IN THE CICHLID, *TILAPIA MOSSAMBICA* (PETERS)

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Changes elicited by methallibure treatment in the hypothalamo- hypophyso-gonadal axis were assessed in a teleost, *Tilapia mossambica*. The treatment resulted in complete disappearance of the neurosecretory material from the nucleus lateralis tuberis with a concomitant inhibition of gonadoropic activity in the cells of the proximal pars distalis. Gonads of the treated animals showed multiplication of the premeiotic germ cells and a suppression and destruction of the meiotic and postmeiotic stages. It is suggested that the inhibition of the pituitary gonadotrops and consequently the regression of gonads in the treated fish are related to the depression in the activity of nucleus lateralis tuberis of the hypothalamus, induced by the drug administered.

INTRODUCTION

The selective blocking of the pituitary gonadotropic activity by the dithiocarbamoylhydrazine derivative Methallibure (ICI 33,828) was initially demonstrated by Paget *et al.* (1961), who successfully used that drug in the rat and suggested that the gonads were inhibited perhaps by a regression of the gonadotrops. This suggestion was substantiated by the investigations in other vertebrates including birds (Sykes 1963), garden lizard (Rangneker and Kulkarni 1969), frog (Kanakaraj and Gangadhara, 1967) and fishes (Hoar *et al.* 1967; Wiebe 1968). However, the cytological manifestation of non-functional gonadotrops was only recently evidenced (Pandey and Leatherland 1970), at least in fishes. It was therefore thought desirable to assess the changes induced by chemical hypophysectomy in the hypothalamo-hypophyso-gonadal axis of the teleost, *Tilapia mossambica* (Peters).

MATERIAL AND METHODS

Forty, young, sexually mature *T. mossambica* were acclimated to laboratory conditions for two weeks. During this period and the subsequent experimental period they were fed live tubifex worms every day. At the commencement of the experiment, the fish ranging in weight from 10–20 g, were starved for 24 hr, weighed and segregated into two matched groups : Control and Experimental. Care was taken to include 10 males and 10 females in each of the two groups. The animals

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belonging to the two groups were maintained separately in four aquaria of identical sizes (60 × 30 × 30 cm), each containing a layer of coarse sand. The ambient waters were changed twice a week and aerated continuously.

The drug methallibure (Batch : PD/AS/4978/67) was injected once a week to the experimental animals intraperitoneally, in the form of a suspension at a dose of 5 mg/0.1 ml/fish. The suspension was prepared according to the method described by Hoar *et al.* (1967). Each of the controls received 0.1 ml of the vehicle. In all, six injections were given and 7 days after the last injection the fish were weighed and sacrificed.

At sacrifice, the gonads were weighed accurately and the middle portions from the left testis or ovary were fixed in aqueous Bouin's fluid. Remaining pieces of these organs were utilised for the determination of the amount of cholesterol in them. The method of Knobil *et al.* (1954) for the estimation of cholesterol was employed. The brain with the attached pituitary was fixed in sublimated Bouin-Hollande fixative. The fixed tissues were routinely processed for paraffin wax (56–58°C) embedding. The gonads were sectioned transversely at 7 μ and stained with Delafield's haematoxylin and eosin and Heidenhain's AZAN (McManus and Mowry 1964) techniques. The brain with attached pituitary was sectioned sagittally at 5 μ and stained with Heidenhains' AZAN, aldehyde fuchsin-periodic acid-Schiff's leucobase-orange G (AF-PAS-OG, Elftman 1959) and celestin blue-toluidine blue-periodic acid-Schiff's leucobase-orange G (TB-PAS-OG, Gurr 1962) methods.

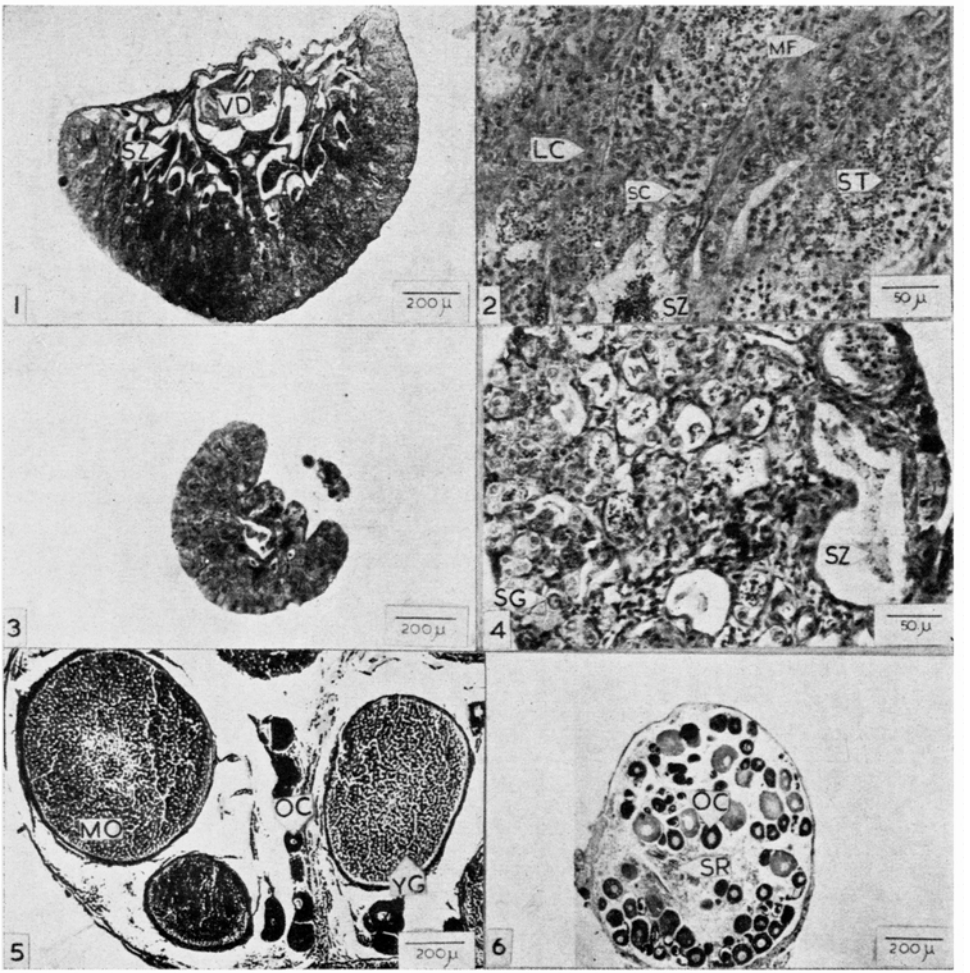
Comparison of gonadal activities of both the groups was made by considering the gonosomatic indices$\left(\frac{\text{Weight of gonad} \times 100}{\text{Weight of animal}}\right)$,gonadal diameters(10 measurements/animal in each group), differential counts of germinal cells and cholesterol contents.

The identification of the testicular cell types was made according to the criteria of Dadzie (1969). Counts of the various/testicular cell types observed in a section were made at a magnification of 450X with the help of an ocular micrometer having 1 sq. cm area divided into quadrants of 0.25 mm^2 each. In each animal's testis counts were made in five areas (each of 1 mm^2) selected at random in each of 10 sections. Table I shows the mean values obtained in the two groups.

The ovarian germinal cells were however counted in an entire transverse section. These observations were made on 10 sections/animal. From the averages thus obtained in each animal the mean values for each group were calculated (Table II).

The proximal pars distalis of the pituitary was studied critically, using several criteria like : area occupied by the proximal pars distalis in the pituitary, staining reactions of its component cells, differential cell counts and changes in their morphology and cytoplasmic granularity. In addition, the hypothalamo-hypophysial systems were also examined histologically to assess alterations, if any, in the secretions of neurosecretory material.

The data obtained after various measurements were tested for statistical significance using the Fisher modification of "Student's" formula for small samples.

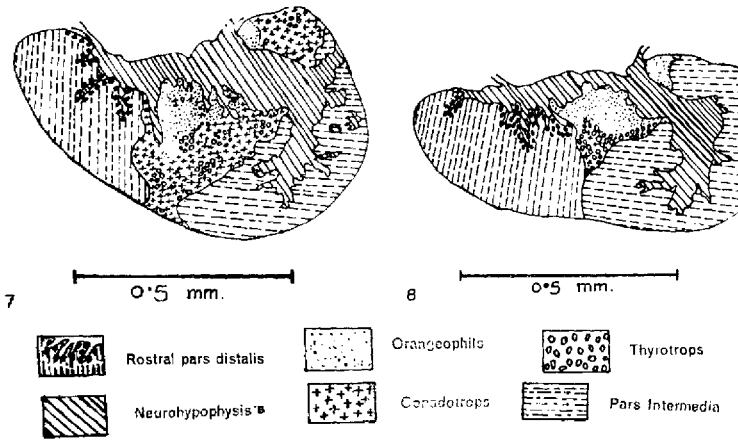


FIGS. 1–6. Photomicrographs of gonads in control and experimental *T. mossambica* showing regressive dhanges following methallibure treatment. 1, T.S. control testis; 2, T.S. control testis showing active spermatogenesis; 3, T.S. experimental testis. Note the reduced size of the organ; 4, T.S. experimental testis showing numerous premeiotic and atrophied postmeiotic germinal elements (*LC*, Leydig cells; *MF*, Mitotic figures; *SC*, Spermatocytes; *SG*, Spermatogonia; *ST*, Spermatids; *SZ*, Spermatozoa; *VD*, Vas deferens); 5, T.S. control ovary. Note the large mature oocytes; 6. T.S. ovary of methalliburated fish showing numerous primary non-yolky oocytes. Note the total absence of primary yolky and mature oocytes (*MO*, Mature oocyte; *OC*, Primary (non-yolky) oocyte; *SR*, Stroma; *YG*, Yolk globules).

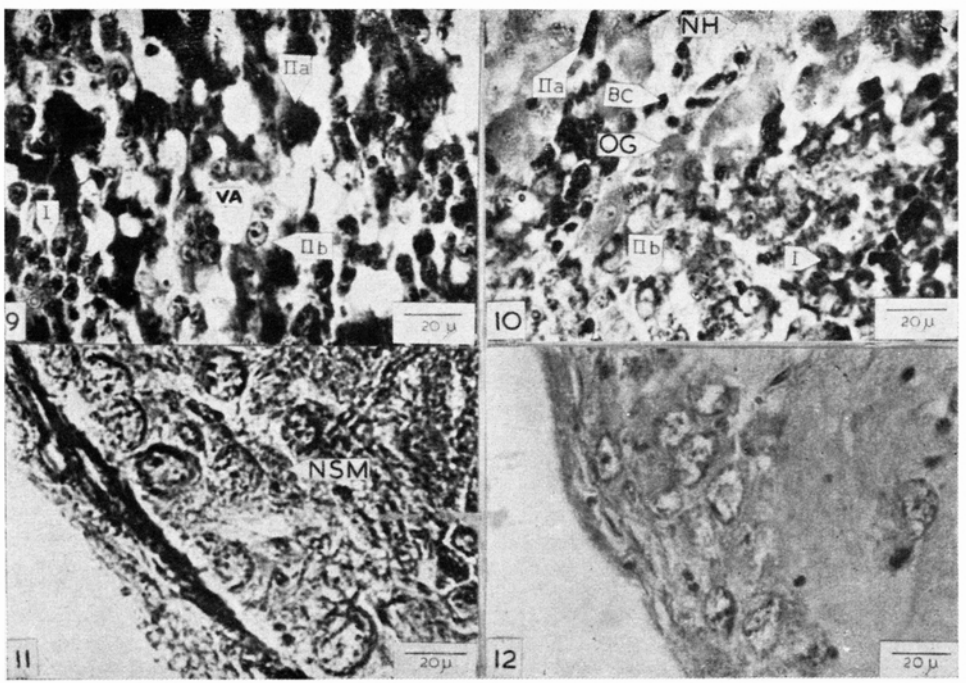
OBSERVATIONS

Gonads

Testis—The testicular structure in normal *T. mossambica* has been described by Dadzie (1969) and our observations are broadly in agreement with those



FIGS. 7–8. Camera lucida drawings of the control and experimental pituitaries to show the gross morphological changes in the proximal pars distalis after methallibure treatment, of *T. mossambica*. 7, Sagittal section of Control pituitary; 8, Sagittal section of Experimental pituitary.



FIGS. 9–12. Photomicrographs of control and experimental proximal pars distalis and nucleus lateralis tuberlis following methallibure treatment of *T. mossambica*; 9, Control proximal pars distalis showing the thyrotrops and gonadotrops; 10, Experimental proximal pars distalis showing regression of gonadot (*BC*, Blood capillary: *NH*, Neurohypophysical branches; *OG*, Organeophils; '*I*', Thyrotrops: '*IIa*', '*IIb*', Gonadotrops; *VA*, Vacunles in '*IIb*' gonadotrops; 11, Control nucleus lateralis. Note the rounded nuclei and the neurosecretory granules; 12, Experimental nucleus lateralis tuberis. Note the distorted nuclei and absence of the neurosecretory granules, *NSM*, Neurosecretory granules).

TABLE I

Effect of methallibure treatment on the testis of T. mossambica

	Group		't'	'p'
	Control	Experimental		
Gonosomatic index	0.836 ± 0.054	0.070 ± 0.011	13.76	<0.0001
Testis diameter (μ)	1883.25 ± 106.00	380.42 ± 24.53	14.30	<0.0001
Number of Germ Cells				
Primary spermatogonia	2.76 ± 0.52	5.72 ± 0.36	4.61	<0.002
Secondary spermatogonia	6.44 ± 0.87	15.36 ± 2.05	4.78	<0.002
Primary spermatocytes	29.76 ± 2.96	23.16 ± 1.29	2.04	Insignificant
Secondary spermatocytes	32.80 ± 2.71	Absent		
Spermatids	77.12 ± 6.10	13.48 ± 1.28	10.21	<0.0001
Spermatozoa	261.88 ± 18.27	96.08 ± 21.12	5.94	<0.0001
Gonadal cholesterol Content (mg/100 mg)	0.465 ± 0.019	1.404 ± 0.143	6.49	<0.0001

Note : Data represent mean values covering 10 animals/group.

recorded by him. Surprisingly enough, no metion has been made of the interstitial cells in the testis of this fish by Dadzie (1969). This tissue formed a major component in the testicular organisation of *T. mossambica* and was formed of distinct cords, 2–9 cells thick, radiating dorso-ventrally among the lobules. These interstitial cells were distinguished from the testicular germinal elements by their distinct cellular outlines and prominent, rounded nuclei. Their cytoplasm took a light blue colour with the AZAN technique.

The control males in the present study were found to be at stage IV of maturity (Dadzie 1969) and their testes (Fig. 1) showed active spermatogenesis (Fig. 2). In the methalliburated males, the regressed state of the testis (Fig. 3) was evidenced by the highly significant reduction in gonosomatic index and the gonadal diameter (Table I). The organ showed a predominance of the premeiotic stages of spermatogenesis (Fig. 4). The primary spermatocytes showed clumped nuclear contents and the secondary spermatocytes were totally absent, indicating very clearly the arrest of reduction division. Very few and small scattered nests of

TABLE II

Effect of methallibure treatment on the Ovary of T. mossambica

	Group		't'	'p'
	Control	Experimental		
Gonosomatic index	2.620 ± 0.286	0.335 ± 0.037	3.29	<0.005
Ovary diameter (μ)	3395.85 ± 166.50	1257.35 ± 129.60	10.13	<0.0001
Number of Germ Cells Oogonia	10.64 ± 1.09	31.81 ± 1.76	10.20	<0.0001
Primary (non-yolky) oocytes	61.52 ± 5.19	87.88 ± 7.48	3.36	<0.005
Primary (yolky) oocytes	4.72 ± 0.37	Absent		
Mature oocytes	8.32 ± 0.88	Absent		
Gonadal cholesterol Content (mg/100 mg)	3.053 ± 0.235	2.638 ± 0.216	1.30	Insignificant

Note : Data represent mean values covering 10 animals/group

spermatids and depleted sperms persisted, but revealed distinct atrophic changes. The sperms in the reduced lumina of the tubules and vas deferens were mostly clumped, had lost their tails and were embedded in an amorphous substance formed perhaps by their disintegration. Presence of some large amoeboid cells among the disintegrating sperms suggests phagocytosis of these germinal cells.

The testicular interstitial tissue of the methalliburated fish showed drastic atrophy, with diminutive, sparsely distributed Leydig cells in contrast to the well organised trabecular interstitial tissue of the control testis. These regressed Leydig cells showed shrunken nature with reduced cytoplasm and small, distorted and pycnotic nuclei.

Ovary—In the control females the paired yellowish ovaries were thick, large, fusiform organs, packed with ripe oocytes. The maturity of the ovaries was further evidenced by the fact that egg-laying occurred twice during the experimental period. As against this, in the methallibuated females the ovaries were reduced to very thin white filaments and showed a very significant reduction in gonosomatic index and ovary diameter (Table II). A lack of the mature stages was quite evident in the organ.

Histologically, a typical transverse section of the control ovary (Fig. 5) revealed all the stages of development : from oogonia and primary oocytes to mature oocytes

and some resorbing and atretic follicles. The oogonia contained clear cytoplasm whereas the larger and more numerous non-yolky primary oocytes were characterised by cytoplasm stained deeply by haematoxylin (blue) and AZAN (purple). Chromatin matter in the nucleus was granular and the single acidophilic nucleolus of the oogonium was replaced by two or more acentric nucleoli. In the larger oocyte, the nucleus stained lightly and some thin chromatin threads were evident. In the perinuclear cytoplasm, varying numbers of lipoid vesicles were present. The yolky oocyte showed its cytoplasm to be restricted to thin peripheral and perinuclear bands. The syncytium-like granulosa showed proliferation and below this a thin, opaque and uniform zona radiata (Dadzie 1968) appeared. A loss of the nuclear membrane and scattering of the acidophilic nucleoli were the changes noticed in the nucleus. In the control ovary, the maturing and ripe oocytes formed about 15 per cent of the total germ cells and they occupied most of the space in the organ due to their large sizes. The stretic follicles revealed many luteal cells.

In contrast to the controls, the ovary in the methalliburated females (Fig. 6) showed great proliferation of the oogonia and a moderate multiplication of the non-yolky primary oocytes (Table II). The latter showed clumped cytoplasm and disorganised chromatin matter. The lipoid vesicles in the larger oocytes showed numerical increase. No maturing or mature oocytes were recognisable due to extensive atrophic changes in them. In the atretic follicles yolky matter was practically absent and a preponderance of the luteal cells was apparent. Several atretic follicles were replaced by stroma made of cellular debris and connective tissue.

It is thus evident that in the methallibure-treated females the differentiation of early stages in the ovary was accelerated, whereas the later stages were suppressed. An active resorption of the yolk already formed and a complete blocking of vitellogenesis were the other events noted.

In the treated fish, the cholesterol content increased very strikingly in the testes (Table I) but no significant variation in this respect was observed in the ovaries (Table II) when compared with the controls.

Pituitary gland

The pituitary of *T. mossambica* is composed of two distinct but closely integrated component parts : adenohypophysis and neurohypophysis. Three regions were distinguished in the adenohypophysis : (*i*) rostral pars distalis, (*ii*) proximal pars distalis, and (*iii*) pars intermedia (Fig. 7). The rostral pars distalis contained two types of acidophil. In the proximal pars distalis, both, acidophils and cyanophils were present. The cells of the pars intermedia were also of two types, cyanophilic and amphiphilic. The changes elicited by methallibure in the proximal pars distalis alone are described, as from the following observations it is clear that the cyanophils related to the gonadotropic function are located in this region (Fig. 8).

Acidophils—These bordered the interdigitations of the neurohypophysis with the proximal pars distalis. They were irregular cells showing affinity to orange G in all the staining sequences used and are termed as orangeophils. A sparse carminophilic or PAS +ve cytoplasmic granulation was observed in them. The nuclei were round to oval and were excentric in position. After methallibure treatment, the orangeophilic zone of the proximal pars distalis showed a moderate

increase in area from 6.9 to 9.9 per cent in addition to an enhanced PAS +ve granulation in its component cells.

Cyanophils—In the control animals, the cyanophilic zone of the proximal pars distalis occupied 25.9 per cent area of the pituitary gland. The criteria as mentioned earlier made it possible to distinguish two cyanophilic types (Fig. 9) in the proximal pars distalis. Type I cyanophils were small (Table III), irregular cells restricted to 4–6 well defined islets in the substance of the proximal pars distalis. Each cell contained distinct cytoplasmic granules in moderate amounts and a round nucleus (3.5 μ) with 1–2 nucleoli and scanty chromatin. The cytoplasmic granules stained blue by the AZAN and TB-PAS-OG and violet by the AF-PAS-OG methods. Some of these cells were scattered among other cells of the proximal pars distalis. In the experimental fish, these cells showed a slight diminution in their cytoplasmic granularity but maintained their size (Table III). Our experiments leading to chemical thyroideotomy in this species (unpublished results) have indicated the type I cyanophils of the proximal pars distalis to be the thyrotrops.

Cells of type II composed the rest of the cyanophilic zone. These were large cells (Table III), strongly aniline blue and PAS +ve. They could be further distinguished into two subtypes. The cell of subtype 'a' was broad and triangular with a round nucleus (3.5 μ) near the base, containing 1–2 nucleoli and scanty chromatin matter. The cytoplasm was packed with granules. The subtype 'b' included irregular cells larger than those of subtype 'a' and were generally vacuolated, but the rest of their cytoplasm was densely packed with granules. The peripherally located vesicular nucleus was larger (6.125 μ) than that observed in the cell of the subtype 'a' and contained a central nucleolus.

In the methalliburated *T. mossambica,* the cells of type II showed a sharp reduction in number and size (Table III) and cytoplasmic granularity. The decrease in size was to such an extent that the cell nucleus alone was visible in the poorly delineated cytoplasm. These type II cyanophils included few cells with a practically normal tinctorial reaction. The degree of reduction in size and number was maximum in 'IIa' cyanophils and consequently they were discernible only occassionally (Fig. 10). The nuclei of the 'IIb' cyanophils were greatly reduced in size (3.5 μ). These regressive changes caused a drastic decrease in the total cyanophilic area of the proximal pars distalis which now constituted only 4.3 per cent of the entire pituitary.

Hypothalamo-hypophysial complex

Of the three staining methods employed, only AZAN and AF-PAS-OG procedures were useful in staining the neurosecretory loci selectively. In the control *T. mossambica,* the nucleus lateralis tuberis (Fig. 11) and the nucleus preopticus were composed of large and small functionally active cells. The neurosecretory granules of the nucleus lateralis tuberis were found to be carminophilic, aniline blue (positive) and AF (negative), while those of the nucleus preopticus were azocarmine (negative) and aniline blue and AF (positive). After six weeks of methallibure treatment the cells constituting the nucleus lateralis tuberis alone showed inhibition as evidenced by shrunken nuclei (Fig. 12). Moreover, the neurosecretory material was found to be absent, as none of the staining methods employed helped

TABLE III

Effect of methallibure treatment on the Proximal Pars Distalis of T. mossambica

	Group		't'	'p'
	Control	Experimental		
Number of cells/Section				
Orangeophils	337 ± 17	364 ± 22	1.83	Insignificant
Cyanophils (I)	107 ± 14	139 ± 22	1.45	Insignificant
Cyanophils (II)	282 ± 14	36 ± 4	11.76	<0.0001
Cell size (μ)				
Orangeophils	6.47 ± 0.72	7.00 ± 0.73	0.28	Insignificant
Cyanophils (I)	5.25 ± 0.55	5.46 ± 0.68	0.33	Insignificant
Cyanophils (IIa)	11.22 ± 0.91	4.79 ± 0.57	6.02	<0.0001
Cyanophils (IIb)	18.20 ± 0.88	8.40 ± 0.90	7.76	<0.0001

NOTE : Data represent mean values covering 10 animals/group.

to reveal the granules in any part of the hypothalamo-hypophysial system of the treated fish.

DISCUSSION

It is well known that the cells in the pituitary responsible for gonadal maturation are the gonadotrops. Withdrawal of the gonadotropins results in atrophic changes leading to destruction of the germ cell stages dependent on meiosis (beyond primary spermatocytes : Hoar *et al.* 1967; beyond primary oocytes : Ball 1968), non-occurrence of steroidogenesis in the gonadal endocrine tissue (Hoar 1969) and a consequent failure of the animals to attain sexual maturity.

Our results in *T. mossambica* show that methallibure treatment induced a very significant reduction in the gonosomatic index and in the size (diameter) of the gonads also. The level of significance in the respect of gonosomatic index was relatively lesser in the females and this suggests that the drug acts slower in the females than in the males. A similar conclusion was also drawn by Hoar *et al.* (1967).

In immature *Gobius paganellus* (Vivien 1941) and the eel *Anguilla anguilla* (Olivereau 1954; cited by Pickford and Atz 1957), surgical hypophysectomy

prevented the development of the gonads, which remained in an infantile state. Further, Ahsan (1966) noted in mature *Couesius plumbeus* that hypophysectomy prevents transformation of spermatogonia into spermatocytes and brings about a marked atrophy of the testes. In fishes, Hoar *et al.* (1967) and Wiebe (1968) were the first to give the evidence in support of non-suppression of the earlier stages of gametogenesis after chemical hypophysectomy resulting from methallibure treatment. Histological observations in the present study showed that, whereas the control gonads exhibited all the gametic stages, these organs in the methallibu-rated fish revealed a total lack of the meiotic and postmeiotic stages, showing thereby that the treatment resulted in a complete blocking of meiosis. It was further evident that the treatment effectively blocked vitellogenesis in the ovary.

In the testis, depletion of the number of secondary spermatocytes has been recorded in methalliburated adult *Carassius auratus* and *Gasterosteus aculeatus* (Hoar *et al.* 1967), *Cymatogaster aggregata* (Wiebe 1968) and *Poecilia reticulata* (Pandey and Leatherland 1970). In *T. mossambica,* the total lack of these germ cells suggests a more pronounced effect of the drug.

The interstitial cells in the male *T. mossambica* showed atrophic changes after methallibure treatment. This observation differs from that made by Pandey (1970) in *Poecilia reticulata,* but is in agreement with that made in other species of fish (Hoar *et al.* 1967; Wiebe 1968). That in fish this tissue is responsible for androgen production is now well established (Hoar 1969).

In the fishes *Esox lucius* (Lofts and Marshall 1957) and *Monopterus albus* (Chan and Phillips 1967), large amounts of cholesterol and lipid are synthesised in the spent testes and these metabolites disappear concomitant with the re-establishment of spermatogenesis. However, investigations carried out in a few other piscean species failed to confirm this finding (Barr 1963; Lofts *et al.,* 1966). Furthermore, in amniotes, a high level of sudanophilia always accompanies suppression of gametogenesis (Lofts 1968). The increase in the cholesterol content of the testis coupled with regressed germinal and interstitial tissues thus indicate the simultaneuos failure of both, gametogenesis and steroidogenesis.

The microscopic structure of the pituitary has been well documented in the teleosts (reviews by Pickford and Atz 1957; Olivereau, 1963; Stahl 1963 ; van Oordt 1968; Ball and Baker 1969). The pituitary of *T. mossambica* conforms to the general teleost pattern.

From all the available accounts, the acidophils of the proximal pars distalis are said to be the somatotrops (e.g., Ball and Baker 1969). The cytological alterations observed in these cells in the methalliburated *T. mossambica* suggest a decreased secretory activity by them.

The pars distalis of the pituitary contains two cyanophiltic cell types. In most of the fishes studied so far, these are distinguished into thyrotrops and gonadotrops. However, Leray and Carlon (1963) and Olivereau (1967) among others have succeeded in distinguishing two types of gonadotrop. That there is an interrelationship between the activity of the gonadotrops and the gonadal cycle is now well recognised (Sundararaj 1959, 1960; Sathyanesan 1963; Stahl 1963; Olivereau and Ball 1964; Rai 1966; Olivereau 1967; van Oordt 1968). Investigations on the pituitary made after the administration of methallibure have shown that the

drug blocks the synthesis of pituitary gonadotropins in several higher vertebrate species (Schmidt-Elmendorff 1962; Brown 1963; Sykes 1963, 1964; Edgren and Peterson 1964; Loraine *et al.* 1965). In the fishes examined by Hoar *et al.* (1967), Wiebe (1968) and Pandey and Leatherland (1970), methallibure treatment resulted in blocking of meiotic and postmeiotic stages of gametogenesis, which depend on the pituitary gonadotropins. Further, Pandey (1970) and Pandey and Leatherland (1970) after studying the effect of methallibure on the pituitary of *P. reticulata,* concluded that the drug brings about a regression of the pituitary gonadotrops. In the present study on *T. mossambica* after methallibure treatment, strongly atrophic changes were noticed in the type II cyanophils of the proximal pars distalis, accompanied by regressive changes in the gonads. These histological changes induced experimentally in the type II cyanophils show these tropic cells to be the gonadotrops. Hoar *et al.* (1967) have suggested that the drug directly affects the synthesis, release or action of the gonadotropins. Our observations on the alterations in the gonadotrops strongly indicate an arrest of biosynthesis of the hormone in them. This is in agreement with the observation made by Pandey (1970) and Pandey and Leatherland (1970).

There is ample evidence to show that in fishes, secretion of gonadotropins is under hypothalamic control. Stiudes on *Poecilia formosa* (Olivereau and Ball 1966) have shown that gonadotrops in the ectopic pituitary transplants undergo regression, proving thereby that a hypothalamic factor is essential for maintaining the normal state of activity in these cells. There has been evidence for some years that the nucleus lateralis tuberis rather than the nucleus pre-opticus is concerned with the regulation of gonadotropic activity in teleosts (Ball and Baker 1969; Peter 1970; Zambrano 1971). These observations together with the fact that in the methalliburated *T. mossambica* there is a complete depletion of neurosecretory material in the nucleus lateralis tuberis and the neurohypophysis, point towards the possibility that the suppression of biosynthetic activity of the gonadotrops may be a direct consequence of the action of the drug on the functional integrity of the nucleus lateralis tuberis.

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