to and after sex differentiation is now required to confirm this hypothesis. Indirect evidence for the possible presence of steroid hormones in the larvae was obtained from the histochemical work. It was shown that the interrenals acquired Δ^5-3β-HSDH and G-6-PO_4 DH activity prior to sex differentiation. This activity persisted throughout the animal's life. The gonads first showed enzyme activity near the beginning of metamorphosis, and thus much later than the interrenals. A more sensitive biochemical method would perhaps indicate whether the enzyme is completely absent or not in the gonads at earlier stages of development. It seems therefore that both interrenals and gonads possess potential power for steroidogenesis prior to the start of metamorphosis. The inhibition by the 2mg/l oestradiol of the Δ^5-3β-HSDH activity in the gonads suggests a direct connection between paradoxical masculinization and inhibition of steroidogenic activity.

The inability of indifferent larval gonads to grow and develop *in vitro* indicates a close relationship between gonads and some other tissue of the larval body, perhaps involving a gonadal growth agent. It is not surprising that oestradiol treatment of the differentiated larval gonads *in vitro* caused no feminization given that by this stage the gonads already have acquired their sex. Successful maintenance of undifferentiated gonads *in vitro* would permit the investigation of whether oestradiol administration will cause sex reversal outside the body.

Presence of radioactivity in the larval liver and the mucosa of the duodenum after the injection of tritiated oestradiol or progesterone suggests that these are the tissues involved in

steroid metabolism and excretion during early life. The persistence of radioactivity in the mucosa of the duodenum after its disappearance from the liver indicates that perhaps the duodenum is the major metabolic-excretory tissue for steroids. The presence of Δ^5-3β-HSDH in the wall of the duodenum supports this suggestion. Pretreatment with cold oestradiol prior to the injection of the tritiated RNA precursor uridine, produced no increase in the accumulation of radioactivity in the gonads or anywhere else in the larval body. This suggests that oestradiol causes sex reversal by stimulating neosynthesis of very small amounts of protein, not possible to detect by autoradiography. The fact that injection of either of the two tritiated steroids into juvenile animals resulted in accumulation of radioactivity mainly in the wall of the kidney tubules, the blood and the choroid plexus of the brain, indicates that after metamorphosis, the brain is actively involved in sexual development and the kidney is the major steroid excretory organ.

From the above it may be concluded that the administered oestradiol and 5α-dihydrotestosterone hormones may both act as sexual inductors when inverting the phenotypic sex of Xenopus.

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APPENDICES

Table i

St. 51 tadpoles

Counts of primordial germ cells (pgs) per 20 sections of each gonad per larva

1		2		3		4		5		6	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
3	1	4	1	5	1	2	0	3	2	5	4
3	2	0	0	5	3	3	0	4	3	4	4
0	1	2	1	3	0	0	2	2	2	3	2
2	2	3	2	4	0	5	0	1	1	4	3
1	1	2	5	5	2	2	1	0	3	4	1
0	2	1	2	2	0	0	3	2	5	2	5
2	1	2	0	3	4	2	5	6	3	1	5
3	1	3	1	2	2	1	2	4	3	0	4
2	2	3	3	1	1	3	5	5	2	5	2
1	1	1	3	3	1	2	3	2	3	5	3
3	5	4	2	2	2	2	5	4	3	4	2
3	2	3	1	0	1	0	3	2	1	1	0
4	4	2	2	0	2	2	1	1	1	2	1
1	3	1	2	0	0	2	1	2	0	0	5
3	3	1	1	1	0	3	4	3	4	1	4
1	2	3	4	0	0	0	5	5	2	2	2
1	1	2	3	2	3	3	5	1	4	4	3
2	2	2	2	2	0	4	4	3	3	3	4
3	2	1	0	2	1	3	2	2	1	5	4
2	1	1	2	2	2	3	3	1	1	2	2
Σ 40	39	41	37	44	24	41	54	53	47	57	60
$\bar{x}$ 2	1.95	2.05	1.85	2.2	1.2	2.05	2.7	2.65	2.35	2.85	3.0

TABLE ii

St. 57 males (alcohol-treated controls)

Spermatogonia counts per 20 sections of each testis per ♂ N tadpoles

1		2		3		4		5		6		7		8		9		10		11	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
7	6	25	19	16	29	25	17	19	35	23	52	14	23	18	24	12	19	14	25	24	12
5	12	36	15	9	10	27	39	21	10	19	36	11	17	32	30	9	12	16	27	27	15
11	5	17	14	12	25	26	23	19	11	18	26	13	6	22	20	8	24	20	32	21	14
9	21	26	30	13	11	14	30	23	17	11	30	15	10	18	19	18	16	25	28	20	10
26	6	22	13	19	9	33	37	30	34	18	37	17	5	15	6	15	17	13	15	27	17
18	9	45	26	11	38	22	12	13	16	23	35	10	11	9	10	21	20	12	19	12	20
10	4	30	25	5	17	28	10	16	20	20	38	15	15	18	17	22	12	17	12	25	18
6	9	15	28	10	24	15	25	17	30	14	22	20	7	21	30	25	15	16	13	20	12
10	4	16	31	19	28	14	16	20	21	26	25	13	9	26	39	24	8	4	22	30	9
13	11	23	33	14	15	20	21	14	32	28	15	14	5	22	35	19	24	11	24	29	18
10	26	18	16	13	17	30	32	25	26	15	13	15	22	28	49	21	15	26	21	14	22
19	17	24	28	9	14	20	25	18	37	16	15	20	30	50	42	11	23	23	19	16	21
15	16	13	8	20	25	8	23	29	15	15	14	13	11	26	36	15	8	20	14	18	25
17	24	14	16	10	8	18	15	24	22	13	12	12	12	30	27	36	15	15	24	12	15
10	12	16	18	14	20	10	13	28	19	30	24	19	11	28	26	17	13	12	17	15	21
16	28	14	16	13	4	21	19	15	28	13	25	15	18	26	25	30	14	13	10	11	13
20	22	19	28	12	10	15	7	16	20	32	21	17	11	32	18	34	17	17	18	13	14
30	20	17	35	18	20	10	18	13	14	15	14	10	20	20	27	20	18	15	24	28	19
20	25	26	27	13	9	10	16	26	22	17	22	23	26	15	12	27	30	12	28	19	10
13	16	30	24	14	12	15	25	19	13	15	21	15	21	13	16	19	16	18	27	12	18
Σ 285	293	449	450	264	345	381	423	405	442	381	497	301	267	469	508	403	336	324	419	393	323
X̄ 15.05	14.65	22.45	22.5	13.2	17.25	19.05	21.15	20.25	22.1	19.05	24.85	15.05	14.5	23.45	25.4	20.15	16.8	16.2	20.95	19.65	16.15

TABLE iiia

St. 57 females (alcohol-treated controls)

Total numbers of oogonia & all oocytes per 20 sections of each ovary per tadpole

1		2		3		4		5		6		7		8		9	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
74	40	60	46	62	58	40	36	62	50	54	47	68	60	52	45	60	50
61	46	62	59	65	73	35	45	68	57	55	43	65	78	48	40	63	48
66	42	70	36	67	83	58	52	70	47	58	49	67	80	45	58	68	55
58	50	74	48	66	70	60	57	78	56	63	55	74	75	44	52	62	47
43	51	34	56	72	81	45	49	62	74	65	58	78	78	49	47	70	62
52	70	43	67	74	68	52	60	80	75	67	64	58	63	54	42	78	68
50	67	42	70	44	58	42	59	78	72	74	70	50	60	48	46	54	72
48	64	72	62	46	52	65	50	76	70	78	65	59	54	56	40	50	65
40	54	70	69	60	64	52	46	65	62	79	62	72	58	50	64	58	50
52	63	59	61	65	76	48	47	64	45	82	68	70	68	53	56	64	70
49	44	63	66	62	85	43	56	60	46	75	60	68	82	62	58	60	68
73	62	80	40	60	75	45	62	55	40	74	64	54	80	64	63	57	52
64	60	69	38	62	65	62	78	64	68	70	58	56	76	66	60	65	54
61	66	66	45	46	80	60	75	72	78	64	50	48	68	68	48	55	50
65	60	69	49	64	65	58	68	76	80	58	48	70	82	60	56	58	49
58	59	60	56	72	74	65	78	75	71	55	54	74	85	54	58	62	53
69	61	70	50	60	68	62	72	68	74	49	56	72	70	56	60	70	58
72	80	64	62	64	78	70	65	65	62	50	62	68	75	62	55	68	54
74	78	72	58	58	72	58	60	69	60	57	68	60	64	68	52	60	69
55	98	65	51	67	70	64	59	74	63	64	65	69	69	65	49	64	50
Σ 1184	1215	1264	1089	1236	1411	1084	1174	1381	1250	1291	1156	1300	1425	1124	1049	1246	1144
$\bar{X}$ 59.2	60.75	63.2	54.45	61.8	70.35	54.2	58.7	69.05	62.5	64.55	57.8	65.00	71.25	56.2	52.45	62.3	57.2

TABLE iiib

St. 57 females (alcohol-treated controls)

Number of oogonia & early oocytes per 20 sections of each ovary per tadpole

1		2		3		4		5		6		7		8		9	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
24	28	18	24	17	16	23	19	10	9	24	19	23	28	19	22	27	30
26	34	23	45	12	18	16	14	15	12	25	16	25	30	24	28	26	35
37	33	33	24	14	19	26	23	10	8	30	17	26	32	35	30	32	40
42	39	21	39	22	16	11	17	7	10	18	16	30	29	37	29	40	47
30	37	17	30	17	15	23	27	20	14	19	22	35	18	30	35	38	39
50	45	22	25	18	15	16	20	12	20	22	24	26	20	32	18	35	42
18	30	34	27	16	14	15	19	18	10	26	30	20	25	28	16	26	40
26	37	31	29	21	22	11	12	20	16	24	20	19	18	26	25	22	38
28	45	30	33	13	17	11	10	18	8	20	22	17	16	18	23	19	27
27	48	18	26	18	17	19	20	14	18	22	19	22	27	19	31	24	25
48	31	23	20	19	21	20	13	15	16	18	17	26	32	27	40	32	20
33	42	24	29	16	23	18	21	14	8	14	16	28	35	32	45	42	29
37	48	26	32	17	20	24	28	8	12	12	20	29	39	35	28	48	32
40	44	24	44	20	15	19	10	12	16	17	22	35	32	40	23	32	29
35	47	28	35	16	17	21	25	9	7	16	23	28	34	42	19	26	34
39	50	30	42	16	14	24	18	8	6	23	14	36	30	39	26	23	36
42	45	26	40	18	20	35	28	11	14	24	12	34	32	35	28	22	40
32	38	34	22	21	19	25	22	16	12	28	11	36	38	36	34	30	28
37	42	30	38	18	15	26	10	20	17	29	17	42	39	26	31	34	24
39	47	32	42	16	17	17	19	22	18	25	19	37	35	22	99	36	22
Σ 690	810	594	646	345	350	400	375	279	251	436	376	574	589	602	560	614	657
$\bar{X}$ 34.5	40.5	26.2	32.3	17.25	17.5	20.0	18.75	13.95	12.55	21.8	18.8	28.7	29.45	30.1	28.0	30.7	32.85

TABLE iiic

St. 57 females (alcohol-controls)

Number of advanced oocytes (zygotene and pachytene) per 20 sections of each ovary per ♀ N tadpole

1		2		3		4		5		6		7		8		9	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
50	12	42	22	45	42	17	17	52	41	30	28	45	32	33	23	33	20
35	12	39	14	53	55	19	31	53	45	30	27	40	48	24	12	37	13
29	9	37	12	53	64	32	29	60	39	28	32	41	48	10	28	36	15
16	11	53	9	44	54	49	40	71	46	45	39	44	46	7	23	22	0
13	14	17	26	55	66	22	22	42	60	46	36	43	60	19	12	32	23
2	25	21	42	56	53	36	40	68	55	45	40	32	43	22	24	43	26
32	37	8	43	28	44	27	40	60	62	48	40	30	35	20	30	28	32
22	27	41	33	25	30	54	38	56	54	54	45	40	36	30	15	28	27
12	9	40	36	47	47	41	36	47	54	59	40	55	42	32	41	39	23
25	15	41	35	47	59	29	27	50	27	60	49	48	41	34	25	40	45
1	13	40	46	43	64	23	43	45	30	57	43	42	50	35	18	28	45
40	20	56	11	44	51	27	41	41	32	60	48	26	45	32	18	15	23
27	12	43	6	45	46	38	50	56	56	58	38	27	37	31	32	17	22
21	22	42	1	26	65	41	65	60	62	47	38	13	36	28	25	22	21
30	13	41	14	48	48	37	43	67	73	42	25	42	48	18	37	32	15
19	9	30	14	56	60	41	60	67	65	32	30	38	55	15	32	39	17
27	16	44	10	52	48	27	44	57	60	25	44	38	38	21	32	48	18
40	42	30	40	43	59	45	43	49	50	22	51	32	37	26	21	38	26
37	36	42	20	30	57	32	50	49	43	28	51	18	25	42	21	26	45
16	51	33	9	51	53	47	40	52	45	39	46	32	34	43	20	28	28
Σ 494	405	740	443	891	1065	684	799	1102	999	855	780	726	780	522	489	632	487
$\bar{X}$ 24.7	20.25	37.0	22.15	44.55	53.25	34.2	39.95	55.1	49.95	42.75	39.0	36.3	39.0	26.1	24.45	31.6	24.35

TABLE iv

Treatment with 5α-dihydrotestosterone, 25µg/l

Number of spermatogonia per 20 sections of each testis per ♂N tadpole

1		2		3		4		5		6		7	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
16	27	8	18	4	11	18	25	7	17	10	19	10	14
9	19	4	3	5	12	10	12	10	9	21	15	14	18
14	17	3	10	4	6	14	19	5	6	6	12	17	12
11	19	13	17	6	13	15	18	20	15	8	10	10	7
9	25	7	3	15	12	25	20	10	20	17	5	8	3
12	15	8	4	5	11	19	25	7	5	19	21	5	9
16	20	19	8	10	5	26	19	18	12	7	4	12	15
11	14	18	7	12	32	30	11	19	16	15	16	17	16
12	15	9	6	17	8	9	5	5	10	7	11	19	14
8	10	6	9	8	21	18	15	25	12	10	7	9	12
12	13	10	11	7	19	11	12	5	4	4	6	6	11
7	16	2	8	10	8	13	27	15	14	3	5	2	12
17	3	13	0	11	14	22	15	11	16	15	12	4	13
22	7	14	2	6	15	16	17	8	7	12	10	6	9
8	8	10	9	9	17	18	9	12	11	18	7	2	7
10	7	6	8	14	18	24	16	19	15	14	10	5	18
16	9	5	9	6	10	13	18	14	18	9	12	4	19
24	4	4	6	9	6	3	15	20	12	14	8	12	14
30	8	5	8	2	3	25	17	5	7	9	10	7	12
23	5	7	9	4	12	22	20	10	9	10	12	9	8
Σ 287	261	171	150	164	253	351	335	245	220	228	212	178	243
$\bar{X}$ 14.35	13.05	8.55	7.75	8.2	12.65	17.55	6.75	12.25	11.0	11.4	10.6	8.9	12.15

TABLE v

Treatment with 5α-dihydrotestosterone, 25µg/l

Number of spermatogonia per 20 sections of each testis per ♂ Subt tadpole

	1		2		3		4		5		6		7		8	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	0	0	2	0	0	1	0	0	2	1	0	4	2	4	3	3
	0	1	0	0	0	1	0	0	0	0	2	5	1	6	2	1
	0	3	0	0	0	2	0	0	1	0	3	3	1	8	0	3
	2	4	2	1	0	0	0	0	1	0	1	2	2	0	1	0
	2	2	2	4	1	0	1	0	2	1	4	0	0	0	0	3
	1	2	1	2	0	0	0	0	0	0	3	0	0	5	3	4
	0	0	1	0	0	0	0	0	0	1	0	4	0	3	0	1
	0	2	1	1	0	0	0	0	0	0	2	6	1	0	1	1
	0	0	1	0	2	0	0	0	1	0	0	3	0	0	2	1
	0	1	4	0	2	0	1	0	0	2	1	0	2	0	3	3
	1	3	2	5	0	0	0	1	0	0	4	2	3	0	3	5
	0	1	3	4	0	0	0	0	1	1	0	0	2	0	0	0
	1	1	0	5	0	1	0	0	2	0	3	1	2	0	0	0
	3	0	0	0	0	2	0	0	0	0	2	3	4	0	0	1
	0	0	2	3	1	0	0	0	0	0	0	0	2	1	0	1
	5	1	1	1	0	0	0	1	1	1	1	0	1	1	0	3
	1	1	2	4	1	1	0	0	0	0	0	2	3	2	0	0
	0	2	1	4	0	3	0	0	2	2	2	1	0	1	0	0
	2	0	2	3	0	1	1	0	0	0	0	0	0	2	0	0
	0	0	1	6	2	0	0	0	1	1	2	3	0	3	3	6
Σ	18	24	28	43	9	12	3	2	14	10	30	39	26	36	21	36
$\bar{X}$	0.9	1.2	1.4	2.15	0.45	0.6	0.15	0.1	0.7	0.50	1.5	1.95	1.3	1.8	1.05	1.8

TABLES vi & vii

Treatment with 5α-dihydrotestosterone, 25μg/l

Number of spermatogonia per 20 sections of each testis per ♂Sub‡ & ♀ tadpoles respectively

	♂Sub‡						♀			
	1		2		3		1		2	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	0	0	1	0	2	3	9	10	3	8
	1	0	2	0	3	2	7	4	6	10
	0	1	6	0	0	1	8	12	2	14
	0	1	5	2	1	2	12	6	4	9
	0	0	2	1	0	0	14	10	8	17
	0	0	3	0	0	1	16	17	6	9
	0	0	1	1	0	0	15	18	16	8
	0	0	2	1	4	0	22	10	5	10
	0	1	0	0	1	0	15	16	11	10
	0	0	3	2	0	2	20	7	10	12
	0	0	1	2	1	0	14	12	9	8
	1	0	0	3	0	4	15	20	8	10
	1	0	0	2	0	0	12	21	9	11
	0	2	2	2	0	0	18	17	17	10
	0	0	1	1	3	1	5	20	13	16
	0	0	4	1	1	2	11	16	7	6
	0	0	0	4	0	0	10	13	12	3
	0	0	0	0	0	2	8	17	11	11
	1	0	0	6	0	0	5	8	10	9
	0	0	3	0	0	1	4	11	9	16
Σ	4	5	36	28	16	21	240	265	176	207
$\bar{X}$	0.2	0.25	1.8	1.4	0.8	1.05	12.0	13.25	8.8	10.35

TABLE viii

Treatment with 5α-dihydrotestosterone, 100µg/l

Number of spermatogonia per 20 sections of each testis per ♂N tadpole

1		2		3		4		5		6	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
21	18	19	16	22	13	20	15	15	12	18	19
22	7	6	16	16	5	24	18	8	10	20	25
17	23	12	21	11	4	17	16	7	13	16	17
14	12	8	26	4	7	14	13	9	25	9	26
13	9	14	8	27	12	25	14	14	18	12	18
26	6	24	14	30	20	10	22	13	15	15	20
25	7	23	12	20	7	15	27	22	16	27	18
6	6	11	9	10	9	5	8	27	25	32	16
12	11	5	15	14	15	19	14	11	14	30	12
0	12	4	14	6	7	22	19	8	5	12	11
19	2	7	5	11	19	27	25	12	9	9	10
18	9	12	4	13	13	30	28	11	16	25	16
27	26	6	13	10	6	20	14	14	15	22	19
31	9	11	16	5	7	18	11	9	10	24	14
25	27	6	19	10	14	10	9	8	7	14	11
19	22	15	16	11	20	8	12	20	10	8	5
33	29	14	8	9	30	13	16	14	16	6	10
28	25	10	20	8	15	12	14	13	15	12	17
20	8	9	21	18	12	18	19	18	19	16	15
14	3	20	11	11	14	17	20	11	20	20	19
Σ 390	271	236	284	266	249	344	334	264	290	347	318
$\bar{X}$ 19.5	13.55	11.8	14.9	13.3	12.45	17.2	16.7	13.2	14.5	17.35	15.9

TABLE ix

Treatment with 5α-dihydrotestosterone, 100µg/l

Number of spermatogonia per 20 sections of each testis per ♂ Abn tadpole

	1		2	
	Left	Right	Left	Right
	13	7	12	8
	14	9	15	10
	16	13	17	11
	7	8	8	14
	11	24	12	17
	21	12	9	12
	13	11	18	13
	12	12	17	8
	14	13	12	9
	15	12	14	7
	10	9	10	15
	12	14	9	16
	15	17	7	14
	9	7	13	13
	12	14	15	6
	6	8	6	5
	5	9	7	8
	8	11	9	7
	12	14	10	9
	13	16	8	5
Σ	238	240	228	207
$\bar{X}$	11.9	12.0	11.4	10.35

SD ??

TABLE x & xi

Treatment with 5α-dihydrotestosterone, 100µg/l

Number of spermatogonia per 20 sections of each testis per ♂ Sub‡ & ♂ Sub‡ tadpoles respectively

♂ Sub‡										♂ Sub‡			
1		2		3		4		5		1		2	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
7	5	6	0	0	0	0	0	0	0	0	0	0	0
4	4	2	4	0	1	0	0	0	0	0	0	1	0
2	0	1	1	3	0	0	3	2	4	0	2	0	0
0	2	0	0	0	0	0	0	0	1	2	0	0	0
0	1	0	0	0	0	0	0	1	2	1	0	0	0
1	0	0	0	0	6	0	0	1	1	0	0	0	0
0	0	0	2	1	3	0	0	0	2	3	2	0	0
0	0	0	0	0	0	0	0	0	1	4	2	2	1
0	0	0	3	0	1	1	2	0	0	0	0	0	0
2	2	0	0	0	0	0	2	3	1	1	1	1	1
0	0	0	0	0	0	0	0	4	0	0	0	0	0
3	0	5	0	0	1	0	0	5	2	0	0	0	0
1	1	0	0	1	2	1	0	6	4	2	0	2	1
0	0	3	6	3	0	0	1	0	3	0	0	0	0
0	0	2	0	0	0	0	0	2	1	0	2	0	0
0	2	4	0	0	0	0	0	2	2	0	0	0	2
1	0	5	0	0	0	0	0	0	0	0	0	0	0
2	1	0	1	4	0	0	0	4	3	2	1	1	0
0	0	3	5	7	0	0	0	5	1	0	0	2	0
1	0	2	1	0	0	0	0	6	3	0	2	0	0
Σ 24	18	35	23	19	14	2	8	41	31	15	12	9	5
$\bar{X}$ 1.2	0.9	1.75	1.15	0.95	0.7	0.1	0.4	2.05	1.55	0.75	0.6	0.45	0.25

TABLE xii

Treatment with 5α-dihydrotestosterone, 100µg/l

Number of gonia per 20 sections of each gonad per ♂ tadpole

	1		2		3		4		5	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	6	5	11	19	7	8	10	8	4	8
	5	7	8	18	9	12	15	5	6	12
	14	12	16	19	19	7	8	9	10	4
	10	8	12	16	5	6	7	12	7	9
	4	14	9	13	12	10	3	14	8	15
	6	11	12	14	10	9	12	11	4	6
	12	8	11	11	16	4	14	6	3	12
	11	12	16	21	9	13	9	3	7	11
	6	7	17	41	11	8	16	9	11	7
	18	15	14	50	10	10	18	15	9	6
	10	17	16	44	17	8	15	17	12	3
	9	15	2	49	8	3	7	14	10	11
	9	10	0	50	12	5	5	13	8	15
	10	17	5	12	11	15	14	10	7	20
	18	14	6	17	14	11	17	6	10	21
	17	13	6	22	5	18	13	7	5	18
	12	19	0	15	9	14	11	6	4	17
	16	20	12	16	8	20	8	5	8	10
	13	19	14	20	10	17	6	12	9	13
	15	18	13	19	13	9	4	14	12	14
Σ	221	261	200	486	215	207	212	196	154	232
$\bar{x}$	11.05	13.05	10.0	24.3	10.75	10.35	10.6	9.8	7.7	11.6

TABLE xiii

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of spermatogonia per 20 sections of each testis per ♂N tadpole

1		2		3	
Left	Right	Left	Right	Left	Right
28	19	11	14	18	17
33	11	25	21	22	27
16	20	17	26	25	26
36	33	22	32	19	22
15	23	27	22	23	24
6	17	24	28	30	28
14	41	18	20	36	32
9	18	12	21	20	18
36	15	7	10	19	25
32	22	19	7	24	18
30	11	9	20	27	16
38	10	18	31	13	14
24	27	10	17	12	20
22	29	16	29	17	9
13	15	10	22	21	19
11	22	20	18	27	29
48	51	24	21	19	32
38	30	14	10	17	30
30	19	12	18	16	12
21	45	5	4	21	31
Σ 500	478	320	391	426	449
$\bar{X}$ 25.0	23.95	16.0	19.55	21.3	22.45

TABLE xiv

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of spermatogonia per 20 sections of each testis per ♂ Abn tadpole

	1		2		3		4		5		6		7	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	22	5	20	17	12	9	20	15	18	12	10	14	10	10
	13	11	23	13	20	11	13	12	25	15	12	9	20	9
	4	10	5	22	22	14	8	10	27	18	13	12	25	8
	9	4	14	15	31	11	14	11	22	14	11	14	14	7
	12	14	17	8	2	15	16	17	13	20	20	16	12	14
	15	7	15	11	6	5	12	13	17	19	9	12	7	19
	14	13	12	20	7	6	20	22	12	8	7	10	9	12
	12	11	18	10	6	17	19	17	9	5	12	11	10	13
	6	10	16	22	10	13	9	8	12	10	16	12	11	8
	5	7	20	19	1	10	7	16	14	17	17	11	8	5
	6	14	6	15	9	7	12	7	20	21	15	12	10	9
	17	9	14	18	19	8	17	5	23	14	5	7	14	10
	14	7	15	15	13	3	6	11	19	12	6	9	17	4
	16	13	7	24	34	12	8	12	10	9	4	3	12	6
	5	12	13	3	7	9	10	9	12	15	12	11	8	13
	6	14	12	4	11	14	12	14	8	9	14	12	4	3
	9	17	14	13	12	2	13	5	7	15	15	10	16	2
	15	8	13	6	10	1	11	9	6	20	17	8	19	4
	13	9	8	9	20	16	8	10	10	11	11	5	21	12
	16	7	5	4	6	3	7	11	12	6	12	16	6	10
Σ	229	202	267	268	258	186	242	234	296	270	238	214	253	178
$\bar{X}$	11.45	10.1	13.35	13.4	12.9	9.3	12.1	11.7	14.8	13.5	11.9	10.7	12.65	8.9

TABLES xv & xvi .

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of spermatogonia per 20 sections of each testis per ♂Sub† & ♂Sub† tadpoles respectively

	♂Sub†								♂Sub†			
	1		2		3		4		1		2	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	0	0	0	0	4	5	2	3	0	1	3	0
	0	2	0	0	0	3	1	1	2	0	1	0
	0	0	0	1	0	2	0	0	0	2	2	7
	0	0	0	0	0	0	0	1	0	0	0	1
	0	0	1	0	6	0	0	2	0	0	1	3
	0	0	0	0	8	5	0	0	0	0	8	0
	0	1	2	2	7	4	3	4	0	0	9	3
	5	0	1	0	0	0	2	1	1	1	7	0
	2	0	2	1	0	0	5	6	0	0	0	0
	0	0	0	0	2	2	4	4	0	0	0	1
	3	0	3	1	0	0	2	1	0	0	0	0
	0	0	4	0	0	1	0	0	3	0	3	2
	2	0	0	0	4	3	0	0	0	0	2	1
	1	2	0	0	5	6	2	1	0	0	1	2
	0	0	0	2	2	1	3	2	0	0	0	0
	0	2	0	1	1	1	3	0	2	0	0	0
	0	0	0	0	2	1	2	1	0	0	0	0
	0	0	0	0	0	0	1	0	0	0	0	0
	0	1	1	1	0	0	2	0	0	0	5	0
	3	5	0	0	4	3	0	2	2	1	0	2
Σ	16	13	14	9	45	37	32	29	10	5	42	22
$\bar{X}$	0.8	0.65	0.7	0.45	2.25	1.85	1.6	1.45	0.5	0.25	2.1	1.1

TABLE xviia

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of gonia per 20 sections of each gonad per ♂ tadpole

1		2		3	
Left	Right	Left	Right	Left	Right
42	22	47	35	25	30
44	27	26	33	32	34
50	20	35	40	44	38
39	29	31	43	47	45
44	28	30	35	38	38
33	27	24	36	32	40
34	25	23	37	30	32
33	26	27	38	28	38
28	29	20	31	24	36
24	23	21	37	22	20
25	24	22	26	20	28
20	18	14	30	18	20
13	30	22	23	16	24
17	29	25	17	20	26
16	15	23	19	24	20
20	19	26	18	18	25
22	21	25	17	16	22
20	22	20	15	23	18
15	30	22	17	20	16
23	29	20	21	17	23
Σ 562	493	503	568	514	573
$\bar{x}$ 28.1	24.65	25.15	28.4	25.7	28.65

TABLE xviib

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of oogonia & early oocytes & zygotene & pachytene oocytes respectively per 20 sections of each gonad per $\overset{\sigma}{+}$ tadpole

oogonia & early oocytes						zygotene & pachytene					
1		2		3		1		2		3	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
7	6	16	15	10	8	35	16	31	20	15	22
6	10	7	19	15	7	38	17	19	14	17	27
10	8	9	10	22	15	40	12	26	30	22	23
10	10	11	15	20	19	29	19	20	28	27	26
5	9	9	13	14	12	39	19	21	22	24	26
10	8	12	17	10	17	23	19	12	19	22	23
21	9	13	12	9	21	13	16	10	25	21	11
14	16	11	16	12	19	19	10	16	22	16	19
13	14	12	14	13	21	15	15	8	17	11	15
16	13	20	27	22	20	8	10	1	10	0	0
12	11	21	14	20	26	5	13	1	12	0	2
15	16	14	11	17	16	0	2	0	19	1	4
13	14	22	23	15	17	4	16	0	0	1	7
13	11	17	13	20	12	0	18	8	4	0	14
16	12	23	16	10	14	0	3	0	3	14	6
20	14	26	18	15	9	9	5	0	0	3	16
13	21	25	17	12	17	0	0	0	0	4	5
20	12	18	14	7	10	1	10	2	1	16	8
14	15	18	16	13	15	3	15	4	1	7	1
20	19	12	21	15	10	13	10	8	0	2	13
Σ 268	248	316	321	291	305	294	245	187	247	223	268
$\bar{X}$ 13.4	12.4	15.8	16.05	14.55	15.25	14.7	12.25	9.35	12.35	11.15	13.40

TABLE xviii

Treatment with 5α-dihydrotestosterone, 500µg/l

Number of oogonia and oocytes per 20 sections of each gonad per ♂-♀ tadpole

oogonia + all oocytes		oogonia + early oocytes		zygotene & pachytene oocytes	
1		1		1	
Left	Right	Left	Right	Left	Right
20	40	10	15	10	25
32	50	12	23	20	27
36	50	15	22	21	28
40	40	22	19	18	21
20	28	12	13	8	15
30	33	7	16	23	17
35	32	17	12	18	20
28	35	10	18	18	17
30	27	9	7	21	20
35	25	10	10	25	15
30	38	15	10	15	28
40	30	15	10	25	20
60	45	10	15	50	30
55	55	15	15	40	40
60	60	10	10	50	50
48	70	20	14	28	56
60	71	15	13	45	58
68	64	18	10	50	54
70	60	16	12	54	48
Σ 797	853	258	264	539	589
$\bar{x}$ 39.85	42.65	12.9	13.2	26.95	29.45
41.25		13.05		28.2	

TABLE xix

Treatment with 5α-dihydrotestosterone, 2mg/l

Number of spermatogonia per 20 sections of each testis per ♂ Abn tadpole

1		2		3		4		5		6		7		8		9	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
10	19	13	26	13	9	20	11	9	17	24	14	12	19	9	22	21	17
14	18	5	15	16	14	24	9	12	19	17	6	18	25	10	18	28	15
6	35	6	24	17	10	18	20	8	25	22	8	15	17	13	15	25	12
12	17	20	12	23	9	16	15	11	27	17	15	8	30	20	27	35	9
8	18	10	13	13	22	12	27	10	16	14	12	7	18	27	30	22	16
9	12	9	12	12	17	12	19	9	22	12	9	9	12	12	12	17	22
5	20	13	15	3	14	14	21	5	17	15	16	11	9	8	11	19	11
2	25	16	10	12	11	9	12	6	21	10	12	22	11	5	6	12	8
8	34	15	12	13	32	15	17	8	29	14	17	23	14	13	14	10	19
9	17	9	29	17	31	12	22	7	20	25	19	16	16	16	9	18	17
20	16	4	26	11	36	23	19	12	18	27	20	8	22	24	14	15	22
30	11	16	30	19	32	24	10	20	17	16	19	5	18	18	15	13	24
19	10	13	12	14	25	27	12	22	24	13	12	13	20	16	20	9	27
14	25	12	9	10	21	11	16	16	17	15	9	18	17	10	11	12	10
16	24	13	8	16	24	15	12	15	22	11	7	19	15	7	6	14	8
18	22	25	18	17	5	19	13	10	16	20	14	21	12	6	18	16	15
20	4	45	7	27	10	7	12	24	13	12	24	25	5	14	21	8	13
9	12	40	11	32	15	10	9	27	10	25	27	7	19	23	17	15	11
27	13	26	3	23	4	13	10	17	9	10	17	6	21	25	15	17	7
23	10	29	18	17	6	20	13	20	12	9	15	14	10	11	12	19	5
Σ 279	352	339	310	325	347	321	299	268	361	328	292	277	330	287	313	345	288
$\bar{X}$ 13.95	17.6	16.95	15.5	16.25	17.35	16.05	14.95	13.4	18.05	16.4	14.6	13.85	16.5	14.3	15.65	17.25	14.4

TABLE xxa

Treatment with 5α-dihydrotestosterone, 2mg/l

Number of oogonia & all oocytes per 20 sections of each gonad per ♂-♀ tadpole

1		2		3		4		5		6		7		8		9		10		11	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
25	45	32	29	95	111	35	46	35	45	28	30	30	50	85	95	40	50	34	30	30	50
34	49	31	36	93	78	36	55	38	50	31	34	38	53	90	100	45	52	28	38	38	54
38	53	32	38	100	73	40	68	44	54	34	38	49	58	94	97	38	62	40	44	48	50
44	46	39	33	90	60	45	79	28	52	37	30	39	42	100	93	42	78	42	40	54	48
18	31	47	56	99	121	41	63	41	38	42	47	40	48	99	85	36	60	47	52	50	38
31	38	36	54	101	124	42	80	42	42	38	52	44	47	95	88	44	69	38	58	48	42
34	39	56	62	104	122	30	83	52	47	50	60	34	50	85	89	40	78	48	60	42	52
26	32	39	35	93	103	27	73	58	45	40	40	38	45	87	90	38	76	52	64	40	60
28	30	35	38	96	99	34	72	50	60	32	38	40	50	93	101	42	70	50	34	38	48
37	27	47	40	81	117	41	78	48	38	42	42	50	62	96	95	47	79	38	42	44	45
28	36	40	36	97	100	35	87	55	46	35	32	52	64	81	80	37	82	44	38	60	55
42	20	32	37	70	71	71	74	60	40	36	30	62	60	97	78	62	76	40	35	64	72
74	67	40	48	51	73	76	64	64	62	44	44	64	68	74	76	74	68	38	43	74	68
51	63	28	30	52	75	80	52	68	65	30	34	68	74	62	70	78	58	44	40	68	74
64	56	37	32	47	88	78	67	72	60	32	30	72	80	60	68	80	64	37	32	58	62
58	71	39	35	50	81	72	70	78	71	37	38	58	68	50	62	76	72	42	42	62	70
64	61	46	40	46	67	68	60	80	78	44	42	64	74	55	60	72	64	40	48	64	68
82	64	47	52	48	65	74	58	85	68	47	50	74	76	62	56	74	60	48	52	60	60
95	70	56	60	45	60	76	70	86	72	50	60	80	69	58	58	77	71	50	60	78	64
76	52	48	50	40	62	77	68	75	64	42	48	74	64	52	60	80	66	54	54	76	62
Σ 949	950	807	841	1498	1750	1078	1367	1179	1097	771	819	1070	1202	1575	1601	1122	1355	854	906	1096	1142
x̄ 47.45	47.5	40.35	42.05	74.9	87.5	53.9	68.3	58.95	54.84	38.55	40.95	53.5	60.1	78.75	80.05	56.1	67.75	42.7	45.3	54.8	57.1

TABLE xxb

Treatment with 5α-dihydrotestosterone, 2mg/l

Numbers of oogonia and early oocytes per 20 sections of each gonad per ♂+♀ tadpole

1		2		3		4		5		6		7		8		9		10		11	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
17	25	14	20	21	19	22	25	29	40	18	22	8	22	25	20	28	24	18	24	25	40
19	27	16	11	16	18	18	20	31	30	15	20	20	25	20	17	17	22	14	18	38	45
11	30	15	17	15	19	19	23	24	23	12	24	12	32	18	24	20	18	15	15	28	35
12	22	20	15	17	14	20	25	18	26	20	18	14	24	22	18	18	27	22	14	28	28
10	30	26	27	21	12	22	21	14	19	24	14	34	28	25	15	24	18	28	22	10	15
30	25	22	23	26	36	12	16	29	35	21	25	30	23	30	35	16	15	24	26	14	24
31	15	15	20	40	44	10	15	12	27	13	15	20	18	45	45	12	14	18	20	15	18
19	25	24	28	24	25	12	17	14	32	22	18	14	21	34	34	13	18	21	18	12	17
22	19	8	20	16	18	14	19	15	20	10	20	32	20	18	22	15	21	12	25	16	14
34	10	7	17	20	21	18	23	16	22	8	14	42	18	24	20	18	24	10	16	21	12
20	22	11	27	18	32	20	25	24	23	9	20	35	14	16	28	14	26	8	20	24	25
37	6	18	6	22	23	17	22	12	17	16	22	12	10	25	20	18	13	7	12	17	27
48	8	19	7	16	14	10	15	14	18	18	22	10	12	19	18	9	17	17	10	13	18
17	11	12	15	21	15	9	12	12	17	14	18	18	8	24	17	20	15	22	8	15	16
12	9	12	5	17	24	18	25	19	18	12	10	20	9	18	21	21	14	14	7	19	17
4	18	15	8	10	29	17	22	12	15	16	8	11	14	14	30	19	15	12	6	21	19
6	12	14	7	9	30	19	17	13	21	13	14	8	12	10	34	15	18	10	15	18	24
9	10	11	4	17	32	20	22	18	20	10	10	7	10	18	36	12	20	12	10	12	22
5	13	9	20	18	29	12	14	8	13	11	12	5	13	20	28	10	15	9	17	10	18
8	11	6	19	10	15	15	13	6	16	8	7	4	14	24	17	13	10	17	19	13	14
Σ 371	348	294	316	374	471	324	391	340	452	290	343	366	347	449	499	332	364	306	322	369	458
$\bar{X}$ 18.55	17.4	14.7	15.8	18.7	23.55	16.2	19.55	17.00	22.60	14.5	16.65	18.3	17.35	22.45	24.95	16.6	18.2	15.3	16.1	18.45	22.9

TABLE xxc

Treatment with 5α-dihydrotestosterone, 2mg/l

Numbers of zygotene & pachytene oocytes per 20 sections of each gonad per $\underset{+}{\sigma}$-♀ tadpole

1		2		3		4		5		6		7		8		9		10		11	
Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
8	20	18	9	74	92	13	21	6	5	10	8	12	28	60	75	12	26	16	6	5	10
15	22	15	25	77	60	18	35	7	20	16	14	18	28	70	83	28	30	14	20	0	9
27	23	17	21	85	54	21	45	20	31	22	14	37	26	76	73	18	44	25	29	20	15
32	24	19	18	73	46	25	54	30	26	17	12	25	18	78	75	24	51	20	26	26	20
8	1	21	29	78	109	19	42	27	19	18	33	6	20	74	70	12	42	19	30	40	23
1	13	14	31	75	86	30	64	13	7	17	27	14	24	65	53	28	54	14	32	34	18
3	24	41	42	64	78	20	68	40	20	37	45	14	32	40	44	28	64	30	40	27	34
7	7	15	7	69	78	15	56	44	13	18	22	24	24	53	56	25	58	31	46	28	43
6	11	27	18	80	81	20	53	35	40	22	18	8	30	75	79	27	49	38	9	22	34
3	17	40	23	61	96	23	55	32	16	34	28	8	44	72	75	29	55	28	26	23	23
8	14	29	9	79	68	15	62	31	23	26	12	17	50	65	52	23	56	36	18	36	30
5	14	14	31	48	48	54	52	48	23	20	8	50	50	72	58	44	63	33	23	47	45
26	59	21	41	35	59	66	49	50	44	26	22	54	56	55	58	65	51	21	33	61	50
34	52	16	15	31	60	71	40	56	48	16	16	50	66	38	53	58	43	22	32	53	58
52	47	25	27	30	64	60	42	53	42	20	20	52	71	42	47	59	50	23	25	39	45
54	52	24	27	40	52	55	48	66	56	21	30	47	54	36	32	57	57	30	36	41	51
58	49	32	33	37	37	49	43	67	57	31	28	56	62	45	26	57	46	30	33	46	44
73	54	36	48	31	33	54	36	67	48	37	40	67	66	44	20	62	40	36	42	48	38
90	57	47	40	27	31	64	56	78	59	39	48	75	56	38	30	67	56	41	43	68	46
68	41	42	31	30	47	62	55	69	48	34	41	70	50	28	43	67	56	41	35	63	48
Σ578	602	513	525	1124	1279	754	976	839	645	481	486	704	855	1126	1102	790	991	548	584	727	684
$\bar{X}$28.9	30.1	25.65	11.51	56.2	63.95	37.7	11.13	41.95	17.22	24.05	24.3	35.2	42.75	56.3	55.1	39.5	49.55	27.4	29.2	36.35	34.2