

BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON THE
REPRODUCTIVE HORMONES OF THE DOGFISH
(*Scyliorhinus canicula* L.)

by

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This is to certify that the work here submitted is the result of the candidate's own investigation under the supervision of Professor J.M. Dodd in the Department of Zoology, University College of North Wales, Bangor, from October 1973 to October 1976. Due acknowledgement has been made in the text of any assistance received.

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CHAPTER 1

REPRODUCTIVE CYCLES IN S. CANICULA

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REPRODUCTIVE CYCLES IN S. CANICULA

Introduction

The survival of any species in a seasonally unstable environment is dependent primarily on that species adjusting its breeding season so that offspring are reared at the most favourable time. In the fishes the teleosts have received by far the greatest amount of attention, not least because of the existence of approximately 20,000 species. Although only a few of this vast number have been investigated, and these are restricted to a few orders, in most teleosts species the process of spawning is limited to a relatively brief time span (Breder and Rosen, 1966). However, one must not generalize, since, as Hoar (1955) has pointed out, the teleosts have exploited the whole gamut of reproductive devices known in vertebrates, and thus as wide a coverage as possible is desirable. Munro et al. (1973) showed that the majority of thirty five species of Caribbean reef fish examined monthly over a period of two years spawned throughout the year, although a peak in spawning is reached when water temperatures are minimal in February, March and April. Furthermore, the biomass of reef fish eggs in the plankton is approximately twelve times greater in the above mentioned period than in the period from June until December.

Hyder (1969a, 1970) encountered males of Tilapia leuosticta, T. nigra and T. zilli in various stages of sexual maturity at all times of the year, although spawning in T. leuosticta corresponds to the period of highest temperatures and the onset of the rainy season. Evidence has been produced which shows a close relationship between flooding and spawning in several tropical species (Khanna, 1958; David, 1959; Tang, 1963). Thus even near the equator, where daylength is approximately



twelve hours throughout the year, and annual temperature fluctuations are minimal, the fish investigated have all shown some degree of annual cyclicity in their reproduction.

Of those species that have been studied intensively enough to establish a definite annual cycle of reproduction, in only a few have the environmental and hormonal factors controlling the cycle been investigated. Major contributions to the effects of environmental factors, mainly temperature and photoperiod, on the reproductive patterns have been provided by Sundararaj and his co-workers, working with the catfish, Heteropneustes fossilis, de Vlaming, on the estuarine gobiid fish, Gillichthys virabilis (see Sundararaj and Vasal, 1976, and de Vlaming, 1974, for reviews) and Baggerman (1969, 1972) with the stickleback, Gasterosteus aculeatus.

There is fairly extensive information available on histophysiological studies of the cyclical changes in the gonads of a number of species of fishes (for specific references see Hoar, 1965), but much less on hormonal annual cycles. The pituitary regulation of the gametogenic activity of teleosts fishes was firmly established in the pioneer studies of Vivien (1938, 1941) and Matthews (1939), and has been described many times since. Successful hypophysectomy has been accomplished in only a few species, but it has demonstrated that the pituitary is essential for the development and maintenance of the larger ova and for the further development of spermatogonia (Pickford and Atz, 1957; Barr, 1963a, b). Numerous individual observations have been made on the gonadotrophic activity of the pituitary of many species of teleosts, but frequent observations throughout the breeding cycle of any one species are rare (Pickford and Atz, 1957). Gerbil'skii, 1940; Kazanski, 1951,



1952; Fontaine and Charvel, 1961; Clemens and Sneed, 1962; and Clemens and Johnson, 1965, using a variety of bioassays, have all shown that pituitary gonadotrophic activity is low following spawning or during gonadal regression, but is relatively high during most other times of the year. Swift and Pickford (1965) stated that maximal gonadotrophic activity in the pituitary of the Perch, Perca fluviatilis, was highest just prior to spawning and lowest during the gonadal quiescent stage; similar observations have been reported in Pleuronectes platessa (Barr and Hobson, 1964) and Mystus vittatus (Singh, 1970). In the bream, Abramis brama, gonadotrophic potency of the pituitary is as great in the spring as in the fall, but cytological investigations suggested that gonadotrophic cells are most active in the spring (Gerbilskii, 1940). De Vlaming (1972) reported that pituitaries obtained from Gillichthys mirabilis with regressing gonads were just as effective as pituitaries from fish undergoing testicular recrudescence in stimulating spermatogenesis in hypophysectomized fish of the same species. Although there may be little difference in gonadotrophin content of pituitaries from Gillichthys with regressed and active gonads, Zambrano (1971) suggest that there may be differences in the ratio of hormone secretion.

However, although most lines of evidence suggest that it is the gonadotrophic principle which is of primary importance to the seasonal development of fish gonads, the above bioassay studies must be viewed in the light of species specificity when different species are used as test and donor animals (Licht and Papkoff, 1976), even within the same order (Licht and Midgley, 1976a). Also Campbell and Idler (1976) have shown that a glycoprotein preparation from teleosts pituitaries, which has the ability to induce oocyte maturation and ovulation in the



hypophysectomized Winter Flounder, Pseudopleuronectes americanus (Campbell, 1975), a classical gonadotrophic activity, fails to stimulate gonadal incorporation of the yolk which accumulated in the serum. In contrast, the nonglycoprotein fraction of these pituitaries stimulated yolk incorporation into the ovary.

Direct evidence for gonadotrophin release during reproductive cycles of fish is limited (Breton et al., 1973). Crim et al. (1975), using an homologous radioimmunoassay for salmon gonadotrophin (Crim et al., 1973), demonstrated that sexual maturity of both males and females was related to significant increases in plasma gonadotrophin concentration, with the highest values observed being females near the time of ovulation. An ovulatory surge of gonadotrophin in the female goldfish was directly confirmed by Breton et al., 1972, using an homologous carp radioimmunoassay.

The effects of exogenous sex steroids, both mammalian and synthetic, on sex accessory organs, secondary sex characters and sexual behaviour in female teleosts have been examined many times. The results of these experiments combined with the effects of ovariectomy leave little doubt that development of secondary sex characters and sexual behaviour is controlled by gonadal steroids (see reviews of Pickford and Atz, 1959; Ball, 1960; Dodd, 1960; Bern and Nandi, 1964; Hoar, 1957, 1965; Nandi, 1967; Raggerman, 1968; Barr, 1968; Liley, 1969, 1972; and Reinboth, 1972, for a summary of this work). In male teleosts injections of testicular extracts, and castration followed by replacement therapy, have established that the testes are a source of androgenic sex hormones upon which the state of development and functional activity of secondary sexual characteristics and sexual behaviour depend (see reviews cited above).



Although there is voluminous literature on the identification, synthesis and effects of sex steroids, there are very few reports of changes in plasma sex steroids accompanying changes in the gonads of teleosts. Changes in androgen levels for both sexes during the spawning process were found in sockeye salmon, Oncorhynchus nerka (Grajcer and Idler, 1961; Schmidt and Idler, 1962; Idler, 1969). Schreck, Flickenger and Hopwood (1972) demonstrated that immature rainbow trout, Salmo gairdnerii, had lower plasma androgen levels than mature trout, and Cedard, Fontaine and Nomura (1961) reported oestrogen concentration of Atlantic salmon, Salmo salar, increases over the period of the spawning migration. Androgen levels of both male and female goldfish clearly followed temporal patterns with the highest levels during spawning (Shreck and Hopwood, 1974) as did the oestrogen levels of the female, although not of the male.

Eleftheriou et al. (1966) have shown that an increase in plasma oestrogens occurs with an increase in ovary weight in the channel catfish. Recently Campbell et al. (1976) have shown that in plasma from male Winter Flounders testosterone concentrations changed only slightly during the annual cycle but 11-ketotestosterone concentrations rose dramatically near the time of spawning. Testosterone levels were highest prior to spawning in plasma from female fish but the concentration of 11-ketotestosterone remained extremely low throughout the year. This contrasts with the results of Wingfield (1973) and Wingfield and Grimm (1974) who found, using radioimmunoassay, that plasma testosterone concentrations fell in both sexes of the plaice, Pleuronectes platessa, prior to spawning.

Besides the sex steroids, a number of other steroids have been implicated in the control of reproductive processes such as meiotic maturation.



tion of teleosts oocytes (see review by Jalabert, 1976). These in vitro study techniques have shown that gonadotrophic action of the pituitary is probably mediated by direct steroid action on oocyte maturation in a number of species. However, according to the species studied and the authors, conclusions are contradictory as to the nature of the active steroids, their site of production, and the link between in vitro maturation and ovulation. These studies gave no attention to annual changes in the steroids until recently, when Campbell et al. (1976) showed that the ratio of cortisol to cortisone concentrations underwent an annual cycle which is similar in plasma from both sexes of the Winter Flounder. However, this cycle was not directly related to the reproductive cycle, and was considered to reflect possible changes in feeding activity.

Elasmobranchs, although they are an extremely ancient class which has been separated from the other vertebrates for more than three hundred million years, have received a good deal of attention from comparative anatomists and histologists (see Perks, 1969, for specific references) though relatively little from physiologists (Dodd, 1975). Living elasmobranch fishes can be divided into two main groups, the Euselachii and the Holocephali. Nothing is known of the reproductive endocrinology in the latter group, and little has been reported for the selachians until recently. These comprise the Hypotremata (skates and rays), which are mainly oviparous, while in the Pleurotremata (sharks and dogfishes) oviparity, ovoviviparity and true viviparity are found (D'Aubrey, 1963), and all these methods, with the possible exception of oviparity in some species, appear to involve well defined reproductive cycles (Dodd, 1972).

Notes on the reproductive cyclicity of oviparous dogfishes and



skates are available for a disparate array of species; most of the data being obtained incidentally, while investigating other species, or as aquarium observations. De Lacy and Chapman (1935) noted that among the San Juan Islands egg-capsules of Raia binoculata may be collected throughout the summer. In early June egg-capsules containing well advanced young were frequently trawled, while in August freshly deposited egg-capsules were not uncommon, thus indicating a possible limited spawning period. However, Clemens and Wilby (1961) believed that the egg-capsules were laid the year round, this being supported by Hitz (1964) who found egg-capsules containing embryos at all stages of maturity in a single haul of a dredge. Nevertheless these observations do not exclude the possibility of a peak season as McEachran (1970) found 45.8% of adult Raia garmani females with egg-capsules in the summer as opposed to 16.7% in the winter. Vladykov (1936) found that the breeding season of Raia diaphanes in Canadian Atlantic waters is during September; this being partly confirmed by Scattergood (1951) who observed females of the same species containing egg-capsules in the same month along the Maine coast. Clark (1922) studied the reproduction of eleven different species of skate and ray occurring at Plymouth. The majority of egg-capsules were found in April, May and June, although these months represent the period of intensive fishing for skates and rays and therefore may not reflect the full extent of the breeding season. Holden et al. (1971) maintained R. clavata, R. brachyura and R. montagui in the Lowestoft aquarium and noted egg-laying in all three species during the spring, although it appeared that R. montagui had a shorter breeding season than either of the other two species; but unfortunately observations were limited to a few individuals.

The most complete account of a reproductive cycle in the skates



and rays comes from Du Boit (1976), who observed between six and seventeen individual females of R. naevus each month over a sixteen month period. She observed egg-capsules throughout the year, contrary to Clark (1922) who found them only from February to June, and by plotting the frequency of five arbitrarily defined developmental stages of oocytes per month throughout the period of study was able to show the absence of any ovarian cycle.

Within the oviparous sharks and dogfishes, Scyliorhinus canicula has received by far the most attention because it is very common around the shores of Great Britain and its small size enables it to be maintained successfully for long periods in aquaria. Ford (1921) examined S. canicula landed by fishermen at Plymouth and presented strong evidence that egg-capsules may be deposited in any month. His results suggested that egg-deposition takes place principally during the spring and summer, and is least during the autumn. The same conclusion was reached by Melton (1939), who found over 50% of the dogfishes examined between December and July had egg-capsules in their oviducts, while less than 5% did during September. Harris (1952) studied the dogfishes supplied to the Department of Zoology at Bristol and concluded that breeding starts in November, when only 18% of the females are carrying egg-capsules, and continues at least until July. All three studies demonstrated that September and October were the months of no or least egg-laying activity, the maximum rate occurring during the spring (see Table 4, for a numerical analysis of these data). Ford (1921) also studied egg-capsules of the Nursehound, Scyliorhinus stellaris, and found the developing embryos were separable into two distinct size groups, so concluded that the breeding season had definite limits. The only other observation on oviparous dogfishes or sharks concerns the

Hornshark, Heterodontus francisci, which deposited egg-capsules during February and March when maintained in an aquarium (Dempster and Herald, 1961).

Most non-oviparous elasmobranchs appear to have a well defined reproductive cycle. In Mustelus canis, a viviparous species living off the east coast of North America, parturition and copulation succeed a gestation period of ten months, and ovulation is said to be copulation-dependent (Hisaw and Abramowitz, 1939). A very similar cycle was found in the Japanese dogfish, Mustelus manazo, by Teshima et al. (1971). The spiny dogfish, Squalus acanthias, is of special interest to biologists because of its unique reproductive cycle. Ford (1921), in his study of the life history of dogfishes of the Northeastern Atlantic, was the first to discover the long gestation period of 22 months. His observation was subsequently confirmed for the Northwestern Atlantic by Templeman (1944) and Hisaw and Albert (1947); for the Northeastern Pacific by Hart (1942) and Bonham et al. (1949); and for the Northwestern Pacific by Yamamoto and Kibezaki (1950). Interestingly, as Ketchen (1972) pointed out, whereas S. acanthias of the North Atlantic and Northeastern Pacific gave birth to their young in the late fall and winter months, these events are delayed until the late spring in the Northwestern Pacific. This difference cannot be readily associated with environmental differences among areas since, superficially at least, the temperature domains of dogfishes in the Sea of Japan, the Northwestern Atlantic and Northern North Sea are not noticeably different.

The viviparous shark Carcharhinus dussumieri showed no definite reproductive season in the Sea of Japan, embryos representing all developmental stages were found during the short collection period (Teshima and



Mizue, 1972), whereas a closely related species, C. milberti, distributed in the waters of the Eastern coast of North America and in the East China Sea, mates in the spring or early summer, with the time of birth being from March until August the following year (Springer, 1960).

Although there is thus a large amount of literature demonstrating elasmobranch reproductive cycles, virtually only a single species, the lesser spotted dogfish, Scyliorhinus canicula, has been studied experimentally, and many important questions are still unresolved. But, as Dodd (1975) points out, there is no case for generalizing to other elasmobranchs from the dogfish.

Of the work on elasmobranch steroids, few mention seasonal variations. Fletcher et al. (1969) quantitated testosterone levels in male and female plasma of the skate Raja radiata caught during summer months and compared them to levels found in the same species by Idler and Truscott (1966) in fish caught in February; the females showing lower levels in the winter, although the males showed no significant change with season. Iupo di Prisco et al. (1967) assessed the plasma levels of various steroids in Torpedo marmorata at several stages of the sexual cycle in females, though their results have been brought into question with a general criticism of the method used for measurement of oestrogens by Eik-Nes and Horning (1968). Recently Dobson (1974) and Sumpter (1976) have demonstrated marked seasonal cycles of both testosterone and oestradiol as measured by radioimmunoassay, in plasma of both sexes of S. canicula.

The elasmobranch pituitary shows an unusually great degree of subdivision into regions (Dodd et al., 1960). Rostral, median, ventral and neurointermediate lobes are easily recognizable, and largely separate



from each other, the latter fusing with the neurohypophysis to form a neurointermediate lobe. The ventral lobe appears to be the main source of gonadotrophic activity (Scanes et al., 1972), and Dobson (1974) has demonstrated by bioassay of total gonadotrophin that the male at least possesses a well marked annual cycle of gonadotrophic content. Recently Firth and Vollrath (1973) have demonstrated that the median lobe contains luteinizing hormone-like gonadotrophic activity, and this also appears to show some seasonal variation in the male but not the female, although the data are less convincing than those of Dobson, being based on only six samples throughout a year.

The thyroid gland has also been implicated in elasmobranch reproduction, although until recently this was based only upon histological studies. Ranzi and Zozza (1936) and Oliverreau (1949a) have reported that gestation in Torpedo marmorata is accompanied by high thyroid activity and hyperaemia of the gland. Oliverreau (1949b) found that the onset of sexual maturity in S. canicula is associated with signs of increased thyroid activity; further, in mature females the thyrosomatic index (T.S.I.) is approximately twice that of males, and there is a cyclical intensification of thyroid activity related to reproductive function. The results of recent experimental work on the effects of thyroidectomy on vitellogenesis (Lewis and Dodd, 1974) appear to indicate that thyroid hormones are implicated in vitellogenesis.

The last major group of fishes are the cyclostomes, which are the most ancient of living vertebrates. The Class comprises two very different Orders: the Myxinoidea or hagfishes, with only two or three living species, and the Petromyzonidae or lampreys with 31 living species. Most contemporary hagfishes live in the deep sea in perpetual darkness and



thermal constancy and thus lack environmental cues by which a reproductive cycle might be regulated. Furthermore, the portal vessels, through which information is transferred to the adenohypophysis, are apparently lacking. Thus the lack of a synchronized cycle of maturation of eggs (Gorbmann et al., 1963) seems to be consistent with the external and internal situation in Polistotrema. Eptatretus burgeri¹, on the other hand, appears to show an annual migration from the deep sea and while in shallow water, where it is exposed to ^{possible photoperiodic} environmental information, the eggs mature. Kobayashi and Uemura (1972) demonstrated a region in the ventral wall of the neurohypophysis of the hagfish which, they state, has all the characteristics of a median eminence, and they believe this system may constitute the co-ordinating mechanism for the seasonal reproductive cycle found in E. burgeri².

Lampreys breed only once and then die, and this is reflected in the fact that the gonads mature only in the last year of the animals' lives and contain only a single crop of germ cells; these show virtually synchronous development (Dodd, 1960; Evennett, 1963). Larsen (unpublished, quoted in Larsen and Rothwell, 1972) believes that the timing of sexual maturity is controlled by temperature and that light plays no part in it, so that central nervous co-ordination may not be required.

In summary, most species of fish studied appear to have a definite reproductive cycle, and this seasonality is reflected in changes within the endocrine system. Some oviparous elasmobranchs appear to possess an extended egg-laying season or continue to lay throughout the year, although the intensity may vary. However, they still appear to show definite endocrine cycles where these have been studied. The only real exception so far uncovered is a single species of hagfish, which is atypical in living in a habitat that appears stable throughout the year.



Materials and Methods

1) Fish Husbandry

Specimens of Scyliorhinus canicula were obtained by trawling in Caernarvon Bay and Liverpool Bay, off the coast of North Wales, at depths between 10 and 50 metres. Immediately after capture six to ten fish were transferred to each shallow-polythene tub (60 x 45 x 24 cm) approximately two-thirds full of fresh seawater. Movement of fish was very restricted in these tubs, and fish usually lie still in close proximity to one another. The water was periodically replaced at sea, especially during the summer months when fish were less liable to survive, more so if crowded. Fishing trips usually lasted one day, although occasionally longer trips were undertaken and in this case the fish were maintained in a single much larger tank (approximately five cubic metres capacity) in which fresh seawater was constantly circulated.

Upon return to the Nuffield Marine Laboratory at Menai Bridge the fish were placed in fibre glass tanks; white, grey or black with approximate dimensions of 1.3 x 0.7 x 0.3 metres depth. A constant supply of recirculated, filtered seawater was run through the tanks which were part of a closed system incorporating two large gravel filters plus an air supply to each tank. Under these conditions survival of fish was very good, and even unpredictable difficulties like the breakdown of pumps and failure of the electricity supply resulted in little or no loss of fish if they were discovered fairly soon.

Fish were also collected on a monthly basis over a period of one year from Plymouth. These were captured by trawling, usually in deeper water than around the coast of Anglesey, maintained alive in tanks on the boat, then deep frozen on arrival at the Marine Biological Station at



Plymouth by members of the station staff. On one occasion five monthly samples, varying between 10 and 18 fish, were collected from Plymouth and driven to Wales, being maintained on ice in transit. On another occasion the remaining monthly samples were examined at Plymouth.

From both collections of fish only those that were sexually mature were used for the annual cycle. In general mature females are above 750 g in weight, although sometimes larger immature fish are found. Thus the state of maturity was assessed by examination of the cloaca. Immature fish have a hymen occluding the oviducts, whereas mature fish have open oviducts.

2) Collection and Preparation of Material

For all annual cycle data fish were sampled on the morning after capture; they had therefore been maintained in the seawater system for approximately 12 hours. Samples were taken approximately one month apart and covered a period of eighteen months in 1974 and 1975. Fish were lightly anaesthetised in an 0.05% solution of MS 222 (Sandoz) in seawater, blotted dry with a towel and weighed to the nearest 1 g on a Mettler P1200 top pan balance. Two ml of blood was withdrawn from the caudal sinus of each as follows. The fish was placed flat, ventral side uppermost, and a 21 G needle attached to a 2 ml plastic syringe inserted into the midline about 3 cm behind the cloaca at a slight angle pointing forwards. It was passed slowly inwards until the base of the vertebral column was reached, then withdrawn a few millimetres and the plunger withdrawn slowly. With experience 2 ml of blood can be collected very easily this way, or if it fails initially slight movements of the needle tip will facilitate collection. The blood was spun immediately in a small Piccolo (Heraeus Christ GMBH, Germany) centrifuge at 1000 rpm



for a few minutes. Clotting did not occur even without the use of heparin and the plasma sample was decanted into shell vials and stored under deep freeze at -20°C until assayed for androgen or oestrogen as described later.

The peritoneal cavity was then opened and the ovary removed with a pair of fine scissors, blotted briefly on filter paper to remove any surface liquid, and weighed to the nearest 0.1 g on the same Mettler P1200 balance. The head of the fish was then severed at the level of the spiracles and a block approximately 2 x 1 cm, containing most of the brain, removed by making two longitudinal cuts just inside each eye socket and a single transverse cut about 2 cm forward from the first cut. The top and sides of this block were then removed using a scapel to leave the brain sitting on the chondrocranium floor in which is embedded the ventral lobe (Figure 1). This lobe, housed in a depression, is intimately attached to a tough connective tissue capsule. It is time consuming and difficult to dissect out this capsule and therefore the fraction of the mass of the ventral lobe comprising the glandular tissues is unknown. Dissection of the neurointermediate lobe (NIL) and rostral plus median lobes (R+ML) was accomplished easily with the aid of fine forceps and scissors.

3) Assay procedures and validation

a) Bioassay of pituitary gonadotrophin content

Bioassay of gonadotrophin levels in the pituitary lobes of dogfish was achieved using the method of Breneman et al. (1962) as modified by Follett and Farmer (1966) and Follett (1970). This technique is a refinement of an earlier method using increase in testicular weight of day old chicks as the parameter of gonadotrophic activity



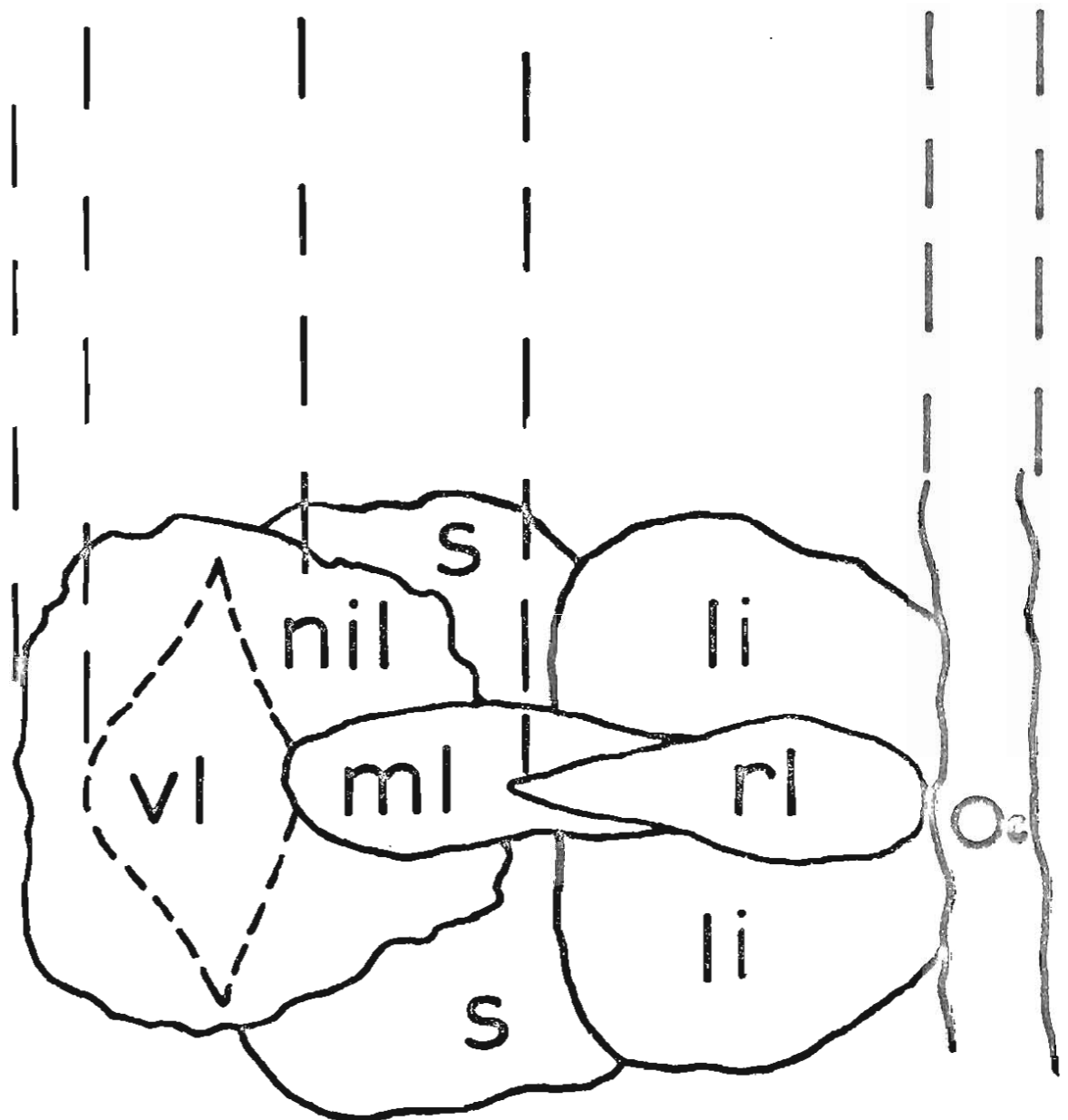
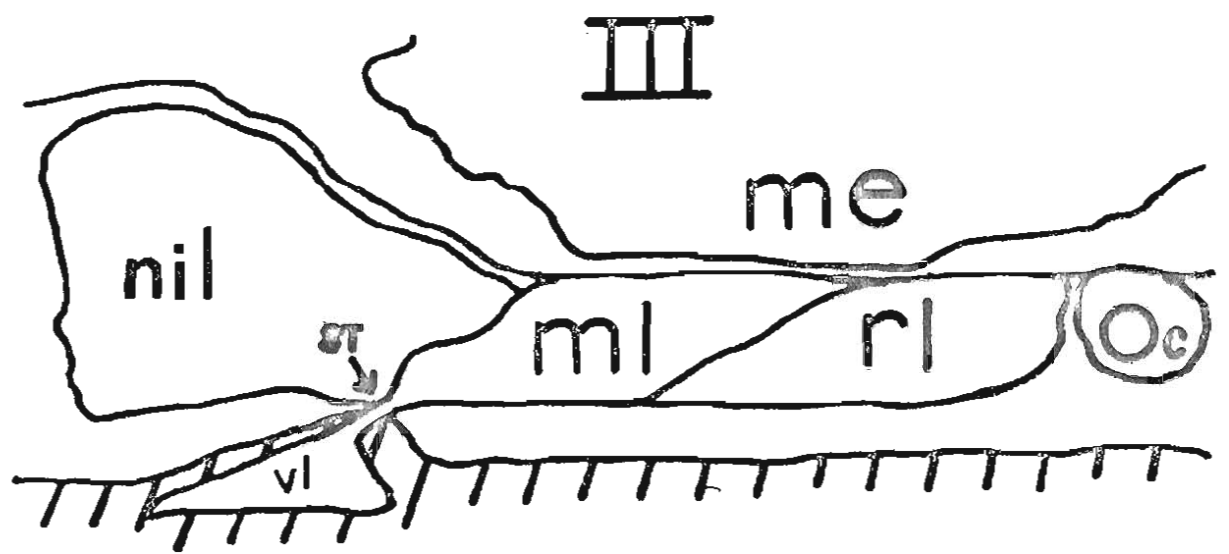
Figure 1:

Arrangement of the pituitary lobes in *Scyliorhinus canicula*.

The upper diagram shows the pituitary lobes in longitudinal section, the lower in ventral aspect.

NIL	=	neurointermediate lobe
ML	=	median lobe
RL	=	rostral lobe
VL	=	ventral lobe
S	=	saccus vasculosus
Li	=	lobi inferiores
Oc	=	optic chiasma
Me	=	median eminence
III	=	third ventricle of the brain
St	=	stalk connecting the ventral and median lobes





(Breneman, Zeller and Beekman, 1959).

Studies with both partially purified mammalian hormones - mostly ovine hormones from the National Institute of Health (NIH) - (e.g. Breneman et al., 1962; Kamiyoshi et al., 1972) and more highly purified hormone preparations (Licht, 1973b) indicate that ^{32}P uptake by chick testes is nonspecific for luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A similar lack of discrimination is also evident for avian gonadotrophins (Follett et al., 1972), but no convincing evidence is available on the specificity of the assay in dogfish (see pp. 114 for discussion of dogfish gonadotrophin(s)). Throughout this work a single batch of NIH-LH-S19 was used as the standard in all assays, but as Dobson (1974) points out, this should not be taken as indicative of LH activity in the elasmobranch pituitary.

The specificity of the technique to gonadotrophins has been demonstrated by several workers (Furr, 1969; Stetson and Erickson, 1970) with respect to mammalian standards. Thyroxine and oestrogen were shown to be ineffective by Furr (1969), though testosterone at a dose of 100 $\mu\text{g}/$ chick gave a significant augmentation of ^{32}P uptake. Stetson and Erickson (1970) demonstrated that prolactin had no effect, and both mammalian prolactin and growth hormone tested in this laboratory (Scanes, unpublished observations) proved ineffective. Licht (1973b) showed that highly purified LH and FSH, essentially free of cross contamination, showed little if any synergism when tested together, so it seems reasonable to conclude that each type of gonadotrophin has intrinsic activity in this assay.

Various different protocols have been reported for the assay, including varying the site of injection, the time interval between stages, or the amount of ^{32}P injected (see Kamiyoshi, 1972, for specific references).



Initially Breneman et al. (1962) obtained an index of precision of 0.1512 ± 0.0299 with between 40 and 52 chicks per dose, but Follett and Farnor (1966), using only 7-10 chicks per dose, obtained values of 0.154 ± 0.044 .

Cockerels (Thorner 909), obtained within 24 hours of hatching from Mytholmroyd Hatcheries Ltd., Hebden Bridge, were kept in continuous light without food or water from the time of collection throughout the assay period. No birds died during the period between injection and sacrifice. Standard gonadotrophin (NIH-1H-S19) was weighed immediately before use on a Cahn Electrobalance to $\pm 1 \mu\text{g}$ and dissolved in 0.9% saline, then serially diluted to give standards at 6.0, 3.0 and 1.5 μg per 0.2 ml. The lyophilized standard was stored in the deep freeze between assays and in this condition has been reported to maintain full potency for a 12 month period by Breneman et al. (1962). Ventral lobes, usually in groups of five, were removed as previously described and four or five transverse cuts made through each lobe. At certain times of the year a single ventral lobe per 2 ml was adequate. The slices were homogenized in a 5 ml hand glass homogenizer containing 2 ml 0.9% saline. This squashes the glandular material out from the cartilage into solution. This was then pipetted out and 0.7 ml diluted by half to give two logarithmically separated doses.

Hormones were injected subcutaneously in the nape of the neck in 0.2 ml, using 6-10 chicks per dose; $1 \mu\text{Ci } ^{32}\text{P}$ was injected by the same route 5 hours later; and the birds sacrificed 22 hours after the hormone injection. Six chicks were injected with 0.9% saline as controls. Groups of chicks were marked with coloured solvent pins. At sacrifice the testes were removed, briefly blotted dry to remove surface body fluids, and weighed on a torsion balance to the nearest 0.1 mg. As killing of



chicks could take up to two hours, they were sacrificed in strict rotation across treatments to counteract variability due to time. After weighing, the tubes were placed on numbered squares of Whatman filter paper and dried under an infra-red lamp for approximately 30 minutes. Dried papers were dropped into previously backgrounded vials containing 5 ml scintillator (4 g PPO and 0.3 g dimethyl POPOP per litre in nitration grade toluene) and counted to an error of $\pm 2.75\%$ (95% confidence limits) in a Nuclear Chicago Unilux III liquid scintillation spectrometer. Quenching was totally absent. Data were expressed as counts per mg wet weight of testes.

Estimates of potency were made with a procedure for four (2:2) balanced factorial assays. If very low gonadotrophin levels were indicated, a 2+1 format was adopted. Analysis of variance was carried out using the method of Bliss (1952), with several unknowns being estimated in a single analysis of variance table to give mean potency and 95% confidence limits. All potencies are expressed as μg equivalents NIH-LH-S19 per lobe. Index of precision was calculated for all assays together with a function indicating departure from parallelism.

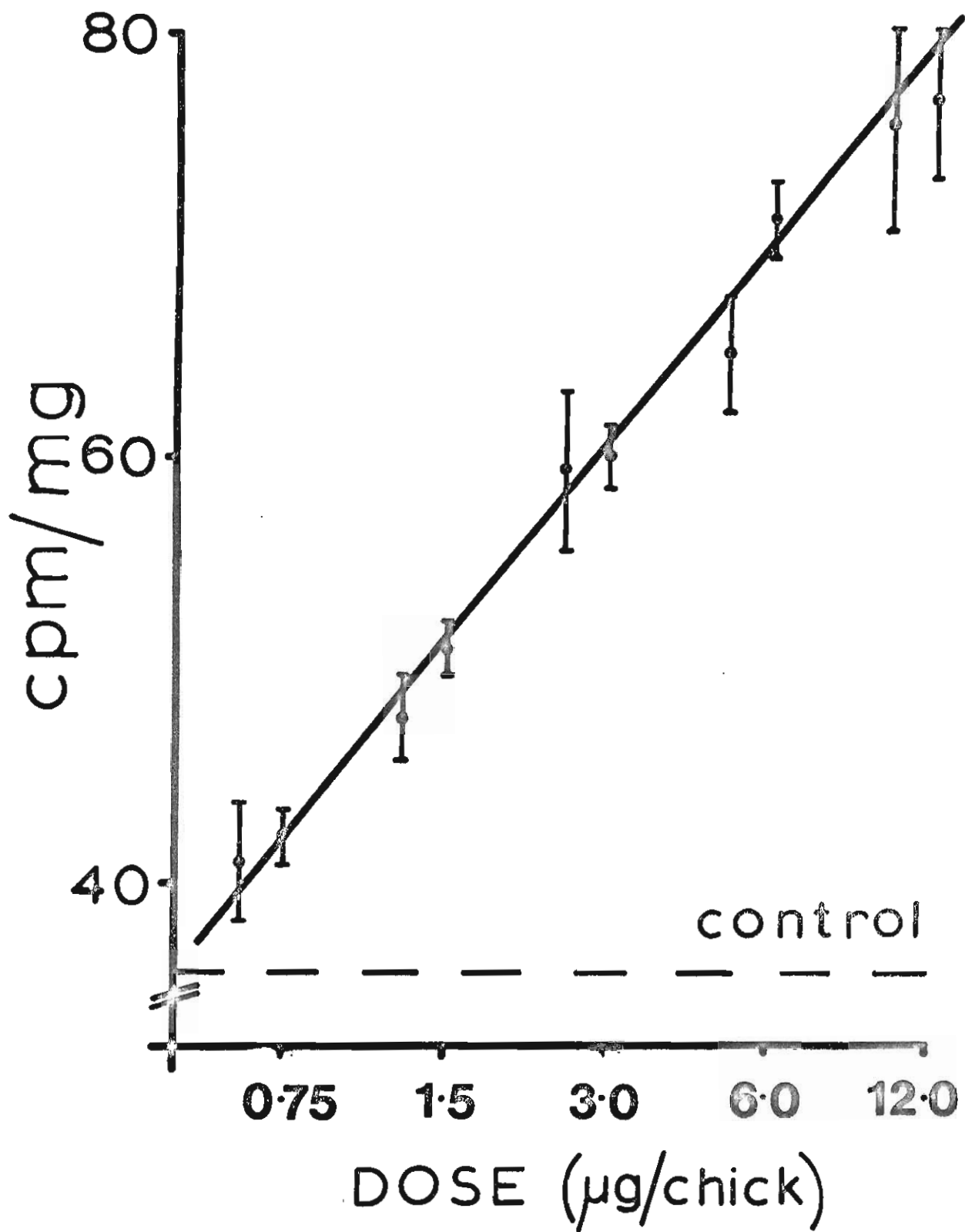
Over a 24-month period the response of the chicks to the standard doses of LH-S19 showed little variation (Figure 2). Mean values (cpm/mg (N)) from all data were: saline 36.06 (296); 0.625 μg NIH-LH-S19 41.13 (56); 0.75 μg 42.02 (176); 1.25 μg 48.70 (64); 1.5 μg 50.92 (232); 2.5 μg 58.53 (64); 3.0 μg 60.10 (232); 5.0 μg 65.01 (64); 6.0 μg 71.19 (208); 10.0 μg 75.64 (48); 12.0 μg 76.82 (80). Between 1.25 and 10.0 μg a log-linear dose-response relationship is obtained which may be represented by the line $y = 44.48 + 3.69 x$. The mean index of precision (λ) of the first twenty assays was 0.263 ± 0.102 (SEM), which is similar



Figure 2:

Composite dose-response curve of mammalian LH (NIH-LH-S19)
based on 42 assays.





to the values of 0.219 ± 0.066 (43) reported by Follett (1970) and 0.228 ± 0.092 (50) reported by Scanes (1972), but higher ($P < 0.001$) than Follett and Farmer (1966) where a λ of 0.154 ± 0.044 (57) was obtained. One must bear in mind, though, that both the source and strain of chicks varied in the different studies.

b) Radioimmunoassay of plasma testosterone

1. Introduction

The radioimmunoassay for plasma testosterone developed by Maung, S.L. (1976) was used. Antitestosterone serum was obtained from Dr. Burton Caldwell of Yale University Medical School. It was prepared in rabbits by injecting testosterone conjugated by an oxime link from carbon 3 of the steroid to bovine serum albumin. In later experiments an antiserum prepared by Dr. B.J.A. Furr (I.C.I. Alderley Edge, Cheshire) was used. This antiserum was prepared in a goat by injecting testosterone-3-carboxy-methyl oxime - BSA.

Assays were usually carried out under conditions where 50% of the labelled testosterone was bound to the antiserum in the absence of unlabelled testosterone (Bound/Free ratio or B/F of 1). Initially, therefore, a serial dilution of the antiserum was carried out to determine the optimal dilution. This proved to be 1:1000 for the Caldwell antiserum and 1:4000 for that from Furr.

2. Extraction of testosterone from plasma

All glassware used in the radioimmunoassay was cleaned as follows. It was first left immersed in a concentrated solution of Pyronex (Diversey Ltd., Barnet, Herts, England), a specialized product for the cleaning of laboratory glassware, for at least 24 hours, then transferred to a bucket through which cold tap water flowed continually overnight, and finally



washed twice in distilled water and twice in crude methanol before drying in a warm oven.

Each plasma to be assayed (usually a 50 μ l aliquot) was added to the bottom of a cleaned centrifuge tube (110 x 15 mm) followed by approximately 500 cpm of ^3H testosterone dissolved in 50 μ l diluent (0.1 M phosphate buffered saline, pH 7.0, containing 0.1% sodium azide). Duplicate 50 μ l samples were also added direct to scintillation vials to enable the exact number of counts added to be calculated. Tubes were mixed briefly and left to stand for 15 minutes. The purpose of adding the ^3H testosterone is to calculate the percentage of steroid extracted from the plasma. 2 mls of a 1:4 mixture of diethyl ether:petroleum spirit (60-80°C), both Analar grade, was then added and the tubes mixed for approximately 2 minutes each, left to stand for 15 minutes, the process repeated, then spun briefly at 1000 rpm to get all the aqueous layer to the bottom of the tube. Tubes were then placed in the deep freeze at -60°C to freeze the aqueous layer, and the ether containing the extracted steroid decanted into similar clean centrifuge tubes. This was dried down in a water bath at 37°C under a stream of air and finally redissolved in 170 μ l of diluent with 0.1% BSA added. With new solvents between 70-90% extraction of testosterone was obtained, although older solvents need redistilling to maintain this percentage extraction. This was usually unnecessary, however, because solvents were used too quickly to warrant redistilling.

Besides plasmas, each assay also contained at least one distilled water blank and both a mature male and female dogfish plasma pool which were included to check the reliability and inter-assay variation. As Maung, S.L. (1976) has demonstrated, it is necessary to use the same volume



of plasma throughout, here 50 μ l, because by increasing the volume of plasma extracted one decreases the level of testosterone measured, e.g. if 50 μ l of plasma was extracted and gave a level of 5 ng/ml, then 500 μ l of the same plasma would give approximately 2.5 ng/ml (Table 2). This is a recurring problem in steroid radioimmunoassays not including a chromatographic step.

3. Preparation of the standard curve and the assay procedure

Step 1: A standard solution of testosterone at a concentration of 2000 pg/50 μ l was prepared using crystalline, analytical grade testosterone (Sigma).

50 μ l aliquots of the standard solution were serially diluted in triplicate in glass tubes (10 x 75 mm, flint glass, no. TF 104, Gallenkamp) to produce 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.82, 3.96 and 1.95 pg/tube. Duplicate aliquots of 50 μ l of the unknowns were added to further tubes, while a final 50 μ l aliquot of the unknown was added directly to a scintillation vial for recovery determination. Three triplicates of blank tubes containing 50 μ l of diluent were also set up. The purpose of the blanks were as follows:

- (a) No antiserum added - these estimate the free counts not absorbed by the charcoal.
- (b) No charcoal added - these measure the total counts added to each tube.
- (c) No unlabelled testosterone - these measure the maximum binding of tritiated testosterone and provide the "100% binding" or the zero point of the standard curve.

Step 2: 50 μ l of ^3H -testosterone (ca. 8000 cpm/50 μ l in diluent) was added to each tube. Very high specific activity (83 Ci/mM) tritiated testosterone was obtained from The Radiochemical Centre (Amersham, Bucks.,

cat. no. TRK 402; 1, 2, 6, 7 (n)-³H-testosterone dissolved in benzene) and stored at -20°C. Stock solutions were prepared by first drying down 15 µl of the ³H-testosterone under an air stream, and then adding 100 ml of diluent. This solution was stored at 4°C and was usable for at least 3 months.

Step 3: The antisera were diluted in assay diluent to a concentration of 1:1000 (Caldwell) or 1:4000 (Furr) and 50 µl added to all tubes except the first pair of blanks which receive 50 µl of diluent. The tubes were briefly spun to get everything to the bottom, thoroughly mixed and left at 4°C either for 4 hrs or overnight, both producing identical standard curves.

Step 4: After incubation 1 ml of dextran-coated charcoal was added to all tubes except the second set of blanks to which 1 ml of diluent was added. The dextran coated charcoal solution contained 125 mg Norit A (Sigma), 12.5 mg Dextran T70 (Pharmacia) and 50 mg gelatin per 100 ml of diluent. It is essential that the charcoal be added rapidly so to achieve this an "Oxford" dispenser, mounted on a magnetic stirrer to ensure an even suspension, was used. Tubes were then mixed and exactly 15 mins after the addition of charcoal to the first tube they were spun at 2500 rpm for 20 mins (GF-6, MSE Ltd., 25/28 Buckingham Gate, London, S.W.1). The supernatants were decanted into scintillation vials to which was added 10 ml of scintillator. 5 ml of scintillator was added to all recovery vials. The vials were left for at least 12 hours and then counted in an automatic liquid scintillation spectrometer with a counting efficiency of 40% for tritium.

As Maung, Z.W. (1976) showed, all the radioactive testosterone in the one ml aliquots was completely extracted from the aqueous phase

into the scintillator within 6 hrs. The advantage of this method, rather than mixing the aqueous phase with a scintillator capable of dissolving water (e.g. dioxan) was the constancy of quenching as shown by the external standard ratio. The small variability meant that no quench correction was necessary.

Results were calculated as "% bindings" where the mean cpm of the blanks containing no unlabelled testosterone are assumed to represent 100%. If more than a single spin is necessary, each one must contain a set of blanks and the results for that spin calculated from these, although little difference was found between spins in an individual assay. It was usual to count the three blanks containing no antisera first, and the mean cpm was then entered into the automatic background subtract of the counter. To check the assay the B/F ratio in the tubes containing no unlabelled testosterone was also calculated. Typical standard curves for each antiserum are shown in Figure 3.

4. Assay specificity

The cross reactions of the antitestosterone sera with other steroids are shown in Table 1. Dobson (1974) used Sephadex LH-20 columns to separate testosterone and DHT in both male and female dogfish plasma. He concluded that the amount of DHT in the normal volumes of plasma used for assay did not justify an attempt at separation.

c) Radioimmunoassay for oestradiol

1. Introduction

The measurement of oestradiol by radioimmunoassay presents special problems in that most antisera raised against the former cross-react appreciably with the other oestrogens oestrone (E1) and oestriol

Figure 3:

Typical standard curves for the testosterone radioimmunoassay, over the range 0.156 to 40 ng/ml (7.8 to 2000 pg/tube), using either the Furr antiserum (top) or the Caldwell antiserum (bottom).

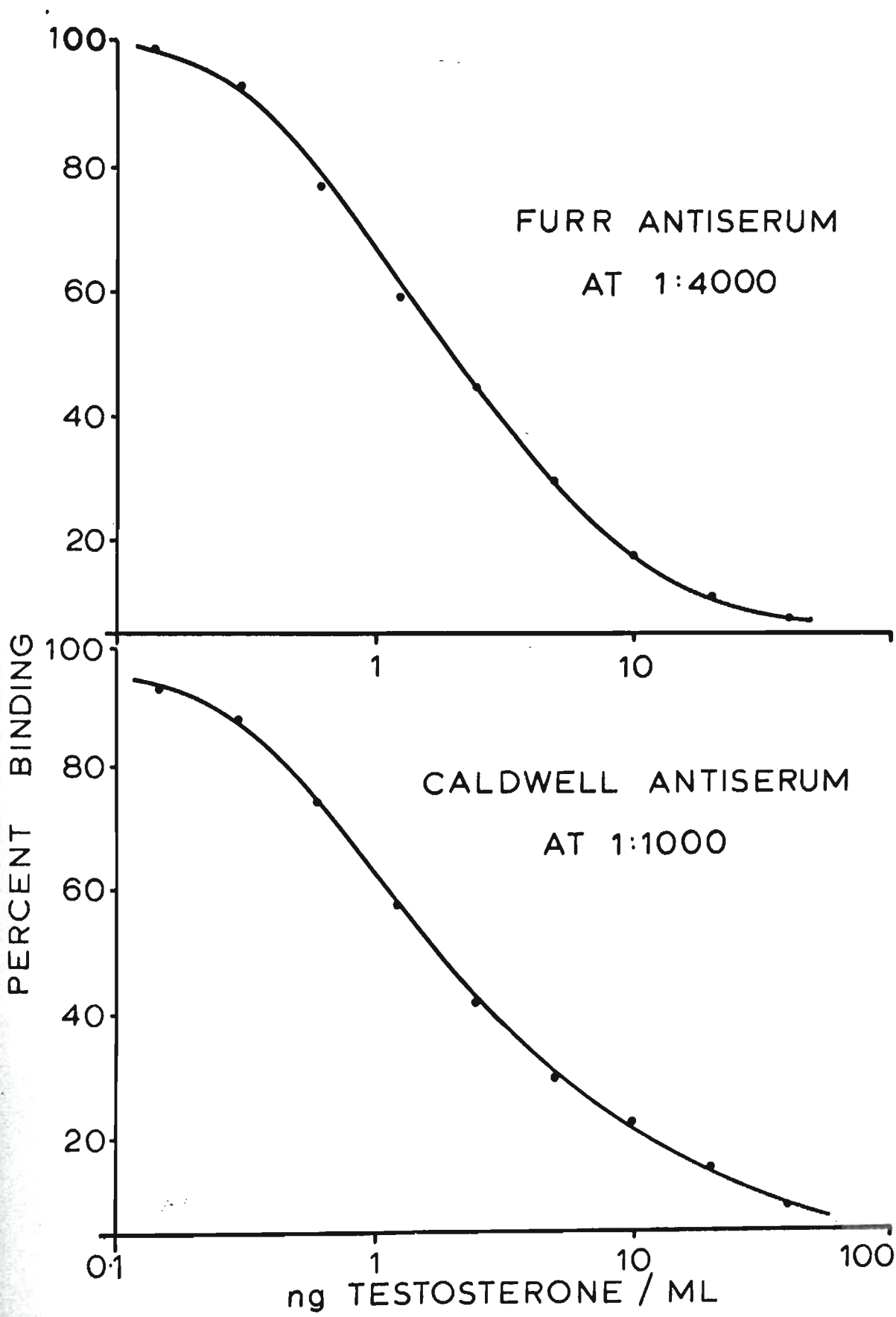


Table 1: Cross-reactions of steroids in the testosterone radioimmunoassay

Steroid	Antiserum	
	Furr	Caldwell
Testosterone	100% ^a	100% ^a
Dihydrotestosterone	66.4 ± 3.8	110
5 β Androstane 3 β -17 β -diol	8.8 ± 1.5	2.75
5 β Androstane 3 α -17 β -diol	13.7 ± 4.0	-
5 α Androstane 3 α -17 β -diol	22.1 ± 4.8	-
5 β Androstane 3 β -17 β -diol	27.4 ± 9.5	-
Nortestosterone	27.3 ± 9.3	-
Androste-4-ene-3:17-dione	0.4 ± 0.1	1.22
5 α Androstane-3:17-dione	0.8 ± 0.9	-
5 β Androstane-3:17-dione	0.6	-
DEHA	0.1	0.06
5 α Androstane-3 β -ol:17-one	6.9	-
5 α Androstane-3 α -ol:17-one	0.2	0.19
5 β Androstane-3 β -ol:17-one	0.3	-
5 β Androstane-3 α -ol:17-one	0.1	-
Cholesterol	0.01	< 0.002
Pregnenolone	0.01	< 0.1
Progesterone	0.04	0.03
Oestrone	0.01	< 0.1
Oestradiol	0.4	0.02
Deoxycortisone	-	0.01
17 α -OH-Progesterone	-	< 0.1
Corticosterone	-	0.002
Cortisol	-	0.002
Aldosterone	-	0.006

^a Expressed as % relative to testosterone.

The data for the Caldwell antiserum and taken from Dobson (1974), those for the Furr antiserum from Etches (1975).



Table 2: Effects of varying plasma volumes on the amount of steroid measured in the testosterone and oestradiol radioimmunoassays

a) Testosterone radioimmunoassay

Sex and maturity	Date caught	Volume of plasma extracted				
		50	100	150	200	250
Mature male	16.4.74	5.20	3.81	2.20	2.77	2.12
Mature male	26.11.74	2.09	2.00	1.61	1.28	1.32
Mature female	7.5.74	2.31	1.71	1.60	1.09	1.13
Mature female	26.11.74	9.82	8.40	5.32	4.98	4.02

b) Oestradiol radioimmunoassay

Sex and maturity	Date caught	Volume of plasma extracted				
		50	100	200	300	500
Mature female	7.5.74	27.70	27.00	26.00	30.56	27.07
Mature female	26.11.74	41.08	56.32	58.97	49.07	51.20

Data for both testosterone and oestradiol radioimmunoassays expressed as ng/ml.



(E₃), although Exley and Woodhams (1976) have shown that highly specific antisera may be produced to the separate oestrogens by conjugation of the antigenic carrier through positions distal to the functional groups of the steroids. The antiserum used in this study, kindly donated by Dr. Burton Caldwell, was prepared in rabbits by oestradiol-17 β conjugated to BSA at carbon 3, and cross-reacts with both oestrone and oestriol (see paragraph on assay specificity). Various chromatographic systems have been employed to purify steroid hormones prior to radioimmunoassay (for review see Abraham, 1974), the two most widely used to separate E₁, E₂ and E₃ being Celite microcolumns e.g. Abraham and Odell, 1970; Robertson *et al.*, 1972; and Sephadex LH-20 columns e.g. Mikhail *et al.*, 1970; Carr *et al.*, 1971; Senior, 1974; Pearson, Murphy and Dix D'Aux, 1975.

This study uses Sephadex LH-20, a lipophilic Sephadex prepared from Sephadex G25, which has been used successfully to separate many steroids, particularly the unconjugated biologically active ones. LH-20 is stable in all solvents which are not strongly acidic and do not contain strong oxidizing agents. Separation depends on molecular size (gel filtration), adsorption, partition, ion exclusion and retardation, and probably mechanisms still unknown. Since it gives very low or negligible blank values it has been especially useful to separate steroids prior to competitive binding assays employing antibodies, transins and receptors. Microcolumns using pipettes have been found advantageous as they can be packed very quickly, the eluate flow can be controlled by input so that stopcocks are unnecessary, and the eluate volume can be made very small.



2. Preparation and testing of microcolumns of Sephadex LH-20

2 ml glass tuberculin syringe barrels, of internal diameter 7 mm fitted with a plastic three-way tap attached to a 21 G Luer stainless hypodermic needle were used to house the columns. 0.4 gram of Sephadex LH-20 was suspended for 1 hr in a mixture of benzene:methanol 90:10 (both Analar grade). The benzene must be freshly redistilled before use or else it gives unacceptably high blank values. The same benzene:methanol mixture was used throughout the procedure for transfer of extracts, elution of columns and preparation of sample aliquots. The empty column was rinsed with solvent which was discarded, then a circle of Whatman GF/B glass filter paper, cut with a No. 7 cork borer, was pushed to the bottom of the syringe barrel. The column was filled with solvent and the Sephadex added, then the tap was opened to let the solvent through and the Sephadex settle. After all the Sephadex had settled, forming a column 2 cm high, a further circle of filter paper is placed on to the top of the column. This prevents the Sephadex floating and drying at the top when the columns are left between assays. Columns were then washed with a further 20 ml of fresh solvent to rid them of impurities which interfere with the assay. Columns are then ready for the addition of the samples.

These small columns stop automatically when the solvent level reaches the top of the gel. Spontaneous stopping depends on the combination of column bore and solvent and is considerably convenient when the columns are operated manually since the taps can be left open throughout a single run. Usually ten columns were run simultaneously, and could be reused for months, provided they are not allowed to dry out between assays, with no significant difference in the behaviour of the steroids over this period.



As no ^3H -oestriol was available, the behaviour of this oestrogen on the LH-20 columns could only be determined by the use of cold hormone and subsequent assay of each separate ml of effluent eluted. As this is the most polar of the oestrogens it is retarded the most on Sephadex LH-20, so is eluted last, as shown by Mikhail et al., 1970. The behaviour of E1 and E2 was traced by applying a small volume of the tritiated steroid to the top of the column, with the tap open, allowing the column to run "dry", then adding successive 200 μl aliquots of solvent, collecting the 200 μl samples eluted directly into scintillation vials, where they were dried down under air, 5 ml of scintillator added and counted immediately. This gave a scan of the elution profile, and showed that the steroids behaved the same whether they were added separately or together and whether the solvent was added in small aliquots or as a single volume using a header tank (Figure 4).

As the antiserum cross-reacts with all three major oestrogens, it is possible to collect the fractions from the Sephadex LH-20 columns containing the cold hormones, and assay each separately using the respective cold hormone in the standard curve (see assay specificity curves). Six mature female plasmas collected in January were chromatographed and assayed as below and gave the following levels of E1 and E2.

Plasma No.	E1 (ng/ml)	E2 (ng/ml)
1	Not detectable	17.36
2	Not detectable	17.61
3	1.5	10.32
4	2.07	21.02
5	Not detectable	12.75
6	Not detectable	11.82

The sensitivity of the oestrone (E1) radioimmunoassay, as defined by Abraham (1974) i.e. the smallest amount of steroid that can be measured in an aliquot of the biological fluid assayed, was 1.44 ng/ml in this assay. Both plasmas that gave positive results were near the detection limit of the assay. As January is a time of fairly high oestradiol levels it would be very difficult to detect oestrone in plasmas throughout the year if they too were near their highest at this time of year. Due to the lack of ^3H oestriol, and also the insensitivity of the antiserum to oestriol (sensitivity of 42 ng/ml) it was impossible to measure plasma oestriol levels, but as Reinboth (1972) pointed out, oestradiol- 17β and oestrone have been found much more frequently than oestriol in the ovaries of teleosts, and he could find no proof for the occurrence of oestriol in several species. Thus it was decided to assay the plasmas for oestradiol alone. Because of the incorporation of a chromatographic step the difficulty caused by varying volumes of plasma, as experienced in the testosterone radioimmunoassay, is absent in the oestradiol assay (see Table 2). Normally 20 μl or 50 μl of plasma was extracted but when levels were at their lowest 200 μl was used.

3. Assay procedure

Excepting the chromatographic step, the basic assay procedure was similar to that employed for the testosterone radioimmunoassay. Initially the antiserum was employed at 1:2000, but subsequently a serial dilution of freshly prepared antiserum showed that a B/F close to 1 could be obtained at a concentration of 1:8000 (Figure 5).

The plasma, with approximately 600 cpm of ^3H oestradiol (6,7- ^3H Oestradiol, Cat. No. TRK 125, 40-60 mCi/mmol., Radiochemical Centre, Amersham) added, was extracted with 3 x 2 ml of diethyl ether.



The combined ether extracts were dried under a stream of air and the steroid redissolved in 200 μ l of benzene:methanol, 90:10, which was applied to the column and allowed to run in by opening the tap. 2.4 ml of the solvent mixture was then added to the top of the column and the eluate discarded. This contained the oestrone. A further 2.9 ml was then added and the eluate, containing the oestradiol, collected in a centrifuge tube. This fraction was evaporated to dryness under a stream of air and redissolved in 170 μ l of Buffer A containing 0.1% bovine gamma globulin. A 50 μ l aliquot was taken directly to a scintillation vial for recovery determination and a further 2 x 50 μ l aliquots to assay tubes. Before further use the columns were washed with at least 20 ml of solvent to clean them ready for the next plasma extract.

50 μ l aliquots of a standard oestradiol solution (Sigma, analytical grade) were serially diluted in triplicate to yield 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25 pg/tube. 50 μ l of ^3H -oestradiol (ca. 8000 cpm/50 μ l in diluent) was added to each tube, followed by 50 μ l of antiserum diluted in diluent to all but the first set of blanks. Tubes were spun briefly, thoroughly mixed and left overnight at 4°C. After incubation 1 ml of dextran-coated charcoal was added, the tubes vortexed and left for 15 minutes, then spun at 2500 rpm (GF/6) for 20 minutes. The supernatants were decanted into backgrounded scintillation vials, 10 ml of scintillator added, left for 12 hours, then counted to an efficiency of \pm 3%. Results were calculated as for the testosterone assay.

4. Assay reliability

Overall 14 assays were carried out using the antiserum at 1:2000, with a mean 50% binding of 9.66 ± 0.68 ng/ml (SEM), and 5 assays



using the antiserum at 1:8000, with a mean 50% binding of 2.29 ± 0.14 ng/ml. The mean B/F ratio of these 19 assays was 1.033 ± 0.11 . Mean recovery was $77.32 \pm 1.94\%$ for a total of 380 plasmas in five assays.

The specificity of the antiserum to a variety of steroid hormones has been described by Dobson (1974). In this study E1, E2 and E3 were tested on a number of occasions and the mean cross-reactions, together with the figures from Dobson (1974) are given in Table 3. Binding of all steroids tested except the oestrogens was less than 0.01% of that of oestradiol- 17β . The sensitivity of the assay, which was approximately 300 pg/ml, depended on the volume of plasma taken and the procedural losses encountered.

The overall precision of the assay was estimated by making five replicate determinations from a pool of mature female plasma. The intra-assay coefficient of variation was 6.21% by this method, and the inter-assay coefficient of variation was 14.75% over 12 determinations of the same plasma pool.

Accuracy was estimated by adding, in duplicate, 1, 5, 10 and 20 ng of oestradiol- 17β to 1 ml aliquots of a pool of plasma taken from an immature female which was found to contain undetectable levels of oestradiol. Results were as follows:

Plasma sample	Recovery of ^3H tracer (%)	E2 added (ng/ml)	E2 recovered (ng/ml)
1	78.8	1	0.78
2	82.4	5	4.36
3	81.9	10	10.21
4	71.4	20	18.92

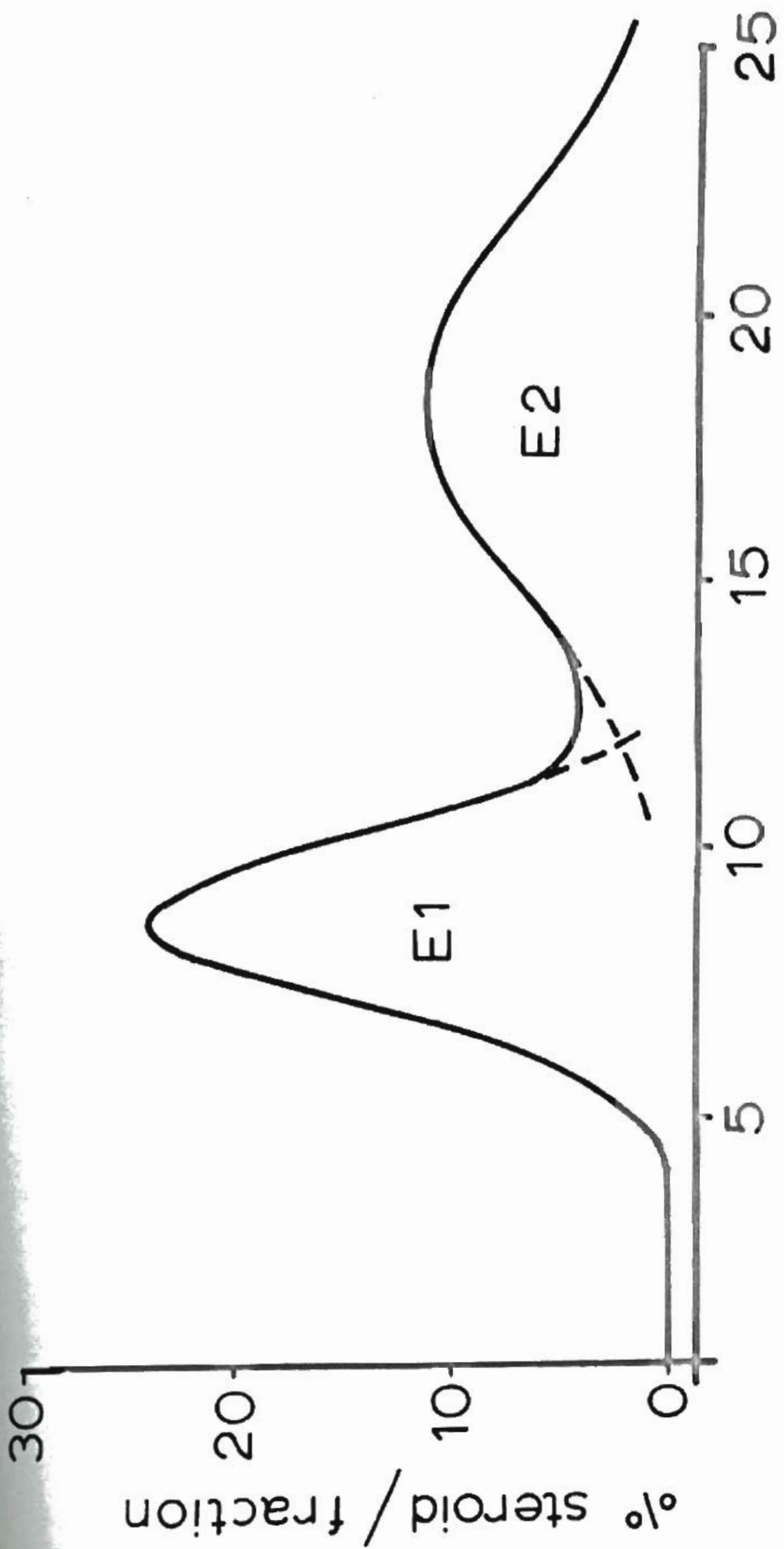
Thus accurate oestradiol determinations in the plasma of the mature female dogfish were possible.



Figure 4:

Separation of Oestrone (E1) and Oestradiol (E2) on
Sephadex LH-20 eluted with benzene:methanol, 90:10.





fraction number (each 200 μl)

Figure 5:

Typical standard curves for the oestradiol radioimmuno-
assay. Initially the antiserum was used at 1:2000 (●—●),
but in later assays at 1:8000 freshly diluted (○—○);
over the range 0.125 - 64 ng/ml (6.25-3200 pg/tube).

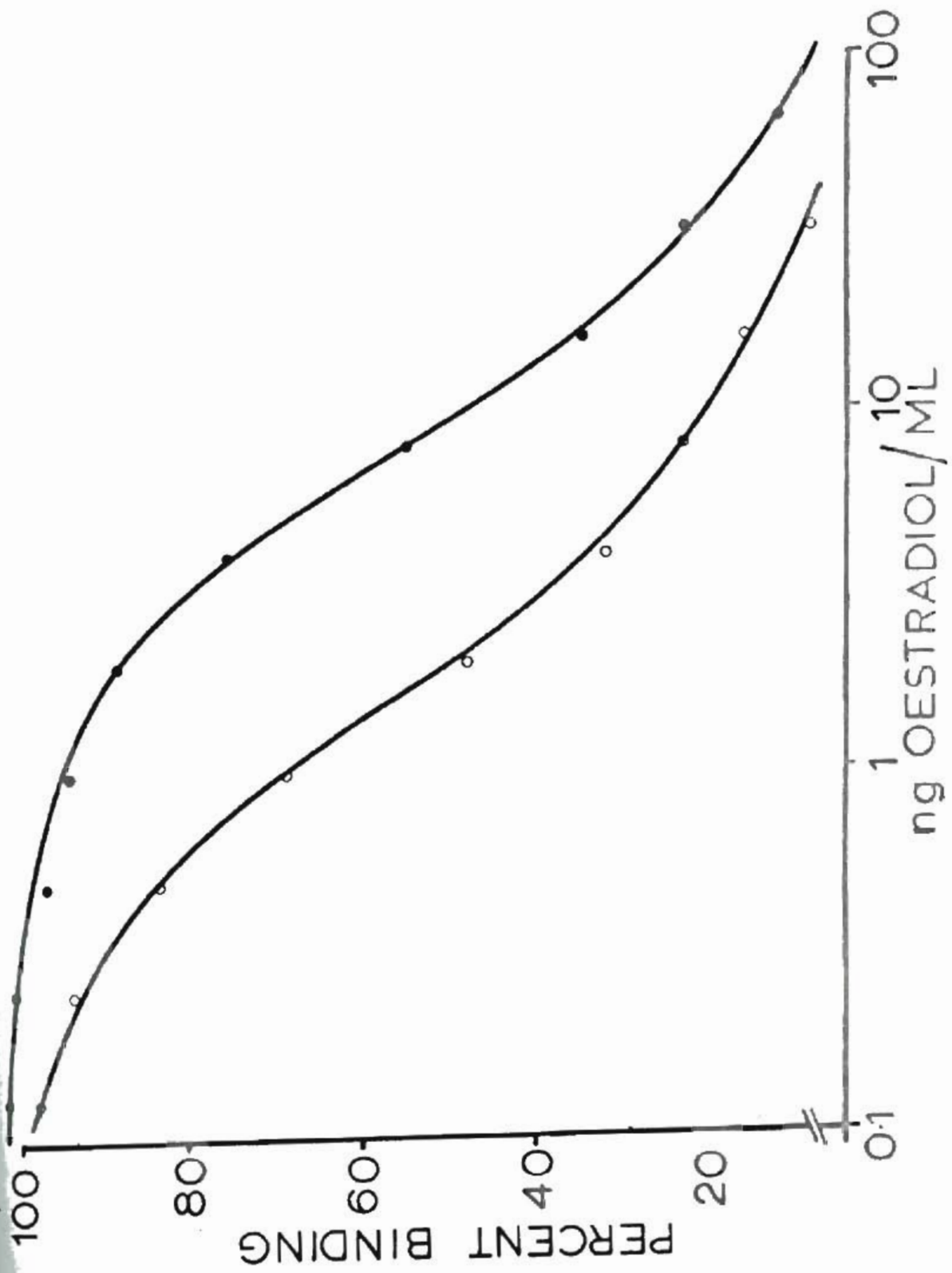


Table 3: Cross reactions of steroids in the oestradiol radioimmunoassay

Steroid	Cross-reaction
Oestradiol-17 β	100 ^a
Oestrone	26.30
Oestriol	1.68
Testosterone	< 0.1
Androstenedione	0.3
Progesterone	0.001
DHA	5.0
DOC	< 0.1
17 α OH progesterone	0.0001
Cholesterol	< 0.1
Corticosterone	0.1
Cortisol	0.1
Stilboestrol	< 1.0
Megoeestrol	< 1.0

^a Expressed as % relative to oestradiol.

The data for E1, E2 and E3 were obtained during the present study, the rest from Dobson (1974).

Results

1) Gravimetric changes throughout the year

The gravimetric data obtained in the present study were collected from August, 1974 until July, 1975, but are plotted in all subsequent tables and figures on a calendar year i.e. January through to December. Thus although strictly speaking July is not followed by August in the data because they are one year apart, it aids comparison with the data obtained by Ford (1921) and Harris (1952), as shown in Table 4. This shows the percentage of females having egg-capsules in the oviducts, on a monthly basis, throughout the year; and also gives the mean gonadosomatic index for the Plymouth and Caernarvon Bay samples obtained in this study, these figures being unavailable for the other two studies. The data from Ford (1921) were obtained from all females above a reasonably large size, while those from Harris (1952) were obtained from a study of all female arriving at Bristol from Ilfracombe. Harris's data tempted him to conclude that all his fish were mature, this assumption being based on the fact that over an 8 or 9 month period of the year there was a constant proportion of females carrying egg-capsules, and the fact that no appreciable size variation has been associated with this condition. He further states, "If, on the other hand, a substantial number of immature females existed in the population during the winter months, the proportion of egg-bearing fish would be expected to rise as the breeding season progresses - which is apparently not true after November". This, however, supposes that the fish mature at a certain critical size, whereas it is equally possible that if a fish has not matured by the beginning of the egg-laying season in late autumn then it will not do so until the next breeding season a

Table 4: Gravimetric data from monthly samples

Month	A			B			C				D			
	N	Carrying eggs		N	Carrying eggs		N	G.S.I.± SEM	Carrying eggs		N	G.S.I.± SEM	Carrying eggs	
		N	%		N	%			N	%			N	%
January	147	18	12.3	172	73	42.4	9	4.64 ± 0.48	5	55.6	4	4.41 ± 0.33	2	50.0
February	113	22	19.5	47	16	34.0	-	-	-	-	21	4.28 ± 0.12	7	33.3
March	-	-	-	186	62	33.3	-	-	-	-	8	5.11 ± 0.10	3	37.5
April	-	-	-	61	20	32.8	5	4.87 ± 0.43	5	100	5	4.22 ± 0.38	2	40.0
May	80	18	22.5	158	47	29.8	9	4.89 ± 0.24	5	55.6	13	4.76 ± 0.27	5	38.5
June	313	74	23.6	61	19	31.1	11	4.51 ± 0.31	8	72.7	32	3.35 ± 0.15	18	56.3
July	109	27	24.8	34	13	38.2	3	4.38 ± 0.19	1	33.3	7	2.67 ± 0.07	3	42.9
August	5	2	40.0	-	-	-	7	3.81 ± 0.47	2	28.6	6	3.04 ± 0.004	1	16.7
September	71	7	9.9	50	0	0	-	-	-	-	12	2.90 ± 0.15	0	0
October	198	19	9.5	44	0	0	8	3.64 ± 0.80	3	37.5	16	3.77 ± 0.16	1	6.25
November	143	24	16.8	197	35	17.8	3	4.66 ± 0.31	2	66.7	8	3.63 ± 0.22	3	37.5
December	138	24	17.4	145	41	28.3	10	5.09 ± 0.31	4	40.0	-	-	-	-

A Data from Ford (1921)

B Data from Harris (1952)

C Data from Plymouth samples taken during this study

D Data from Caernarvon Bay samples taken during this study

Figure 6:

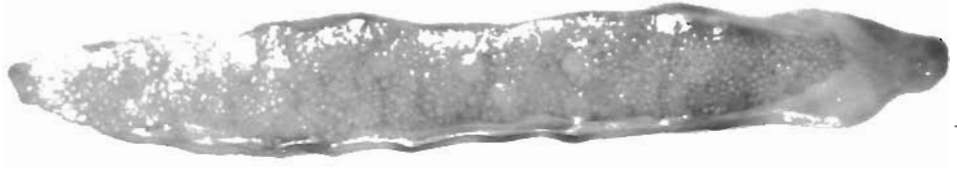
A selection of ovaries removed from fish caught in late October, 1976, demonstrating the range from a completely immature ovary (A) to an ovary taken from a fish containing a pair of egg-capsules in the oviducts (E).

All fish were over 850 g.

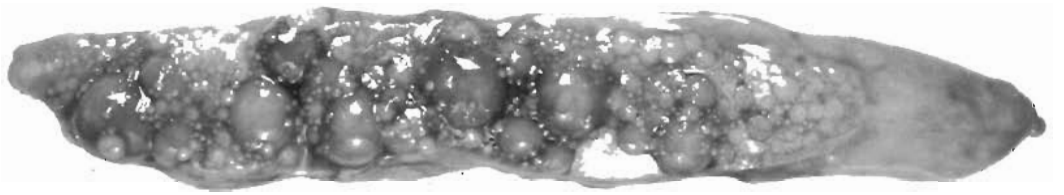
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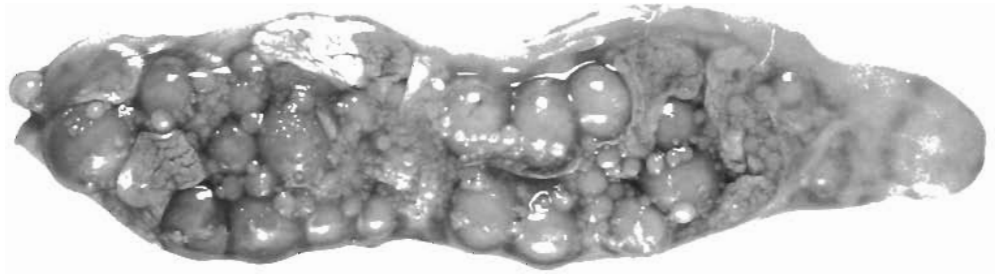
B



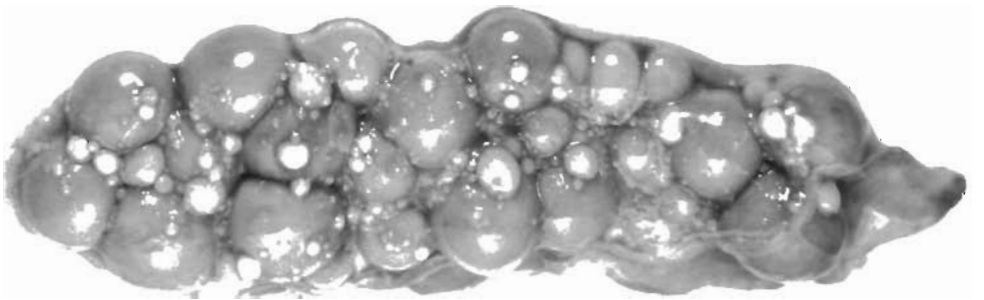
C



D



E



year hence, although other fish the same size may well be mature. Thus Harris's sample may contain a proportion of large immature fish. Figure 6 shows a selection of ovaries obtained from a sample of fish caught in late October, 1976. Although all fish were over 850 g, and most were mature as expected, they showed a range of ovary development stages from one that has no sign of vitellogenesis and appears completely immature, through some showing a little vitellogenesis and sometimes atretic follicles, to the fully mature ovary containing many vitellogenic follicles of varying sizes. In the present study the great majority of female fish over 700 g were found to be mature, whatever time of year the sample was taken, having ovaries containing vitellogenic follicles and the oviducts being open; but a number of fish above this weight were immature. Dodd (personal communication) recalls a sample of very large females, obtained from Morecombe Bay, which were all apparently immature when dissected. Thus size alone cannot determine the state of sexual maturity of a female, but open oviducts at least indicate that the fish once was, and almost certainly still is, sexually mature.

A further complicating factor is the presence of atresia in the follicles during the late summer months. The degree of atresia appears to vary from year to year, during the summers of 1974 and 1975 very little atresia was apparent in either the Caernarvon Bay or Plymouth fish. In a sample of twelve mature females taken in late July, 1975, only four showed any atresia at all, and of these two had only the two largest pairs of follicles undergoing atresia, although the other two showed a more advanced condition where many follicles were undergoing atresia. Atresia seems to occur in the largest vitellogenic follicles first, and as it advances so smaller vitellogenic follicles are affected.

Figure 7:

The percentage of females carrying fully formed egg-capsules each month.

- A. Data taken from Ford (1921) who examined fish landed by fishermen at Plymouth.
- B. Data taken from Harris (1952) who examined fish caught at Ilfracombe and supplied to the Zoology Department of Bristol University.
- C. Data from Plymouth samples taken during this study.
- D. Data from Anglesey samples taken during this study.



% FISH WITH EGGS IN OVIDUCTS

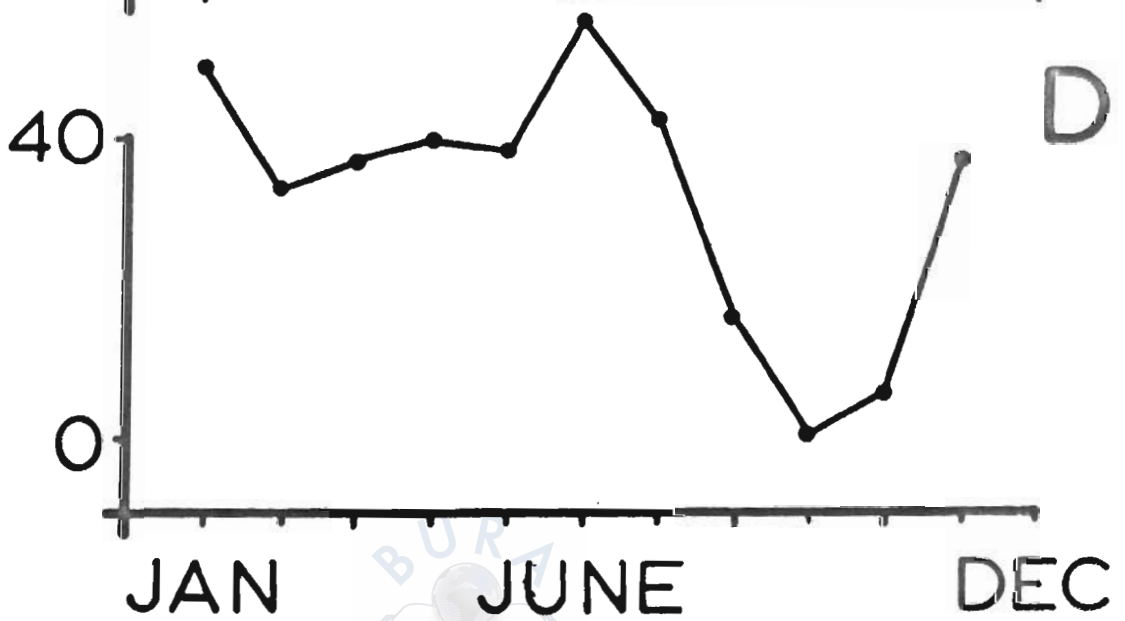
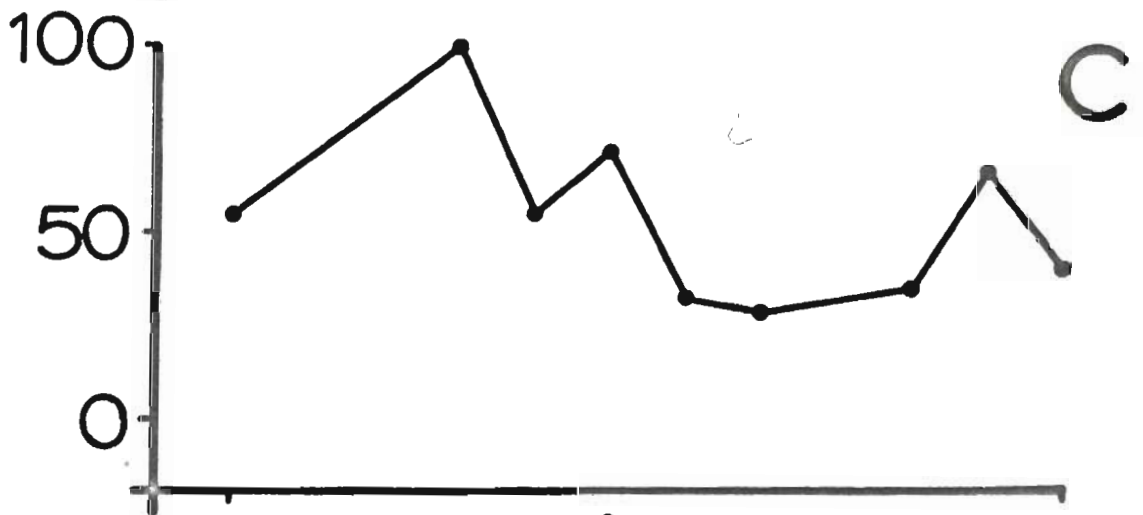
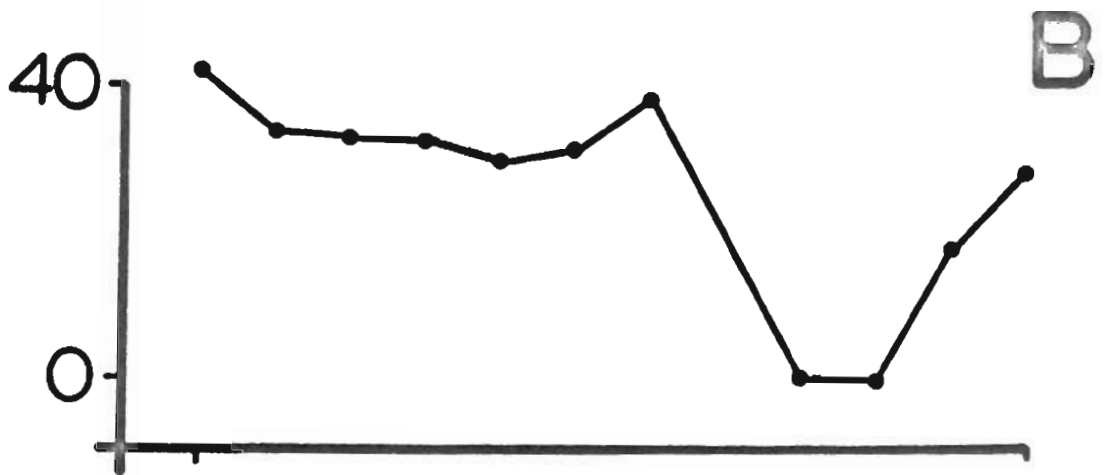
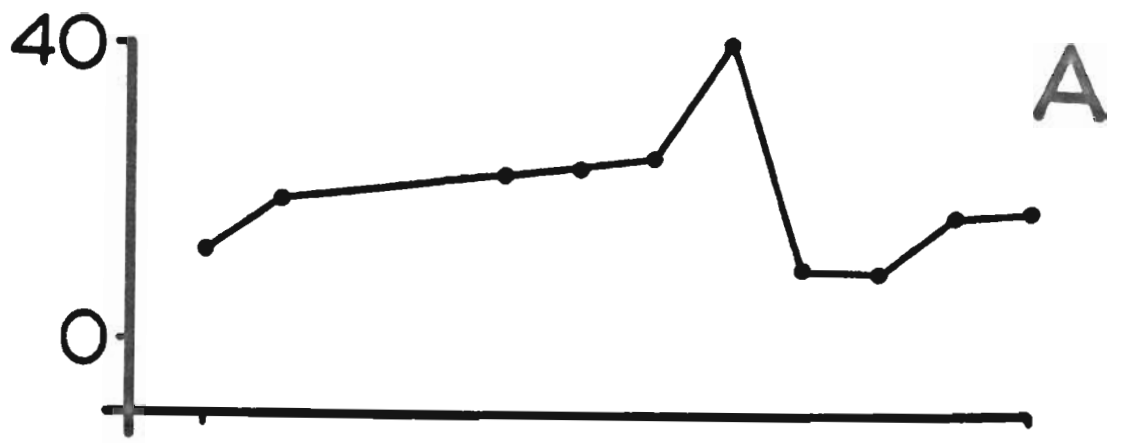


Figure 8:

Changes in the gonadosomatic index (G.S.I.) of mature females throughout the year. Each point represents the mean \pm SEM.

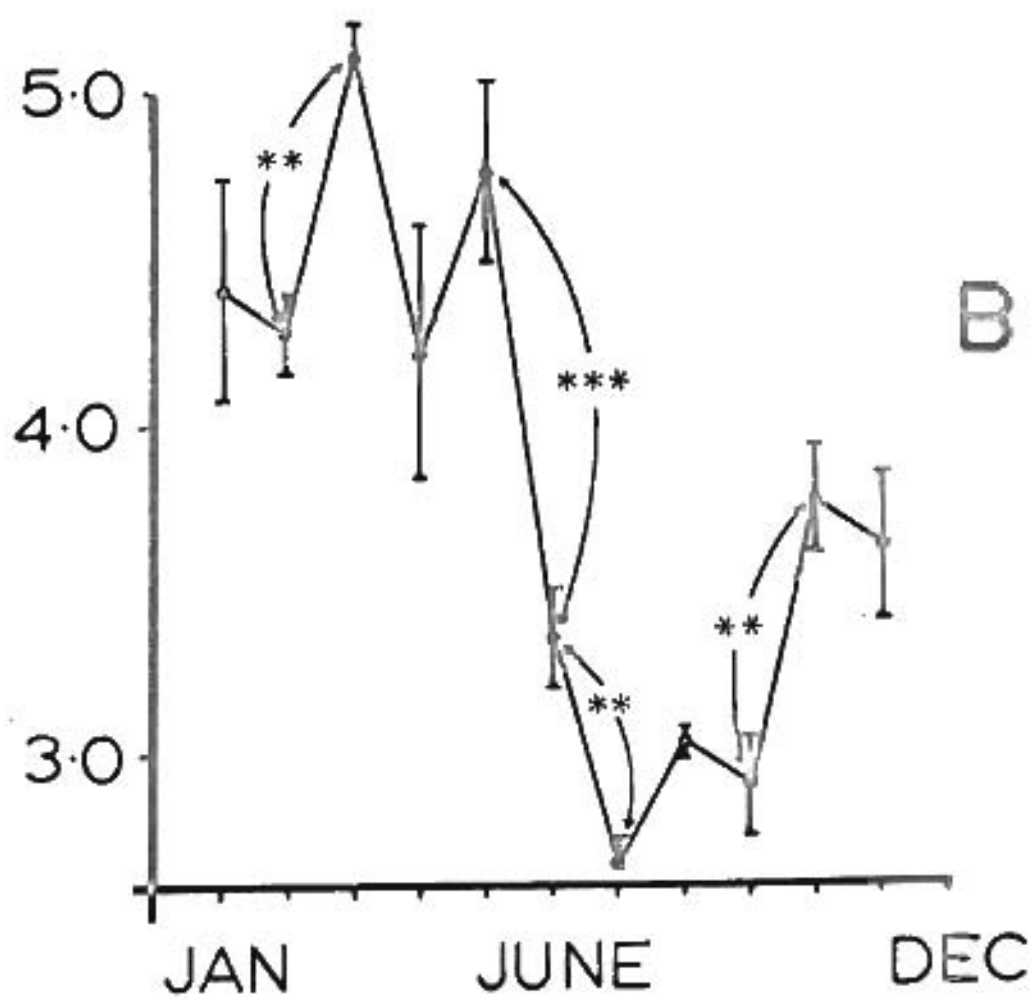
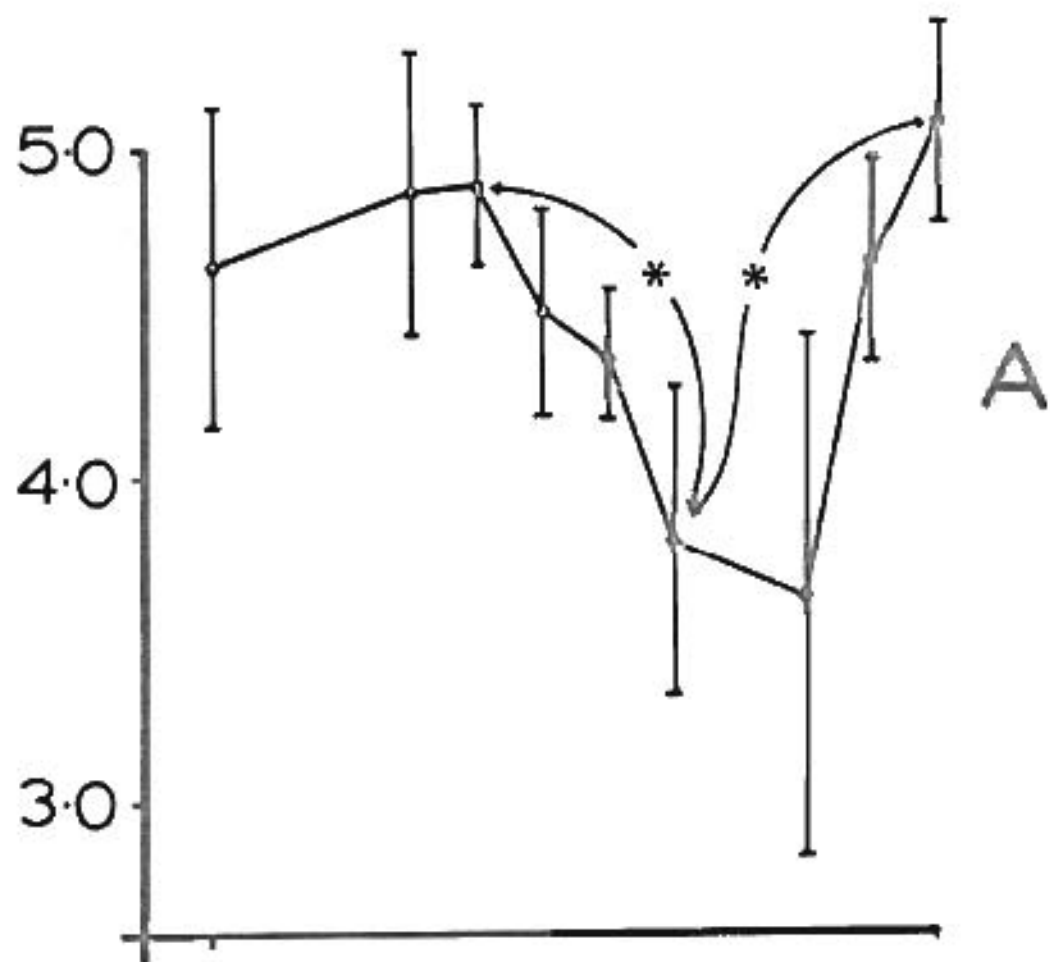
- A. Fish obtained from Plymouth
- B. Fish obtained around the coast of Anglesey.

* P < 0.05, ** P < 0.01, *** P < 0.001

Independent Students "t" test.



GONADOSOMATIC INDEX



This is reflected in the fact that egg-laying is at its lowest rate in late summer in all four surveys conducted (see Figure 7), when atresia would have occurred if any were present that year. Observations in 1976 demonstrated that a very large percentage, if not all, of the ovaries from mature females were undergoing atresia, although to various degrees, in both September and October. The fall in the gonadosomatic index from May until September, which appears more marked in the Caernarvon Bay than Plymouth samples (see Figure 8), could be due either to the larger follicles becoming atretic while smaller follicles fail to develop fast enough to maintain the ovarian weight, or can be explained by the larger vitellogenic follicles being ovulated, with again the smaller follicles failing to develop fast enough; and thus the G.S.I. falls without the need for atresia.

2) Changes in the ventral lobe gonadotrophin content throughout a year

The potencies of the ventral lobe pools from mature females are given in Table 5, and plotted graphically in Figure 9 where the data obtained by Dobson (1974) are given as well. In most cases five ventral lobes per pool were adequate, but during the peak of the egg-laying season only two or three ventral lobes were necessary. With this small number of ventral lobes per pool, if the individual variation is large then the mean potency obtained may differ significantly from that obtained from a larger pool. The only indication that the variation is not large comes from a comparison of the data of Dobson and those obtained in the present study, and also by comparing the potency obtained for the same month in different years, although the latter may not reflect individual variation but rather natural variation caused by different environmental conditions. Three samples were assayed in February 1975



Table 5:

Potencies of monthly samples of ventral lobes from females assayed by the
³²P chick bioassay*

Date	Gonadotrophic potency/ventral lobe (µg equivalents NIH-IH-S18)
December 1970	41.48 (16.21 - 106.11)
April 1971	47.43 (22.81 - 98.63)
July 1971	< 1.79 (N.D.)
October 1971	8.65 (5.01 - 16.18)
December 1971	8.88 (4.96 - 13.55)
February 1972(3)	13.62 (8.48 - 21.03)
March 1972	11.62 (6.73 - 20.92)
April 1972	20.36 (9.48 - 73.13)
June 1972	13.98 (6.31 - 33.33)
August 1972	< 1.50 (N.D.)
September 1972	6.85 (3.72 - 12.10)
20th August 1974	12.09 (6.32 - 18.71)
27th August 1974	8.01 (5.79 - 28.32)
7th September 1974	10.24 (8.27 - 86.16)
27th September 1974	15.59 (9.41 - 37.20)
30th September 1974	12.09 (7.77 - 18.34)
30th October 1974	8.33 (5.71 - 19.18)
27th November 1974	24.11 (0.56 - 343.1)
5th December 1974	17.82 (4.72 - 39.57)
17th December 1974	21.51 (16.56 - 27.93)
24th January 1975 (3)	29.82 (11.61 - 64.31)
6th February 1975 (3)	41.54 (26.82 - 91.77)
11th February 1975 (3)	48.69 (31.57 - 69.70)
25th February 1975 (3)	39.14 (6.73 - 79.01)
25th March 1975 (2)	82.49 (53.40 - 127.00)
16th April 1975 (3)	30.82 (17.50 - 54.28)
21st April 1975 (3)	84.25 (53.64 - 198.19)
20th May 1975 (4)	51.32 (16.83 - 97.98)
9th June 1975	21.31 (7.82 - 49.64)
22nd July 1975	N.D. (< 3.0 µg)

/Continued



Table 5 (Continued):

* Potencies are expressed per ventral lobe with 95% confidence limits in parenthesis.

N.D. = Not detectable, figures given are the lowest standard used multiplied by the fraction of ventral lobe per chick.

Figures in brackets after the date indicate the number of fish used where this differs from five.

Data for December 1970 until September 1972 are taken from Dobson (1974).

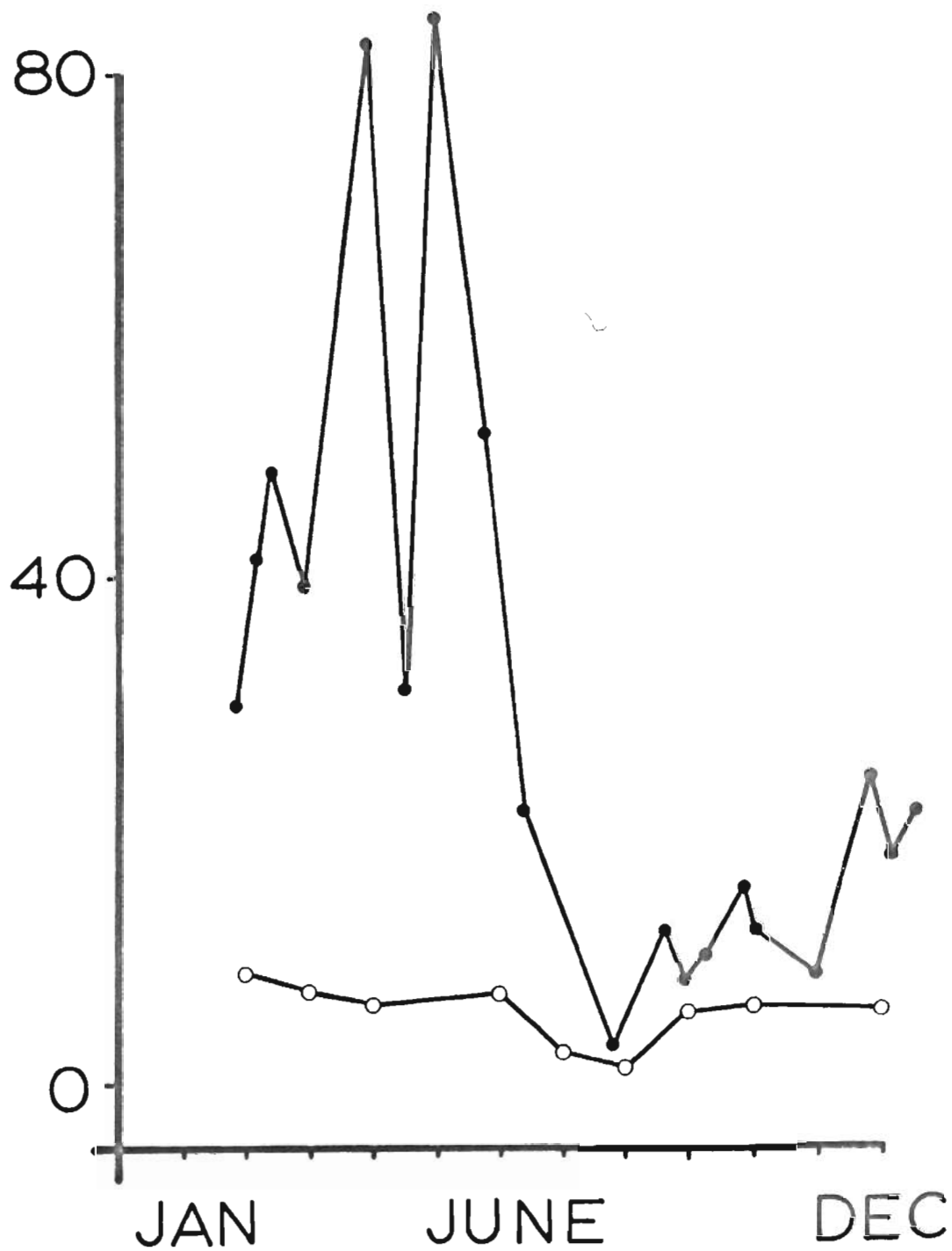


Figure 9:

Gonadotrophic potencies, expressed as ug equivalents, of ventral lobes from male (o—o) and female (●—●) dogfish. Data for the male taken from Dobson (1974). The August sample for the male and the late July sample for the female were both below the detection limit of the assay and are plotted as the maximum possible level.



μg EQUIVALENTS NIH-LH-S19 / VL



which proved to be not significantly different from one another, which indicates a lack of marked individual variation; although on two occasions very large swollen ventral lobes were found, one of these giving a potency of 112 $\mu\text{g}/\text{lobe}$, which was approximately ten times the September mean, when the particular fish was caught. As the minimum detection limit of the assay is 1.5 $\mu\text{g}/\text{chick}$, which corresponds to 1.25 $\mu\text{g}/\text{ventral lobe}$ with the protocol used, it is only possible to say that the July female sample and the August male sample are $< 1.25 \mu\text{g}$ equivalents/ventral lobe. However, as part of the purification of dogfish gonadotropin (see later), 200 ventral lobes from fish of mixed sex were assayed and gave a potency of $< 0.75 \mu\text{g}/\text{ventral lobe}$. This is well below the value for immature males of 1.72 (1.14-3.12) $\mu\text{g}/\text{lobe}$ obtained by Lewis (1975) and 1.33 (0.66-2.79) $\mu\text{g}/\text{lobe}$ for immature females obtained during the present study; although both these later figures depend upon the visual assessment of maturity of the fish used.

3) Changes in plasma testosterone and oestradiol concentrations throughout a year

The annual changes in plasma testosterone and oestradiol in mature females are shown in figures 10 and 11 respectively, while figure 12 shows both plasma steroid levels in relation to that of the plasma testosterone levels of the mature male. Eleven plasmas from immature females ranging in size from 480-916 g, collected randomly over a year, all gave non-detectable levels of both oestradiol and testosterone. Two fish that were judged to be maturing by the presence of only a few small vitellogenic follicles in the ovary had plasma steroid levels within the same range as those of the mature fish at the same time of the year. A selection of plasmas from mature females were also assayed

Figure 10:

Plasma testosterone levels of mature female dogfish throughout the year. Each point represents the mean of between 4 and 10 individuals \pm SEM.

** P < 0.01, Students "t" test.

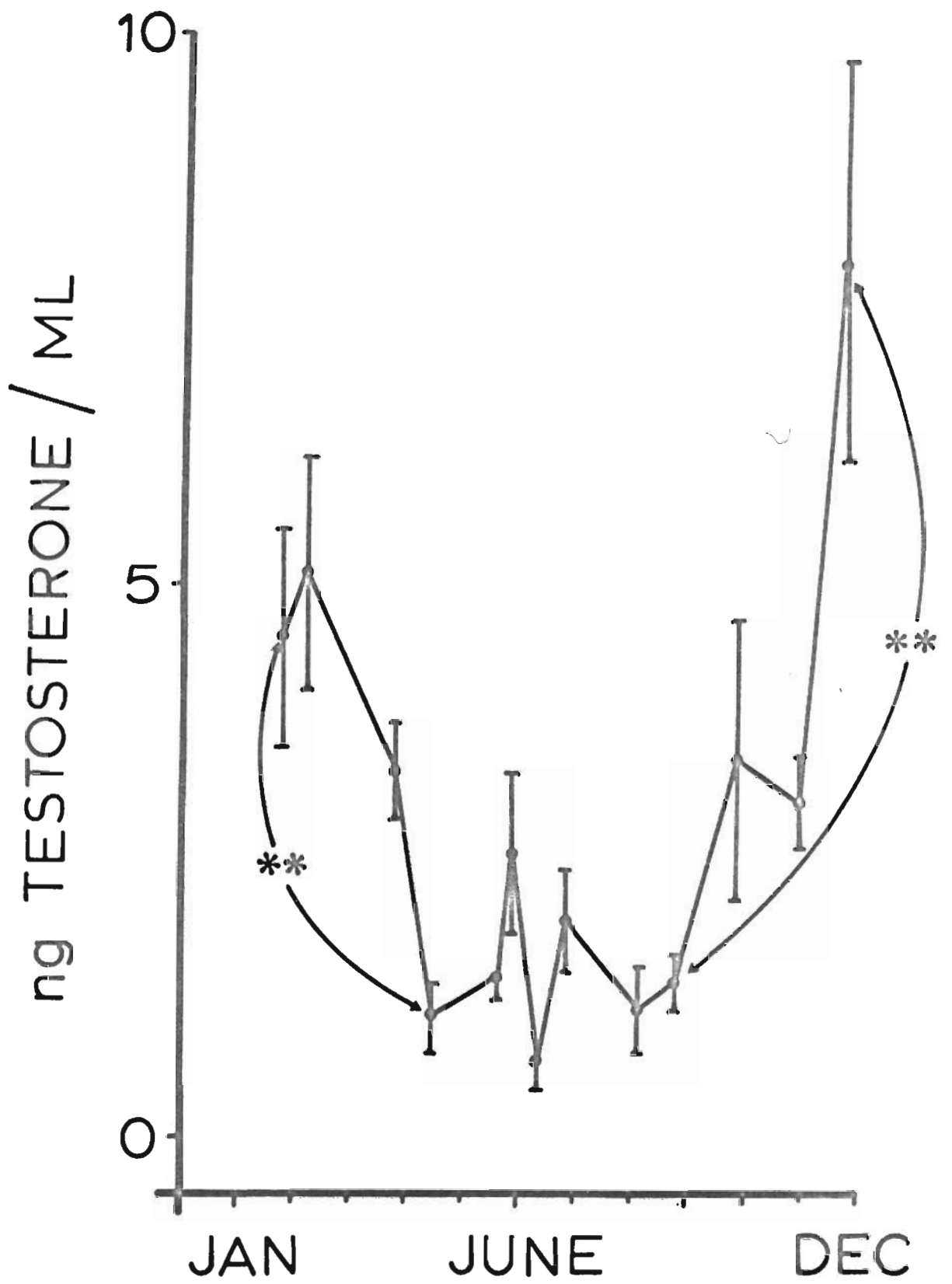


Figure 11:

Plasma oestradiol levels of mature female dogfish throughout the year. Each point represents the mean of between 4 and 10 individuals \pm SEM

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;

Students "t" test.

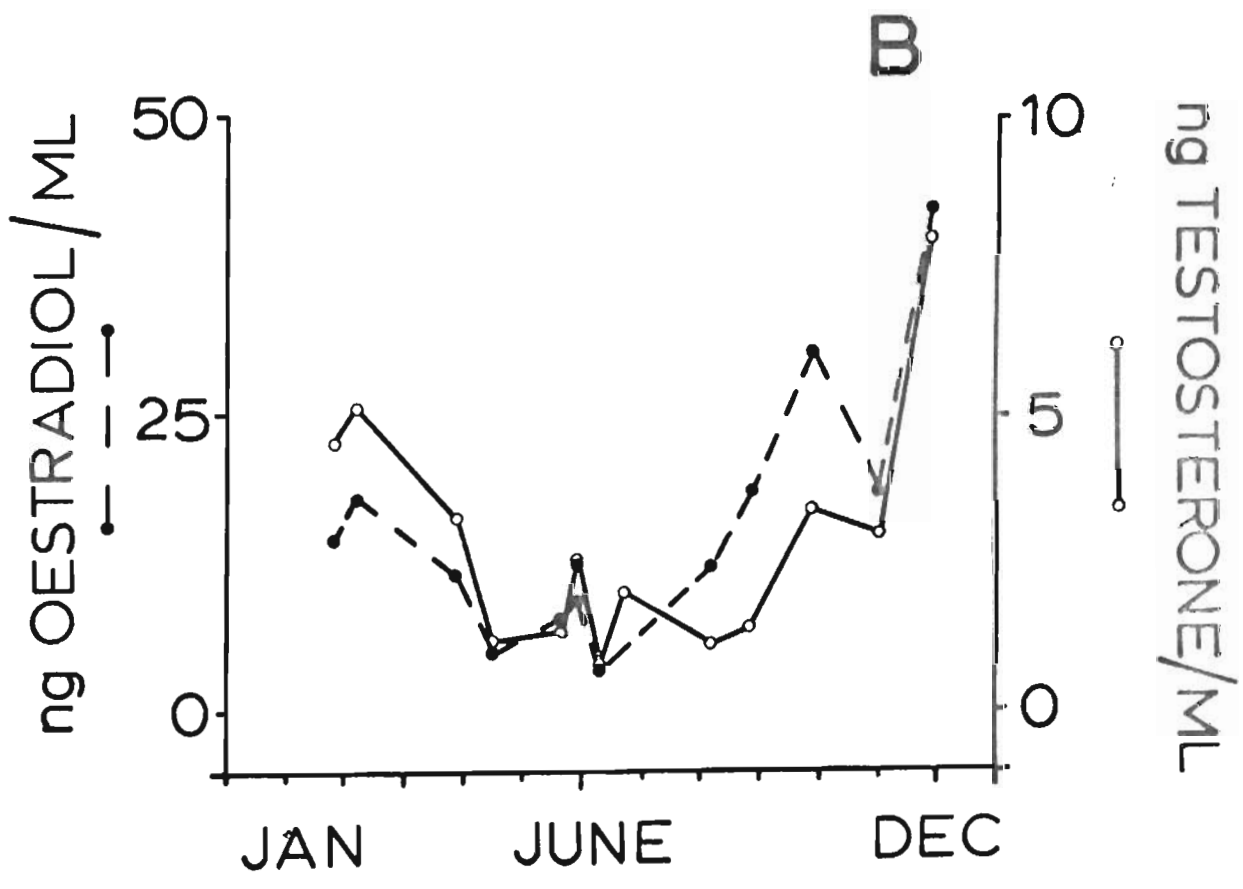
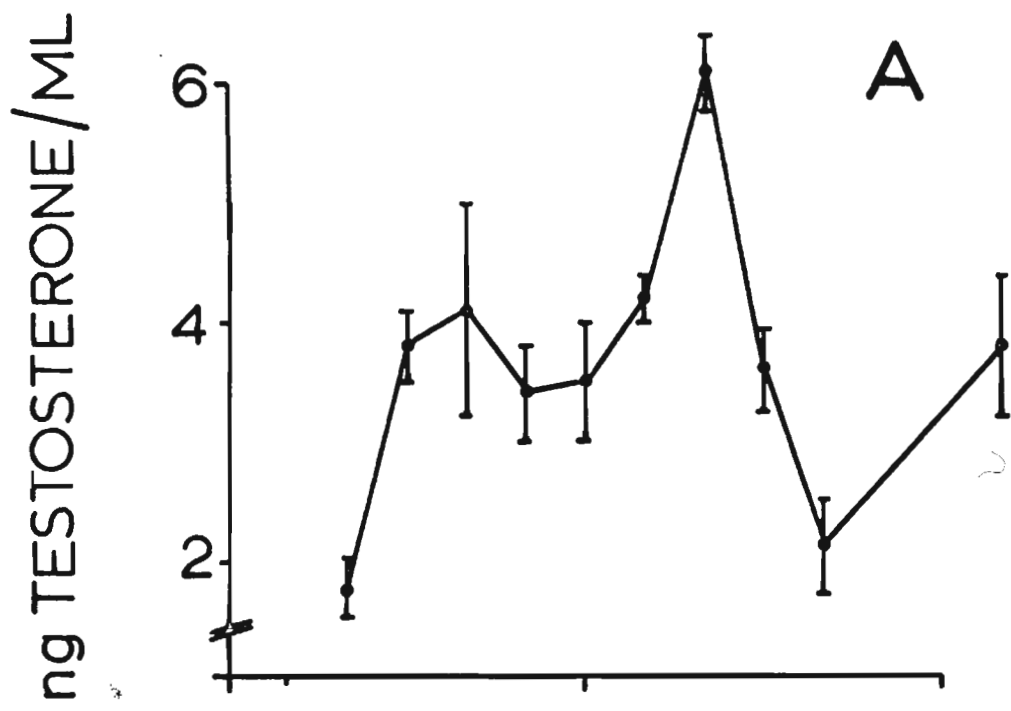
Figure 12:

A: plasma testosterone levels of mature male dogfish throughout the year. Data from Dobson (1974).

B: plasma testosterone and oestradiol levels of mature females throughout the year.

Each point represents the mean of between four and ten individuals \pm SEM (in A only).





for oestrone but in every case the level proved undetectable, although the antiserum is less sensitive to this steroid, the minimum detectable level of oestrone being 1.6 ng/ml. No detectable levels of oestradiol could be found in a sample of six male plasmas collected during August when testosterone levels are highest in this sex. No attempt was made to measure oestriol in the plasmas because of the lack of sensitivity of the assay.

Discussion

All the available existing (or published) data indicate that the dogfish has a very extended, if not continuous, egg-laying season, although the rate of egg-laying either decreases markedly, or stops completely, for a short time in late summer. Although on average around 40% of mature females contain egg-capsules in the oviducts throughout most of the year, it is impossible to obtain a precise figure for the rate of egg-laying from any of the studies. However observation of mature females held in captivity have shown that they are very reluctant to lay eggs in our aquarium, possibly because they lack the visual stimulus of the seaweed on which they attach their egg-capsules in nature. If a batch of freshly caught mature females is transferred to the aquarium those already having egg-capsules in the oviducts, which can be felt by palpating the fishes, will lay these in the first few days, but then egg-laying will cease completely for at least a month and only then will it start again, but at a very slow rate, some females not laying at all while others will lay pairs of eggs once every two weeks or so. Rays, on the other hand, appear not to be so affected by captivity, possibly because they normally lay their egg-capsules on the bottom unattached to any objects, and so probably do not require the visual stimulus the dog-

fish appears to need. Holden et al. (1971) observed two maximum rates of egg-laying, 0.5 per day for R. brachyura and R. montagui, and one egg per day for R. clavata. Clark (1922) observed a slightly higher rate of 0.61 egg per day for one R. brachyura kept in the aquarium at Plymouth for 41 days. No other observations have been made on British rays, but Libby (1959) observed that R. eglanteria laid two egg capsules every four days in an aquarium. Observations by Richards et al. (1963) indicate that the rate of egg-laying of R. erinacea may be lower; these authors kept nine females in a tank for 71 days, during which time 65 eggs were laid. By palpating the females they were able to show that probably only five of them produced eggs and that of these five fish three probably produced only one pair of egg-capsules each. The remaining 59 egg-capsules were, therefore, probably produced by only two females, a rate of 0.42 egg-capsules per female per day, which corresponds closely to the minimum time interval of five days which Richards et al. observed between the laying of two successive pairs of eggs. Holden (1971) therefore concludes there is considerable variation in the rate of egg-laying of rays, although this could be due to the time of year the studies were undertaken and the conditions under which the fish were maintained. One further interesting fact to emerge from the egg-laying observations was that females lay fertile eggs at least nine months after their last contact with a male.

Craik (1976) studied the endocrine control of vitellogenesis of the dogfish in this laboratory, and showed that whereas other female egg-laying vertebrates show highly elevated levels of vitellogenin just before the breeding season, the dogfish differs both quantitatively, in the much smaller amount of vitellogenin present in the female plasma

during vitellogenesis, and qualitatively, in that the vitellogenin is present throughout the year. Thus a clear distinction emerges between a typical vertebrate with a brief breeding season, intense vitellogenesis and a short period of high plasma vitellogenin, and the dogfish type, which although it may produce the same weight of yolk in a year, does so over almost the entire year, with a corresponding lower overall rate of vitellogenesis and a lower level of vitellogenin in the plasma.

There are, however, strong reasons for believing that the rate of vitellogenesis undergoes annual variation. Craik (1976) found that the hepatosomatic index (liver weight as percentage of body weight) of adult female dogfish undergoes a considerable annual variation, reaching a maximum of $8.82 \pm 0.38\%$ (SEM) early in the laying season (November) and a minimum of 5.14 ± 0.49 when egg-laying is slowest during August. The male liver undergoes a much less marked variation, the corresponding figures being 5.67 ± 0.4 and $5.33 \pm 0.60\%$. The female gonadosomatic index reaches a maximum (5.1 ± 0.2) in March-May and a minimum (2.6 ± 0.2) in July-September. Thus the gonad cycle distinctly lags behind the liver cycle, the maxima being separated by about three months. It appears that the liver builds up a reservoir of nutrients which is metabolised steadily over 3 months while the ovary grows to its maximum size by yolk deposition. Examination of the liver by Craik (personal communication) at times near its maximum and minimum size showed a significant difference in the lipid content (39.7 ± 1.2 and $24.1 \pm 3.5\%$ of wet weight respectively). Calculation based on these figures shows that 67% of the seasonal increase in liver weight can be attributed to lipid. Probably the remaining third is due to other reserves such as glycogen, together with the cell architecture necessary for converting the reserves into vitellogenin.

The changes in the liver show a close correlation with the seasonal changes in plasma oestradiol, which also reaches a maximum in November - December and a minimum in May - June. This is further evidence for the implication of oestrogens as the causative agent of vitellogenesis and of the liver as its target organ. We may envisage that vitellogenin, plasma oestradiol and liver size all reach maxima in midwinter while the ovary is growing, and have begun to decline by March - April when the ovary reaches its maximum size. This was partly verified by the fact that oestradiol injection into mature females caused a several-fold increase of vitellogenin, while radioimmunoassay estimate of the circulating oestradiol levels of the treated fish showed these were two to three times those normally found in winter fish. It was concluded that these effects were due to hormone acting at physiological levels comparable to those found in nature rather than to aberrant responses caused by abnormally high circulating pharmacological levels of hormone.

It is more difficult to fit the ventral lobe gonadotrophin annual profile into this picture. Because of the wide limits involved in bioassays in general, it is impossible to detect subtle changes from month to month, but between August - September, when levels are at their lowest, and April - May, when they are highest, there is around an eighty-fold change in the ventral lobe content of gonadotrophin. Thus, although plasma steroids rise from July onwards and remain elevated all winter, presumably in response to change in circulating levels of gonadotrophin, pituitary gonadotrophin levels rise steadily but later. It is possible to explain this by supposing that in mid summer the secretion of gonadotrophin from the ventral lobe increases, the rate of secretion being



greater than the rate of synthesis, so pituitary levels drop while the circulating level increases. This in turn increases steroid synthesis and secretion so levels of the latter rise. It is only after some months that the rate of synthesis of gonadotrophin catches up with the rate of secretion and so the pituitary level stops falling and begins to increase as the rate of synthesis rises above the rate of secretion. In the male, where changes in pituitary gonadotrophin content are much less marked, the maximum level reached being approximately one tenth of that achieved by the females; this being reflected in the size of the ventral lobe at certain times of the year.

An ultrastructural study by Knowles *et al.* (1975) has shown that the part of the pars distalis adjacent to the hypothalamus consists of two distinct regions, the rostral and median lobes, and that there is a morphological similarity between some cells of the median and ventral lobes, which they speculate may indicate the presence of the same hormone in both lobes. Firth and Vollrath (1973), using a bioassay, based on the stimulation of meiosis in *Xenopus* oocytes, which is specific for LH amongst purified anterior pituitary hormones of mammals, also concluded that the median lobe possessed a gonadotrophin-like activity, which was present in four out of six of the bimonthly samples tested and absent in April and August. This demonstration of a gonadotrophin in the median lobe, if validated, is clearly of great importance, though more work is required before its existence is established, its functional significance can be assessed and its relationship to the ventral lobe gonadotrophin can be established. However, using a very sensitive bioassay based on the production of testosterone from isolated quail interstitial cells (Maung, Z.W., 1976) which is specific for LH amongst



mammalian and avian hormones, only very low concentrations of gonadotrophin could be detected in the median lobe (for a more concise discussion see Chapter 3). A further complication arises in view of the report by Campbell and Idler (1976) that the nonglycoprotein fraction, obtained from the pituitary of the Winter Flounder, stimulated increase of labelled yolk in serum and induced uptake of this material into the gonad, with a concurrent stimulation of gonadal growth. In contrast, the glycoprotein preparation had neither of these effects, and neither of the pituitary hormone fractions affected the labelling of liver yolk. Thus one of the classic actions of gonadotrophins appears to be carried out by a nonglycoprotein in this fish. Until dogfish gonadotrophin has been purified to an extent where either a sensitive bioassay or radioimmunoassay can be developed to measure circulating gonadotrophin levels, it will be difficult, or impossible, to determine how pituitary gonadotrophin content is correlated with the sexual cycle, or how the secretion of the gonadal steroids is controlled.

There is now some evidence that the thyroid gland plays a role in elasmobranch reproduction. Oliverseau (1949b) found that the onset of sexual maturity in S. canicula is associated with signs of increased thyroid activity, and further that in mature females the thyrosomatic index (T.S.I.) is approximately twice that of the male, and there is a cyclical intensification of thyroid activity related to reproductive function. Lewis (1975) and Lewis and Dodd (1974) have recently verified and extended these findings by an experimental approach as well as by observation on thyroid weights, both absolute and related to body weight in different reproductive states. For example, at the time of first sexual maturity (puberty), the absolute weight of the thyroid in the

female is greater than at any other time in the animal's life. Furthermore, among females, the T.S.I. is lowest in immature specimens and, although it is lower in mature egg-laying specimens than in fish at the time of puberty, it shows a rise in the autumn, the time at which atretic ovaries are undergoing a new wave of vitellogenesis. These data support the suggested role of the thyroid in reproduction and some recent experimental work on the effects of thyroidectomy on vitellogenesis (Lewis and Dodd, 1974) has provided direct evidence for such a function. Three experiments were carried out, at autopsy all sham operated fish contained vitellogenic follicles of varying sizes up to a diameter of 12 mm (i.e. the size at which ovulation takes place) in addition to pre-vitellogenic follicles and early and late corpora atretica, whereas in the completely thyroidectomised animals none of the follicles was undergoing vitellogenesis. The results appear to indicate that thyroid hormones are implicated in vitellogenesis, but as Craik (1976) has demonstrated that mobilization of liver lipid and protein for vitellogenin is under the control of oestrogens, then the thyroid hormones are more likely to have a direct action on the ovary.

Earlier work on steroids in nonmammalian vertebrates has shown that plasma testosterone levels about tenfold those found in man occur in the thorny skate, R. radiata, some salmonids and two amphibians (see Ozon, 1972, for a review). Idler and Truscott (1966), investigating the skates R. radiata and R. ocellata, were the first to report the isolation and conclusive identification of testosterone from the plasma of elasmobranchs. Plasma concentrations of the steroid, measured by G.L.C. or the aromatase enzyme method, of 76 skates were as follows: free testosterone in male R. radiata ranged from 2.8 to 10.2 $\mu\text{g}/100\text{ ml}$ (mean 7.4)



and from 0.02 to 0.6 $\mu\text{g}/100\text{ ml}$ (mean 0.47) in females. In R. ocellata males 2.2 to 20.8 $\mu\text{g}/100\text{ ml}$ (mean 10.0) and in females (from a pooled sample of four fish) the level was 0.59 $\mu\text{g}/100\text{ ml}$. The glucuronide of testosterone was quantitated in R. radiata at 4.3 to 6.7 $\mu\text{g}/100\text{ ml}$ (mean 5.5) in males and 0.13 $\mu\text{g}/100\text{ ml}$ in 8 females. Fletcher et al. (1969) again measured testosterone levels in skate and found that whilst the values for males were the same as those found by Idler and Truscott (1966), the values for females were much higher, being not significantly different from those of the male. This was explained as seasonal variation. Darrow and Fletcher (1972) demonstrated that testosterone is produced by the testes in vivo, the levels being higher in testicular effluent plasma than in peripheral plasma. Assay of peripheral and ovarian effluent plasmas from a single fish gave levels of 2.3 and 2.6 $\mu\text{g}/100\text{ ml}$ of free testosterone and glucuronide respectively in peripheral plasma and 6.4 and 15.2 $\mu\text{g}/100\text{ ml}$ in ovarian effluent plasma, thus the ovary seems to be at least one, if not the only, source of plasma androgen. Lupo di Prisco et al. (1967) identified testosterone in the plasma of T. marmorata and measured its concentration at different stages of the reproductive cycle. Levels found were 3.5 $\mu\text{g}/100\text{ ml}$ in immature animals and 1.56, 0.80 and 2.36 $\mu\text{g}/100\text{ ml}$ in pregestating, gestating and postgestating animals respectively.

Campbell et al. (1976) have recently studied fluctuation in plasma steroids of the Winter Flounder over the seasonal reproductive cycle. Testosterone concentrations were significantly higher in plasma collected from female fish during September than in plasma from spent fish in July or from the November sample. Testosterone increased during the overwintering period to reach a peak in the plasma of pre-maturational

fish in June. In male fish the same cycle was found but the fluctuations were less extreme. Concentrations of 11-ketotestosterone, an androgen found only in male fish (Idler, 1969) were low in July, September and November. In April the range of values obtained is extremely large but may consist of two groupings, one with concentrations of 2-4 $\mu\text{g}/100$ ml and the other of approximately 17 $\mu\text{g}/100$ ml; the value for the pooled sample from prespawning fish was 17.9 $\mu\text{g}/100$ ml. These authors suggest that 11-ketotestosterone in male Winter Flounders may be involved primarily in the later stages of sexual maturation.

Studies on the synthesis and circulating levels of oestrogen are few. Simpson et al. (1968) incubated the component tissues of the ovary of S. canicula with DEHA or androstenedione as substrate. Oestradiol-17 β was produced only by the follicular membrane of developing eggs and by the ovarian stroma. Corpora atretica and their membranes produced oestrone only and this steroid was also produced by the membranes of developing follicles. Corpora lutea produced a striking amount of testosterone glucuronide whilst a 17-hydroxy steroid dehydrogenase seems to be localised in all tissues. The authors, whilst pointing out the difficulties of extrapolation from in vitro to in vivo studies, suggest that stroma and corpora lutea play little part in steroid production in vivo and that the steroidogenesis of the corpus atreticum declines rapidly after its initial formation. Thus it seems that the developing follicle is responsible for the major steroidogenesis in the elasmobranch ovary, as in higher vertebrates, and that oestradiol-17 β and oestrone are its major products. The identification of oestriol by Chieffi and Iupo (1963) must be viewed with reserve because their method would no longer be accepted as conclusive.

The steroid levels reported here are in close agreement with those reported by Dobson (1974), both studies utilizing radioimmunoassay, although the purification steps were different in the separate studies. In males the mean testosterone levels for monthly samples ranged from 1.7 to 6.2 ng/ml, females reaching the slightly higher level of 7.95 ± 1.9 ng/ml. These levels are at the lower range of other studies on elasmobranchs, being much lower than Idler and Truscott's values for the rays R. radiata and R. ocellata.

Studies of the present type yield no information on the dynamics of steroid metabolism. The teleost ovary has been shown to convert testosterone to oestradiol by Lambert et al. (1971) and oestrogen is capable of stimulating vitellogenesis in S. canicula, which suggest that the testosterone detected in females in this study might serve as a precursor for oestrogen during vitellogenesis. In this context it is interesting to note that fluctuations in both testosterone and oestradiol in the female rise and fall perfectly together, so that at all times the ratio of oestradiol to testosterone is close to five. There is a subtle change in this ratio in that when steroid levels are falling from February until May the ratio is less than five, whereas when steroid levels are rising the ratio is greater than five. Thus when the reproductive cycle is beginning in late summer there is relatively more oestradiol to testosterone than is found when the cycle is regressing, possibly because the demand for oestradiol is greater at the beginning of the season when vitellogenesis begins and so testosterone is converted more rapidly into oestradiol.

Martin (1975) demonstrated an elasmobranch steroid-binding protein in S. canicula that binds both sex steroids (C_{18} - C_{19} steroids)

and C₂₁ steroids and, therefore, its specificity is quite different from the sex steroid binding protein and corticosteroid binding proteins described in serum of teleost fishes, amphibians, reptiles, birds and mammals. Presently the more important function of the association steroids-serum proteins is seen as the regulation of the physiological activity of the steroids. The steroid-protein complex forms a biologically inert reservoir where the steroid is protected from metabolism or excretion (for a review see Sandberg et al., 1966). The steroid is made available by dissociation and may be considered as a physiologically active agent. It is apparent that serum protein-steroids complex may play an important role in steroid metabolism in elasmobranchs. Thus Martin (1975) suggests the high steroids-binding capacities of elasmobranch steroid binding protein may explain why elasmobranchs are able to tolerate high steroid levels.

As we have seen, the dogfish appears to be cyclical in its reproductive physiology and it seems reasonable to suppose that the cycles are environmentally controlled. Dobson and Dodd (1976) suggest that water temperature may be the primary, if not the only, environmental cue with regard to spermatogenesis in the male. This suggestion was based partly on the indirect evidence of the correlation between gonadosomatic index and water temperature, and also the ventral lobe gonadotrophin content and water temperature. Direct evidence came from the appearance of a "zone of breakdown" in the testes of ventral lobectomized fish kept under various light and temperature regimes. This "zone of breakdown" only appeared if fish were maintained at high temperatures (15°C), irrespective of the light regime. This temperature is reached during mid summer around the coast of Anglesey, when plasma testosterone levels

begin to fall in the male, and only rise again when the water begins to cool in autumn. Almost the reverse pattern is seen with both testosterone and oestradiol in the female, however, and thus it is difficult to correlate changes in steroid levels in both sexes with any single environmental factor.

CHAPTER 2

THE PITUITARY-GONADAL AXIS IN S. CANICULA

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THE PITUITARY-GONADAL AXIS IN S. CANICULA

Introduction

Some pituitary regulation of certain aspects of reproduction has been demonstrated in all vertebrate animals. However, the available data suggest that this control is sometimes less precise and embraces fewer aspects of reproduction among the fishes. Differences occur between the various groups of fishes, the control being less complete in cyclostomes than in the Selachii and Teleostii (Larsen, 1973). In a variety of teleost species a correlation between basophils (gonadotrophic cells) of the proximal pars distalis and the gonadal cycle can be observed. This cell type shows hyperplasia, hypertrophy and other signs of increased activity in association with the ripening of the gonads (for specific references see review by de Vlaming, 1974).

The dependence of gonadal function on the pituitary in teleosts was first demonstrated by Vivien (1938, 1939 a,b,c, 1941) and Matthews (1939). Investigation of the gonadotrophic activities of the pituitary gland in the Agnatha awaited the investigations of Dodd and his associates in the early sixties (Dodd et al., 1960; Evannett and Dodd, 1963). In the gnathostomes, hypophysectomy is followed not only by an arrest of ovarian development, but also by a general atresia of the follicles, while spermatogenesis is completely blocked in the testes. In the lamprey, however, spermatogenesis, spermiogenesis and ovarian growth seem to be autonomous processes; they are retarded but not suppressed in the absence of the pituitary. The subject is comprehensively reviewed by de Vlaming (1974) who considers the effects of hypophysectomy reported probably vary as a function of the gonadal condition at the time of surgery (season) and with temperature of maintenance. The discrepancies reported as to



the exact stages of spermatogenesis and vitellogenesis affected by hypophysectomy are probably due, in part, to variation in these parameters.

Although the many reports of the effects of hypophysectomy in fishes demonstrated the indispensable nature of the pituitary for reproduction, as Barr (1968) points out, hypophysectomy alters the entire hormonal milieu and thus the direct dependence of the gonads on the pituitary is difficult to establish by this surgical approach. Investigations of the pituitary functions of fishes have sometimes also been hampered by the technical difficulties of hypophysectomy. Some authors have circumvented this problem by using Methallibure as a chemical blocking agent of gonadotrophic functions (reviewed by Hyder, 1972). The addition of methallibure to the water inhibited spermatogenesis in Gasterosteus aculeatus and Carassius auratus (Hoar, Wiebe and Wai, 1967; Wiebe, 1968, 1969) and Poecilia reticulata (Billard, Breton and Jalabert, 1970; Martin and ^(com)Boerage, 1970; Pandey, 1970; Pandey and Leatherland, 1970). When methallibure-treated goldfish were injected with carp whole pituitary extract, spermatogenesis resumed and spermiation occurred (Billard, Breton and Eiscaffre, 1971). This suggested the drug acted by suppressing the synthesis or release of gonadotrophins at the hypothalamo-hypophysial level and not by blocking their action peripherally. In an ultrastructural study Leatherland (1969) confirmed that methallibure could block gonadotrophin synthesis.

In male Tilapia methallibure effectively suppresses gonadotrophin production, leading to the disappearance of all spermatocytes and spermatids (Hyder, 1972). In the female Tilapia methallibure administration depresses ovarian weight, G.S.I., vitellogenesis and thecal cell proliferation (Hyder, 1972), and resembles in other species the effects of surgical



hypophysectomy. Breton, Jalabert and Billard (1973) directly confirmed that methallibure suppressed plasma gonadotrophin levels in goldfish, although the pituitary level remained at the pretreatment level.

Clomiphene citrate is another synthetic drug that has recently been used in studies on the pituitary-gonadal axis of fish. It is an analogue of a non-steroidal oestrogen and is either oestrogenic or anti-oestrogenic in different mammals. Both Pandey and Stacey (1975) and Breton, Jalabert and Fostier (1975) have considered clomiphene to act as an anti-oestrogen by competing with the natural oestrogen for receptor or binding sites in the hypothalamo-hypophyseal axis and thus effectively displacing the natural oestrogen from receptor sites. Breton, Jalabert and Fostier (1975) demonstrated that administration of clomiphene citrate to carp induces a large discharge of gonadotrophin into the plasma. This gonadotrophin surge induced by clomiphene may be responsible for the induction of ovulation in goldfish (Pandey and Hoar, 1972; Pandey *et al.*, 1973). A similar rise in plasma gonadotrophin levels has recently been observed in the rainbow trout after castration (Billard, Richard and Breton, 1976). This short term elevation of gonadotrophin concentration is the most direct evidence of gonadal feedback, although indirect evidence was given by Atz (1955) and McBride and Van Overbeke (1969), who showed modification in the gonadotrophin cells in the pituitary after physiological or surgical castration. There are also some suggestions that steroids are involved in this regulation process (Febvre and Lafaurie, 1971; Sasayama and Takahashi, 1972; Breton, Jalabert, Fostier and Billard, 1975).

The last, and perhaps now the most widely used, method of studying the control of the gonads by the pituitary is by the use of



gonadotrophic preparations. Until the fairly recent purification of fish gonadotrophins, mammalian gonadotrophins and TSH were used by many authors when studying fish reproduction. When mammalian gonadotrophins do exert favourable action on one of the phases of reproduction, it is generally the LH which is the most active (Pickford and Atz, 1957; Dodd, 1960; Hoar, 1966). While in the male it is possible to obtain spermatogenesis and spermiation by mammalian hormones, in the female of the same species a piscine hormone is usually required. Thus, as early as 1936 (Fontaine, 1936) the complete maturation of a male Anguilla anguilla was obtained with human chorionic gonadotrophin (HCG). In order to obtain complete maturation in the female eel, it was necessary to use pituitary extracts of carp (Fontaine et al., 1964). In general, the action of mammalian gonadotrophins is much less certain than that of piscine gonadotrophic hormones and will not be further pursued here.

With the purification of fish gonadotrophic hormones it was possible to show clearly and conclusively their controlling action on the gonad. The purified hormone of Oncorhynchus tshawytscha (Yamazaki and Donaldson, 1968a), onc-GTH, induces complete sexual maturation in the juvenile male pink salmon (O. gorbuscha) in the years of hatching (Funk and Donaldson, 1972) and reinitiates and restores oogenesis and vitellogenesis in the hypophysectomized catfish (Sundararaj and Anand, 1972; Sundararaj et al., 1972). The onc-GTH also induces ovulation in the goldfish (Yamazaki and Donaldson, 1968b) and in the catfish (Sundararaj et al., 1972) and moreover is capable of inducing maturation of oocytes in vitro in Salmo gairdneri (Jalabert et al., 1973), but not ovulation. Purer preparations of salmon gonadotrophin (Idler et al., 1975 b,c) are capable of stimulating cAMP production in immature rainbow trout ovaries

and testes (Idler et al., 1975a).

The hormone isolated from Cyprinus carpio pituitaries (Fontaine and Gérard, 1963; Burzawa-Gérard, 1971) is capable of restoring vitellogenesis in a hypophysectomized teleost. In hypophysectomized male Carassius onc-GTH and c-GTH induce spermatogenesis and spermiation (Yamazaki and Donaldson, 1968a,b; Billard et al., 1970). Leydig cells are activated by onc-GTH, suggesting to Sundararaj et al. (1971) that onc-GTH acts by way of androgens secreted by these cells.

The action of other pituitary hormones on reproduction is much less certain. Thus mammalian TSH can bring on the maturation of the male eel (Olivereau, 1961) but only a slight increase of the gonadosomatic ratio and of the ovular diameter of the female eel. This increase can be observed in hypophysectomized eels (Bertrand and Fontaine, unpublished observations, mentioned in Fontaine, 1968), which tends to prove that the effect is not associated with the pituitary. Fontaine (1976) concludes that it does not seem impossible that TSH acts directly on the gonads and also that TSH may play a more important role in the sexual organs than the thyroid hormones. Prolactin and growth hormone (GH) have also been implicated in fish reproduction. Pickford et al. (1972) observed that mammalian GH stimulated spermatogonial proliferation in hypophysectomized Fundulus heteroclitus. GH and prolactin are also probably involved, together with androgens, in the control of seminal vesicles in the catfish (Sundararaj and Goswami, 1965; Sundararaj and Nayer, 1969) and in Gillichthys mirabilis (de Vlaming and Sundararaj, 1972). The abundance of serotonin in the male reproductive tract of the Selachian Squalus acanthias suggested a role for this hormone in the reproductive process, specially at the time of copulation (Mann, 1960). There is no doubt that

neurohypophysial (Perks, 1969) and urophysial (Bern, 1969) hormones affect reproduction, specially spawning and egg-laying (Iederis, 1972; Heller, 1972), although they have not yet been implicated in the earlier processes of reproduction.

In summary, all investigators agree that the pituitary is required for gonadal maturation, after either physiological or surgical hypophysectomy vitellogenesis is suppressed with atresia of the larger developing oocytes, spermatogenesis is blocked at the spermatogonia-spermatocyte stage, and steroidogenesis does not occur in the gonadal endocrine tissues. Although these general findings are consistent, there are still many unresolved details. It is not clear whether gonadotrophin is required for multiplication of the spermatogonia, specifically triggering the reduction divisions, spermiation or ovulation, nor is it certain to what degree the Sertoli cells and other supporting tissues in the gonads are dependent on the pituitary. The later stages of spermatogenesis and spermiogenesis, if well under way, seem to continue with production of mature sperm but spermiation is usually not observed in the absence of the pituitary, and the mature sperm disappear through phagocytosis or resorption. Spermiation has been reported in the hypophysectomized plaice (Barr, 1963b) and lake chub (Ahsan, 1966); these differences may depend on the stage of sexual maturity at the time of surgery.

The changes in ovarian histology which follow hypophysectomy have been much less frequently examined. However, the findings are consistent with those recorded by Dodd *et al.* (1960) for the elasmobranchs. These indicate that after hypophysectomy a slow but inevitable resorption of ova may be expected, vitellogenesis comes to an end, new ova fail to



form, and ovulation and spawning do not occur unless yolk deposition was complete prior to the surgery.

The present study of the pituitary-gonadal axis in the dogfish continues that begun by Dodd et al. (1960) and continued by Dobson (1974). The latter author could detect no effect of hypophysectomy on plasma testosterone levels in males, and argued for a degree of autonomy of the dogfish testis not found in higher vertebrates. One of the major aims of this study was to see if the reverse could be obtained, i.e. could elevated steroid levels be obtained by replacement therapy in hypophysectomized fish.

Materials and Methods

1) Surgical Techniques

Surgery was performed on fish anaesthetised with methane tricaine sulphonate (MS 222; Sandoz) at a concentration of approximately 1 part in 5000 parts of seawater. Ten minutes in the anaesthetic proved sufficient for the operations which lasted 10 to 15 minutes. Recovery in fresh, recirculating seawater occurred within 30 minutes of completion of the operation.

Operative procedures were carried out with the fish out of water in a 'V'-shaped wooden trough. The mouth was held open using two rubber bands. A Zeiss epitechnoscope with shadow-free light source and a Kaltenbach and Voigt (Germany) portable dental drill were used for hypophysectomy.

a) Ventral lobectomy (VLX)

A small median incision (1.5 cm) was made in the mucous membrane of the roof of the mouth and kept open with retractors. This reveals the

carotid anastomosis which is used as a guide (Figure 13). A small crescent shaped area of the cartilage anterior to the vessels was pared away with a dental burr to reveal the ventral surface of the connective tissue capsule containing the lobe. The capsule, a tough, flexible, white sheet, was cut from one side to the other with the point of a scalpel blade and gently opened. Colloid contained within the lobe flowed out and the capsule deflated. Cellular tissue contained within was removed by swabbing with small rolls of cotton wool wound on to the points of fine forceps and pushed gently into the extremities of the cavity, which are not visible.

b) Rostral and Median Lobectomy (R+MLX) and Neurointermediate Lobectomy (NILX)

Ablation of these pituitary lobes requires a different approach (Chevins and Dodd, 1970; Dobson, 1974). After a similar incision in the mucus membrane, the cranial cavity was entered by making two longitudinal incisions through the cartilage of the brain case using a No. 12 scalpel blade as shown in Figure 13. Care must be taken to ensure that these incisions are lateral to the medial end of the internal carotid arteries and far enough anterior to avoid disturbing the ventral lobe. A further incision was made to connect the lateral ones at their anterior ends forming a three-sided flap of cartilage. The cartilage flap was then reflected backward and held away from the cavity by a hypodermic needle pushed gently into the cartilage. If the cuts are made obliquely, the bevelled edges help to prevent retraction of the flap into the brain cavity and possible brain damage when the flap is replaced after the operation. Care must also be taken that the sides of the flap are not cut too far from the midline, too wide a flap causes bleeding from cut vertebral arteries.



Figure 13: (After Dobson, 1974)

A. Ventral lobectomy operation

Diagram of the operation site

C = Carotid anastomosis

I = Inter-orbital vein

V = Ventral lobe; this is not visible during the operation as it lies on the cartilage inside the cranial cavity.

B. Surgical approach to the median, rostral and neurointermediate lobes

A flap has been cut in the cartilage of the base of the cranium and lifted up and backwards as described in the text.

C = Carotid anastomosis

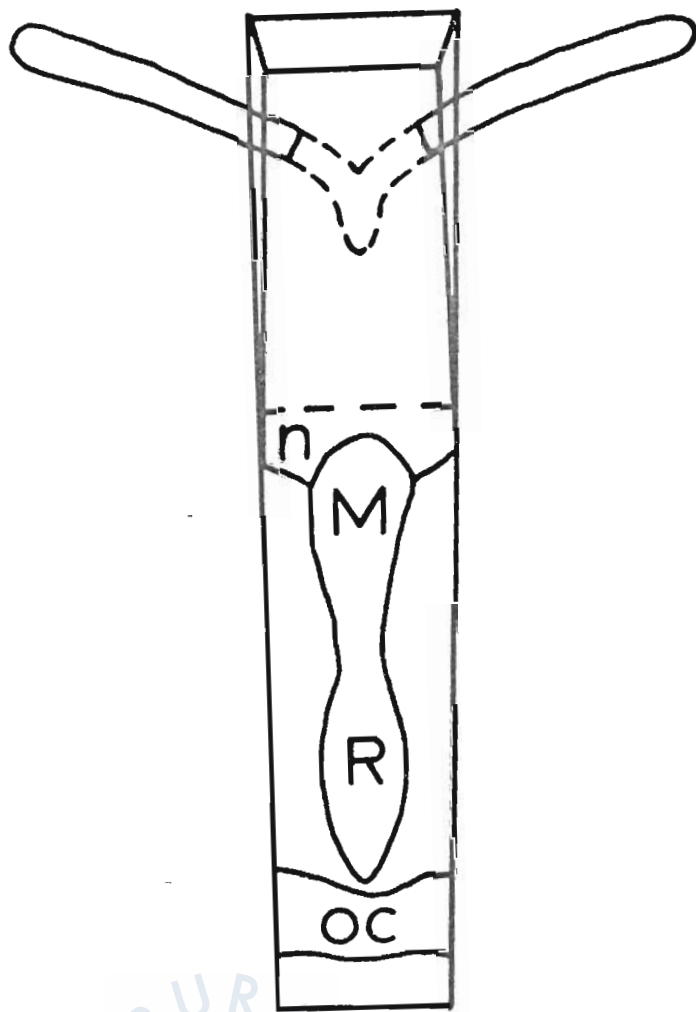
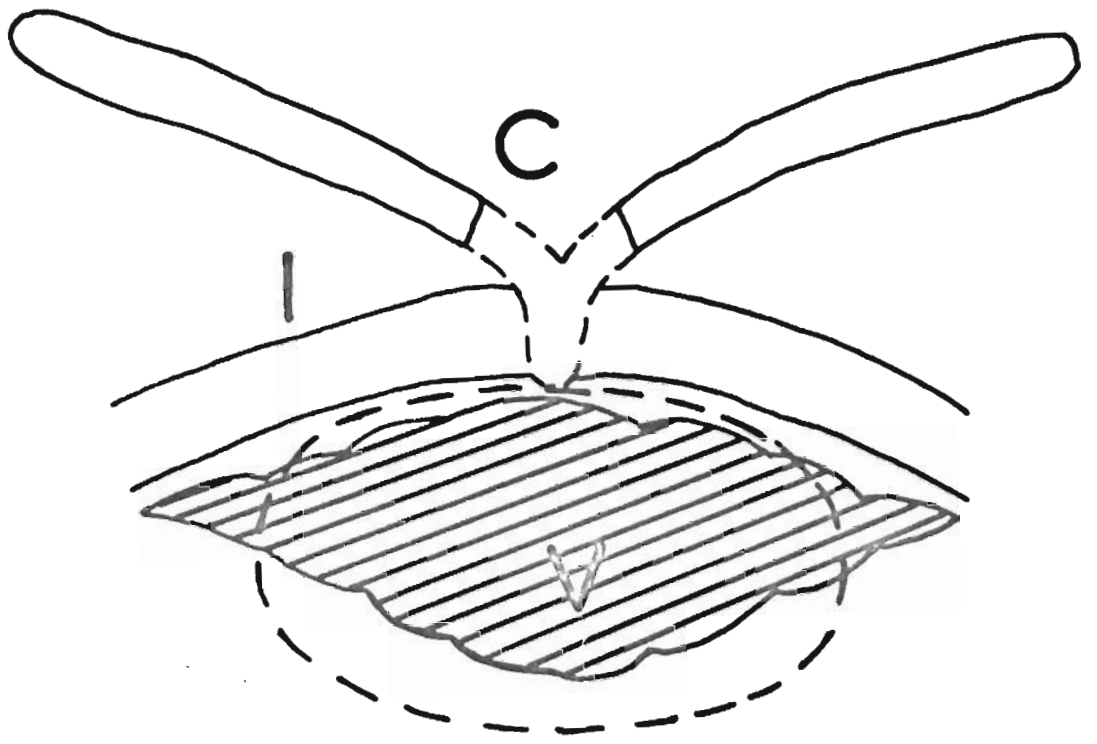
M = Median lobe

R = Rostral lobe

N = Neurointermediate lobe

OC = Optic chiasma





Rostral and median lobes were always removed together in this work. The anterior tip of the rostral lobe was picked up in fine forceps and lifted. Using fine scissors, the lobe was separated from the underlying brain tissue, working posteriorly towards the neurointermediate lobe. The rostral and median lobes were removed in one piece.

The neurointermediate lobe is slightly more difficult to remove, but this can be effected if the hinge of the flap is made as far back as possible, without damaging the ventral lobe. This allows the "pituitary stalk" (between the median and ventral lobes) to be cut. Removal of the neurointermediate lobe is then achieved by firstly separating the median lobe from the neurointermediate lobe using fine scissors, and then separation of the neurointermediate lobe from the saccus vasculosus.

When both the ventral and other lobes are removed at the same time the flap is cut only after removal of the ventral lobe. After such an operation the flap must be stitched into place at its anterior end because weakening of the "hinge", caused by drilling, results in an unstable flap. If the ventral lobe is not removed this is unnecessary. In one group of fish, after removal of the rostral and median lobe, the hypothalamus was completely destroyed by heat cautery. In all pituitary operations suture of the mucus membrane with fine monofilament nylon (Armour Pharmaceutical Company Ltd., size I (5/0)) completed the surgery. Healing of the membrane is slow in cold weather but begins within a few days in summer and healing of the incision is accomplished in about 3 weeks.

Completeness of the operation can be assessed visually when rostral, median and neurointermediate lobes are removed. Dobson (1974) serially sectioned the brain after these operations and showed that no



tissue is left adhering to the former. Serial section of ventral lobe areas indicated that few cells survive the operation, leading Dobson (1974) to conclude "a monolayer of pituitary cells adhering to the dorsal wall of the cavity show no sign of regenerating the ventral after 100 days post-operation and these cells seem unable to carry out the normal function of the ventral lobe".

No damage to any other structure occurred with ventralobectomy. The removal of the rostral and median lobes usually caused damage to the hypothalamus which produces irreversible darkening (Hogben, 1936; Wilson, 1972). Neurointermediate lobectomy caused irreversible paling (Wilson, 1972). Damage to the sacculus vasculosus causes some bleeding but has no apparent effect on the survival of the fish. Survival of operated and control fish (taken drilling for ventral lobectomy and the cutting and lifting of a flap for the other operations) was approximately equal except for the case of total hypophysectomy where more operated fish died. Details of survival are given for each group in the appropriate section.

c) Thyroidectomy (TX)

Thyroidectomy was performed as described by Matty (1954) and Lewis (1975). Fish were again anaesthetised and placed ventral surface uppermost in a wooden trough. The mouth was opened and the lower jaw pulled back and held by a strong rubber band. This revealed the soft white mucosa on the ventral surface of the tongue. Using a scalpel, a medial incision was made through the mucosa. From this stage onwards the operation was carried out using blunt dissection. The sub-dermal connective tissue was separated, with curved blunt forceps, and the coracohyoid muscles, lying ventral to the thyroid gland, were revealed. These muscles were separated medially for about 2 cm posterior to their



origin at the edge of the lower jaw. Occasionally this resulted in a considerable loss of blood as the sinus in which the thyroid gland lies was ruptured, although bleeding ceased rapidly and the operation was resumed after swabbing. The arms of a retractor were inserted under the muscles and the thyroid sinus opened slightly, exposing the thyroid gland showing up orange or yellow against the light pink of the surrounding tissue. The tail of the thyroid gland was fully exposed by further blunt dissection at the inner surfaces of the coracohyoid muscles. The thyroid gland was "peeled" back posteriorly by holding the gland in one pair of forceps and severing connective tissue with another pair. If possible the whole of the gland was removed in one piece and the cavity was carefully inspected and swabbed out with cotton wool. Any fragments of thyroid adhering to connective tissue were easily distinguished by their colour and were picked off. A large, loose stitch was tied to draw the muscles together, and the mucosa then stitched together over the muscles to close the wound.

2) Force Feeding

Force feeding of experimental fish was carried out using a mechanical feeding device as described by Dodd et al. (1959). This consisted basically of a metal cylinder that was filled with minced fresh whiting, and a mechanically operated plunger to force this food out through a plastic tube at the bottom. Feeding can be carried out by a single operator who, holding the anaesthetised fish vertically, opens its mouth and inserts the plastic tube gently until the latter reaches the stomach. He then turns the plunger handle the requisite number of times until the desired amount of food has been delivered. In this way fifty dogfish can be fed in one hour; each receiving the



same amount of food. Dodd et al. (1959) noted that females fed in this way lay normal looking eggs at regular intervals throughout the egg laying season. In the course of this study fish were fed approximately once every 2 weeks with about 40 g of minced whiting. All the egg-laying experiments were conducted during the winter months when the fish's metabolism is lower than during the summer months of higher water temperature. The mean weight loss over the four month span of most egg-laying experiments was 5.8%.

3) Monitoring of egg-laying

In all experiments involving monitoring of egg-laying after operation, the fish were divided so that between 6 and 8 animals were present in each tank at the beginning of the experiment, with no other fish present. Fish were distributed so that all animals present in an individual tank had received the same operation. As far as possible groups of fish were distributed randomly with this limit to attempt to minimise any effects due to light or temperature differences within the tank system. Every day, usually in the morning, all tanks were inspected for egg cases, which were removed and kept separately (see below). If an egg case was seen protruding from the cloaca of a fish it was removed by gentle pulling. In this way it was possible to ascribe about half the total number of eggs laid to individual fish. Egg cases laid in the tank, usually wound around the stand pipes, could only be ascribed to a group of animals. Besides these pipes nothing else was present in the tanks around which the fish could wrap the tendrils of their egg cases.

All eggs collected were transferred to floating plastic trays divided into numbered compartments by nylon twine so that the fertility of each pair could be checked subsequently.



4) Intravenous Injection of Hormones or Pituitary Extracts

All test substances administered were injected intravenously into the caudal sinus. The needle of the plastic syringe, already loaded with the test substance, was inserted into the caudal sinus as described in Chapter 1 and some blood withdrawn into the syringe to check that the needle was correctly positioned. This small volume of blood, together with the test substance in 1 ml, was then carefully injected into the sinus. Very little, if any, leakage of the test substance occurred if the syringe needle was left in the caudal sinus for about half a minute after injection of the test substance. Subsequent blood samples were collected as described previously (Chapter 1).

In the initial experiment involving injection of a ventral lobe extract into mature male S. canicula, the four experimental fish each received an extract equivalent to 8 ventral lobes. These lobes were collected from males during April, and 32 were homogenized in 5 ml 0.9% saline as described previously (Chapter 1). The extract was spun and 1 ml of the supernatant injected into each fish (= 8 ventral lobes), while the remaining 1 ml was frozen and later assayed for its biological gonadotrophic potency in the ^{32}P chick assay.

In the later experiment involving injection of an extract of the various pituitary lobes into hypophysectomized mature male dogfish, all fish received an extract equivalent to 8 lobes. The various lobes were collected from a single batch of 40 fish caught during April, of mixed sex. The pituitary lobes were separated from one another and frozen until the experiment, when they were thawed and homogenized in 0.9% saline as above. In this case none of the extracts were subsequently bioassayed for their gonadotrophic potency.

Ovine follicle-stimulating hormone (NIH-FSH-S10) and ovine luteinizing hormone (NIH-LH-S19) were weighed out on a Cahn gram Electrobalance (Cahn Instrument Company, California, U.S.A.), dissolved in 0.9% saline and injected as before.

5) Steroid Radioimmunoassays

These were carried out as described in Chapter 1.

Results

1) Effects of pituitary extracts on plasma steroid levels.

As Dobson (1974) had failed to show an effect of hypophysectomy on plasma testosterone levels in mature males, he argued for a degree of autonomy of the testis not found in higher vertebrates, although his experiments were by no means exhaustive. In view of this finding it seemed worthwhile to see if elevated steroid levels could be obtained in adults by replacement therapy.

The initial experiment was conducted primarily to establish if steroid levels could be altered experimentally, and if so, to ascertain a time course for this change. Seven mature intact males, captured three days previously in early August, were used. The four receiving an injection of a ventral lobe extract all showed a rise in plasma testosterone concentration an hour after the injection (Figure 14). The level was still increasing 90 minutes after the injection of the ventral lobe extract, at the end of the experiment. This increase in plasma testosterone concentration was highly significant ($P < 0.01$) between the preinjection and 90 minute post-injection bleeds in the four fish receiving the ventral lobe extract, but there was no significant change in the plasma testosterone level in the saline injected fish throughout the experiment. Estimation

Figure 14:

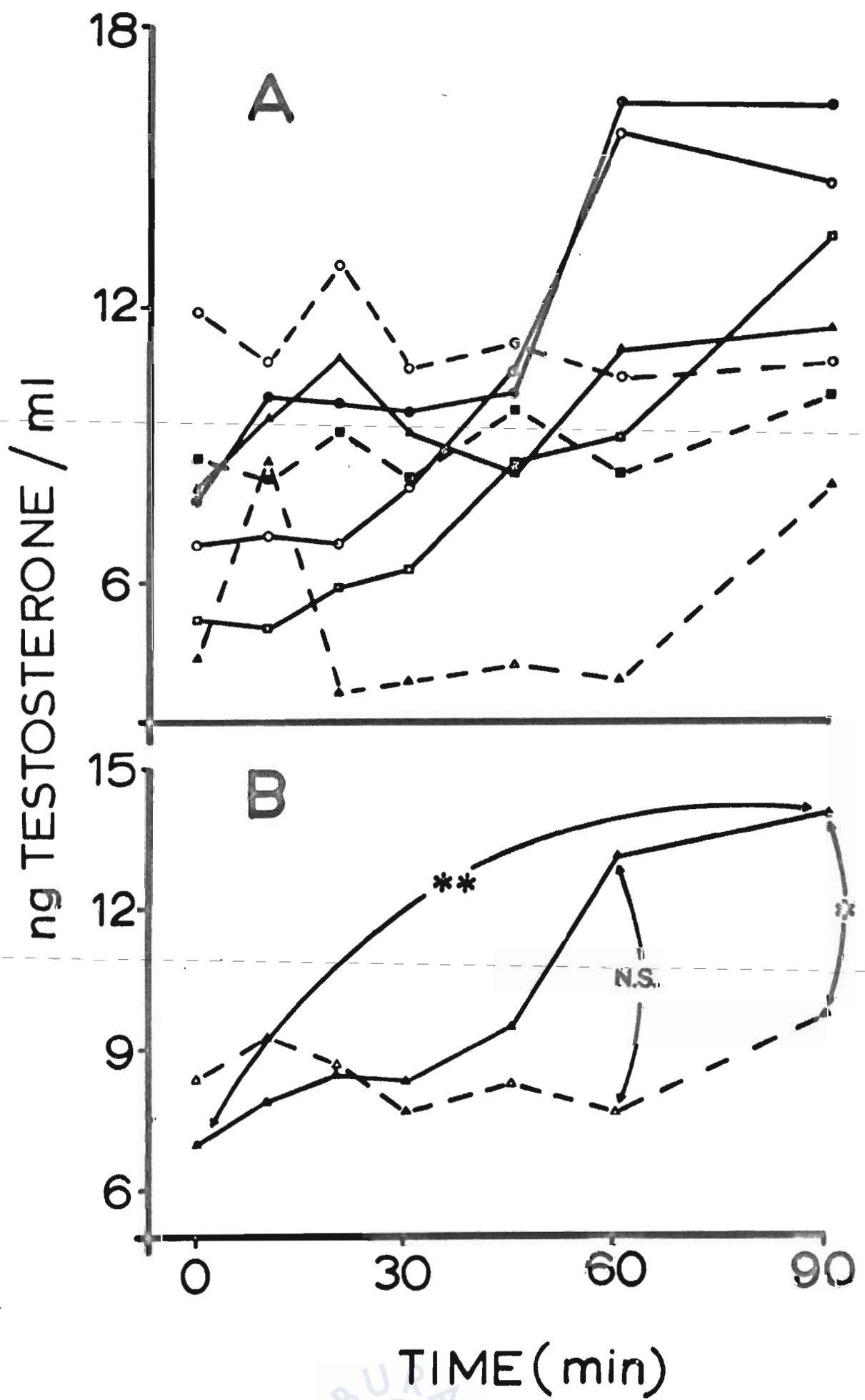
Elevation of plasma testosterone levels in mature male S. canicula after an intravenous injection of either a ventral lobe extract (solid lines) or saline (dotted lines). Each fish received the equivalent of eight ventral lobes or a similar volume of saline.

A: Individual fish, B: Mean values for control and experimental groups.

NS = Not significant; * P < 0.05; ** P < 0.01;

Students "t" test.





of the gonadotrophic potency of the ventral lobe extract in the ^{32}P chick assay showed that each fish received 60.8 (31.2 - 129.6) μg equivalents NIH-LH-S19. Although the experiment was conducted on only a small number of intact fish, also presumably containing endogenous circulating gonadotrophin, it convincingly demonstrated that plasma testosterone levels could be elevated in intact mature males, and also gave an indication that although the response had begun after 1 hr, levels were still rising after $1\frac{1}{2}$ hours.

After the success of the initial experiment, a larger experiment was conducted to determine which of the various pituitary lobes were capable of stimulating this increase in testosterone levels. Fifteen mature males, caught on the 17th September, were completely hypophysectomized two days later. All fish showed marked paling following the operation. They were split into three equal groups two days after the operation. After the initial post-operative bleed (time 0 h), one group received a rostral and median lobe extract, one a neurointermediate lobe extract and the last group a ventral lobe extract, all lobes having come from a separate single batch of fish. Those injected with the neurointermediate lobe extract went very dark after approximately 30 minutes. All fish survived until the end of the experiment although one died very shortly after the final sample was collected.

The first interesting feature to emerge was the fact that the plasma testosterone levels halved between the pre-operation bleed and the first post-operation bleed two days later. This fall was highly significant ($P < 0.001$) over the whole group of fifteen experimental fish. As well as altering the initial testosterone levels the operation also lowered the variability between individuals. Those fish receiving an extract of






rostral and median lobes showed no significant change in plasma testosterone concentration throughout the course of the post-operative bleeds (Figure 15). However there was a steady, non-significant, rise in testosterone concentration up to four hours post-injection, followed by a fall back to pre-injection levels after 24 hours. Fish receiving an extract of neurointermediate lobe showed a significant ($P < 0.05$) rise four hours after the injection, which was maintained at five hours post-injection, but had fallen significantly ($P < 0.05$) after 48 hours, back to pre-injection levels. At no time were the testosterone concentrations in fish receiving either rostral and median or neurointermediate lobe extracts significantly different from one another.

Injection of a ventral lobe extract caused a significant ($P < 0.05$) rise in testosterone concentrations within one hour, and by five hours this was highly significant ($P < 0.001$). Plasma testosterone concentration in fish injected with ventral lobe extract were significantly higher ($P < 0.05$) than those injected with rostral and median lobe extract two hours after the injection, and were highly significant ($P < 0.001$) after five hours. Four hours after the injection fish that received a ventral lobe extract had significantly higher ($P < 0.05$) testosterone concentrations than those fish that received the neurointermediate lobe extract, this difference being more significant ($P < 0.01$) after 24 hours. Even 72 hours after the injections the fish that received the ventral lobe extract had a significantly ($P < 0.05$) higher plasma testosterone concentration than either of the two groups receiving rostral and median or neurointermediate lobe extracts, both the latter two groups having returned to pre-injection levels (time 0 hours), although the concentration had decreased markedly ($P < 0.05$) between five and 48 hours post-injection in the



Figure 15:

Elevation of plasma testosterone levels in hypophysectomized mature males following the intravenous injection of rostral plus median lobe extract (), ventral lobe extract (), or neurointermediate lobe extract (). Each fish received the equivalent of eight lobes.

Each column represents the mean \pm SEM (n = 5) ng/ml.

NS = Not significant; * P < 0.05; ** P < 0.01;

*** P < 0.001.



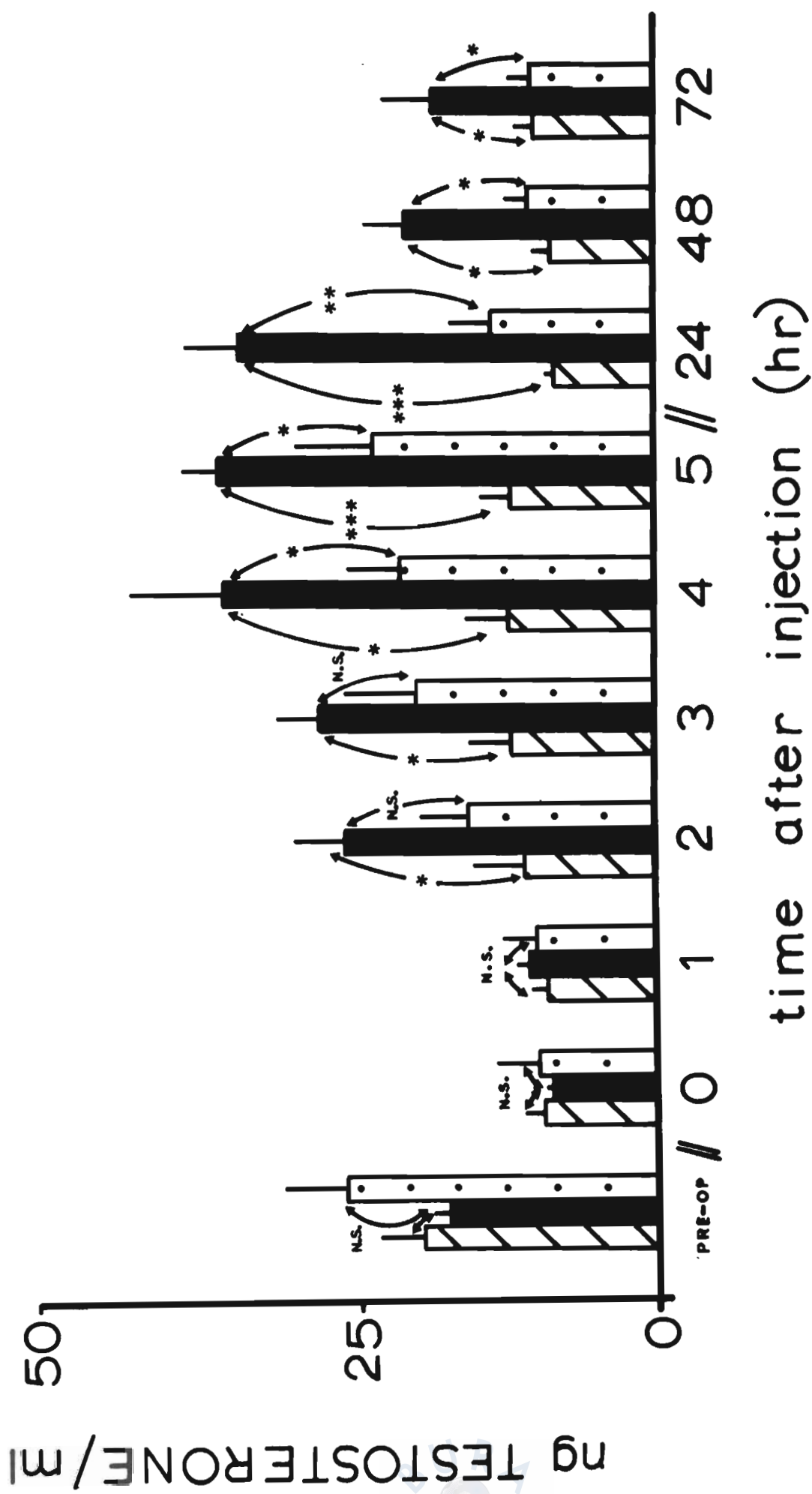


Table 6: Changes in plasma testosterone concentrations following the intravenous injection of various pituitary lobe extracts

Pituitary lobe extract injected	Plasma testosterone concentrations ng/ml (\pm SEM), n = 5 in each case									
	Time after injection (hours)									
	Pre-op	0	1	2	3	4	5	24	48	72
Rostral + median lobe	18.43 \pm 3.81	8.91 \pm 1.51*	8.37 \pm 1.76	10.62 \pm 3.81	11.60 \pm 3.59	11.88 \pm 3.45	11.66 \pm 2.39	7.89 \pm 0.78	8.50 \pm 1.33	9.45 \pm 1.81
	←→							→ N.S.		
Ventral lobe	16.44 \pm 1.53	8.19 \pm 0.64*	10.21 \pm 0.95	25.19 \pm 4.08	26.96 \pm 4.62	34.81 \pm 7.67	35.14 \pm 3.09	33.47 \pm 4.61	20.25 \pm 3.29*	17.76 \pm 3.10
	←→		←→					←→		
							→ ***			
Neurointermediate lobe	24.87 \pm 5.69	9.59 \pm 2.37**	9.71 \pm 2.51	14.85 \pm 4.07	19.22 \pm 6.02	20.57 \pm 4.58	22.71 \pm 6.67	13.41 \pm 3.26	10.37 \pm 1.79	9.97 \pm 1.73*
	←→						←→ *			



former fish. These results are tabulated in Table 6.

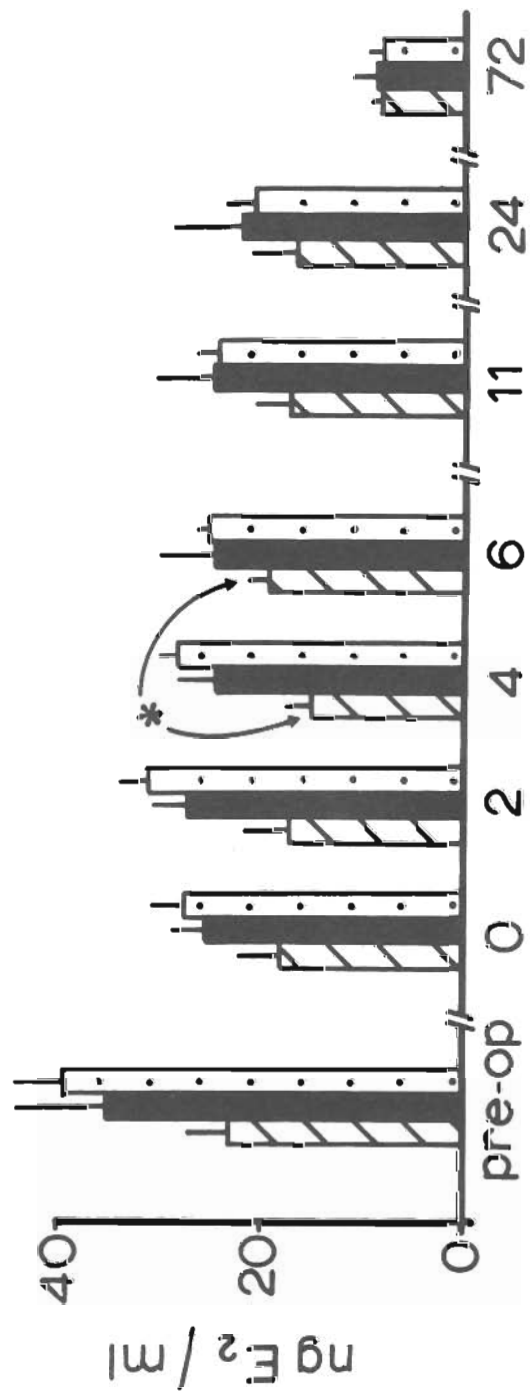
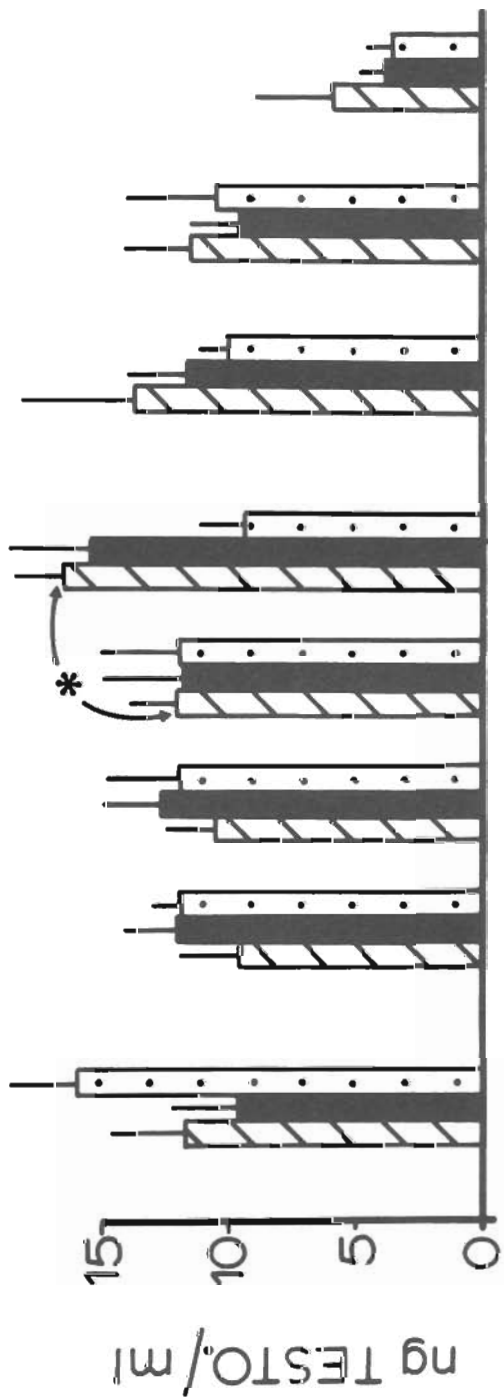
2) Effect of mammalian gonadotrophins on plasma steroid levels

The effects of mammalian gonadotrophins were studied on a group of mature females caught in late February and completely hypophysectomized over the following two days. The time scale adopted followed fairly closely to that used in the experiment described above. There was no significant fall in either plasma testosterone or oestradiol concentration between the pre-operative and pre-injection (time 0 hours) bleeds five days later. Injection of either ovine LH (NIH-LH-S19, 100 µg/fish) or saline did not cause any increase in either plasma testosterone or oestradiol concentrations (Figure 16). Ovine PSH (NIH-PSH-S10, 100 µg/fish) caused a significant ($P < 0.05$) rise in both testosterone and oestradiol concentration between 4 and 6 hours post-injection (Figure 16). There was a highly significant ($P < 0.001$) fall in both plasma steroid concentrations between 24 and 72 hours post-injection in the 13 fish remaining alive at the end of the experiment, when considered as a single group.

To attempt to determine whether this almost complete failure to respond to ovine gonadotrophins was due to an intrinsic inability of mammalian hormones to stimulate steroid secretion in the dogfish, or whether the fish themselves were unresponsive and unable to respond to any gonadotrophin, five of the fish, selected at random but including at least one from each of the three groups, were injected with a ventral lobe extract equivalent to 40 µg equivalents NIH-LH-S19 as determined in the ^{32}P chick assay. These five fish were pre-bled before the injection and subsequently bled 2, 4, 6 and 24 hours after the injection. Only the

Figure 16:

Levels of plasma testosterone (top graph) and oestradiol (bottom graph) in hypophysectomized mature females following the intravenous injection of either ovine follicle-stimulating hormone (NIH-FSH-S10, 100 µg/fish, ▨), ovine luteinizing hormone (NIH-LH-S19, 100 µg/fish, ▩), or saline (◻). Each column represents the mean \pm SEM (n = 4, 5 or 6) ng/ml. * P < 0.05.



time after injection (hr)

oestradiol levels were measured in these plasmas, but no significant rise occurred, in fact there was a slight fall over the experimental period. However, this experiment was conducted 8 days after the initial experiment involving injection of ovine gonadotrophins.

3) Effect of removal of pituitary lobes on plasma steroid levels

Although Dobson (1974) had failed to detect an effect of hypophysectomy on testosterone concentrations in mature male dogfish, the experiment conducted here involving injection of pituitary lobe extracts into males had shown a highly significant fall in plasma testosterone concentration over two days in a group of 15 completely hypophysectomized fish. To see if this could be repeated a group of ten freshly captured mature females were operated on in late October; six were ventral lobectomized and four were rostral and median lobectomized, and subsequently bled over the next two weeks. The results, shown in Figure 17, demonstrate that the oestradiol concentration falls rapidly over the first four days ($P < 0.001$ for both groups combined) and then levels out, although still falling, between days 4 and 14 post-operation ($P < 0.05$ for both groups combined). However, at no time is there any significant difference between the groups, which received the different types of pituitary operation.

A more thorough experiment involved three groups of 10 mature male fish, one ventral lobectomized, another rostral and median lobectomized and the last neurointermediate lobectomized. The fish were all part of a single batch caught on the 17th October and operated on over the 22nd and 23rd October. All subsequent blood sampling was carried out between 9 and 11 a.m., following the same time scale adopted in the previous experiments. As can be seen in Figure 18, the plasma testosterone levels of



Figure 17:

Fall in plasma oestradiol levels in mature female S. canicula following ventral lobectomy (●—●) and rostral and median lobectomy (○-----○). Each point represents the mean \pm SEM ng/ml, n = 6 for VLX and 4 for R + MLX.



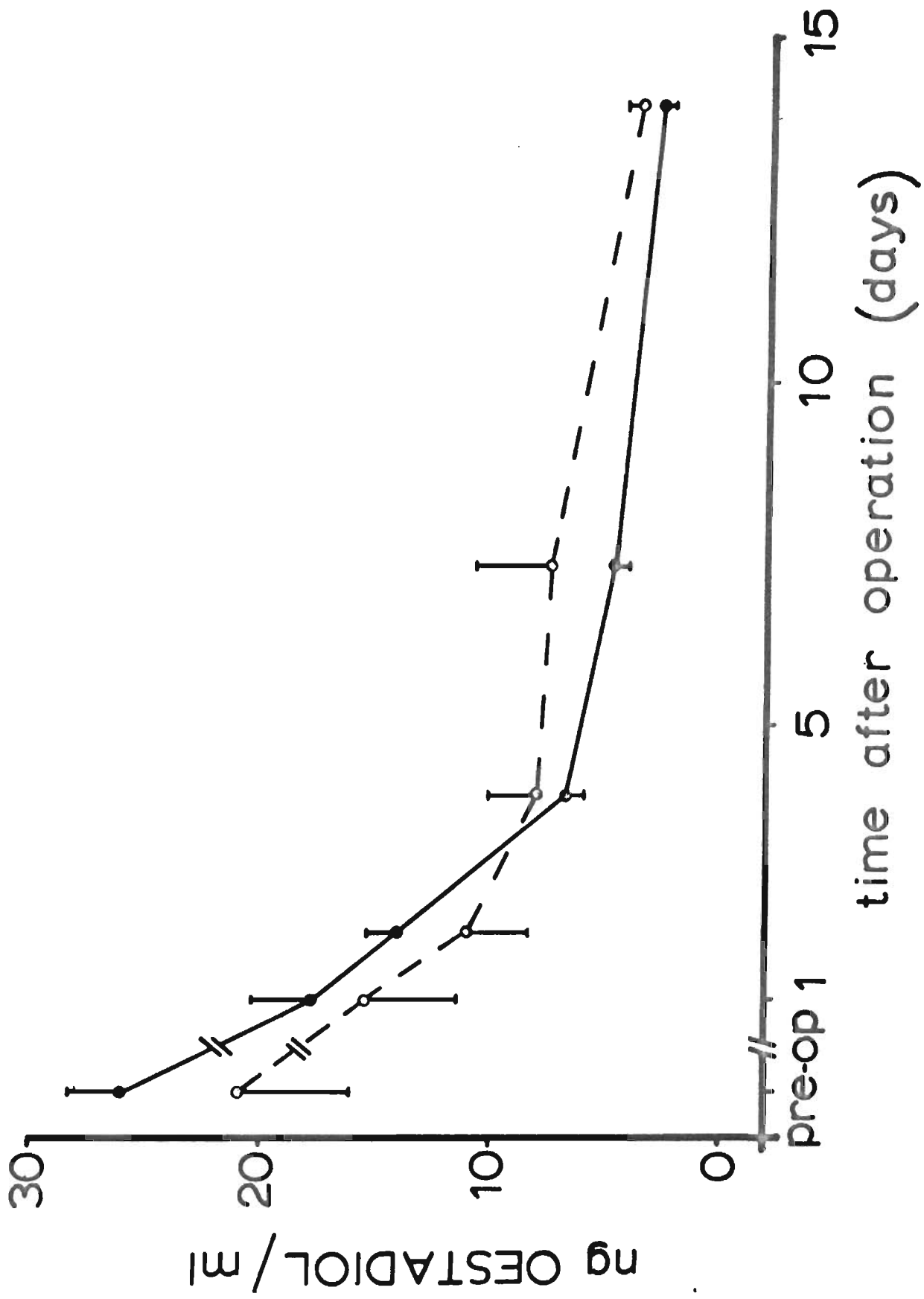





Figure 18:

Fall of plasma testosterone levels in mature males following ventralobectomy (), rostral plus medianlobectomy () and neurointermediate-lobectomy (). Each column represents the mean \pm SEM (n = 10) ng/ml.





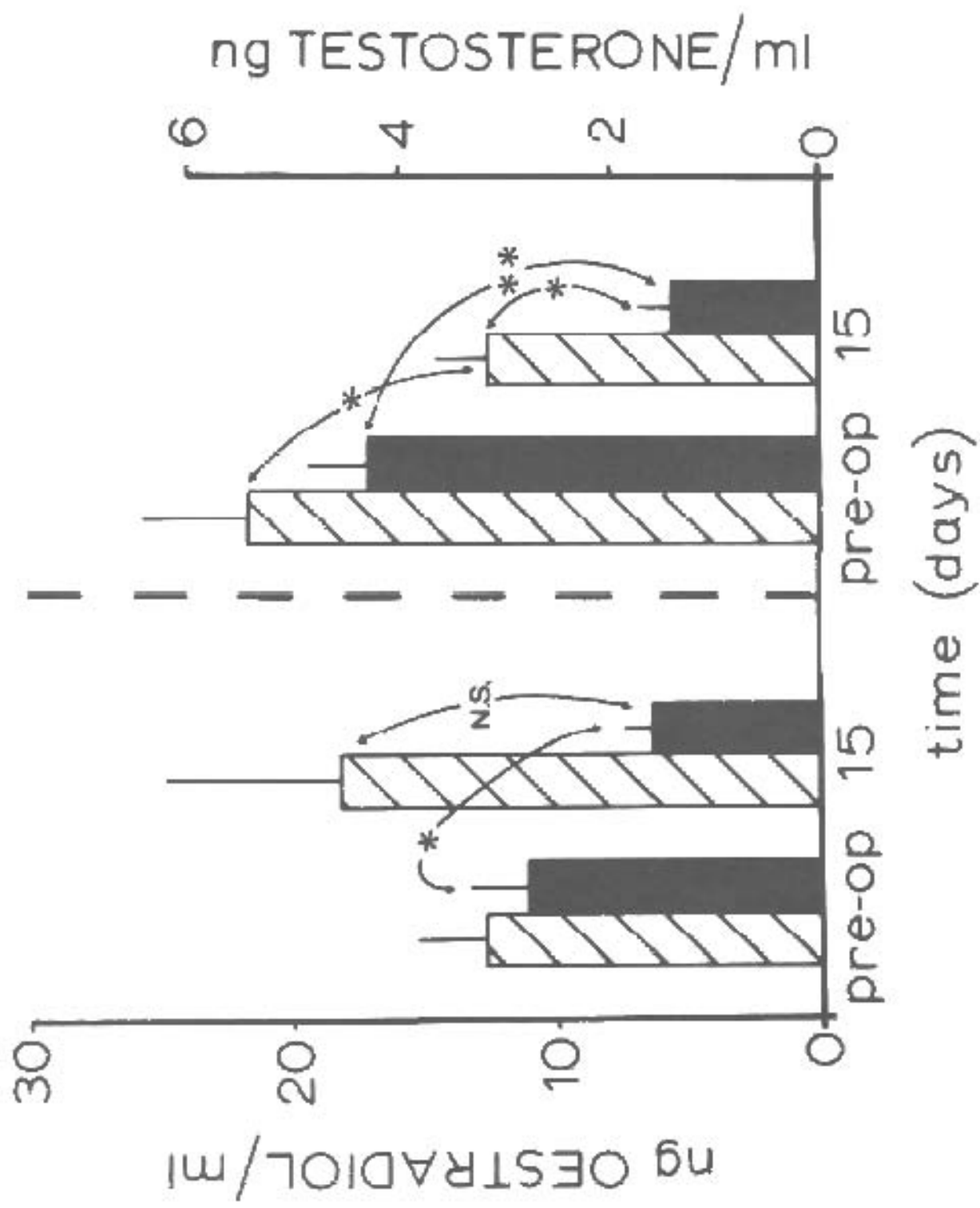
all three groups fell steadily throughout the duration of the experiment. Once again the rate of fall was more rapid in the first four days than in the latter ten days, although it fell significantly between any bleed and the following one in all three groups. To avoid confusion Figure 18 only shows the statistical significance of the testosterone concentration fall in the group of fish that were rostral and median lobectomized. On days 2 and 4 the plasma testosterone concentration was significantly lower ($P < 0.05$) in the ventral lobectomized group than it is in the rostral and median lobectomized fish, although there is no significant difference between these two groups at the beginning or end of the experiment. At no time is there any significant difference between the testosterone concentrations of the ventral lobectomized and neurointermediate lobectomized groups.

Plasma testosterone and oestradiol concentrations were also measured in ventral lobectomized and control fish operated by Dr. Craik. These mature females, split into two groups of six, were captured on the 26th September and operated on the 1st October. The fish were fed at the time of operation and again on the 8th, 12th and 15th October and a second blood sample collected on the 16th October (+ 15 days). Plasma oestradiol concentration did not change significantly over this period in the control fish, but fell significantly ($P < 0.05$) in the ventral lobectomized group. Testosterone levels fell in both groups of fish, although the fall was greater in the ventral lobectomized fish ($P < 0.01$) than in the control fish ($P < 0.05$) (Figure 19). After the 15 days of the experiment the plasma testosterone concentration was significantly ($P < 0.05$) lower in the ventral lobectomized fish than in the control fish, but there was no significant difference in the oestradiol levels at this time.



Figure 19:

Plasma testosterone and oestradiol levels in ventral lobectomised () and sham ventral lobectomized () mature female S. canicula. Fish were fed after operation and again on days +7, +11 and +14.



4; Effect of removal of pituitary lobes on the rate of egg-laying

Results of the effect of various pituitary operations on the rate of egg-laying of mature females are fairly difficult to interpret because confinement of the fish over a long period of time in the tanks appears to have a fairly drastic effect on their egg-laying capabilities. By monitoring the egg-laying of individual fish it was possible to demonstrate that some fish appeared either incapable or reluctant to lay egg-capsules at all, while others lay only sporadically. However, a number of conclusions can still be drawn from the available data. Firstly, by palpating the fish while anaesthetized for operation, it was possible to feel if any egg capsules were present in the oviducts, and if so, this was recorded. All of these egg capsules were subsequently collected within approximately one week of the operation. Anaesthetizing the fish for operation often causes egg-capsules to be oviposited if they are already present in the posterior portion of the oviduct.

All groups of fish appeared to have a burst of egg-laying activity shortly after the operation, and this was always followed by a period, ranging in length from 19 to 48 days depending on the group of fish, when no egg capsules at all were laid. Subsequent to this period of complete inactivity in relation to egg-laying, all groups of fish excepting those where the ventral lobe had been removed commenced egg-laying again, although at a reduced rate. After the initial burst of egg-laying shortly after the operation, which can probably be accounted for by egg-capsules already formed, those fish that had been ventral lobectomized ceased egg-laying completely (see Figures 20 and 21). A visual examination of the ovaries of these fish at the end of the experiment showed that all the large yolky vitellogenic follicles had become

Figure 20:

Rate of egg-laying and survivorship of operated and control mature female S. canicula over a 5 month period.

- A. Group of 20 control rostral plus medianlobectomies (R + MLC), where a flap was opened.
- B. Group of 25 rostral plus medianlobectomies (R + MLX).
- C. Group of 20 control ventrallobectomies (VLC), with token drilling.
- D. Group of 24 ventrallobectomies (VLX).

Groups A and B were operated between 21st and 25th October, 1973, and groups C and D between 3rd and 9th November, 1973.

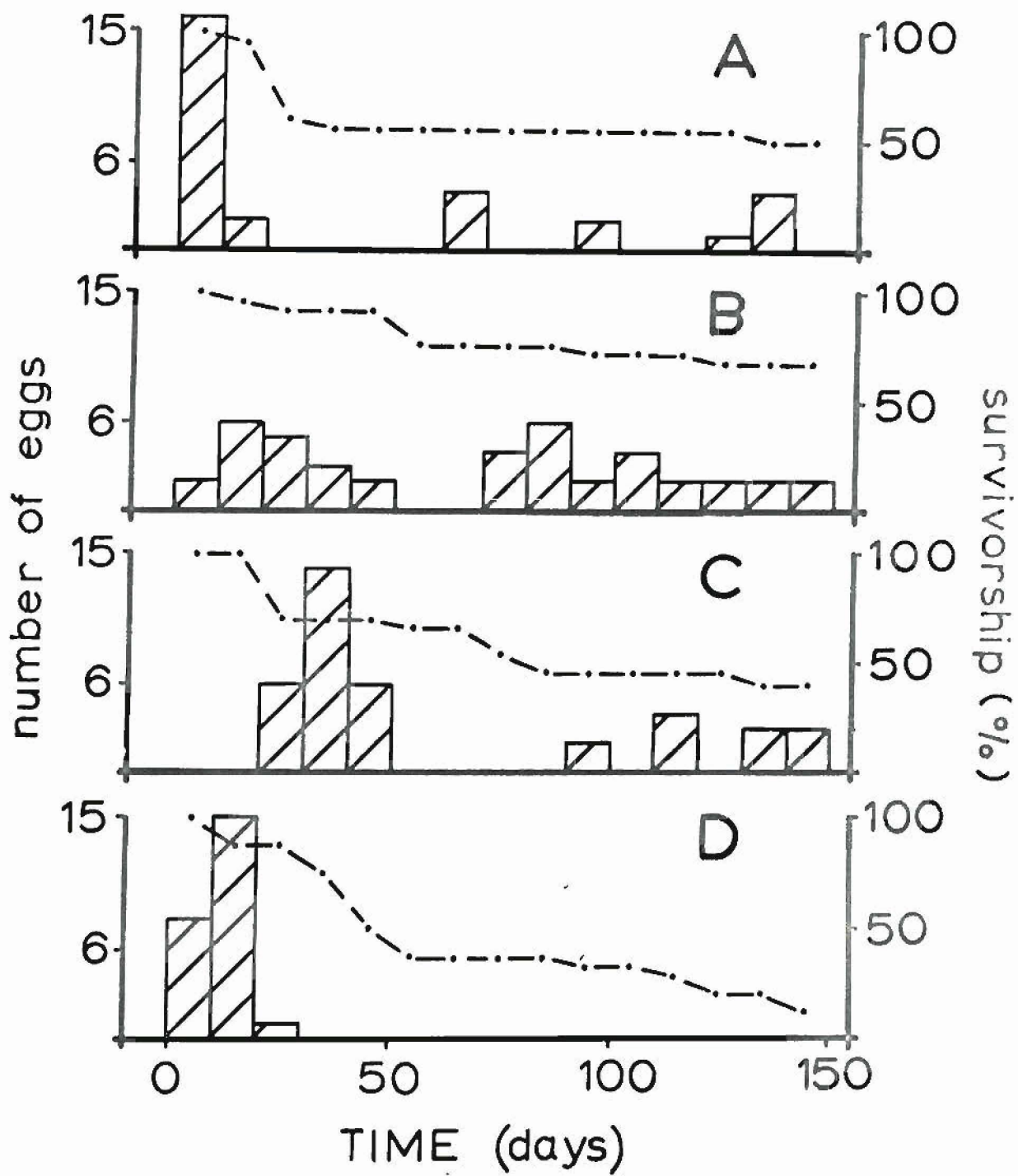
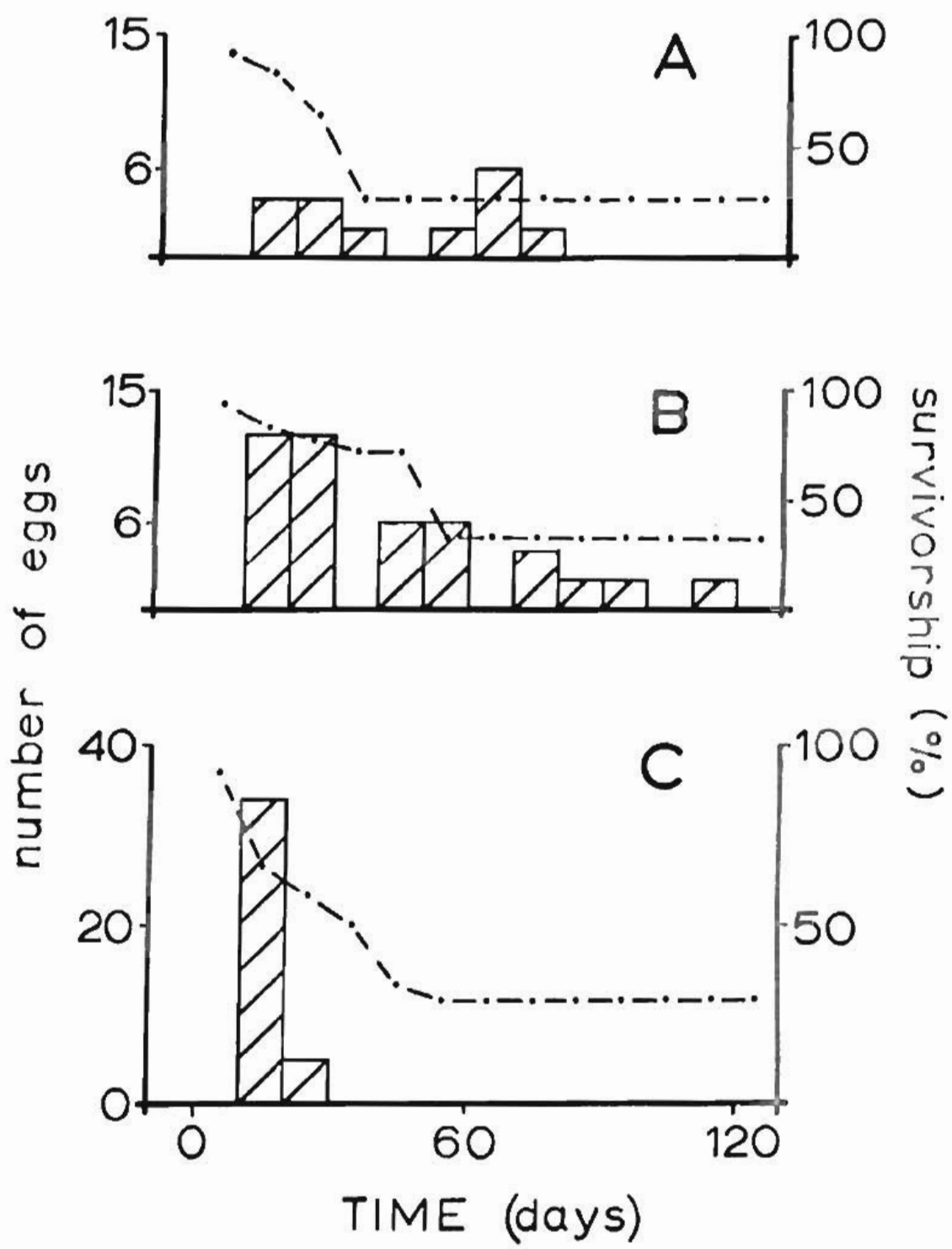


Figure 21:

Rate of egg-laying and survivorship of operated and control mature female S. canicula over a 4 month period.

- A. Group of 11 control rostral plus medianlobectomies (R + MLC), where a flap was opened.
- B. Group of 16 rostral plus medianlobectomies plus heat cautery of the hypothalamus (R + MLX + cautery).
- C. Group of 24 ventralobectomized plus thyroidectomized fish.

Group A and B were operated on 22nd February, 1974 and group C on 24th January, 1974.



atretic, some being in an advanced state of atresia. Examination of the ovaries of fish that had either been control operated or rostral and median lobectomized showed that most fish had some atretic follicles in the ovary, although this atresia was not usually as advanced as in the ventral lobectomized fish.

In almost all cases egg-capsules were obviously in pairs, whether they were extracted directly from the cloaca of a fish or laid in the tank. When only a single egg-capsule was collected on one day it was usually possible to pair it with another single egg-capsule laid a day either side. Nearly all eggs appeared fertile, with no difference in the percentage of fertile eggs between the various operations. Even seven months after any contact with a male the four females still laying eggs were producing ones.

Discussion

As has been pointed out elsewhere (Dodd, 1955), elasmobranch fish afford special opportunities for studying reproductive physiology. The Order contains closely related species which are oviparous, ovo-viviparous and viviparous. External secondary sexual characters in the form of claspers are present in the males and a well defined breeding cycle is characteristic of many species. Moreover, the pituitary gland is well developed, accessible and consists of three distinct and largely separate lobes. In spite of these attractive features, there are still many unresolved problems relating to the sex and reproduction in these fish. A great deal of the work that has been carried out concerned with the endocrinology of reproduction in the elasmobranchs has been conducted by Dodd and his co-workers (reviewed in Dodd, 1960; Dodd, 1975) on the

dogfish, Scyliorhinus canicula, as the latter is an excellent experimental animal.

Vivien (1941) published details of experimental surgery on S. canicula, but, unfortunately, his material and records were destroyed and no histological details could be given. Although the paper, in consequence, is discursive rather than precise, several interesting results are recorded in it. Pituitary extracts and implants caused resumption of gonadal activity during the resting period of the sexual cycle, though, in females, injection or implantation was effective in this role only when it was carried out close to the time at which gonadal activity would normally be re-initiated. This appears to be the only previous report on the effects of replacement therapy on reproductive physiology in the dogfish.

The experiments reported here, involving elevation of plasma testosterone concentrations in mature males, either intact or completely hypophysectomized, after injection of pituitary lobe extracts, were conducted in the autumn when plasma testosterone concentrations were falling rapidly in mature males in nature (Dobson, 1974). Bioassayable gonadotrophic potency of the male ventral lobe is lowest in August (Dobson, 1974), but rises through September and October, although circulating gonadotrophin levels are unknown. Dobson (1974) also states that the low point of spermatocytogenesis is in October, and thus fish used for the replacement therapy experiments reported here probably had relatively inactive testes in comparison with other times of the year. In the hypophysectomized males a four-fold increase in plasma testosterone concentration was obtained four hours after injection with an extract of ventral lobe. Although the biological gonadotrophic potency of this particular extract

was not measured, the intact males of the first experiment received the same number of lobes from the same pool, this extract having a gonadotrophic potency of 60 μg equivalents NIH-LH-S19/fish. According to Dobson (1974) mature male ventral lobes contain 5.8 μg equivalents ovine LH at the time of year the experiments were conducted. As measurement of circulating gonadotrophin concentration is not possible at the moment, it is impossible to know the circulating gonadotrophin levels in the intact and injected fish.

Although a significant response was obtained after 90 minutes, the maximum response did not occur until four hours after administration of the ventral lobe extract. Maung, S.L. (1976) found that intravenous injection of ovine LH into mature male quail caused a maximum elevation of plasma testosterone concentration after only 15 minutes, which had fallen again after 1 hour. The difference in timing is probably to be expected between a cold blooded fish that shows extreme lethargy within the confines of a tank, and an active bird having a high metabolic rate. In the dogfish, the plasma testosterone concentration is still maximally elevated 24 hours after the injection of the ventral lobe extract but has decreased significantly after 48 hours. At first glance this appears to imply that the half-life of the circulating gonadotrophin is longer in the case of the dogfish. However, this may not be the case, as several independent factors may lead to the plasma testosterone concentration remaining elevated for 24 hours. The testosterone clearance rate may be different between birds and fish. Plasma testosterone in two male dogfish castrated in July fell only slightly over the first two days post-operation, but then fell more rapidly over the next 6 days to reach a concentration one quarter of that circulating initially (Dobson, 1974).

Jenkins (personal communication) has recently shown that testosterone concentrations in ovariectomized mature dogfish falls to undetectable levels within two days, although the oestradiol concentration of the same fish did not fall as rapidly, and had not dropped significantly until 7 days after the operation. These fish were operated in December. Recently Jenkins (personal communication) has also demonstrated that dogfish injected with a dogfish anti-gonadotrophin (anti-GM2, 787-3, see Chapter 3 of this thesis) show a highly significant fall in plasma testosterone concentration after 6 hours, the latter rising again after a further 6 hours. These experiments, all of which presumably cause a cessation of androgen and oestrogen secretion from the gonad, illustrate that the steroid clearance rate from the plasma may well be variable depending on the time of the experiments, and so no definitive conclusions can be drawn as to the half life of the injected dogfish gonadotrophin.

Interestingly the hypophysectomized males injected with an extract of neurointermediate lobes also showed a significant elevation of plasma testosterone concentration. This rise followed the same time course as that induced by the ventral lobe extract, although in amplitude it was only about one half. It is not possible to quantify the relative amount of gonadotrophin in the two extracts that caused a significant rise in testosterone concentration. As the maximum response under the experimental conditions is unknown, it is impossible to know if the ventral lobe extract caused maximal secretion, and if it did how much less extract would have given the same response. If one assumes that the testosterone secreted originated from the testis, which the results of castration of both male and female appear to demonstrate, then the elevation of plasma testosterone concentration after an injection of a pituitary extract is

the first demonstration that the gonadotrophin present in the pituitary is capable of stimulating steroidogenesis of the dogfish in the classical way.

At one time or another all lobes of the dogfish pituitary have been implicated in gonadotrophin synthesis. The ventral lobe is certainly the main, if not the only, lobe containing gonadotrophin. This has been confirmed both surgically (Dodd et al., 1960) and using different gonadotrophin bioassays (Scanes et al., 1972; Firth and Vollrath, 1973). However, the median lobe has also been implicated, both by bioassay (Firth and Vollrath, 1973) and by ultrastructural studies (Knowles et al., 1975), as has the neurointermediate lobe (Goddard and Dodd, in Dodd, 1955), although neither of these have been confirmed surgically.

Biochemical evidence presented in Chapter 3 shows that, using standard mammalian extraction and purification techniques designed for glycoproteins, a large batch of neurointermediate lobes contained only 1% of the bioassayable gonadotrophic activity present in the ventral lobes from the same fish. It was therefore quite surprising to find that the neurointermediate lobe extract was capable of stimulating steroidogenesis to the degree that it did. There are a number of possible reasons to explain this result, the most obvious being that the neurointermediate lobe extract injected into the dogfish contained a gonadotrophin that was very active in stimulating steroidogenesis in the dogfish, but was much less potent in either the ^{32}P chick assay or the quail interstitial cell assay, which also employ the amount of testosterone secreted as its end point. This seems rather unlikely and there appears no logical explanation as to why the neurointermediate lobe extract should be so potent in stimulating steroidogenesis in the homologous species but show little if any

gonadotrophic activity otherwise. Campbell and Idler (1976) recently showed that a glycoprotein preparation from teleost pituitaries, which should contain any classical gonadotrophin, failed to stimulate gonadal incorporation of the yolk which accumulated in the serum. In contrast, the nonglycoprotein fraction of these pituitaries stimulated yolk incorporation into the ovary. In the Winter Flounder a typical gonadotrophin stimulated response is thus initiated by a non-glycoprotein, and a situation analogous to this may occur in the dogfish, although it seems highly unlikely, especially in view of the fact that the ventral lobe also stimulates steroidogenesis, and this lobe certainly contains the majority of the glycoprotein present in the dogfish pituitary (see Chapter 3).

Elasmobranchs might be expected to yield information of considerable interest on the question of hormone specificity, but there are few data available as yet on this aspect of the problem. Carlisle (1954), using the male dogfish, has investigated the effects of certain mammalian hormones on the spermiation reaction. Freshly caught mature dogfish can be made to yield spermatozoa at the urinogenital papilla by lightly stimulating the belly region, whereas this reaction can no longer be elicited from males which have been starved for some time. However, Carlisle found that the spermiation reaction could be re-initiated in such fish by certain mammalian hormones. Chorionic gonadotrophin induced spermiation at a dose level of 1-2 mg per fish, but was inactive at 0.5 mg; luteotrophic hormone, extracted from human post-partum urine, was effective at 2 mg per fish, and anterior lobe pituitary gonadotrophin (Praephyson) stimulated spermiation when 105 i.u. were injected into each fish, but was ineffective at a dose level of 35 i.u. Vivien (1941) found extracts of mammalian pituitary material were effective in causing resump-

tion of gonadal activity during the resting period of the sexual cycle, although the amounts required were up to three hundred times as much mammalian as homologous material. A small but significant increase in the weight of ovary and oviducts of immature specimens of Raja radiata was reported by Dodd (1955) in response to serum gonadotrophin (Gestyl, Organon) administration.

The single experiment reported in this study in which mammalian gonadotrophins were injected does nothing to clarify the situation. Of the two ovine gonadotrophins injected, only the follicle-stimulating hormone caused any significant increase in steroid concentrations. This increase, however, occurred in both plasma testosterone and oestradiol concentrations at the same time, between four and six hours post-injection, at the time of maximum plasma testosterone concentration in fish injected with ventral lobe extract. As NIH-FSH is considered to be approximately 2% pure (Licht, 1973b), whereas NIH-LH is approximately 50% pure, by administration of equal quantities of pure hormone, or varying amounts of each of the NIH hormones so that both doses contain equal amounts of biologically active hormone, it may be possible to show that mammalian FSH is steroidogenic in the female dogfish. Subsequent to the injection of the ovine gonadotrophic hormones, some of the same experimental fish failed to respond to a ventral lobe extract. This could either be because from the onset of the first experiment the fish were unresponsive to gonadotrophin stimulation of steroidogenesis, and if so dogfish may respond to mammalian gonadotrophins at other times of the year, or else by the time of the second injection, that of the ventral lobe extract, the fish had lost their ability to respond to gonadotrophin stimulation although they were responsive earlier, at the time of the injection of mammalian gonado-



trophin. In this case the failure to respond would be a true inability of mammalian gonadotrophins to stimulate steroidogenesis in the female dogfish.

A final possibility is that the ventral lobe extract injected into some of the fish, subsequent to the ovine hormones, did not contain enough gonadotrophic activity to stimulate steroidogenesis. Bioassay of an aliquot showed that each fish received the equivalent of 40 μ g activity, which is only two-thirds of the activity used in the other experiments involving males. Possibly this dose is not enough in the case of the female. In this respect it is worth noting that the female ventral lobe contains in excess of 40 μ g equivalents of gonadotrophic activity throughout much of the egg-laying season, when this experiment was conducted, so the injected dose was only equivalent to one lobe or less, whereas the males showing highly significant responses to ventral lobe extracts had received around ten times the gonadotrophin content normally present in the male ventral lobe at the time of the experiments.

As Dobson (1974) had obtained no significant difference in plasma testosterone concentrations in males following rostral and median lobectomy, or ventral lobectomy over control fish, and only completely hypophysectomized fish showed a significant fall in testosterone concentration, and then only marginally, it was surprising to find that complete hypophysectomy produced a halving of plasma testosterone concentration in males over two days. However, a more thorough appraisal of oestradiol concentrations in either ventral lobectomized or control operated fish showed that in both groups the plasma oestradiol concentration fell at the same rate over the two weekly periods of the experiment, confirming Dobson's results. When oestradiol concentrations in either



R + MIX, NILX or VLX females were compared, the levels in all three groups fell throughout the course of the experiment, with little difference between the three groups. Jenkins (personal communication) has recently demonstrated that steroid levels rise during the first day of captivity, possibly due to stress, and subsequently fall at the same rate as shown in this work. It may be best to look for an effect of ventral lobectomy on steroid levels after the fish have been allowed to settle in the tanks for a while. Alternatively the fall in steroid levels in fish held in captivity may be due to inanition as they are very reluctant to feed, especially over the colder months. This was partially confirmed by measuring both testosterone and oestradiol concentrations in hypophysectomized and control fish that were force fed regularly throughout the experiment. Steroid levels fell but not as fast as in the previous experiments, and those of the hypophysectomized fish fell significantly faster.

The time of year that the experiment is conducted, the condition of the fish prior to operation, and the subsequent treatment of these fish all appear to be important when considering the effects of hypophysectomy. Dodd et al. (1960) showed that removal of the ventral lobe in males caused a breakdown of the ampullae lying between those containing spermatogonia and those containing primary spermatocytes. Ventral lobectomy obviously, therefore, has a marked effect on the testes but whether this is related to its steroidogenic capabilities remains to be demonstrated. The ventral lobe of the pituitary plays a similarly important role in the maintenance of the dogfish ovary. Ventral lobectomy results in atrophy of all eggs in which vitellogenesis has started, the eggs being converted into corpora atretica. Effects are first noticed about three weeks after hypophyse-



tomy; follicles containing large eggs become hyperaemic and change colour to orange-yellow. They also lose their spherical shape and become flaccid. The process of atresia continues during the succeeding weeks and considerable folding of the egg surface and removal of broken down contents produces marked shrinkage (Dodd, 1960).

Hsaw and Albert (quoted in Dodd, 1955) have shown that removal of the pituitary gland during ovulation in the smooth dogfish (Mustelus canis) stops the process, but it can be re-initiated by implantation of homoplastic pituitary material. No attempt was made to localize the region of the pituitary responsible for controlling ovulation. However Dodd et al. (1960) have shown that ovulation in Scyliorhinus canicula is controlled by the ventral lobe of the pituitary. No ventral lobectomized fish laid eggs more than ten days after removal of the ventral lobe; such eggs as have been laid were undoubtedly either ovulated or about to be ovulated at the time of the operation. Control fish in captivity laid pairs of eggs regularly throughout the experiment period at intervals of approximately three weeks. As is the case in male dogfish from which the rostral or neurointermediate lobes, or both, have been removed, the reproductive processes and structures appeared to be entirely unaffected. From this surgical work Dodd et al. (1960) concluded that the ventral lobe is the seat of gonadotrophin production in dogfish.

The results of egg-laying experiments reported in this work confirm the results of Dodd and his associates. Only after ventral lobectomy did egg-laying cease completely, and was not reinitiated over the five month experimental period, extending over the greater part of a full egg-laying season. It is not possible to say unequivocally that none of the other pituitary operations affected egg-laying because sub-

sequent to the operation, and following a period in all experiments where no eggs were oviposited, only some of the fish recommenced egg-laying. Those that did begin egg-laying again did so at approximately three weekly intervals, as experienced by Dodd et al. (1960). When sacrificed these fish had normal looking ovaries for that time of year, with little or no sign of atresia. Fish that did not recommence egg-laying were found to have ovaries containing varying degrees of atresia, although very rarely as advanced as that found in ventral lobectomized fish. This may well be because the fish suffered inanition over the course of the experiment, although they were force fed every two weeks and the average weight loss of all fish over the five months of the experiment was only 5%.

Although the fish that do recommence egg-laying appear to average a pair of egg-capsules once every three weeks, this seems to be an extended period between pairs of eggs when one considers that on average 30% of freshly caught mature female fish contain egg-capsules in their oviducts when autopsied (Chapter 1). Although this last fact does not confirm a faster egg-laying rate in nature, it certainly points strongly towards it. If so fish held in captivity never recover completely to lay egg-capsules at the natural rate. This may well be linked with the rapid fall in circulating testosterone and oestradiol concentrations when the fish are held in captivity, to level out after about ten days. The high initial levels recorded, however, may be elevated due to stress and the subsequent lower levels later may be the true circulating concentrations found in nature. This would require confirming by measurement of steroid concentrations of fish bled very soon after capture before steroid concentrations could rise. Interestingly, some of the fish

that had been extensively heat cauterised in the anterior hypothalamic region laid egg-capsules at a much faster rate than that seen in any other operated or captive fish; pairs of eggs being laid only three days apart from one another. Phillips and van Tienhoven (1960) showed that reproduction in wild ducks is often inhibited in captivity. This reproductive failure of captive ducks was due to reduced pituitary gonadotrophin output. These authors postulated that the pituitary depression was a result of constant fear or anxiety acting by way of the hypothalamus. Later Phillips (1964) demonstrated that lesions in the ventral medial archistriatum and tractus occipito-mesencephalicus of captive Mallards greatly reduced escape behaviour or fear. These Mallards 'tamed' by lesions in the archistriatum or its projections had significantly larger ovaries and oviducts than did intact controls. One possibility is that captive dogfish show a similar phenomenon, and cauterisation of the hypothalamus removes the nervous inhibition to egg-laying and the latter recommences at its natural rate.

CHAPTER 3

THE PURIFICATION AND PROPERTIES OF DOGFISH GONADOTROPHIN

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THE PURIFICATION AND PROPERTIES OF DOGFISH GONADOTROPHIN

Introduction

Interest in purifying the pituitary gonadotrophins of non-mammalian vertebrates, and in determining their chemical structure and biological actions, has increased greatly in the past few years. This has occurred for a number of reasons, some of which are practical and technical, while others are more academic. There is considerable interest, for example, in using the purified gonadotrophins directly to improve fertility in a number of fish species and in marine turtles (see review by Fontaine, 1976, for the use of hormones for the control of reproduction in aquaculture). The technical improvements in chromatography now allow the purification of hormones from animals available only in relatively small numbers. The academic interest derives largely from a desire to understand the evolution of the gonadotrophic hormones themselves, as they constitute a closely related group of hormones present throughout the vertebrates, and equally to investigate the evolution of their target organs. Without doubt a great impetus has come from the recent finding that the three mammalian glycoprotein hormones secreted by the anterior pituitary gland (luteinizing, follicle-stimulating and thyroid-stimulating hormones) have different biological actions, but there are similarities in their chemical compositions and molecular weights. Since Li and Starman (1964) first showed that purified sheep luteinizing hormone dissociates into two sub-units in acid solution, studies on pituitary luteinizing hormone from other mammals have confirmed that the subunit structure exists in all species examined thus far (Stockell Hartree, 1975). One subunit, α , is common to all three hormones and has been referred to as the species-specific subunit, while the



other subunit, β , is of different structure in each case and conveys the information that dictates the specificity of each hormone-specific subunit. Studies of these gonadotrophin subunits (reviewed by Pierce, 1975) show that although the β -subunit of thyroid-stimulating hormone, luteinizing hormone and human chorionic gonadotrophin have different sequences, they evolved, in all probability, from a single polypeptide chain. Follicle-stimulating hormone from the pituitary apparently also consists of two subunits (Reichert and Ward, 1974), but their structural relations to those of the other hormones are not yet clarified. These similarities in structure raise a host of questions pertaining to the synthesis of the glycoprotein hormones, and to the biological specificity of "LH" and "FSH" in different vertebrate groups.

A final and important reason for purifying the gonadotrophins has been to develop radioimmunoassays for the hormones. The application of radioimmunoassay techniques to the measurement of protein hormones in biological fluids has greatly contributed to progress in endocrinology. Protein hormone immunoassays are characterized by showing high species specificity and it is only rarely that antisera will cross-react with the hormones of another group.

Knowledge about non-mammalian vertebrate gonadotrophins, however, is still extremely limited. They have been purified from only two bird species (Stockell Hartree and Cunningham, 1969; Scanes and Follett, 1972; Furuya and Ishii, 1974; Farmer, Papkoff and Licht, 1975a), three reptiles (Licht and Papkoff, 1974b; Papkoff, Farmer and Licht, 1976a, Licht, Farmer and Papkoff, 1976) two amphibians (Licht and Papkoff, 1974a; Licht, Farmer and Papkoff, 1975) and five species of bony fishes (carp: Fontaine and Gérard, 1963; Burzawa-Gérard, 1971; two species of salmon: Donaldson,

Yamazaki, Dye and Philleo, 1972; Idler, Bazar and Hwang, 1975b; sturgeon: Burzawa-Gérard, Goncharov and Fontaine, 1975a; trout: Breton, Jalabert and Reinaud, 1976). Incomplete data are available for a few other fish species (Sundararaj, Anand and Sinha, 1972; Hattingh and du Toit, 1973; Haider and Blum, 1976; Sundararaj and Samy, 1974). No information exists either for the cartilaginous fishes, or for the Agnatha.

As Donaldson (1973) points out, the main stumbling block in studies on the isolation of gonadotrophin from the pituitary gland of fishes has been the development of suitable bioassays, more so because purified non-mammalian hormones are relatively inactive in mammalian bioassays (Burzawa-Gérard, 1969; Licht, Müller and Tsui, 1976; Papkoff, Farmer and Licht, 1976b).

Witschi (1955) demonstrated that the pituitary gland of salmon contains both luteinizing activity, measured in the Weaver-finch assay, and follicle-stimulating activity, measured by vaginal cornification in the adult rat. A quantitative assay for piscine gonadotrophic hormones based on the weight increase of gonads and accessory sexual organs, in either male or female immature mice, was developed by Otsuka (1956a, b). A year later Robertson and Rinfret (1957) extracted gonadotrophin from the salmon Oncorhynchus tshawytscha pituitary glands using a mixture of acetone and acetic acid dissolved in water. The gonadotrophin was precipitated by increasing the concentration of acetone, and the biological activity assayed by its effect on the growth rate of immature trout testes. Purification of salmon gonadotrophin was continued using this assay by Schmidt et al. (1965), although the assay had the disadvantage that it required a two week period of treatment. An early attempt at

the purification of carp gonadotrophin by Clemens et al. (1964) used the goldfish testicular hydration assay (Clemens and Grant, 1964) to monitor the potency of the various fractions. Although this assay took only 24 hours to perform, it required removal of the gonads with consequent death of the test animal. This gonadotrophin was referred to by Clemens and co-workers as the "gonadal hydration factor".

The release of spermatozoa (spermiation) from the testes of mature Anurans has been used extensively for assaying and studying the physiology of gonadotrophins. It has received wide attention for the study of zoological specificity among vertebrate gonadotrophins (reviewed by Dodd, 1960; Witschi and Chang, 1959), and it has been applied as a routine bioassay for the purification of gonadotrophin from fish pituitaries (Fontaine and Gérard, 1963; Burzawa-Gérard, 1971; Burzawa-Gérard, Goncharov and Fontaine, 1975a). Unfortunately, it shows seasonal variation in response to mammalian LH (Burzawa-Gérard and Fontaine, 1965). In spite of some disparities there had been a tendency to consider the Anuran spermiation response as being dependent primarily on an LH-like factor (van Oordt and de Kort, 1969; Burgos and Vitale-Calpi, 1967) until Licht (1973a) re-examined the question of its gonadotrophic specificity with some of the more highly purified preparations of PSH and LH available. He states, "... it seems reasonable to conclude, on the basis of these comparative studies in three diverse species, that the anuran spermiation response is not specific for either mammalian pituitary gonadotropin. Since there is no apparent discrimination between purified mammalian PSH and LH in the spermiation test, the demonstration that pituitary materials possess activity in this test only identifies the presence of 'gonadotrophic' factor and provides no evidence re-



garding whether it is FSH or LH like in its biological properties". Licht (1973a) went on to demonstrate a pronounced discrepancy between the potency ratios of the Papkoff and NIH preparations of ovine LH between the amuran spermiation response and other assays of these same gonadotrophin preparations. These included assay in the lizard, Anolis carolinensis. Whatever the basis for the pronounced discrepancy in estimation of relative potencies in Amuran and other test systems, Licht's data obviously have important implications for the use of the Amuran spermiation test for monitoring hormone purification.

In their early experiments on the purification of salmon gonadotrophin (see Donaldson and Yamazaki, 1968; Donaldson et al., 1972), Yamazaki and Donaldson (1968a) developed an assay based on the spermiation response of hypophysectomized mature male goldfish. In this assay a response was obtained in only 24 hours, and human chorionic gonadotrophin (HCG), although not equine LH or ovine FSH, could be used to standardize the assay. In 1970 they changed from the goldfish assay to the ^{32}P -uptake in day old chick testes (Breneman et al., 1962; Follett and Farner, 1966) which has the advantages of rapidity, sensitivity, precision and the ability to respond to mammalian LH as well as to salmon gonadotrophin (Donaldson et al., 1972; Idler et al., 1975b), dogfish gonadotrophin (Scanes et al., 1972), amphibian gonadotrophin (Donaldson et al., unpublished, quoted in Donaldson, 1973; Licht and Papkoff, 1974a), reptilian gonadotrophin (Licht and Papkoff, 1974b) and avian gonadotrophins (Follett and Farner, 1966). Again, however, this assay shows a lack of discrimination between the two types of gonadotrophins in both birds (Furr and Cunningham, 1970) and mammals (Licht, 1973b).

The in vitro maturation and ovulation of oocytes from a variety



of amphibians, especially the African clawed toad, Xenopus laevis, has been widely used as a bioassay in gonadotrophin purification. The relatively high specificity for LH from a wide variety of tetrapod species (reviewed by Licht and Papkoff, 1976) has made the assay particularly valuable for comparative studies on pituitary gonadotrophins. Licht and his colleagues have relied heavily upon this assay to identify and quantify LH during the purification of pituitary hormones from birds, reptiles and amphibians (briefly reviewed by Farmer, Papkoff and Licht, 1975b), although in the course of their studies they have observed considerable variation in the relative potencies of crude pituitary extracts and comparably purified preparations of LH from different species. This has applied even with a single class or order. Burzawa-Gérard et al. (1975a) used oocytes from three different species of Amphibian as well as extending the assay to fishes by utilizing the oocytes from a Chondrosteian, the sturgeon (Acipenser stellatus), to assay gonadotrophic activity during their purification of Acipenser gonadotrophin, thus avoiding the problems of species-specificity. As Jalabert et al. (1974) demonstrated, the maturation of oocytes in vitro can be considered as representative of a typical fish gonadotrophic activity since only one gonadotrophin seems to promote both follicular growth (vitellogenesis) and ovulation in fishes (Sundararaj et al., 1972; Burzawa-Gerard and Fontaine, 1972). Recently, Jalabert (1976) has carried out a detailed study of the in vitro maturation and ovulation of rainbow trout (Salmo gairdnerii) oocytes, and extended his model to the control of oocyte maturation in the northern pike (Esox lucius) and goldfish (Carassius auratus). Rainbow trout oocytes were also used by Breton et al. (1976) during the purification of rainbow trout gonadotrophin.

Finally, Idler et al. (1975a) developed a bioassay based on the stimulation of cyclic AMP (cAMP) levels in immature trout gonadal tissue of both sexes, to aid them in their purification of salmon gonadotrophins. This assay was sensitive in the range 0.3 to 10.5 SG units (1 SG unit = 1 µg NIH-LH-S18 in the ^{32}P -chick bioassay). It was developed from the observations of Fontaine et al. (1972) that the pituitary gonadotrophin from salmon stimulated adenyl cyclase activity in ovarian homogenates of goldfish.

One major disadvantage of most bioassays is that they require comparatively large amounts of hormone and thus a significant amount of activity may have to be used in monitoring purifications from animals available only in relatively small numbers. Recently a number of mammalian bioassays have been developed that are at least as sensitive as the respective radioimmunoassays and have the advantage that they measure the biological activity of the gonadotrophin. Dufau et al. (1976) developed an in vitro assay based upon the ability of dispersed rat testis interstitial cell suspensions to synthesize testosterone in the presence of "LH"-like gonadotrophins, which Garfink et al. (1976) showed should prove useful for the study of non-vertebrate "LH"-like gonadotrophins, including that from a trout pituitary extract. Radioiodinated human FSH (^{125}I -hFSH) was used by Licht and Midgley (1976a,b) to study the specificity of the gonadotrophin-binding sites of various reptilian, avian and mammalian gonadal tissues by examining competitive interactions with several non-mammalian gonadotrophins. They demonstrated that a radioligand receptor assay employing heterologous reagents can be used as a gonadotrophin assay, but experienced considerable difficulty in defining the FSH-LH specificity of the assay in terms of mammalian hormones.



The actual purification of fish gonadotrophins has been greatly influenced by the concept of two gonadotrophins existing in lower vertebrates. This arose from the fact that this duality had been demonstrated in mammals. Since the mammalian pituitary gland contains several proteins which are useful for clinical and research purposes, purification procedures have been developed so that several hormones can be isolated from the same pituitary tissue. The glycoprotein hormones can be separated from other pituitary hormones by fractionation with aqueous ethanol or $(\text{NH}_4)_2\text{SO}_4$ solutions, with further purification of each of the three glycoprotein hormones being performed by steps which include ion-exchange chromatography and gel filtration.

Carp gonadotrophin (c-GTH) was the first fish gonadotrophin to be extensively purified (Fontaine and Gérard, 1963), the steps being summarized in Figure 22. The recent addition of a preparative electrophoresis step on polyacrylamide gel led to a better purification (Burzawa-Gérard, 1971). The biologically active peak was eluted with an Rf of about 0.50, and separated from an inactive material (Rf = 0.80). After recovery on Sephadex G-100, the lyophilized powder was about 20 times more active than the starting material. Its activity was about 1.5 x NIH-LH-S1 (measured on frog spermiation in October), with a yield in activity of about 40%. A very similar technique was used by Burzawa-Gérard, Goncharov and Fontaine (1975a) for sturgeon (Acipenser stellatus) gonadotrophin, and by Breton, Jalabert and Reinaud (1976) for trout (Salmo gairdnerii) gonadotrophin. Although there are slight differences between the three purifications, all involved an initial alcohol extraction, according either to Bates et al. (1959) or Schmidt et al. (1965), followed



Figure 22:

Flow diagram for the purification procedure of carp pituitary gonadotrophin (from Burzawa-Gérard, 1971).

Acetonic powder of carp pituitary glands



Extraction by alcoholic percolation



Fraction soluble in EtOH 57%, NaCl 2%

Sephadex G-50, Tris HCl, pH 7.8



Unretarded fraction

Chromatography on DEAE-Cellulose, Tris HCl, pH 7.8



Adsorbed fraction eluted by gradient of ionic strength

Sephadex G-100, tris phosphate, pH 7.2



Fraction of $K_D = 0.28$

Preparative electrophoresis



Fraction of $R_p = 0.50 - 0.54$

Sephadex G-100, NH_4HCO_3



Fraction of $K_D = 0.28$

Lyophilisation \longrightarrow c-GTH



by gel filtration, ion exchange chromatography on DEAE-cellulose, and a final gel filtration.

Gonadotrophin has been purified from two species of salmon, the chinook (Oncorhynchus tshawytscha) by Donaldson and Yamazaki, 1968; Donaldson et al., 1972; and the chum (Oncorhynchus keta) by Idler, Bazar and Hwang (1975b). The former used techniques resembling those of the French workers (see Figure 23): an alcohol extraction followed by two filtrations on Sephadex G-100, and eventually DEAE-cellulose chromatography. This yielded 260 mg of SG-G100 per 100 g of frozen pituitary glands, with an activity of 0.1 x NIH-LH-S16 in the ³²P-chick assay. 1 mg of SG-G100 was equivalent to 2150 I.U. of HCG in the goldfish spermiation bioassay, while the most active DEAE fraction was approximately five times more potent than SG-G100 in the same assay.

Idler, Bazar and Hwang (1975b) described a rapid purification procedure employing mild conditions which included an initial affinity chromatography step using Con A-Sepharose (see Figure 24). This was first successfully applied to the fractionation of glycoprotein hormones by Dufau et al. (1972). After affinity chromatography Idler et al. (1975 b, c) filtered the gonadotrophin on Sephadex G-75 and followed this with ion-exchange chromatography on DEAE-Bio Gel A. They isolated two proteins with gonadotrophic activity that exhibited distinct behaviour, both chemically on polyacrylamide gel electrophoresis and Sephadex G-75 (superfine), and biologically, as assessed by the ratio of cAMP stimulation in immature rainbow trout ovaries and testes.

Biochemical studies on the purified fish gonadotrophins have demonstrated that the physico-chemical characteristics of the hormones are similar as regards molecular weight, determined on Sephadex G-100 or

Figure 23:

Flow diagram for the purification procedure of salmon
pituitary gonadotrophin (from Donaldson et al., 1972).

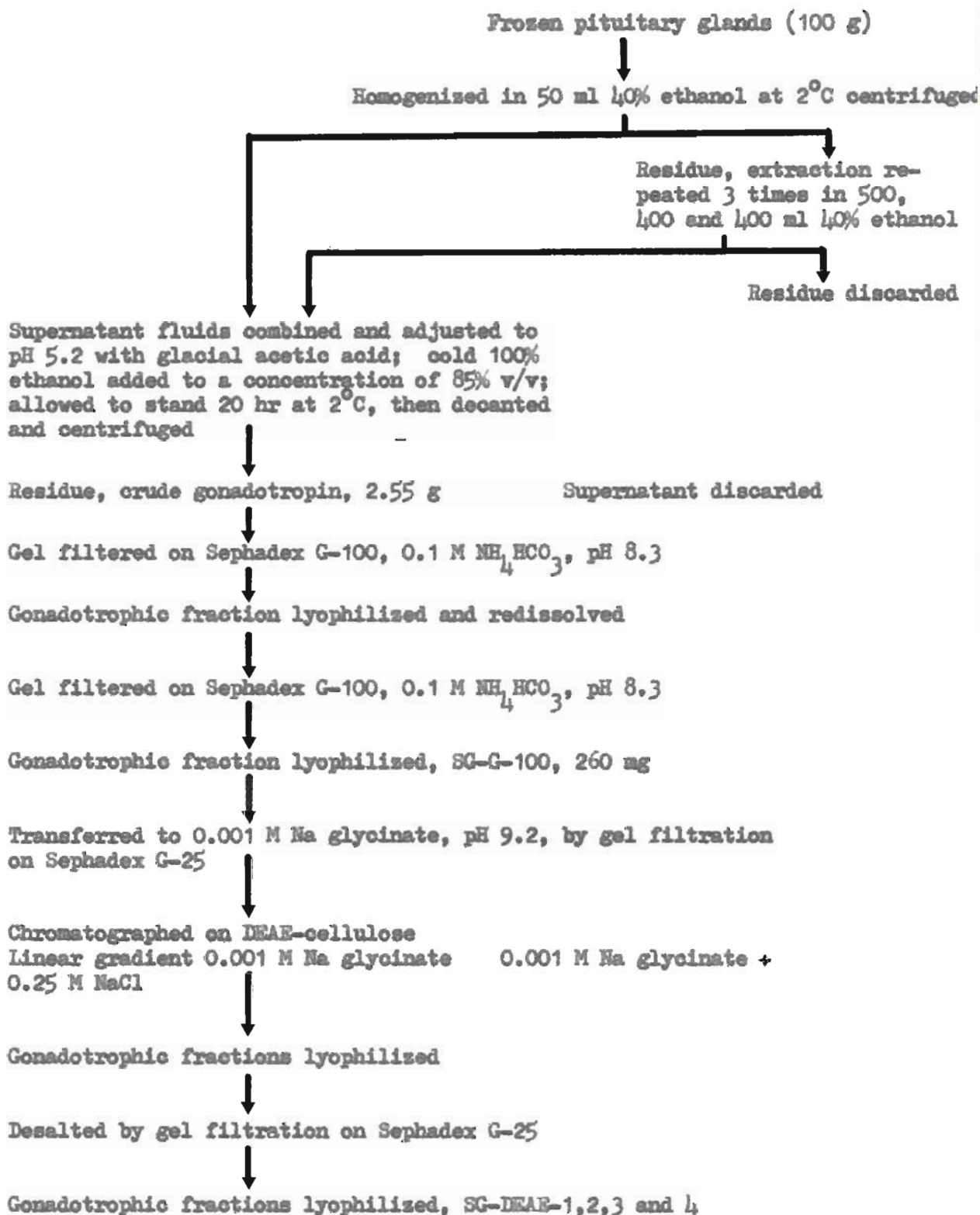


Figure 24:

Flow diagram for the purification of salmon (Oncorhynchus keta)
pituitary gonadotrophin (from Idler, Bazar and Hwang, 1975b,c)

Frozen pituitary glands (50 g)



Homogenized in 200 ml of Buffer B, centrifuged and supernatant filtered through cheesecloth



Affinity chromatography on ConA-Sepharose
The adsorbed glycoprotein fraction eluted with Buffer B



Concentration by ultrafiltration

Gel filtration on Sephadex G-75 superfine



Concentration by ultrafiltration and dialysis

Ion exchange chromatography on DEAE-BioGela



Initial linear gradient (500 ml) from 0.03 to 0.06 M NH_4HCO_3 , then elution continued with 0.06 M NH_4HCO_3 (100 ml), followed by a second linear gradient from 0.06 M to 0.2 M NH_4HCO_3 (450 ml). Individual fractions DEAE-1, 2 and 3 concentrated by ultrafiltration

Sephadex G-75 superfine → DEAE 1: G-75

DEAE 2: G-75

DEAE 3: G-75

by equilibrium ultracentrifugation, and sedimentation coefficient. The values obtained are comparable to those of ovine LH and FSH (Papkoff, 1971; Cahill and Hart, 1969). Like mammalian hormones, carp (α -GTH), trout (β -GTH), sturgeon (α -GTH) and salmon gonadotrophin appear to consist of two subunits that show little or none of the biological activity of the intact native molecule (Burzawa-Gérard, Goncharov, Dumas and Fontaine, 1976; Breton, Jalabert and Reinaud, 1976; Pierce, Faith and Donaldson, 1976). The two subunits of α -GTH, when incubated together even for a short period of time, regain much of the biological activity of the native α -GTH. Burzawa-Gérard and Fontaine (1976) formed a hybrid molecule by the association of the α -subunit of bovine LH with the β -subunit of carp gonadotrophin which was about one hundred times less active than either α -GTH or bovine LH on the ovarian adenylyl cyclase activity of the carp and rat respectively, but was considerably more effective than either on frog spermiation in vivo, or adenylyl cyclase activity in vitro.

The aminoacid composition of α -GTH, α -GTH, β -GTH and two related salmon fractions, SG-CM11 and SG-DEAE1 (Pierce, Faith and Donaldson, 1976) have been determined, and are shown in Figure 25. A comparison of the numbers of residues of each aminoacid for the fish gonadotrophins reveals notable differences which, besides suggesting different sequences, may explain the immunological and biological specificity of the fish gonadotrophins. These values are also different from those obtained for mammalian LH and FSH. Analysis of carbohydrate and sialic acid contents in the fish proteins has not been possible due to the amounts produced being too small.

A different approach to the study of the biochemical similarities

Figure 25:

Aminoacid composition of fish glycoprotein fractions compared to highly purified mammalian LH and FSH^a.

- a For comparison, values are expressed as residues per 200 residues for the fish gonadotrophin fractions. This figure was chosen because there are about 200 residues in the mammalian gonadotrophins.
- b Data from Pierce, Faith and Donaldson (1976).
- c Data from Burzawa-Gérard, Goncharov and Fontaine (1975b).
- d Data from Breton, Jalabert and Reinaud (1976).

Residue	Bovine LH ^b	Human FSH ^b	c-GTH ^c	t-GTH ₂ ^d	aci-GTH ^c	SG-CM2 ^b	SG-DEAE-1 ^b
Lysine	12	13	13.4	12.3	13.2	11.8	11.4
Histidine	6	6	6.4	7.9	4.4	5.4	3.2
Arginine	11	8	8.2	8.6	6.0	4.8	5.4
Aspartic acid	11	15	16.8	22.2	20.0	19.6	22.6
Threonine	16	22	15.4	19.0	14.4	22.4	22.6
Serine	14	14	13.6	11.1	17.2	11.8	13.0
Glutamic acid	14	22	18.4	19.7	23.6	20.6	19.4
Proline	27	11	16.6	14.5	16.0	16.6	14.0
Glycine	11	6	7.8	8.8	9.2	9.4	11.4
Alanine	15	12	7.6	7.2	7.4	7.6	6.4
Half-cystine	22	22	13.8	15.0	15.4	24.2	23.8
Valine	13	13	23.0	15.0	11.0	13.0	11.8
Methionine	7	4	3.0	4.6	3.2	4.2	4.8
Isoleucine	7	7	5.6	8.8	8.4	6.2	8.0
Leucine	14	9	14.8	12.5	18.0	8.2	9.2
Tyrosine	7	11	9.6	6.2	5.0	8.0	7.6
Phenylalanine	8	8	5.6	6.8	7.0	5.4	5.4
	215	204	200	200	200	200	200

between fish and mammalian gonadotrophins was adopted by Pierce, Faith and Donaldson (1976). They showed that by disrupting the tertiary structure of salmon gonadotrophin fractions their immunological determinants are sufficiently similar to the mammalian α -subunits to demonstrate cross-reactivity. By extrapolating data from the correlation between immunological cross-reactivity and the differences in sequence found in other globular proteins, they concluded that the number of amino-acid replacements between the presumed α -subunit of the salmon and of mammals is quite large. The sequence differences, as reflected in antigenic determinants, between β -subunits of the salmon and their mammalian counterparts must be very great as negligible cross-reactivity was found by immunodiffusion, though use of more sensitive techniques might reveal significant immunological relationships.

Materials and Methods

1) Collection of pituitary glands

Pituitary glands were dissected from fresh, locally caught mature adult dogfish, Scyliorhinus canicula (as described in Chapter 1) and separated into their various lobes. Three batches of tissue were processed during the course of this study. Purification 1 was conducted on a batch of 990 ventral lobes obtained from fish caught during January and February, 1976; purification 2 used a batch of 1300 ventral lobes obtained during April and May, 1976; and purification 3 used 380 ventral lobes collected during November, 1976. These batches of fish contained approximately equal numbers of both sexes. The purification of possible rostral- and median lobe-gonadotrophins, and neurointermediate lobe-gonadotrophins, were both conducted on batches of 1060 lobes collected

between January and March, 1976. After dissection the various pituitary lobes were stored at -20°C .

2) Fractionation procedures

a) Glycoprotein extraction

All procedures were performed at 4°C with the exception of the Sephadex B-100 fractionations in purification 2 which were run at room temperature. The initial glycoprotein extract (GTN) from purifications 1 and 2 was prepared according to the method of Stockell Hartree (1966), where the more soluble glycoprotein hormones are separated from growth hormone by a method developed initially for extraction of sheep pituitary gonadotrophins, but which has since been modified for glycoprotein hormones from other species. The frozen pieces of cartilage containing the ventral lobes were cut into thin slices as described in Chapter 1 and homogenized in batches of 100 for approximately five minutes in a mortar and pestle with 20 ml 10% (w/v) ammonium acetate (pH 5.1) adjusted with glacial acetic acid:ethanol, 60:40 (v/v). Each batch was centrifuged immediately after extraction and the supernatant decanted. The pieces of cartilage were again extracted with a further 20 ml as above and the supernatants combined. Supernatants from each batch of 100 glands were pooled and the glycoprotein precipitated by slowly adding 2.5 times the volume of cold ethanol with stirring. This was continued for 30 minutes after addition of ethanol. The mixture was then left at 4°C for 24 hours to complete precipitation of glycoprotein hormones, after which it was centrifuged for 30 minutes at 1500 g. The precipitate, containing glycoprotein hormones, was washed twice with cold ethanol, twice with cold diethyl ether, centrifuged after each wash, and finally dried in a vacuum desiccator. This method of precipitation and drying of the glyco-



proteins, by making the solution 85% with respect to ethanol, at pH 5.1, was used for all fractions in the subsequent chromatographic steps.

b) Affinity chromatography on ConA-Sepharose

Affinity chromatography on ConA-Sepharose (Pharmacia) was carried out according to the method of Idler *et al.* (1975b). The GTN extract was dissolved in 10 ml cold modified Buffer B (0.05 M Tris-Cl, pH 7.7; 0.5 M NaCl; 0.2 mM dithiothreitol; 1 mM $MnCl_2$; 1 mM $CaCl_2$) using a small glass homogenizer, centrifuged, and the precipitate again extracted with 10 ml Buffer B. The supernatants were combined and applied to a 1.25 x 15 cm column of ConA-Sepharose, previously equilibrated with Buffer B. After entering the column, the sample was eluted with Buffer B until the absorbance at 276 nm of the eluate approached baseline values. The adsorbed glycoprotein fraction was then eluted with Buffer B containing 0.15 M α -Methyl-D-glucoside (Buffer C). Contents of tubes containing measurable protein concentrations at 276 nm were pooled to form the ConA-2 fraction.

c) Ion exchange chromatography of ConA-2 fraction on carboxymethylcellulose

Whatman CM-32 (carboxymethyl cellulose) was cleaned, recycled and equilibrated by a modification of the manufacturer's instructions (Stockell, Hartree, 1975). This procedure is simple and all operations could be performed in a beaker. A weighed amount of the ion exchanger was stirred into 15 volumes of 0.5M NaOH in a beaker and left for between 30 minutes and 2 hours. The supernatant was discarded and the resin re-suspended in 0.5M HCl. It was then left for 30 minutes before decantation and resuspension of the resin in 15 volumes of distilled water - allowing the suspension to settle 1 minute for each 2 cm height of the suspension -

before decanting to remove "fines". This procedure was repeated twice more. Finally, the resin was suspended in the starting buffer, 4 mM ammonium acetate (4 ml of 1 M ammonium acetate, pH 5.5, diluted to 1 litre), and, after mixing, the pH of the mixture was adjusted to 5.5 with glacial acetic acid, and this was followed by settling and decantation. This was repeated several times before pouring the column, giving a fairly rapid equilibration of the ion exchange resin. After pouring the column, equilibration was continued until the pH of the effluent was the same as that of the starting buffer.

The ConA-2 fraction was dissolved in the starting buffer, as described previously, and applied to the column. After collection of the unadsorbed fraction (CM 1) and the absorption of the column effluent at 276 nm had returned to baseline levels, the adsorbed fraction (CM 2) was eluted by passing 1 M ammonium acetate through the column. Both fractions were precipitated and dried as described previously.

d) Gel filtration on Sephadex G-100

The ConA-2 fraction, originating from purification 2, was dissolved in 5 ml 0.05 M Na phosphate, pH 7.5, and applied to a 2.5 x 100 cm column of Sephadex G-100, previously equilibrated with the same buffer. The non-retarded protein peak was precipitated from solution to yield the G-100 fraction. All subsequent gel filtrations were conducted at 4°C and eluted with 0.05 M Na phosphate/0.9% NaCl on the advice of Stockell Hartree (personal communication) as lesser concentrations sometimes leave gonadotrophins on the G-100, thus reducing the yield. The void volume (V₀) of the G-100 columns, i.e. the point at which the non-retarded material emerges, was checked after each experimental run by the use of Blue Dextran 2000 (Pharmacia) at a concentration of 2 mg/ml.



3) Protein determination

Where it was not possible to measure the protein concentration of eluted fractions directly using a spectrophotometer, they were estimated by a modification of the method of Lowry et al. (1951) that allowed as little as 2.5 μg protein per 50 μl volume to be detected. Bovine serum albumin (Sigma) was used as the standard at concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.56 $\mu\text{g}/50 \mu\text{l}$. Aliquots of the standard or unknown (50 μl) were placed at the bottom of clean glass centrifuge tubes (110 x 15 mm) followed by 50 μl 0.1 M NaOH and 1000 μl reagent C (see below), mixed and held 10 minutes at room temperature. Reagent D (100 μl , see below) was then added and the tubes mixed immediately and held for 30 minutes at room temperature. After this period 1 ml distilled water was added and the optical density of the samples read at 700 nm in 10 mm cuvettes in a Unicam SP 1800 Ultraviolet spectrophotometer.

Reagents:

- A. 2% Na_2CO_3 in 0.1 M NaOH.
- B. 5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium citrate.
- C. Mix 50 ml A with 1 ml B just prior to use.
- D. Dilute 1 ml Folin Ciocalteau with 1.3 ml H_2O .

4) Characterization of biological activity

a) Bioassay techniques for gonadotrophins

1. ^{32}P -uptake by chick testes

Gonadotrophic activity was monitored in all fractions by the use of the ^{32}P chick bioassay as described in Chapter 1.

2. Quail interstitial cell assay

This assay is based on the ability of isolated quail inter-

stitial cells to secrete testosterone in the presence of an LH-like gonadotrophin, and was developed by Maung, Z.W. (1976) in this Department. The preparation and incubation of the interstitial cell suspension is summarized in Figure 26; full details can be found in Maung, Z.W. (1976).

3. Stimulation of testosterone secretion in quail in vivo

Dogfish gonadotrophin fractions from purification 1 and NIH-LH-S19 as standard were dissolved in 0.9% saline and injected subcutaneously into sexually mature male quail in a volume of 0.2 ml. Blood samples were collected serially from the wing veins just before injection and 3 hours later plasma testosterone concentration was measured by radioimmunoassay as described in Chapter 1.

b) Bioassay technique for thyrotrophin

1. ^{32}P -uptake by chick thyroids

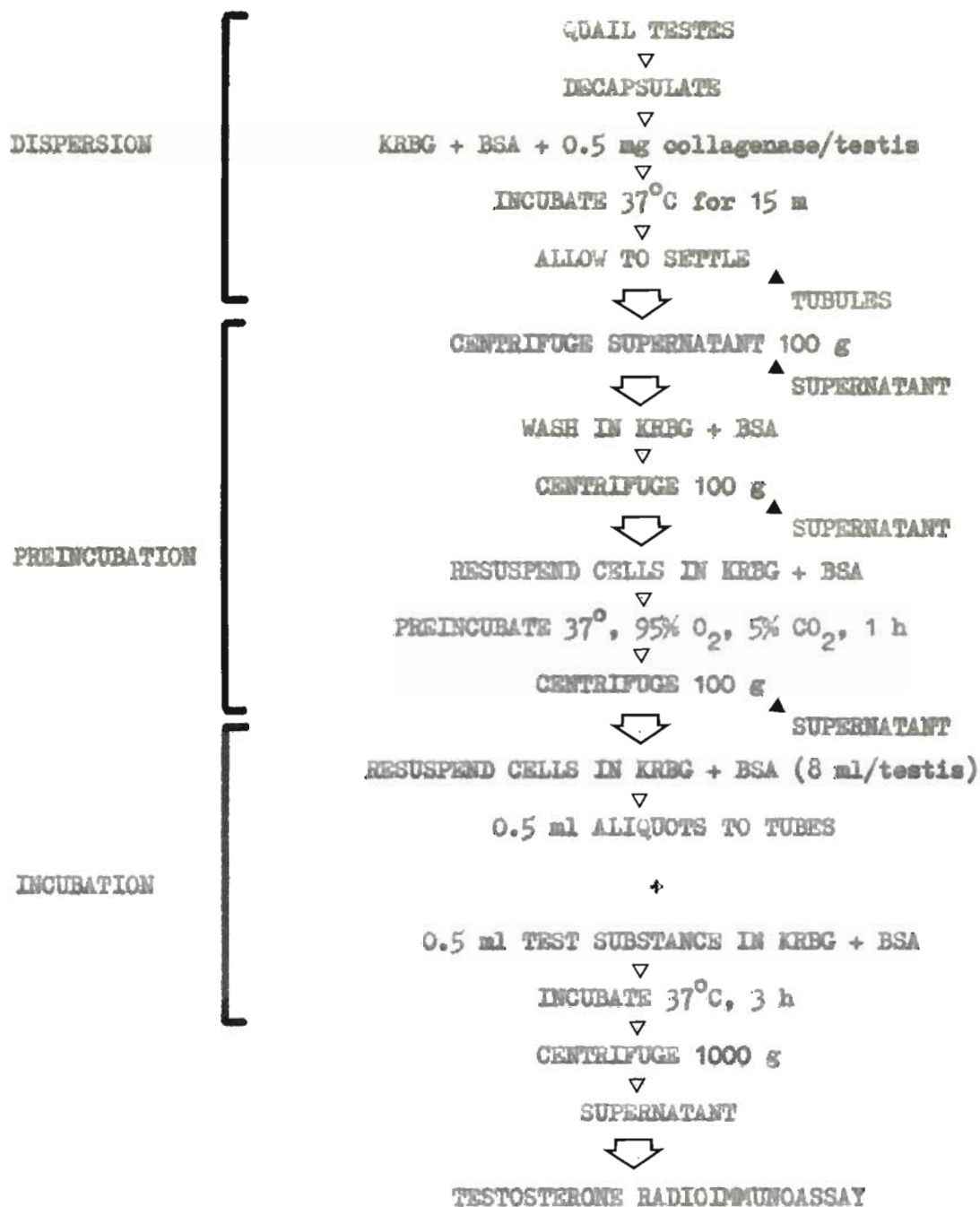
Thyrotrophin was estimated by its stimulation of the uptake of ^{32}P by the thyroids of one day old chicks, following the protocol of Scanes and Follett (1972). NIH-TSH-S8, injected over a range 1.0-4.0 μg per chick, was employed as the standard. Standards and unknowns, dissolved in 0.2 ml 0.9% saline were injected into the nape of the neck, followed 6 hours later by 0.75 μCi carrier-free ^{32}P -phosphate, also in 0.2 ml 0.9% saline. The birds were killed one hour later, the thyroids dissected out, placed on preweighed filter paper disc and weighed. After drying, the discs were counted in the same way as in the total gonadotrophin assay. The data were expressed as cpm/mg and the mean potency and 95% confidence limits were calculated as before (Bliss, 1952).

Figure 26:

Summary of the preparation and incubation of dispersed
quail interstitial cells (from Maung, Z.W., 1976).

THE PREPARATION AND INCUBATION OF THE INTERSTITIAL

CELL SUSPENSION



c) Radioimmunoassay techniques (RIA)

1. Immunization schedules

Antisera were raised in New Zealand white rabbits by the method of Ross *et al.* (1971), against three preparations of dogfish gonadotrophin of varying degrees of purity, namely fractions GTN, ConA-2 and GM2 (from purification 1).

A 10² cm area of fur was shaved on both flanks of the rabbit. Immunogens were dissolved in 0.9% saline and mixed with 2 volumes Freund's complete adjuvant (Difco) by repeated ejection from a hypodermic syringe until a drop of emulsion was contained completely when placed on water. Each rabbit was then injected intradermally with 2 ml emulsion at 30-50 sites on the back. Two rabbits (776 and 777) received 200 µg GTN, a further two (778 and 779) received 200 µg ConA-2 and one other (787) received 200 µg GM2. Rabbits were bled on day 30, boosted on day 40 as for the primary immunization with 10-20% of the quantity of antigen used initially, and bled thereafter at 10-day intervals, i.e. days 50, 60, 70 etc. after the primary immunization. Blood was allowed to clot for 1 hour at room temperature, the clot separated from the glass and allowed to retract at 4°C overnight. The clots were removed before centrifugation and separation of the serum. The antigonadotrophic properties of some of the antisera were tested by their ability to block the increase in ³²P-uptake into chick testes caused by fresh dogfish ventral lobe extracts.

2. Preparation of ¹²⁵I-labelled proteins

Dogfish gonadotrophins ConA-2 (from both purifications 2 and 3) and GM2, and NIAMDD-rat-FSH-I-1 were iodinated according to the method of Greenwood, Hunter and Glover (1963) with Na¹²⁵I (IMS 30, Radiochemical Centre, Amersham). In all cases, 2.5 µg hormone were reacted with 0.5 mCi

Na¹²⁵I and 10 µl Chloramine T (50 mg in 10 ml 0.05 M Na phosphate), the reaction being buffered with 25 µl 0.5 M Na phosphate. The reaction was stopped after 30 seconds by adding 100 µl Na metabisulphite (25 mg in 10 ml 0.05 M Na phosphate) and 200 µl KI/bovine serum albumin (50 mg of each in 5 ml 0.05 M Na phosphate). Labelled hormone was isolated from the reaction mixture by gel filtration on a 1 x 15 cm column of Sephadex G-25 fine, previously washed with 40 mg bovine serum albumin. 1 ml of the active protein peak was then chromatographed on an 0.6 x 30 cm column of Sephadex G-100 to improve the immunological activity of the label.

3. Hormonal measurement by radioimmunoassay

The method adopted was a typical post-precipitation double antibody technique (see reviews by Midgley, Niswender and Rebar, 1969; Kirkham and Hunter, 1971). In an assay 50 µl diluent (phosphate-buffered saline containing 0.1% sodium azide, 0.01 M-EDTA and 0.5% lyophilized egg albumin), standard, pituitary extract or plasma were incubated for 24 hours at 4°C with 50 µl of the antiserum (diluted with diluent containing 1:200 normal rabbit serum to a final concentration such that 30-50% of the label was bound to the antibody in the absence of unlabelled hormone).

Labelled dogfish gonadotrophin was then added (5000 cpm/tube in 50 µl diluent) followed 24 hours later by 50 µl 1:16 anti-rabbit gamma-globulin (Wellcome). The tubes were centrifuged 24 hours later at 1500 g for 30 minutes, the supernatants aspirated and the tubes, containing the precipitates, counted in an automatic gamma-spectrometer.

Results

1) Initial extraction of glycoproteins from pituitaries

Before the work of Idler et al. (1975b) initial extraction of



non-mammalian glycoprotein hormones from either frozen or acetone-dried pituitary glands was usually carried out either by some form of ethanoic extraction (Bates et al., 1959; Stockell Hartree, 1966) or an alkaline extraction followed by ammonium sulphate precipitation (Licht and Papkoff, 1974a,b). Both of these techniques were employed early in the purification studies of dogfish gonadotrophin in case one proved more successful than the other.

One hundred ventral lobes were extracted in a mortar and pestle in 40 ml 0.9% saline. Six ml of this extract was retained and the remainder divided into two equal fractions. To one fraction 10 ml 30% (w/v) ammonium acetate was added, giving a final solution of 30 ml 10% (w/v) ammonium acetate, the pH was adjusted to 5.1 with glacial acetic acid and 20 ml 90% ethanol added. To the other fraction a saturated solution of $\text{Ca}(\text{OH})_2$ was added until the pH reached 9.5. This alkaline extract was spun and the supernatant, together with that from the Stockell Hartree extraction procedure, lyophilized. This dried the alkaline extract, but not the ethanoic solution, so the latter was completed by rotary evaporation. Both dry powders were then redissolved in appropriate volumes of 0.9% saline and bioassayed, together with the initial saline extract. The Papkoff method extracted 47% of the gonadotrophic activity originally present in the fraction before addition of $\text{Ca}(\text{OH})_2$. Thus, using this initial extraction procedure, plus lyophilization, more than 50% of the biological activity was lost. Unfortunately, the Stockell Hartree extract killed the chicks shortly after injection, so it was impossible to compare the two procedures. However, it was decided to continue with the Stockell Hartree extraction technique for a while, carrying it one stage further to the precipitation of the glycoprotein fraction by



addition of 2.5 times the volume of cold ethanol.

After two further trials, a shortened technique was perfected which allowed all of the biological activity initially present in the ventral lobes to be extracted and recovered in the GTN fraction. This technique omitted the stirring of the glands with 10% ammonium acetate: ethanol, 60:40 for 24 hours as described for human pituitary glands (Stockell Hartree, 1966), because homogenization in a mortar and pestle extracted all the glycoprotein into solution within a few minutes. This solution was centrifuged at 4000 rpm (MSE Mistral 2L centrifuge) at 4°C for 10 minutes and the supernatant, containing the glycoprotein, decanted. Three volumes of cold ethanol were immediately added to precipitate the glycoprotein (GTN) fraction.

Because a number of authors have commented on the apparent loss of biological activity following lyophilization (e.g. Idler et al., 1975 b), it was decided to investigate this using dogfish gonadotrophin. Twenty ventral lobes were extracted by homogenization in 8 ml 0.9% saline. One half of the extract was stored at -20°C while the other was lyophilized and then redissolved in 4 ml 0.9% saline. Both fractions were assayed immediately afterwards. The lyophilized material contained 44% of the biological activity of the freshly frozen saline extract. A pool of twenty acetone-dried ventral lobes were also compared to a comparable pool of frozen lobes. After homogenization and recovery of a GTN extract for each group by ethanolic precipitation, there was no significant difference in the amount of biological activity recovered between the two groups.

At this time the work of Idler et al. (1975 a,b,c) was published on the purification of salmon gonadotrophin using affinity chromatography on ConA-Sepharose. After a number of increasingly successful minor



attempts at purification on ConA-Sephadex, and a modification of Idler's Buffer B as described in the Methods and Materials Section, 80% of the initial biological activity of the ventral lobes could be recovered by an ethanolic extraction followed by chromatography on ConA-Sephadex. As it is impossible to weigh a ventral lobe because of the surrounding cartilage from which it is not easily separable, it was not possible to determine the increase in potency following the two steps, although the increase must have been about 20 fold between the GTN and ConA-2 fractions. Very little gonadotrophic activity was lost.

2) Purification 1

The shortened Stockell Hartree glycoprotein extraction procedure yielded 366 mg GTN from the 990 ventral lobes, with a biological activity of 0.033 x NIH-LH-S19 in the chick assay. Using a small glass homogenizer to dissolve the GTN fraction prior to affinity chromatography on ConA-Sephadex, it was found that approximately 40% of this fraction was insoluble in the modified Buffer B. However, this insoluble fraction had no biological activity when assayed in chicks. Following chromatography of 340 mg GTN on a 1.2 x 5 cm column of ConA-Sephadex (see Figure 27), fraction ConA-1 had a biological activity of 0.044 x NIH-LH-S19 and fraction ConA-2 an activity of 0.288 x NIH-LH-S19. Although achieving a 9-fold increase in potency of ConA-2 over the GTN fraction, only 35% of the gonadotrophic activity applied to the column was recovered in the former fraction. A further 46% of the gonadotrophic activity was present in ConA-1, giving a total recovery of 81% of the gonadotrophic activity.

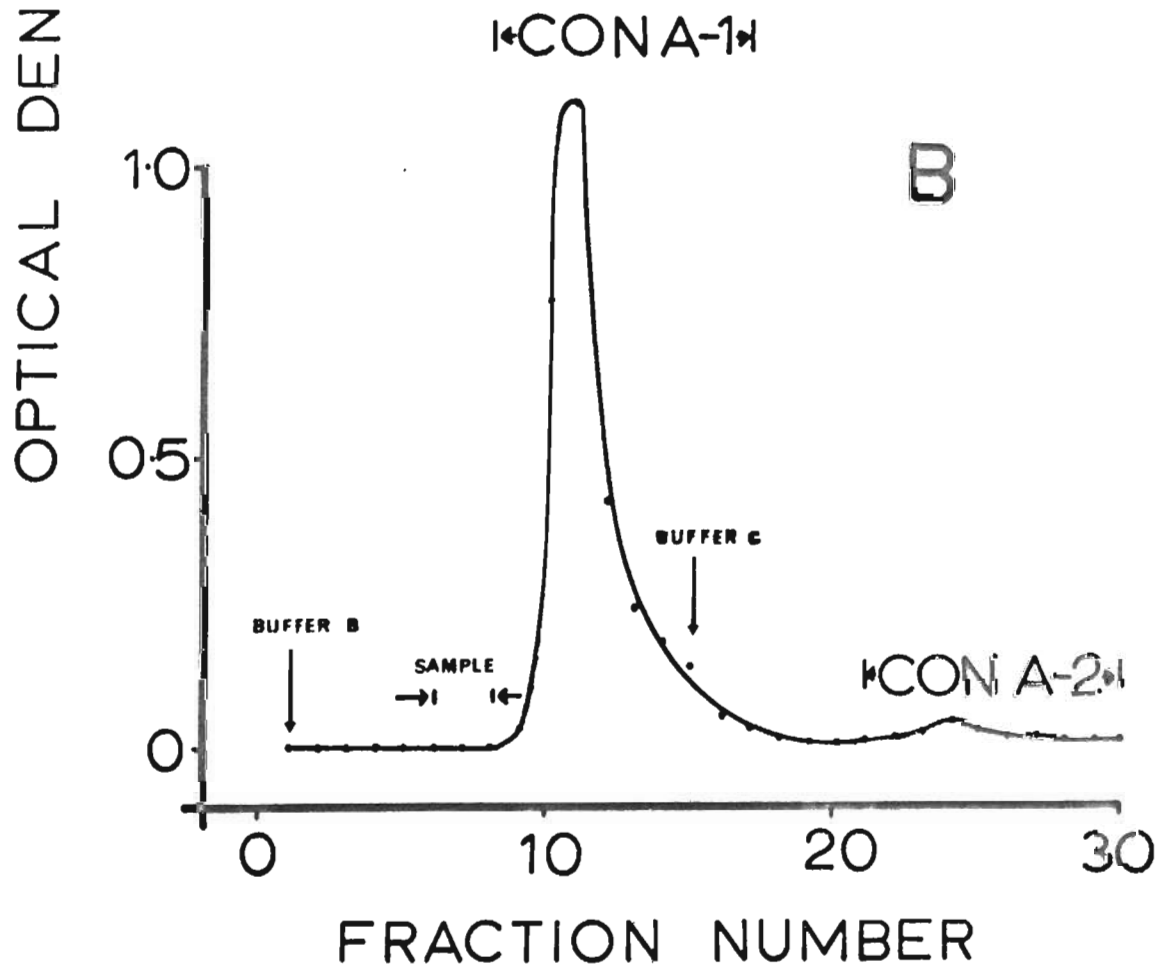
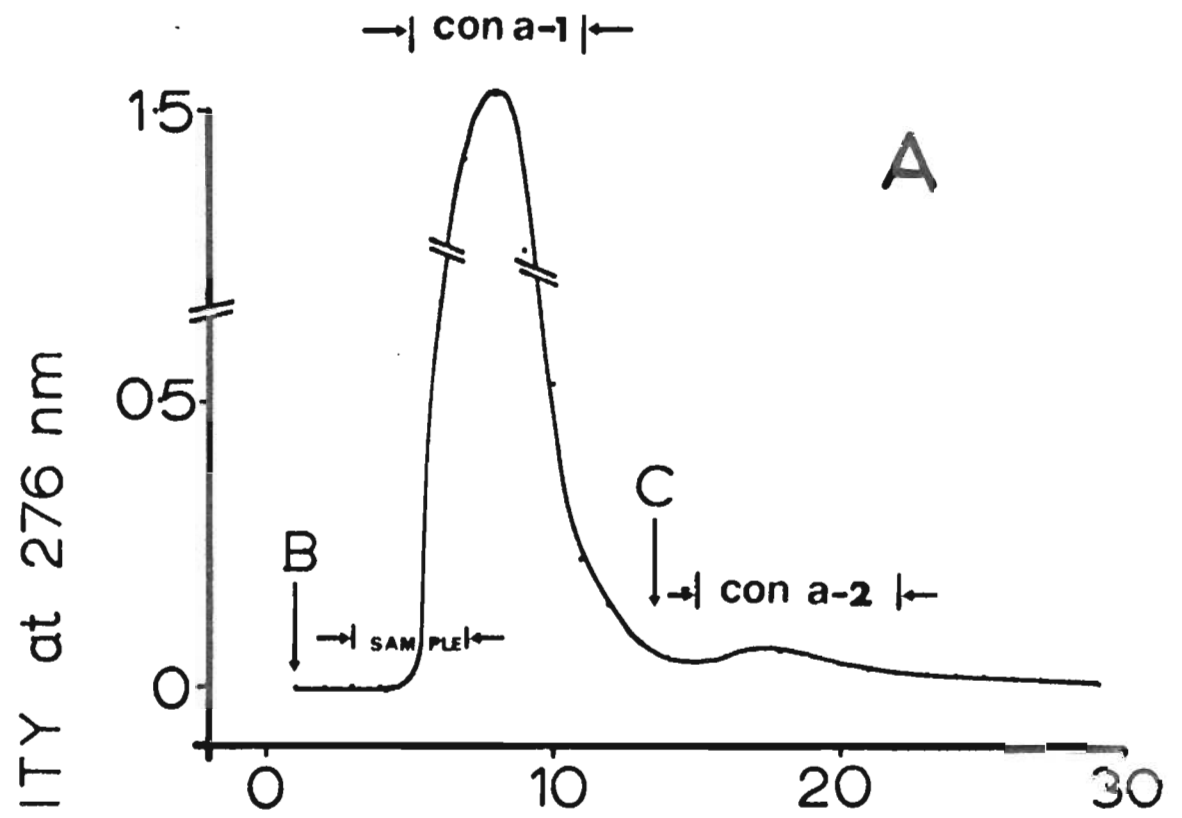
As the ConA-1 fraction still contained a lot of biological activity (\approx 5 mg NIH-LH-S19), it was rechromatographed on a larger (1.2 x 15 cm) ConA-Sephadex column to see if this would separate the

Figure 27:

A: Elution profile of a glycoprotein extract of 990 ventral lobes from a 1.2 x 5 cm column of ConA-Sepharose. Fractions of 3 ml were collected at a flow rate of 10 ml/hr. Selected fractions were pooled as indicated.

B: Elution profile of the rerun of ConA-1 from above down a larger, 1.2 x 15 cm, column of ConA-Sepharose to extract the remaining gonadotrophin. Fractions of 3 ml were collected at a flow rate of 6 ml/hr. Selected fractions were pooled as indicated.





glycoproteins. It seemed more likely that the original column had been overloaded rather than the dogfish gonadotrophin was not a glycoprotein, and therefore would not bind to ConA-Sepharose, especially as previous experience indicated that all the biologically-active gonadotrophin in the chick assay would bind to the gel. When 100 mg ConA-1 was chromatographed again it yielded 40.6 mg of a further ConA-1 fraction and 6.85 mg ConA-2, having gonadotrophic activities of 0.017 and 0.235 x NIH-LH-S19 respectively in the chick assay (see Figure 27). The yield in activity was not as good from the second column as from the first, being 53% of that applied, although a much larger part of it, 70%, was in the ConA-2 fraction. Pooling of the two ConA-2 fractions at this stage gave 19 mg of a material with a potency of 0.275 x NIH-LH-S19.

Estimates of the potencies of the GTN and ConA-1 and 2 fractions from the first ConA-Sepharose column by the in vivo stimulation of testosterone secretion in quail were slightly lower than the values obtained from studies using the chick assay, although the relative potencies of the three fractions appeared to be the same in both assays. Potency estimates of the GTN and ConA-1 fractions could not be obtained because the dose of hormone employed did not increase plasma testosterone concentrations as much as the lowest dose of standard LH employed. The potencies of the GTN and ConA-1 fractions could only be calculated as <0.022 and <0.040 x NIH-LH-S19 respectively (see Table 7).

Thyrotrophic potency estimates of some of the fractions obtained thus far gave values of 0.0032 (mean of two determinations) for GTN, 0.0042 for ConA-1 from the second ConA-Sepharose column, and 0.0116 for pooled ConA-2, all expressed relative to NIH-TSH-S8. Thus, although there had been a 9-fold increase in potency of the ConA-2 fraction over

Table 7: Effect of dogfish gonadotrophin fractions from purification 1 on plasma testosterone concentrations of mature male quail

Bird No.	Treatment	Testosterone concentration			
		Pre-bleed		3 h post-injection	
		Individual	Mean \pm SEM	Individual	Mean \pm SEM
1	NIH-LH-S19 40 μ g/bird	4.17	6.26 \pm 0.85	11.44	10.46 \pm 1.15 P < 0.01 ^(a)
2		5.63		8.44	
3		4.69		10.20	
4		6.06		8.43	
5		7.03		8.58	
6		10.00		15.67	
7	NIH-LH-S19 20 μ g/bird	9.84	3.91 \pm 1.27	15.60	13.23 \pm 1.40 P < 0.001
8		4.36		16.57	
9		3.03		12.67	
10		3.28		15.02	
11		1.93		7.11	
12		1.04		12.39	
13	NIH-LH-S19 10 μ g/bird	6.92	5.65 \pm 1.38	16.06	9.75 \pm 1.34 N.S.
14		7.60		8.23	
15		10.70		9.08	
16		3.88		6.64	
17		3.24		10.04	
18		1.54		8.47	
19	OTN 452 μ g/bird	4.67	5.98 \pm 1.80	4.34	8.31 \pm 2.32 N.S.
20		1.85		4.77	
21		6.07		7.04	
22		9.77		10.14	
23		12.38		18.98	
24		1.14		4.59	
25	ConA-1 253 μ g/bird	3.80	2.25 \pm 0.33	5.69	7.14 \pm 0.78 P < 0.01
26		1.73		6.49	
27		2.29		7.92	
28		1.66		4.46	
29		2.29		9.36	
30		1.75		8.90	
31	ConA-2 68.5 μ g/bird	1.70	2.83 \pm 0.55	7.94	12.64 \pm 1.68 P < 0.01
32		1.69		9.25	
33		2.90		15.08	
34		2.05		19.23	
35		3.43		11.26	
36		5.19		13.09	

(a) Students "t" test against pre-bleed concentrations.

the GTN with respect to gonadotrophin, the ratio was only 4-fold with respect to thyrotrophin, a higher percentage of TSH failing to bind to ConA-Sepharose. However, the overall recovery of gonadotrophin was higher than for thyrotrophin.

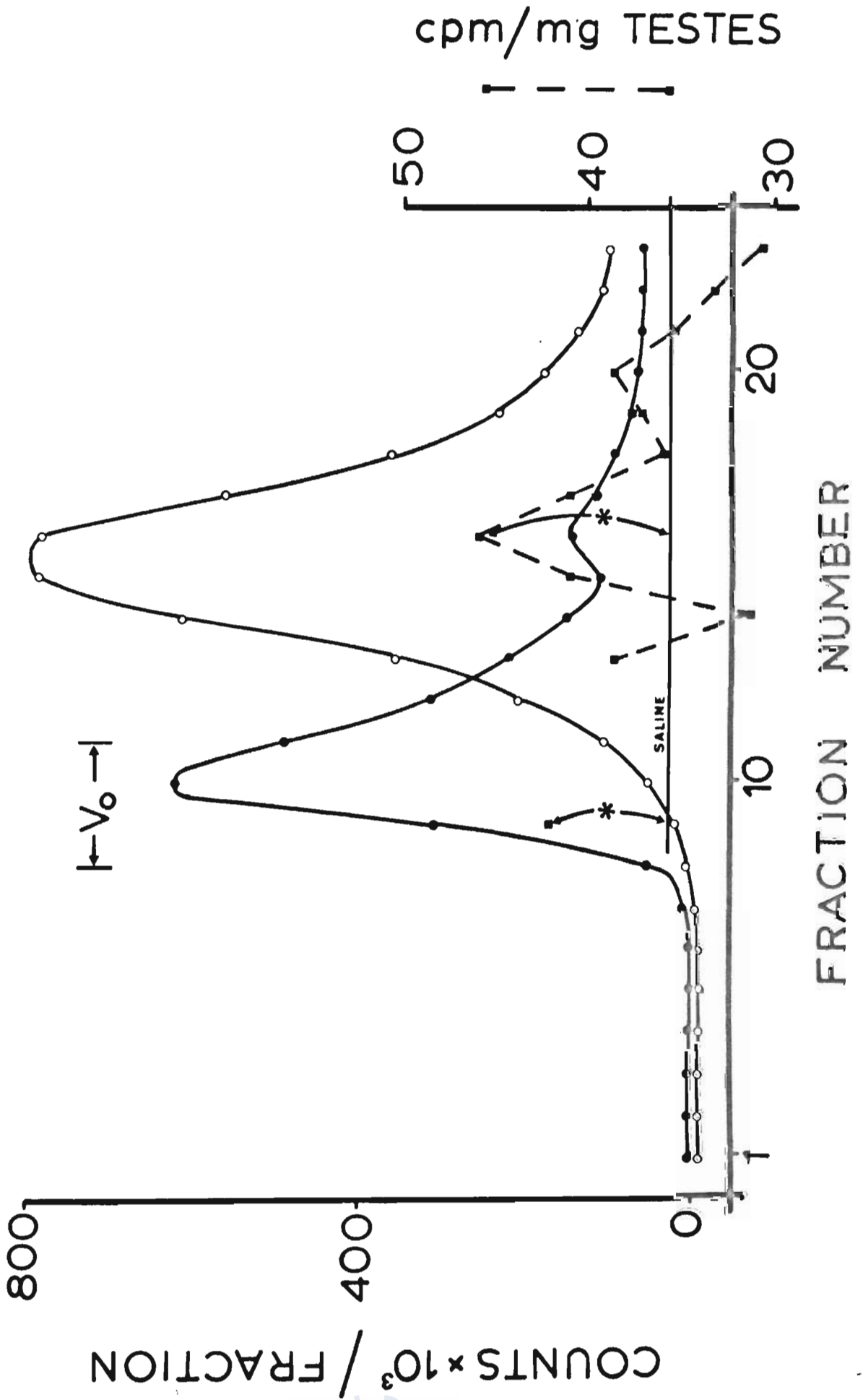
As Idler et al. (1975b) had demonstrated that the adsorbed ConA-2 fraction, when chromatographed on Sephadex G-75 superfine, yielded two protein peaks with the bulk of the salmon gonadotrophic activity in the ascending part of the second peak, the next logical step was to gel filter the dogfish ConA-2 fraction to remove any high molecular weight contaminants. An initial trial with 300 µg pooled ConA-2 on G-100 yielded a single protein peak at the void volume (V_0) of the column. Only one of the fractions at the V_0 was assayed for gonadotrophic activity, and this showed a small but significant gonadotrophic titre, corresponding to approximately 6% of the activity applied. A biological scan of fractions 13 to 23 showed a small peak of activity in fractions 15 to 17, which contained approximately 20% of the activity applied (see Figure 28). Either there had been a considerable loss of activity on the column or else, as became a distinct possibility after further work, much of the biological activity was not retarded on G-100 and probably emerged in fractions 10-12 which had not been assayed for their gonadotrophic activity. Confirmation that the column itself was capable of retarding gonadotrophins was demonstrated using lightly iodinated rat FSH, which emerged many fractions removed from the V_0 , and showed a peak in fractions 15 and 16. As shown in Figure 28, pooled ConA-2 iodinated under the same conditions emerged at the V_0 of the column, with only a very minor peak occurring in the same position of rat FSH. A reassay of pooled ConA-2 in the chick assay at this time showed that its potency had not significantly altered.



Figure 28:

Radioactivity elution profiles of gently iodinated pooled ConA-2 (●—●) and NIAMDD rat PSH (○—○) from a 0.6 x 35 cm column of Sephadex G-100. The figure also indicates the biological elution profile of 300 µg of pooled ConA-2 (■---■), measured by the ³²P-chick assay. In all cases the sample was applied in 1 ml and the column eluted with 0.05 M Na phosphate/0.9% NaCl at a flow rate of 12 ml/hr, collecting 1 ml fractions. The void volume of the column (V₀) was determined using blue dextran before and after the three experimental runs and had not changed significantly.

* P < 0.05, Students "t" test.



Attention was now focussed on whether there were one or two gonadotrophins in the ConA-2 fraction. As CM-cellulose has been widely used to separate FSH from LH and TSH in mammalian species (Stockell Hartree, 1975), and successfully used also for avian gonadotrophins (Scanes and Follett, 1972), this ion exchange resin was chosen. Chromatography of 660 μ g ConA-2 yielded two protein peaks, as determined by measuring protein in 50 μ l aliquots of each fraction eluted (the second buffer, 1 M ammonium acetate (pH 5.5) interfered with the protein determinations in the later fractions). The first fraction, the protein peak not adsorbed on to the resin, contained 27% of the gonadotrophic activity applied to the column, and the second fraction eluted with 1 M ammonium acetate, 29% (see Figure 29). As these results were intriguing and the recovery from the column reasonably good for an ion-exchange resin, 11.8 mg ConA-2 was fractionated on a 1.2 x 5 cm column of CM-32 under the same conditions. As shown in Figure 29, this yielded 6.5 mg CM1, with a potency of 0.081 x NIH-LH-S19, and 700 μ g CM2, with a potency of 1.041 x NIH-LH-S19, as measured by the chick assay. The total biological activities of CM1 and CM2 represent 17 and 23% respectively of that applied. The ratios of total gonadotrophic activity CM1:CM2 were 0.95 and 0.74 in the two runs. The potencies of the various partially purified gonadotrophic fractions in the chick assay are shown graphically in Figure 30. In the in vitro quail interstitial cell assay, CM1 had a potency of 0.055 x NIH-LH-S19, while CM2 was equipotent with the standard. Both unknowns and standard were tested in duplicate at four different dose levels, the results being shown graphically in Figure 31. Table 8 summarises the gonadotrophic and thyrotrophic potencies of the various fractions obtained during purification 1.



Figure 29:

A: Elution profile of 660 μg of pooled ConA-2 on carboxymethyl cellulose (CM 32) pre-equilibrated with the starting buffer (4 mM ammonium acetate, pH 5.5). The protein concentration of each fraction (\bullet — \bullet) was measured by the method of Lowry et al. (1951) and the biological activity (\circ — — \circ) using the ^{32}P -chick assay. Protein concentrations of fractions 29 onwards could not be measured because the 1 M NH_4 acetate buffer interfered with the determinations.

*** $P < 0.001$, Students "t" test.

B: Elution profile of 11.8 mg of pooled ConA-2 on a 1.2 x 5 cm column of CM 32 pre-equilibrated with the starting buffer (4 mM ammonium acetate, pH 5.5). Fractions of 2.5 ml were collected at a flow rate of 20 ml/hr. Selected fractions were pooled as indicated.

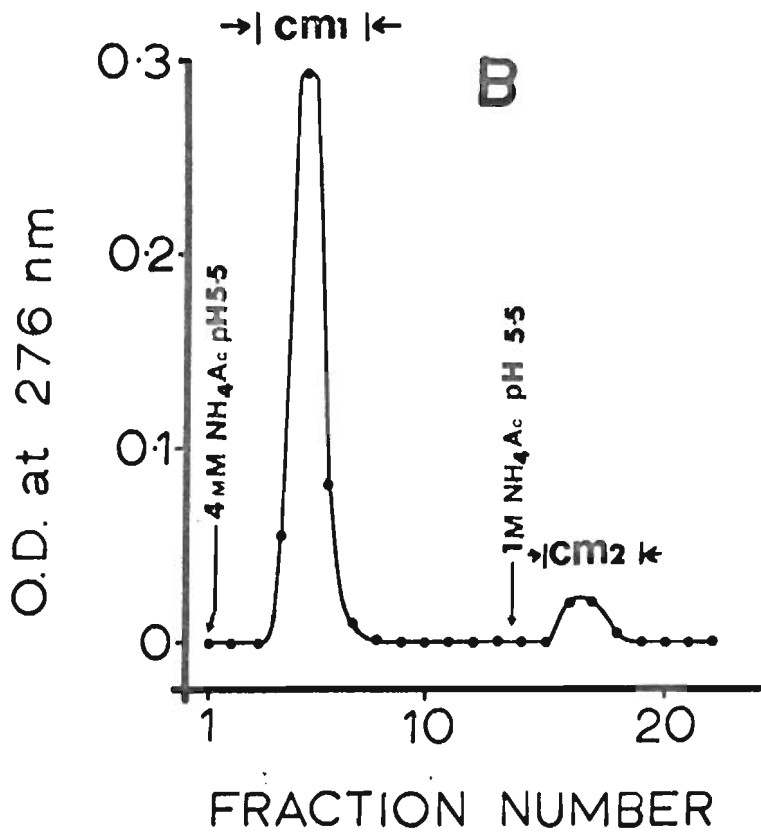
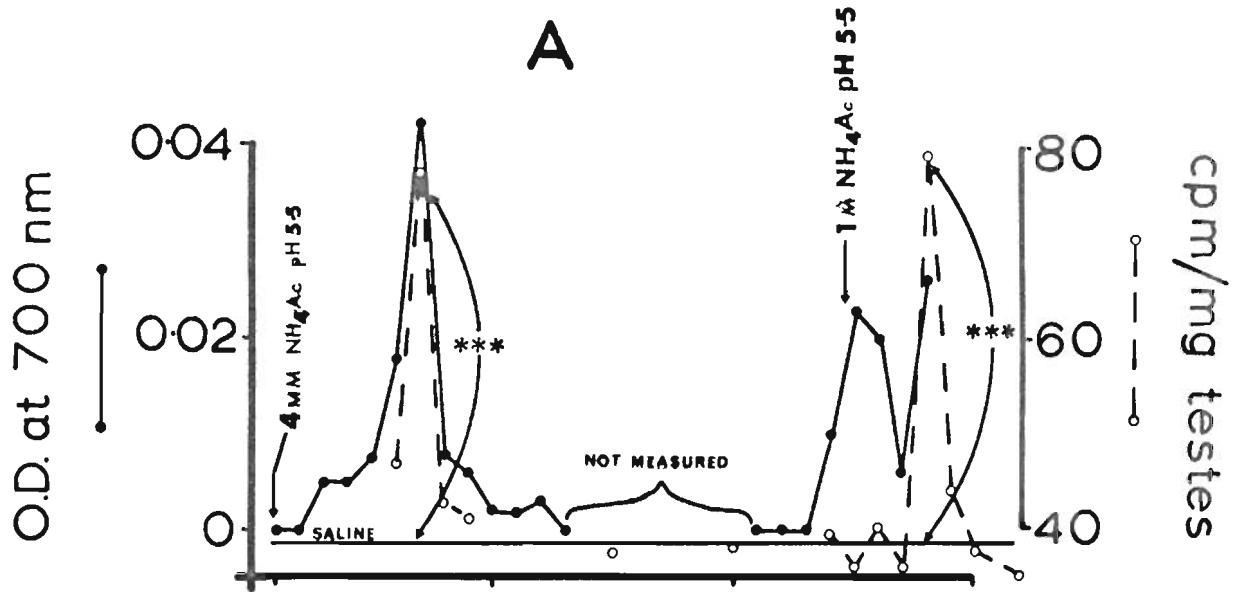


Figure 30:

Dose-response curves in chick ^{32}P -gonadotrophin assay for various dogfish gonadotrophic fractions from purification 1.

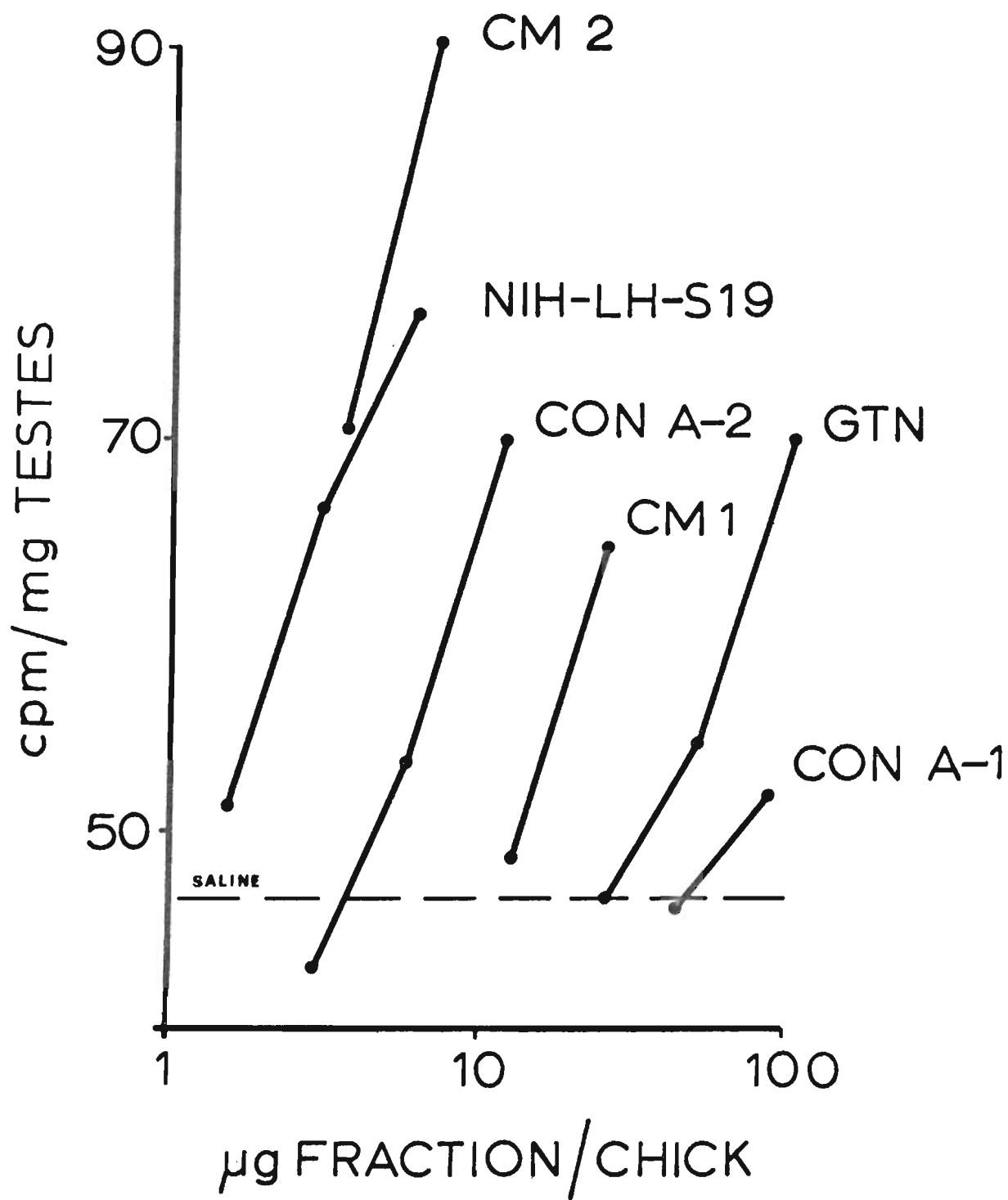


Figure 31:

Effects of partially purified dogfish gonadotrophins (fractions GM1 and GM2) and ovine LH (NIH-LH-S19) on testosterone release from quail interstitial cells.

The mean results of duplicate tubes are shown.



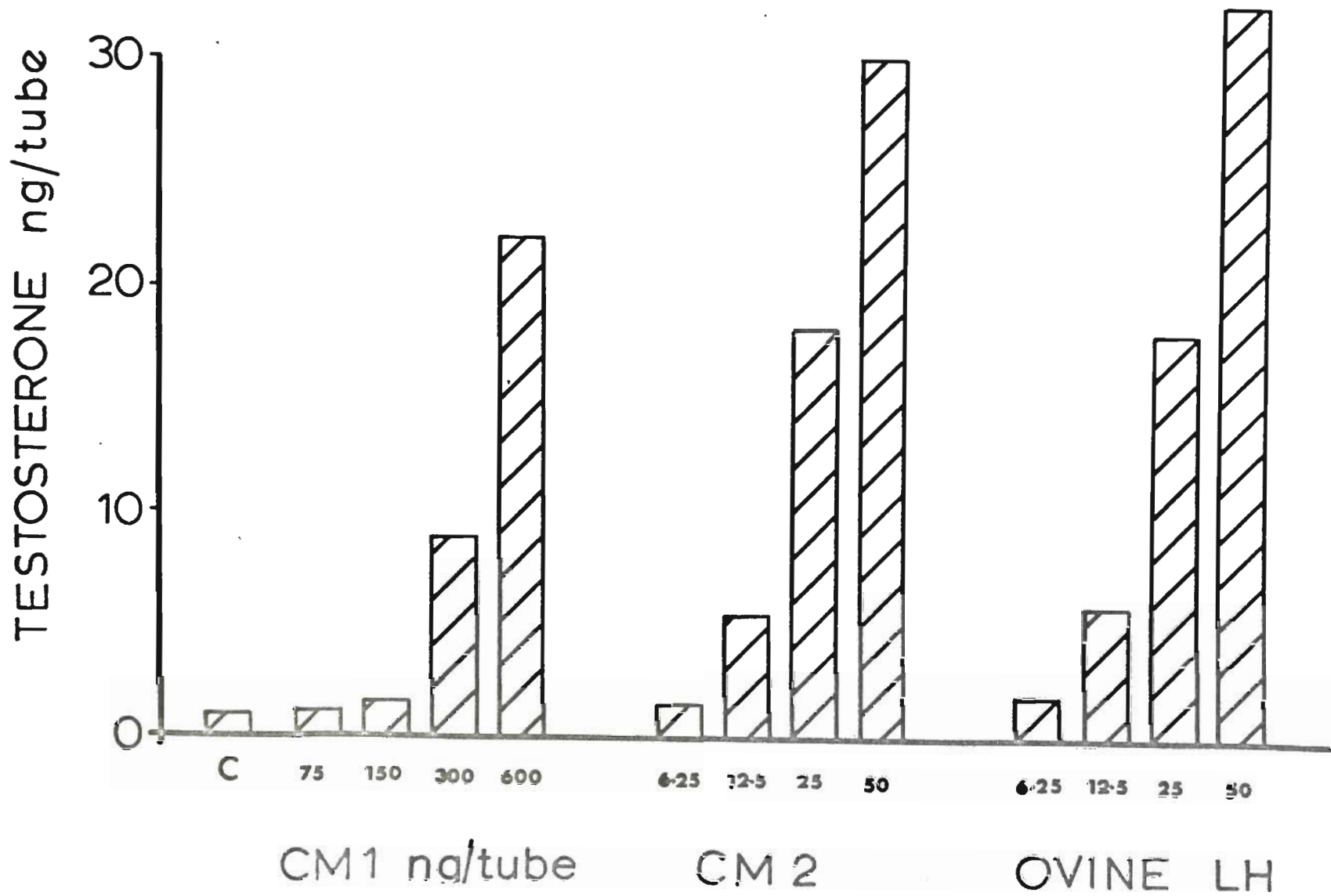


Table 8: Weight yields and gonadotrophic and thyrotrophic potencies of dogfish ventral lobe fractions from the first purification

Fraction	Weight (mg)	Gonadotrophin (a)	λ (b)	Thyrotrophin (c)	λ (b)
Glycoprotein precipitate (GPN) from 990 ventral lobes	365.9	32.6 (21.2-49.3) 29.7 (22.1-40.0)	0.213 0.218	3.79 (3.37-4.27) 2.59 (1.91-3.52)	0.118 0.241
<u>ConA-Sepharose chromatography</u>					
ConA-1	115.0	47.0 (30.5-138.0) 40.0 (5.36-103.9)	0.252 0.364		
ConA-2	13.63	288.1 (186.3-433.6) (215.1 using quail <u>in vivo</u> assay)	0.213		
<u>Rechromatography of ConA-1 on ConA-Sepharose</u>					
ConA-1	40.56	16.9 (0.7-29.3)	0.322	4.18 (3.13-5.58)	0.241
ConA-2	6.85	234.5 (143.6-248.2)	0.231		
Pooled ConA-2	18.96	275.4 (211.8-358.1)	0.218	11.57 (9.07-14.75)	0.241
<u>CM-Cellulose chromatography</u>					
CM1	6.6	80.5 (47.1-94.7) (54.5 in quail interstitial cell assay)	0.233		
CM2	0.70	1041 (967.5-1194) (1000 in quail interstitial cell assay)	0.147		

(a) Gonadotrophic activity expressed in μg equivalents NIH-LH-S19 per mg, with 95% confidence limits shown in brackets.

(b) λ - Index of precision (a/b).

(c) Thyrotrophic activity expressed in μg equivalents NIH-TSH-S8 per mg, with 95% confidence limits shown in brackets.

3) Purification 2

Initially, this followed the methods employed in the first purification, with a shortened ethanolic extraction followed by precipitation of the GTN extract with ethanol, and subsequent chromatography on ConA-Sepharose. The 1300 ventral lobes yielded 540 mg GTN with a potency of 0.020 x NIH-LH-S19 in the chick assay. Affinity chromatography of 500 mg of this material on a 1.2 x 15 cm column of ConA-Sepharose yielded 72 mg ConA-1 with a gonadotrophic activity of 0.011 x NIH-LH-S19, and 25.1 mg ConA-2 with a gonadotrophic activity of 0.485 x NIH-LH-S19, a 25-fold increase in potency over the GTN extract. Although the GTN extract is only slightly greater than half as potent as the corresponding fraction from the previous purification, possibly because the glands were collected later in the year, when ventral lobe gonadotrophin content is falling, (see Figure 9 of Chapter 1) the ConA-1 and ConA-2 fractions contain 7.6 and 115% respectively of the activity applied to the ConA-Sepharose column. This yield of approximately 120% is well within the limits of the potency estimates of the various fractions, but does indicate that very little if any activity was lost during ConA-Sepharose chromatography.

It was not possible to calculate similar figures for the thyrotrophin (TSH) content after ConA-Sepharose chromatography because the TSH content of the GTN extract was not determined. However, the potencies of ConA-1 and ConA-2 were 0.0041 and 0.026 x NIH-TSH-S8, respectively. Thus the gonadotrophic ratio of CM2:CM1 was 43:1, while the TSH ratio was only 4.3:1, again suggesting a preferential purification of gonadotrophin over thyrotrophin. The data are summarised in Table 9.

Chromatography on ConA-Sepharose was followed three and a half



Table 9: Weight yields and gonadotrophic and thyrotrophic potencies of dogfish ventral lobe fractions from the second purification

Fraction	Weight (mg)	Gonadotrophin (a)	λ (b)	Thyrotrophin (c)	λ (b)
Glycoprotein precipitate (GM) from 1300 ventral lobes	510.8	19.6 (8.5-28.8)	0.222		
<u>ConA-Sepharose chromatography</u>					
ConA-1	72.3	11.2 (0.0002-22.31)	0.306	4.1 (3.6-4.6)	0.102
ConA-2	25.1	485.0 (340.8-799.6)	0.205	25.8 (22.5-29.6)	0.102
<u>Sehadex G-100 chromatography</u>					
G-100 Fraction 1	12.85	99.6 (34.1-152.0)	0.222		

- (a) Gonadotrophic activity expressed in μg equivalents NIH-1H-519 per mg, with 95% confidence limits shown in brackets.
- (b) λ - Index of precision (s/b).
- (c) Thyrotrophic activity expressed in μg equivalents NIH-TSH-58 per mg, with 95% confidence limits shown in brackets.

months later by gel filtration of 19.7 mg ConA-2 on a 2.5 x 100 cm column of Sephadex G-100, as shown in Figure 32. This yielded only a single protein-peak, emerging at the V_0 of the column, which when precipitated weighed 12.8 mg and had a gonadotrophic activity of 0.100 x NIH-LH-S19. There appeared to have been nearly a 5-fold drop in potency, with almost 90% of the gonadotrophic activity not being recovered from the column. Subsequent pooling and bioassay of the fractions eluted after the V_0 failed to reveal any gonadotrophic activity other than that already detected, and not retarded on the G-100 column. Even though the column was eluted at room temperature with 0.05 M Na phosphate (not the best choice in retrospect!), this seemed a rather large loss of biological activity. Elution off the same column, under the same conditions, with both freshly iodinated-rat FSH and with ovalbumin (Koch-Light Laboratories Ltd., 5 x crystallised) confirmed that the rat gonadotrophin as shown in Figure 32, was retarded on the column and that ovalbumin was eluted just prior to the rat FSH. Interestingly, iodinated rat FSH that had been stored at 4°C for 40 days gave a major radioactive peak at the V_0 of the G-100 column, with a minor one later at the position where all of the fresh material had been eluted.

A reassay of ConA-2 three weeks after the major gel filtration run gave a potency of 0.129 x NIH-LH-S19, as opposed to 0.485 x NIH-LH-S19 when first assayed. If the ConA-2 fraction had lost potency prior to the main G-100 run, then the recovery of gonadotrophic activity may have been much greater than the 13% first calculated. Reassay of the major gonadotrophic fractions from both purifications 1 and 2 showed that nearly all the fractions had lost potency on storage as dry powders at -20°C (see Table 10). The most potent fractions reassayed, ConA-2 from



Figure 32:

Elution profile of 19.7 mg of ConA-2 (2nd purification) on a 2.5 x 110 cm column of Sephadex G-100. For comparison, the radioactivity profile of freshly iodinated NIAMDD rat FSH is included. In both cases the column was eluted with 0.05 M Na phosphate at a flow rate of 30 ml/hr and 4 ml fractions were collected. The void volume (V_0) was determined after both runs by the use of blue dextran. All the biological activity recovered after eluting the ConA-2 was in the non-retarded protein peak emerging at the V_0 .



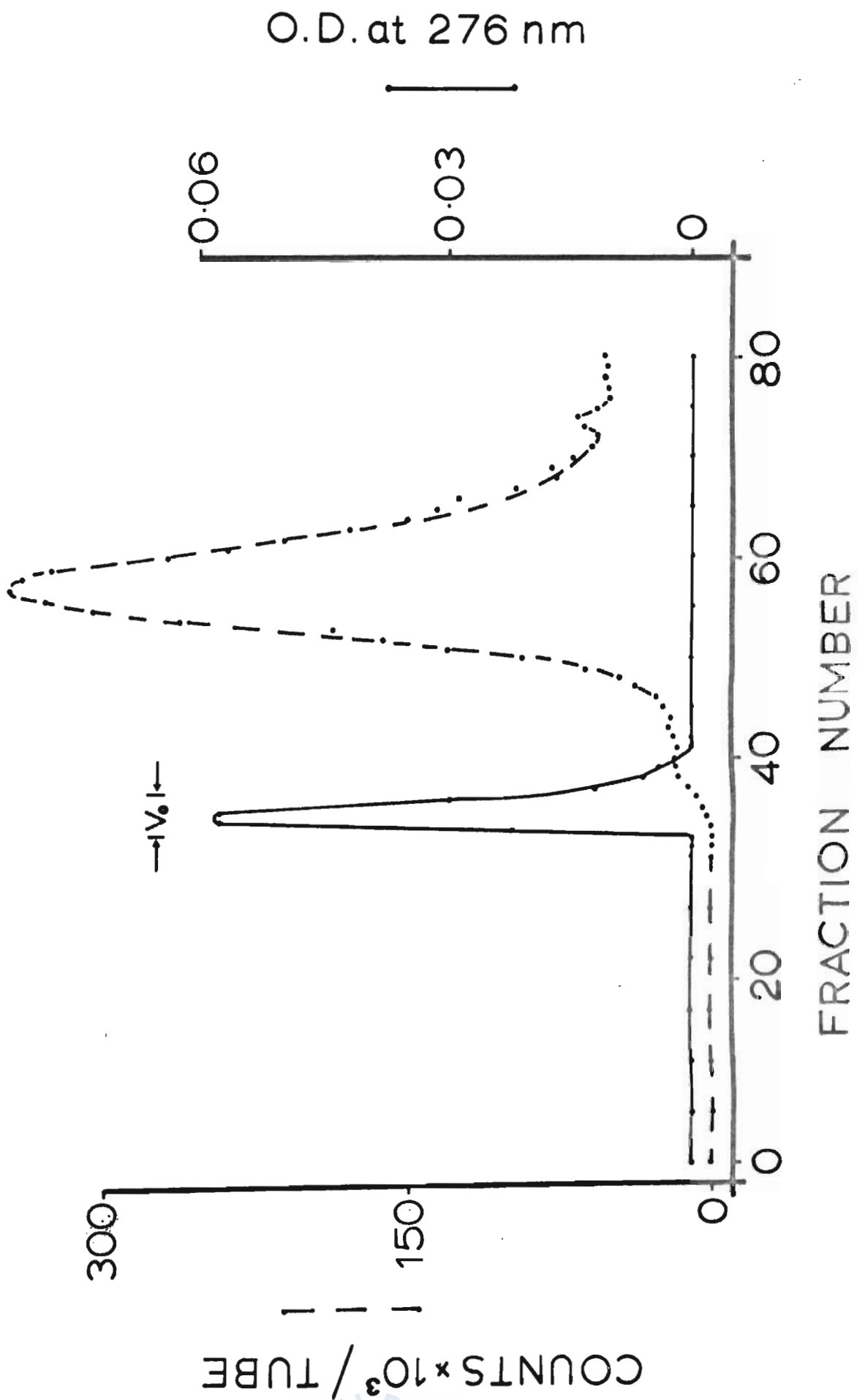


Table 10: Change in potency ^(a) with time of various dogfish gonadotrophin fractions as measured in the ³²P-chick assay

	Date						
	7/5/76	13/5/76	21/5/76	26/8/76	10/9/76	16/9/76	30/9/76
Pooled ConA-2 (1st purification)	275.4 (211.8-358.1)						79.5 (38.6-166.2)
ConA-2 (2nd purification)		485.0 (340.8-799.6)				128.7 (107.6-154.0)	165.6 (144.4-208.7)
CM1			80.5 (47.1-94.7)				80.4 (50.4-172.6)
CM2			104.1 (967.5-1194.0)				
G-100 (2nd purification)				96.6 (34.1-152.0)	92.4 (59.7-139.4)	65.0 (46.1-92.9)	59.4 (21.5-97.5)

(a) Total gonadotrophic activity per fraction expressed in µg equivalents NIH-LH-S19 per ng.

λ is 0.199 (\pm 0.010), n = 12.

Figure 33:

A: Biological activity profile of a saline extract of 25 ventral lobes on a 1.2 x 60 cm column of Sephadex G-100. All fractions were assayed using the ^{32}P -chick bioassay.

B: Radioactivity profiles of freshly iodinated GM2 (●—●) and NIAMDD rat FSH (●---●) on the same column of G-100. In all cases the column was eluted with 0.05 M Na phosphate/0.9% NaCl at a flow rate of 25 ml/hr and 4.2 ml fractions were collected. All samples were applied in a volume of 2.5 ml.



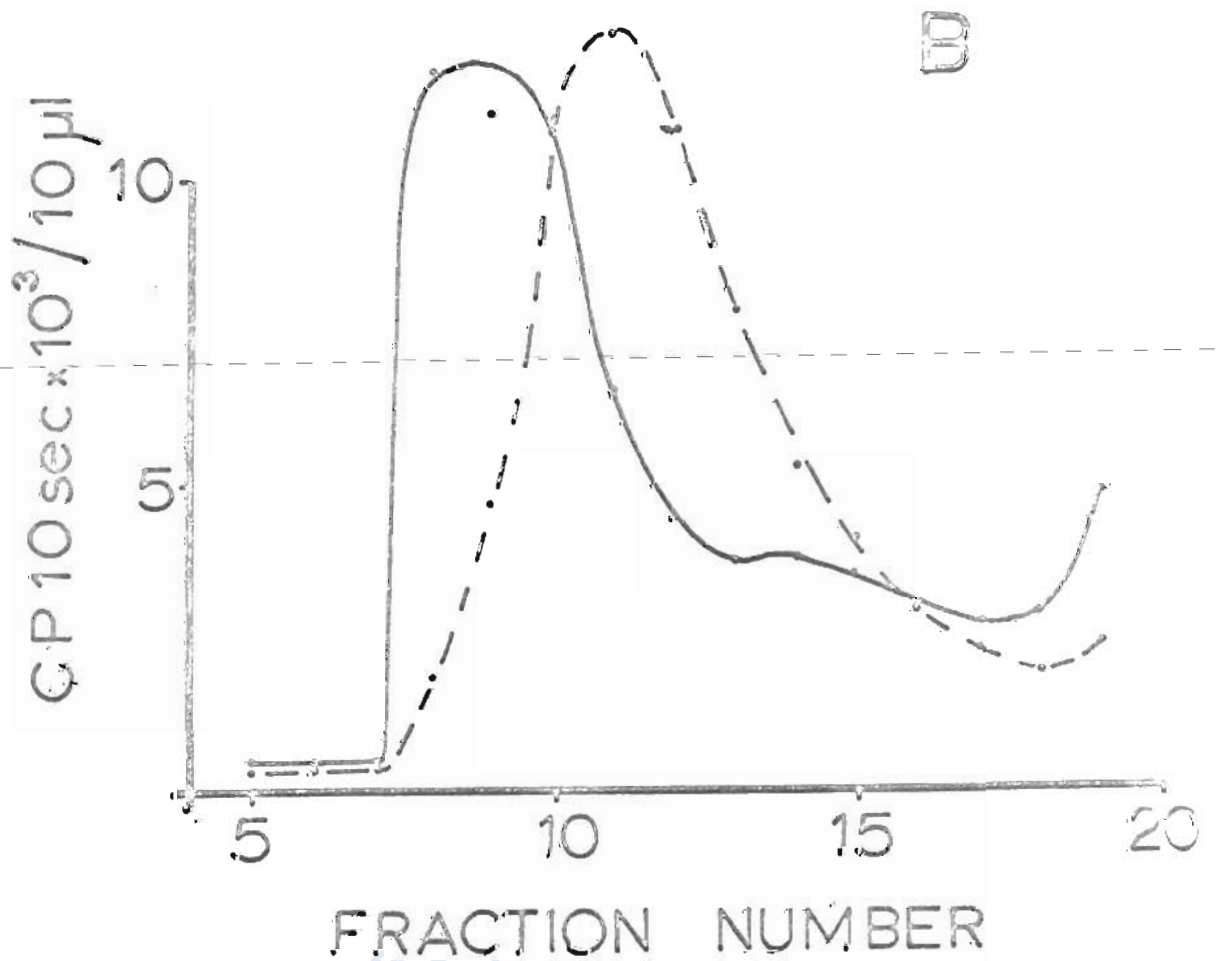
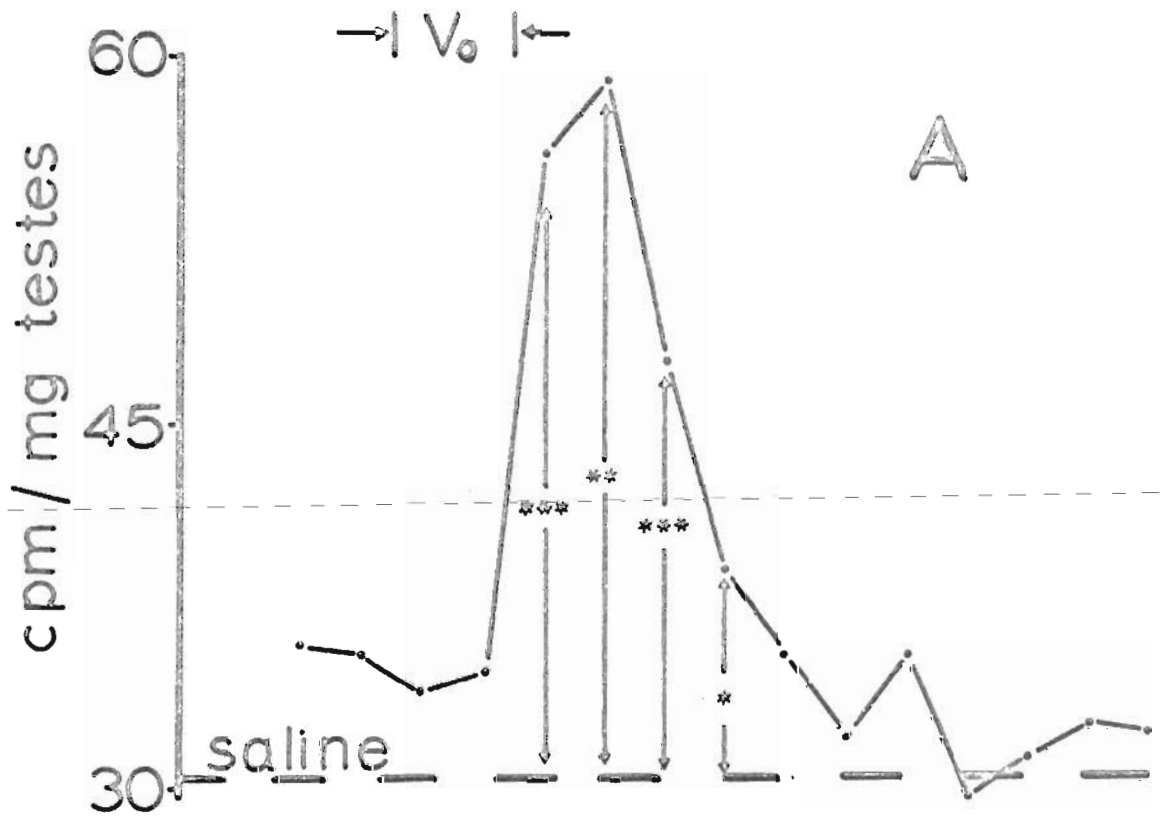
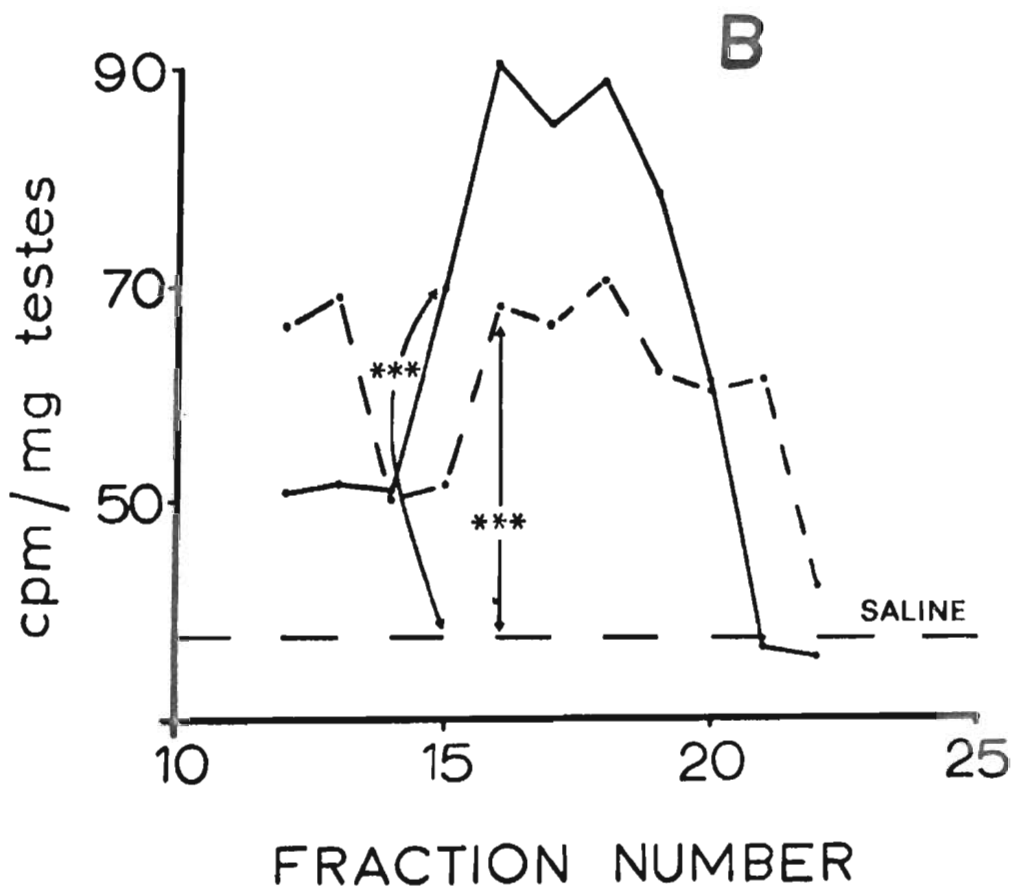
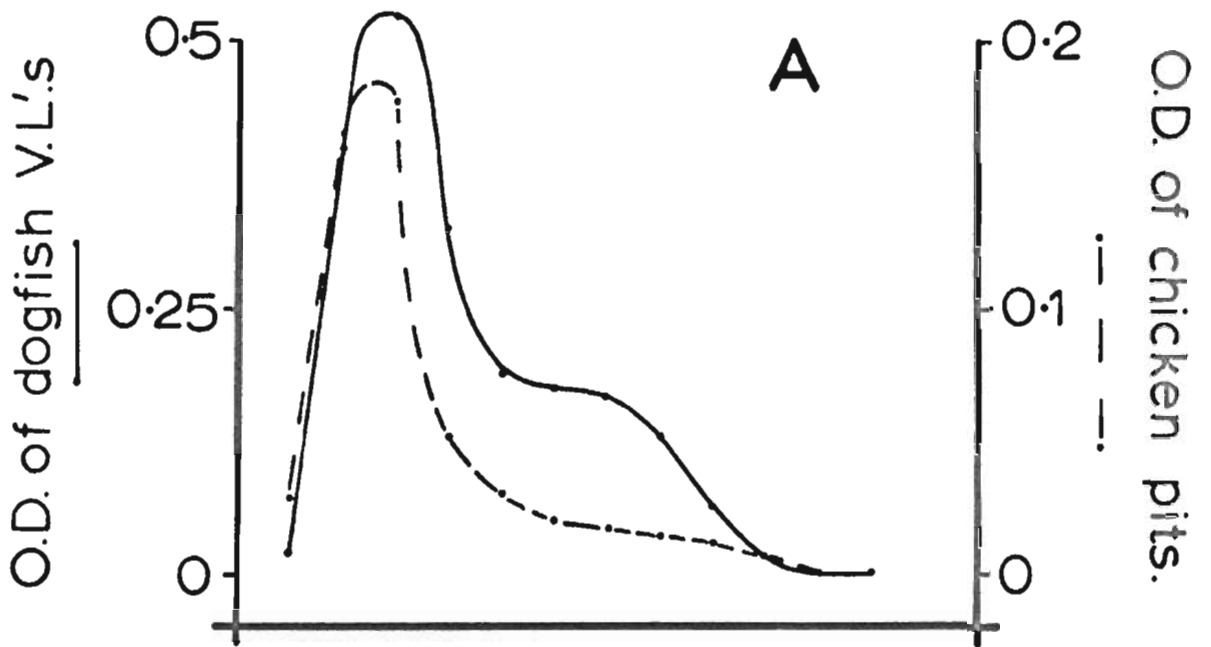


Figure 34:

A: Protein concentration profiles of a saline extract of either 18 dogfish ventral lobes (●—●) or 4 chicken pituitaries (●---●) on a 1.2 x 60 cm column of Sephadex G-100.

B: Biological activity profiles of the fractions collected from the two experiments above, assayed in the ^{32}P -chick bioassay. In both cases the column was eluted with 0.05 M Na phosphate/0.9% NaCl at a flow rate of 22 ml/hr, collecting 2.2 ml fractions.



was grossly overloaded with activity, both dogfish and chicken gonadotrophins were retarded to the same extent on the column. Also, in both cases, a high percentage of the gonadotrophic activity applied to the column was recovered. Use of a larger (2.5 x 100 cm) column of G-100, with a much slower flow rate, retarded the gonadotrophin of fresh ventral lobes to the extent that twenty 4 ml fractions separated the void volume from the peak of gonadotrophic activity. Under the same conditions iodinated salmon gonadotrophin (SG-DEAE-3, a gift from Dr. E.M. Donaldson) was eluted at the same position as the gonadotrophic activity of the ventral lobes.

4) Purification 3

Ten ml of a saline extract of 380 ventral lobes was filtered on Sephadex G-100, producing the elution profile shown in Figure 35. Fractions were pooled as indicated and the proteins precipitated with ethanol. Fractions 2, 3 and 4 yielded 240, 497 and 603 mg respectively, with gonadotrophic activities of 0.0012, 0.0040 and 0.00085 x NIH-LH-S19. These three fractions contained 96% of the activity applied to the column. Fraction 3, which contained 71% of the activity, was applied to a 1.2 x 15 cm column of ConA-Sepharose and yielded 139 mg ConA-1 and 3.0 mg ConA-2, with potencies of 0.002 and 0.376 x NIH-LH-S19 respectively. 73% of the gonadotrophic activity was recovered off the ConA-Sepharose column, of which 57% was in ConA-2. Potencies of the various fractions are summarised in Table 11, while Table 12 shows the percentage yields of all three major purifications at various stages.

5) Purification of gonadotrophin from other pituitary lobes

A single batch of 1060 lobes of either rostral plus median, or neurointermediate, yielded 94.4 and 416 mg GTN extract respectively.



Figure 35:

Elution profile of a saline extract of 380 ventral lobes on a 2.5 x 110 cm column of Sephadex G-100. The column was eluted with 0.05 M Na phosphate/ 0.9% NaCl at a flow rate of 10 ml/hr, and fractions of 4.3 ml were collected. The respective fractions were pooled as indicated and assayed in the ^{32}P -chick bioassay, the results being expressed as μg equivalents per mg NIH-LH-S19.

N.D. = Not detectable, < 0.20 $\mu\text{g}/\text{mg}$.

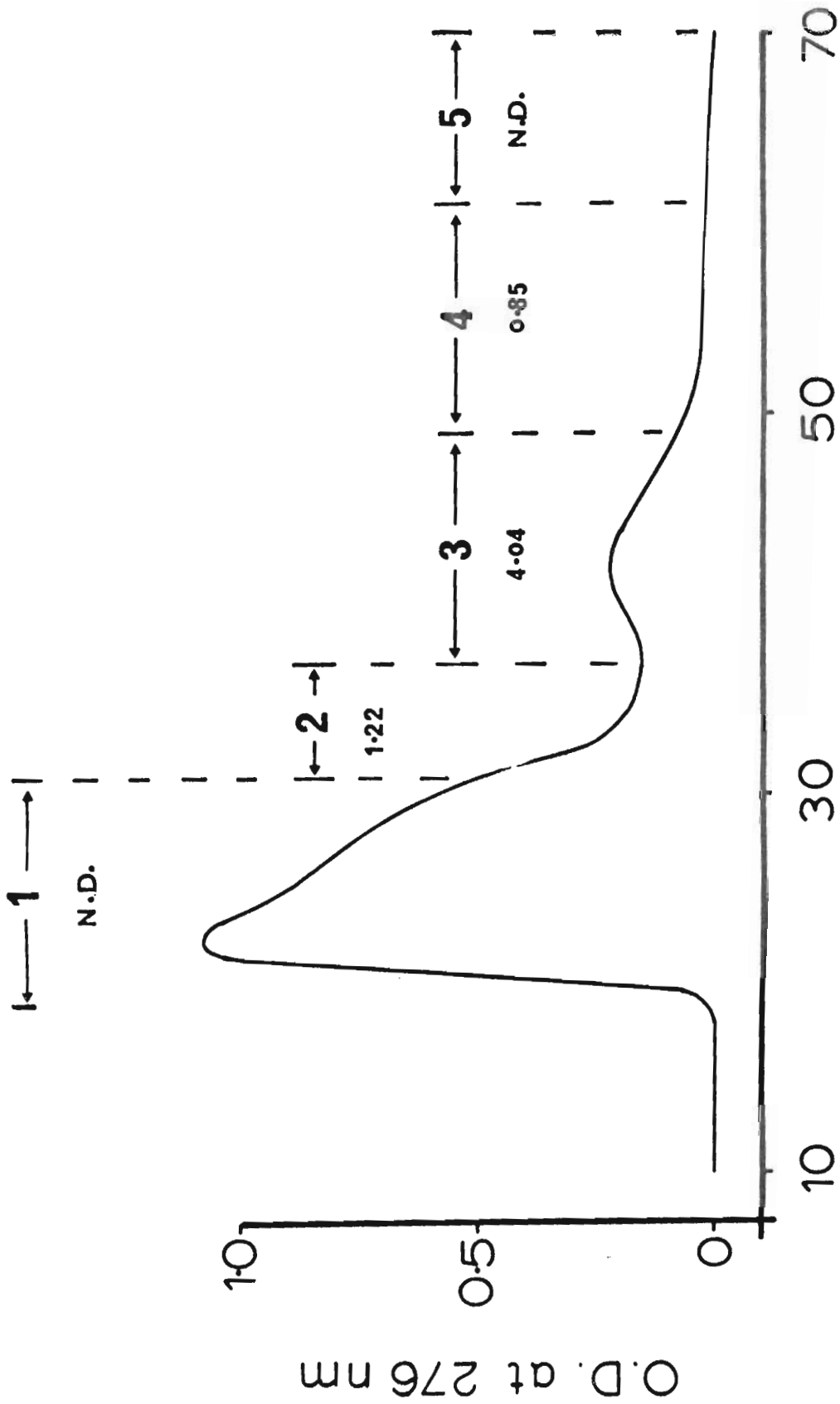


Table 11: Weight yields and gonadotrophic potencies of dogfish ventral lobe fractions from the third purification

Fraction	Weight (mg)	Gonadotrophin (a)	λ (b)
Saline extract from 380 ventral lobes (o)	-	3405.78 (2562-3859) (Total activity present)	0.190
<u>Sephadex G-100 chromatography</u>			
Fraction 2	239.5	1.42 (1.01-1.78)	0.096
Fraction 3	496.6	1.01 (0.71-1.44)	0.326
Fraction 4	602.7	4.04 (3.26-5.12)	0.106
<u>ConA-Sephadex chromatography</u>			
ConA-1	139.3	0.85 (0.70-1.29)	0.326
ConA-2	3.0	2.25 (1.52-3.99)	0.190
		376.2 (191.6-512.8)	0.245

(a) Gonadotrophic activity expressed in μ g equivalents NIH-1H-519 per mg, with 95% confidence limits shown in brackets.

(b) λ - Index of precision (a/b).

(c) Total gonadotrophic activity in the 10 ml of extract.



Table 12: Gonadotrophin yields during all three purifications

	Purification 1		Purification 2		Purification 3	
	Total activity (a)	% recovery (b)	Total activity	% recovery (b)	Total activity	% recovery (b)
Saline extract	11398	100	10600	100	2918	100
Glycoprotein precipitate (GTM)						
Sephader G-100 chromatography						
Fraction 2					291.0	10.0
Fraction 3					2006	68.8
Fraction 4					512.3	17.6
ConA-Sepharose chromatography						
ConA-1	685.5 (d)	6.01	809.8	7.6	313.4	9.2
ConA-2	5221.6 (d)	45.8	12174	114.8	1128.6	33.1
CM-Cellulose chromatography						
CM1	531.3	4.7				
CM2	728.7	6.4				
Sephader G-100 chromatography						
Fraction 1			1277	12.0		

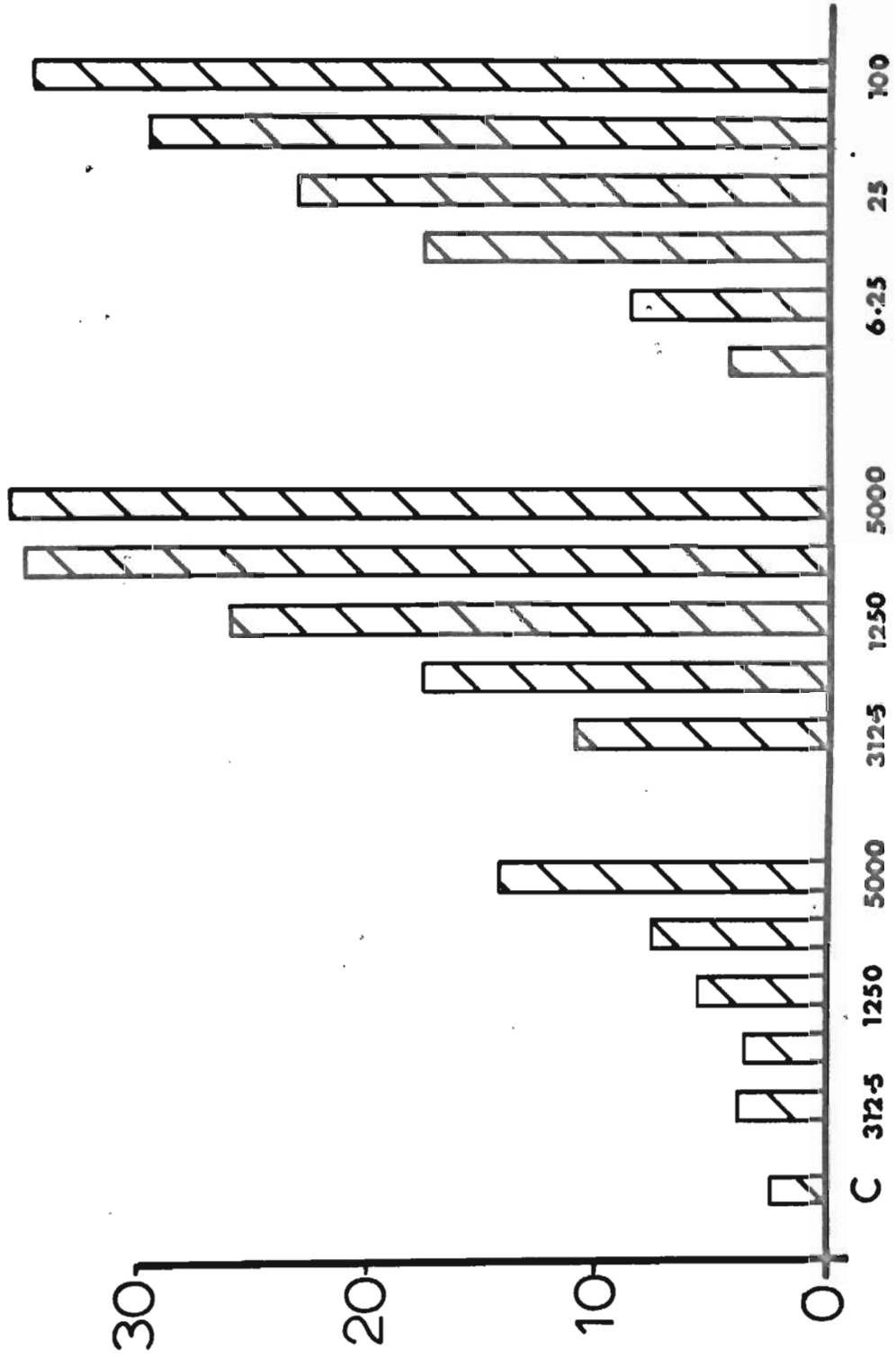
- (a) Total gonadotrophic activity per fraction expressed in μg equivalents NIH-IR-S19 per μg .
- (b) Percentage recovery of gonadotrophic activity relative to initial extract, the latter being by definition 100.
- (c) Percentage recovery of gonadotrophic activity relative to the amount of activity applied to the individual columns.
- (d) Figures represent recovery from the second ConA-Sepharose column of purification 1.

In both cases this material was chromatographed on a 1.2 x 15 cm ConA-Sepharose column. The vast majority of the material was not adsorbed to the column. The rostral plus median lobes yielded 35.5 mg ConA-1 and 7.6 mg ConA-2, while the neurointermediate lobes yielded 182 and 4.5 mg respectively. When assayed in chicks neither the glycoprotein fraction from the rostral plus median lobes (R + ML/ConA-2), at a dose of 105 µg/chick, nor from neurointermediate lobes (NIL/ConA-2), at a dose of 46 µg/chick, elicited a response, giving potencies of < 0.007 x NIH-LH-S19 for R + ML/ConA-2 and < 0.016 for NIL/ConA-2). When tested in the in vitro quail interstitial cell assay, both fractions elicited a response, as shown in Figure 36, giving potencies of 0.028 x NIH-LH-S19 for NIL/ConA-2 and 0.002 x NIH-LH-S19 for R + ML/ConA-2. The total gonadotrophic activity yields, according to this assay, were therefore 17 and 125 µg equivalents NIH-LH-S19 for the GTN extract from rostral plus median and neurointermediate lobes respectively. Both groups of lobes comprised a large percentage of the pituitary pool from 1300 fish used in purification 2, where a similar GTN extract of the ventral lobes contained a total of 11400 µg equivalents NIH-LH-S19 as determined in the chick assay. Considering the yield from the ventral lobes to be 100%, then that from the rostral plus median lobes was 0.18%, and from the neurointermediate lobes 1.1%. Expressed in a different way a single average ventral lobe in purification 2 contained 8.7 µg activity, while a rostral plus median lobe contained 0.013 µg and a neurointermediate lobe 0.10 µg gonadotrophic activity. However, as the figures for the ventral lobe purification were determined using the chick assay, while those for the other two lobes were determined using the quail interstitial cell assay, they may not be comparable, although both fractions CM1 and CM2 gave similar potency estimates in the two assays.

Figure 36:

Effects of the ConA-2 fraction from both rostral and median lobes (R + ML) and neurointermediate lobes (NIL) on testosterone release from quail interstitial cells. The mean results of duplicate tubes are shown.

ng TESTOSTERONE / tube



R+ML/Con A 2 NIL
ng/tube

OVINE LH



6) Immunoassay of dogfish gonadotrophin

A few antisera were tested for their ability to block the increased ^{32}P incorporation into day-old chick testes which follows administration of gonadotrophins. The same regime was used as in the bioassay except that the antiserum (20-50 μl diluted to 200 μl in saline) was injected subcutaneously 30 minutes prior to the hormone.

A dose of 20 μl anti-ConA-2 (purification 1) did not affect testicular ^{32}P -phosphate uptake in saline-injected control chicks, or in those given ovine luteinizing hormone (6.0 and 3.0 $\mu\text{g}/\text{chick}$), or the dogfish gonadotrophin fraction pooled ConA-2 (a dose equivalent to 3.0 μg NIH-LH-S19). Similarly, a dose of 50 μl anti-CM2 had no effect on testicular ^{32}P -uptake in chicks given ovine luteinizing hormone. However, there was a significant ($P < 0.05$) reduction in the response to ventral lobe extracts (Table 13); 50 μl anti-CM2 neutralizing approximately 2 μg equivalents ventral lobe extract.

Iodination of CM2, followed by chromatography on a small G-25 column to separate free iodide, and subsequent filtration of the protein peak on a 2.5 x 100 cm column of G-100 yielded three distinct peaks, although the relative size of the first two peaks varied considerably over seven iodinations. The first peak, emerging at the V_0 of the column, bound strongly to both anti-ConA-2 and anti-CM2, giving a maximum Bound/Free (B/F) ratio of approximately 7 in the presence of excess antiserum. The second peak, retarded on the column, although not to the same extent as iodinated rat FSH, was only tested for its binding capacity to anti-ConA-2, where a maximum B/F ratio of 0.25 was obtained. The third peak, very strongly retarded on G-100, did not bind to the antiserum at any dilution of the latter. Subsequent cellulose acetate

Table 13: The effect of antisera in vivo on gonadotrophic activity

Treatment/chick	Response + SEM (cpm/mg)	
6.0 µg NIH-LH-S19	109.7 ± 7.0	
3.0 µg NIH-LH-S19	90.5 ± 2.0	
1.5 µg NIH-LH-S19	72.3 ± 7.4	
0.3 Ventral lobe	95.6 ± 7.2	} N.S.
0.3 Ventral lobe + 50 µl N.R.S.	94.7 ± 3.8	
0.3 Ventral lobe + 50 µl anti-CM2	77.7 ± 5.3	} P < 0.05
0.15 Ventral lobe	77.5 ± 3.6	
0.15 Ventral lobe + 50 µl anti-CM2	59.1 ± 4.7	} P < 0.01
Saline	44.5 ± 3.4	
6.0 µg NIH-LH-S19	77.5 ± 4.2	
3.0 µg NIH-LH-S19	72.4 ± 2.2	
1.5 µg NIH-LH-S19	59.0 ± 4.7	
0.27 Ventral lobe	88.9 ± 2.5	} N.S.
0.27 Ventral lobe + 50 µl N.R.S.	87.5 ± 3.3	
0.27 Ventral lobe + 50 µl anti-CM2	75.4 ± 6.5	} P < 0.05
0.13 Ventral lobe	76.6 ± 4.4	
0.13 Ventral lobe + 50 µl N.R.S.	73.0 ± 3.9	} N.S.
0.13 Ventral lobe + 50 µl anti-CM2	58.1 ± 3.5	
3.0 µg NIH-LH-S19 + 50 µl N.R.S.	65.0 ± 3.7	} N.S.
3.0 µg NIH-LH-S19 + 50 µl anti-CM2	74.6 ± 2.9	
Saline	40.8 ± 2.6	

The first group of figures refers to anti-CM2, 2nd bleed and the second group to anti-CM2, 3rd bleed.



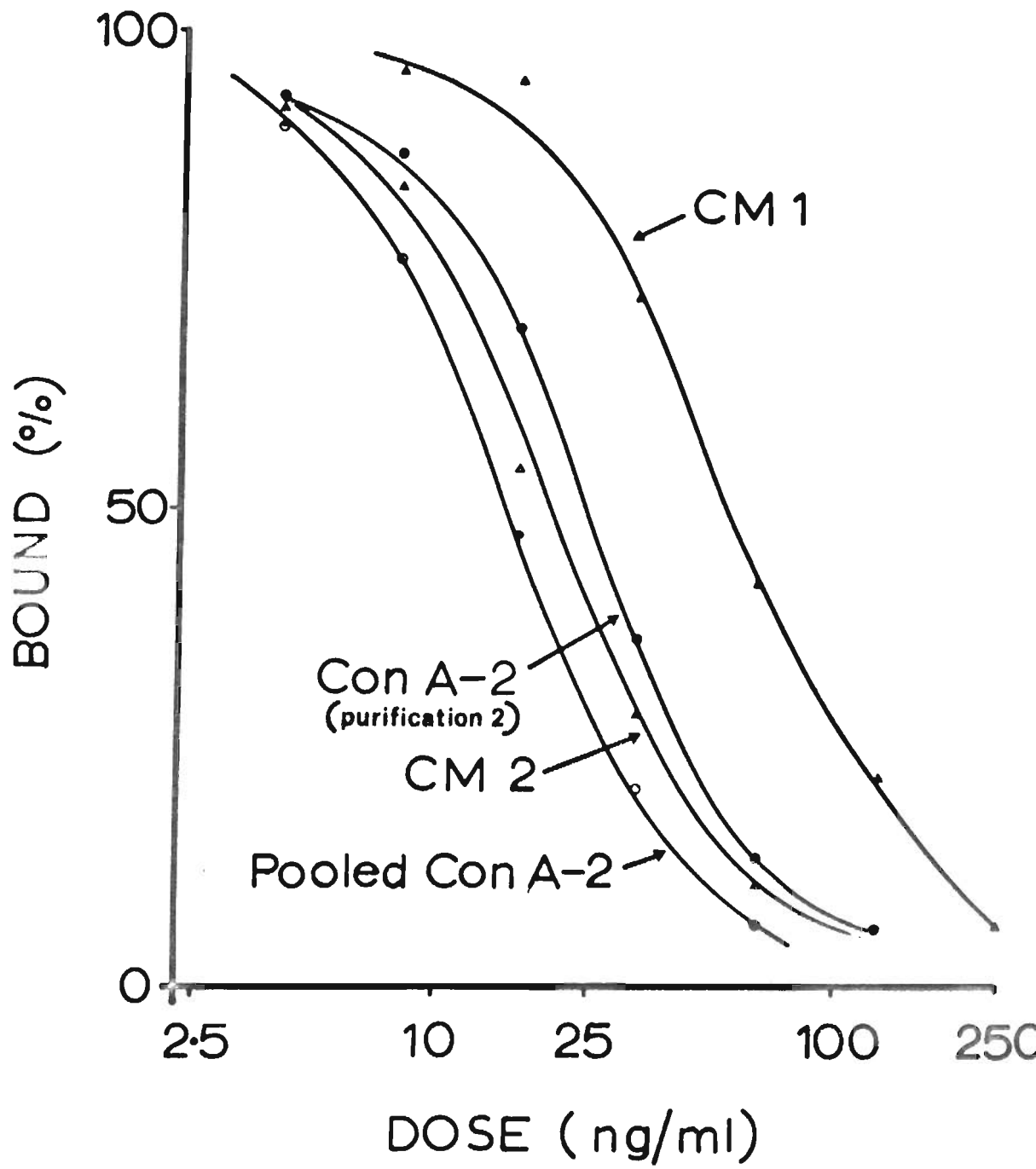
electrophoresis of the peak showed that it migrated at the same rate as free iodide. The first peak remained at the origin and the second migrated a short distance. Iodinated pooled ConA-2 gave a single peak emerging at the V_0 when chromatographed on a smaller (0.6 x 35 cm) column of G-100, whereas rat FSE was again retarded (Figure 28).

Choosing a dilution of anti-ConA-2 that gave a B/F ratio near to 1, the ability of various dogfish gonadotrophin fractions to inhibit the binding of labelled CM2, (peak 1 from G-100) was tested. Figure 37 shows that pooled ConA-2 proved the most immunoreactive in the assay, producing the most sensitive standard curve, followed in order by CM2, ConA-2 (purification 2) and CM1. In contrast, the biological gonadotrophic activities had been in the order CM2 > ConA-2 > Pooled ConA-2 > CM1. All dogfish plasmas tested, whether from mature, immature or hypophysectomized fish, were able to inhibit completely the binding between label and antiserum in this assay system, even when the plasmas were serially diluted 10 times, giving a final dilution of 1:512. Serial dilution of a ventral lobe extract from a mature fish gave a curve non-parallel to the standard. The same plasmas assayed in a homologous chicken LH assay run routinely in this department (Follett, Scanes and Cunningham, 1972) did not alter the binding of labelled chicken LH to anti-chicken LH. Similarly dogfish plasmas had no activity in an homologous salmon gonadotrophin assay (Crim, Meyer and Donaldson, 1973; Tan, 1976) and a heterologous avian follicle-stimulating hormone assay (Follett, 1976).

By filtering a saline extract of 55 fresh ventral lobes, followed by bioassay of the fractions collected, it was possible to demonstrate that dogfish gonadotrophin was retarded on G-100. The major protein peak,

Figure 37:

Dose-response curves obtained in the dogfish "immunoassay" with various partially purified gonadotrophin fractions. Each point represents the mean of duplicate determinations.



eluted at the V_0 , had no biological gonadotrophic activity, while later fractions were shown to contain activity in the ^{32}P chick assay. Immunoassay of these same fractions showed that the protein peak eluted at the V_0 of the column gave parallel dose response curves to the standard, while these fractions containing biologically active gonadotrophin gave dose-response curves non-parallel to the standard. Thus the assay as it stood appeared to be measuring a high molecular weight contaminant present in the iodinated material, and also present in very high concentrations in all plasma. Use of either antiConA-2 or anti-CM2, the latter containing antigonadotrophic activity, produced very similar results.

Purification 3 was undertaken primarily to produce a biologically active gonadotrophic fraction that was retarded on G-100. Hopefully, therefore, the high molecular weight contaminant found in all fractions so far could be separated from the gonadotrophic activity. Iodinated ConA-2 from the purification, with a biological activity of 0.376 x NIH-LH-S19, was indeed retarded on G-100 to the same extent as rat FSH. However, it only weakly bound to the best antiserum, anti-CM2. Even in the presence of excess antiserum the B/F ratio obtained was only 0.25. All dogfish gonadotrophic fractions tested were able to inhibit this binding. The amount of cold hormone needed to reduce the binding by 50% was 1 ng pooled ConA-2, 2 ng ConA-2 (purification 2), 8 ng G-100 and approximately 10 ng ConA-2 (purification 3). These results are very similar to those obtained previously where the label employed, CM2, was not retarded on G-100. Plasmas also had the same effect, completely inhibiting binding between label and antiserum at all dilutions of the plasma tested, as did extracts of either the various pituitary lobes or brain tissue.



To try and determine what it was in dogfish plasma that cross-reacted in the assay, 1 ml of plasma from a mature female was filtered on a 1.2 x 60 cm column of G-100 and all fractions immunoassayed. Using anti-GM2 and labelled ConA-2 from purification 3 (retarded on G-100), all fractions from the V_0 onwards showed very high immunological activity, until the V_T of the column was reached, where subsequent fractions showed little, if any, immunological activity.

Discussion

1) The purification and properties of fish gonadotrophins

A reproducible procedure has been developed for the preparation of highly purified gonadotrophin from small batches of dogfish ventral lobes. Homogenization of the ventral lobes in a mortar and pestle, followed by precipitation of a glycoprotein extract, GTN, appeared to extract all of the biologically active gonadotrophin of the glands. Subsequent affinity chromatography of this fraction on ConA-Sepharose as described by Idler, Bazar and Hwang (1975b) concentrated the vast majority of the gonadotrophin into fraction ConA-2. In their purification procedure B for salmon gonadotrophin, only 2.1% of the gonadotrophic activity recovered was in ConA-1, while in the dogfish purifications 1, 2 and 3, 30%, 5% and 30% respectively of the activity recovered was in ConA-1. As the capacity of ConA-Sepharose is not given by the manufacturers, and because a 1.2 x 5 cm column proved too small in purification 1, a larger 1.2 x 15 cm column was used subsequently.

In procedure B of Idler, Bazar and Hwang (1975b) the ratio of gonadotrophic activity applied to bed volume of column was 646:1 (i.e. 646 units of salmon gonadotrophic activity per ml bed volume), whereas in

purification 2, where the largest amount of dogfish gonadotrophin was applied to the column, the corresponding ratio was 532:1, both activities measured in the ^{32}P chick assay. Thus the ConA-Sepharose column of Idler *et al.* (1975b) was more saturated with respect to gonadotrophin than were any of the columns employed in the present studies on dogfish gonadotrophin. However, some dogfish TSH was also eluted with the ConA-2 fraction whereas Idler *et al.* (1975b) could not detect any TSH activity in their fractions using the same assay. Thus it appears that dogfish gonadotrophin binds less well to Con-A-Sepharose than salmon gonadotrophin under the same conditions. This conclusion does not preclude the possibility of one gonadotrophin being intrinsically more active in the chick assay than the other. Also either the salmon or the dogfish fractions applied to ConA-Sepharose may have contained relatively more material of non-gonadotrophin or TSH origin that is also adsorbed on to the gel. Whatever the reason, it would seem reasonable to conclude that a slightly larger column of ConA-Sepharose may be more effective.

The affinity chromatography step was highly successful in eliminating unwanted protein and achieving a marked increase in gonadotrophic activity. In general, the protein yields were less than 5%, recoveries of gonadotrophic activity were high, and on average a 45-fold purification was achieved in the adsorbed glycoprotein fraction. Recently Pierce, Faith and Donaldson (1976) have employed ConA-Sepharose in the further purification of Donaldson's SG-G-100 fraction, and again much of the biological activity was found in the glycoprotein fraction eluted from the resin, but, as they did not give the specific activities of fractions ConA-1 and ConA-2 eluted from the column, it is not possible to

determine the yield of biological activity or the percentage of the latter present in ConA-2. Comparing the three studies of purification of fish gonadotrophin on ConA-Sepharose, that of Idler, Bazar and Hwang (1975b), procedure B, with a 50% recovery of biological activity, almost all of which was present in ConA-2, and purification 2 of this study, where there was an apparent 120% recovery of biological activity, with 11½% of this present in ConA-2, produced the best results.

Idler et al. (1975b) also reported that after adsorbing the glycoprotein to ConA-Sepharose, a linear gradient from 0 to 0.4M α -methyl-D-glucoside in Buffer C did not resolve the salmon gonadotrophin into more than one peak with gonadotrophic activity. Thus, although ConA-Sepharose chromatography was an important step in their purification scheme, it did not separate two gonadotrophins one from the other. In the present purifications, there is some evidence that the gonadotrophin has been preferentially purified with respect to TSH, the former binding more strongly to ConA-Sepharose than the latter. For example, in purification 2, only 6% of the gonadotrophic activity was in ConA-1, whereas the corresponding figure for TSH was 31%. Idler, Bazar and Hwang (1975b) report that using the same assay as employed here, no TSH activity was detected in any of their G-75 fractions. As they presumably only tested these fractions, it is impossible to know if the earlier ConA-fractions contained TSH activity that was subsequently separated from the G-75 gonadotrophic fractions. Interestingly, Fontaine and Fontaine (1956) showed that two salmonid species had very low concentrations of TSH in the pituitary.

The detection and separation of TSH from gonadotrophin has proved very difficult in the case of fish preparations, although DEAE-



cellulose is used successfully in mammalian (Stockell Hartree, 1975) and avian (Scanes, 1972) studies. Although few data are available, as many of the purifications have ignored the problem, possibly because TSH was not the main focus of the studies, non-mammalian TSH appears to be a glycoprotein so is usually purified along with the gonadotrophins. Licht and Papkoff (1974b) showed that TSH activity was evident in all their final gonadotrophin fractions; not being effectively separated from either gonadotrophin. The relatively low TSH potency in any one fraction was explained as probably being related to the wide distribution of this activity into many different fractions. The total separation of GTH and TSH was difficult in the case of the carp, even though they were shown to be different entities. Whereas CM-cellulose has a very high binding capacity for most TSH's (Bates and Condliffe, 1966), including that from the eel (Fontaine and Condliffe, 1963), this was not the case with carp material, where the TSH activity was present in both CM1 and CM2 (Burzawa-Gérard, 1974). Partial separation of c-GTH and c-TSH was, however, achieved on DEAE-cellulose, where TSH was eluted slightly later than c-GTH under the conditions employed (Burzawa-Gérard, 1971). No mention of TSH activity is found in reports on the purification of trout GTH (Breton, Jalabert and Reinaud, 1976), sturgeon GTH (Burzawa-Gérard, Goncharov and Fontaine, 1975a) or salmon, Onchorynchus tshawytscha GTH (Donaldson, Yamazaki, Dye and Philleo, 1972). Donaldson and McBride (1974) later demonstrated that the salmon gonadotrophin preparation SG-G-100 possessed thyrotrophic activity. Unfortunately it was impossible to measure the TSH activity of the dogfish fractions CM1 and CM2 because of the limited amount of material available, although it would be surprising if CM2 did not contain TSH activity.



2) The presence of one or two gonadotrophins in fish

There is still contention as to whether one or two gonadotrophins are present in the pituitary gland of fishes. Two gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are found in the pituitary glands of all mammals. The primary action of FSH is on the development of ovarian follicles in the female, LH is responsible for transformation of this structure after ovulation into the corpus luteum. In the male FSH stimulates the development of seminiferous tubules, whereas LH stimulates the production of androgens by the Leydig cells (Sairam and Papkoff, 1974). Recent fractionation studies have demonstrated two separate gonadotrophin molecules in representatives of all three classes of non-mammalian tetrapods, including anuran amphibians (Licht and Papkoff, 1974a); two representatives of chelonian (Licht and Papkoff, 1974b and Licht, Farmer and Papkoff, 1976) and one of crocodilian (Licht, Farmer and Papkoff, 1976) reptiles and several galliform birds (Stockell Hartree and Cunningham, 1969; Scanes and Follett, 1972; Farmer, Papkoff and Licht, 1975a). Information obtained on the chemical nature and biological profiles of these hormones during fractionation and preliminary immunological studies (Licht, Papkoff, Goldman, Follett and Scanes, 1974) indicate that the two gonadotrophins are homologous to the follicle-stimulating and luteinizing hormones of the mammalian pituitary. While these data suggest that the existence of two separate gonadotrophins is a primitive feature in tetrapod evolution, the studies so far on fish species seem to indicate that probably only one gonadotrophin is present in the pituitary, having both biological 'LH' and 'FSH' like actions in a variety of test systems.

Until recently the major attempts at purification of fish gonadotrophin had all relied upon some form of ethanolic precipitation of the crude homogenate followed by gel filtration and ion-exchange chromatography on DEAE-cellulose. From these studies pituitaries of the carp, Cyprinus carpio (Fontaine and Gérard, 1963; Burzawa-Gérard, 1971), trout, Salmo gairdnerii (Breton, Jalabert and Reinaud, 1976), salmon, Oncorhynchus tshawytscha (Donaldson, Yamazaki, Dye and Philleo, 1972) and the sturgeon, Acipenser stellatus (Burzawa-Gérard, Goncharov and Fontaine, 1975a,b) were all believed to contain only one gonadotrophin. Although DEAE-cellulose has been shown to separate successfully the two gonadotrophins in mammalian material (Roos, 1968), fractionation of the above species yielded only one fraction with gonadotrophic activity. These single fractions from both the carp and salmon pituitaries are able to stimulate the various functional aspects of gonadal activity, including those in hypophysectomized fish, leading to the hypothesis that only one gonadotrophin exists in the teleost pituitary (Burzawa-Gérard and Fontaine, 1965, 1966, 1972; Donaldson et al., 1972; Burzawa-Gérard, 1974).

Idler, Bazar and Hwang (1975c), however, obtained some evidence for the existence of two or more distinct gonadotrophins in chum salmon pituitaries. Rechromatography of their fraction G-75 II on Sephadex G-75 superfine gave a symmetrical protein peak with a coincident cAMP activity profile. Repeated freezing and thawing elicited a shift in this cAMP activity profile toward the trailing edge of the protein peak. Employing similar elution conditions to Licht and Papkoff (1974b), who obtained two separate gonadotrophins from the pituitary gland of the snapping turtle by ion exchange chromatography on DEAE-cellulose, the

salmon material also showed two active peaks. Peak DEAE-I differed from DEAE-II and III (the latter two are the ascending and descending parts of the second active peak) in that it stimulated cAMP production in ovaries more than in testes, and its gonadotrophic activity significantly decreased following chromatography on Sephadex G-75 superfine. Idler, Bazar and Hwang (1975c) also suggested that DEAE-I is extremely labile to ethanolic extraction and the subsequent arduous procedures required to prepare the purified extract for chromatography. This may explain why the previous studies have reported the presence of only one gonadotrophin. Gel electrophoresis of the DEAE-G-75 fractions also confirmed an inherent charge difference between the fractions.

Carboxymethyl cellulose was used in an attempt to separate the ConA-2 fraction into two gonadotrophins because, along with DEAE-cellulose, constitutes one of the few methods which will separate LH from FSH in higher vertebrates. At first glance the data suggest the dogfish pituitary does contain two gonadotrophins as in both cases the biological activity recovered from the column was split equally between the two fractions CM1 and CM2. However, as about 90% of the protein was not adsorbed on to the column and was eluted with the first fraction (CM1), this had a specific activity much lower than CM2. Bioassays based upon testosterone release from quail interstitial cells, which appears to be specific for 'LH' when tested with ovine and chicken gonadotrophins (Maung, Z.W., 1976), suggest the contrary. Both CM1 and CM2 were assayed against the same NIH ovine LH standard in the ³²P-uptake in chicks and interstitial cell assays, with CM1 having activities (relative to NIH-LH-S19) of 0.081 and 0.055 respectively in the two assays, while CM2 had potencies of 1.04 and 1.00. This suggests that

the activity present in CM1 represents 'LH' that did not bind to the CM-cellulose column, rather than a separate 'FSH'. However, this does require independent confirmation by other bioassays. Stockell Hartree (1975) recommends a bed volume of 80 ml CM-cellulose per g of glyco-protein fraction, so my column of 6 ml bed volume contained eight times the minimum volume of gel required for mammalian pituitary extracts, and so overloading of the column was probably not a problem.

In the fractionation of mammalian and avian LH and FSH using CM-cellulose, over 80% of the FSH activity recovered was in the CM1 fraction (horse: Stockell Hartree, Mills, Welch and Thomas, 1968; dog: Stockell Hartree, Bell, Christie and Kirkham, 1972; chicken: Stockell Hartree and Cumingham, 1969), although the percentage of the LH activity recovered present in the CM2 fraction varied from 38% for the chicken hormone to 95% for the dog. Thus CM-cellulose is relatively poor at separating the two gonadotrophins in the chicken, with 91% of the FSH and 62% of the LH that being eluted from the column in CM1, although the adsorbed CM2 fraction contained little FSH activity. Approximately 45% of the biological activity recovered off the CM-cellulose column eluted with the dogfish ConA-2 fraction was not adsorbed to the ion exchanger, this percentage being comparable to the 'LH' like gonadotrophin not adsorbed in mammalian and avian studies. Burzawa-Gérard (1974) briefly mentions that 70% of carp gonadotrophic activity recovered from CM-cellulose in the non-adsorbed fraction, CM1, although the TSH activity was also present in both fractions. She abandoned this method and routinely separates carp-GTH and TSH on DEAE-cellulose at pH 7.8. However, a satisfactory separation of eel TSH and gonadotrophin was accomplished by Fontaine (1969) using CM-cellulose under the same



conditions, the TSH binding strongly to the resin. Thus both carp and eel gonadotrophin are acidic in nature, not being adsorbed to the weakly acidic cation exchanger CM-cellulose. Trout, sturgeon and salmon gonadotrophin are also acidic in nature judging by their behaviour on DEAE-cellulose. Dogfish gonadotrophin, on the other hand, may well be basic, as more than half of the gonadotrophic activity recovered off CM-cellulose resides in CM2, in a comparable way to mammalian and avian LH. In summary, the biochemical data from CM-cellulose in no way precludes the possibility that the dogfish possesses only a single gonadotrophin which is 'LH' like in its behaviour on ion exchange resins.

Very recently Pierce, Faith and Donaldson (1976) have re-examined the earlier salmon gonadotrophin fractions purified by Donaldson, Yamazaki, Dye and Philleo (1972). The starting material for further purification was SG-G-100, which was subjected to ConA-Sepharose chromatography to yield a glycoprotein fraction which was subsequently applied to CM-cellulose under the conditions described by Stockell Hartree (1966). Of 40 mg ConA-2 applied, 25 mg CM1 was recovered with a specific activity of $0.03 \times S19$, and 7.8 mg CM2 (both 2a and 2b). While the potency of CM2a was not given, CM2b had a potency of $0.014 \times NIH-LH-S19$. The potency of the original ConA-2 fraction chromatographed on CM-cellulose was not given either, making it impossible to calculate the proportion of the activity recovered relative to that applied. However, 90% of the activity recovered was not adsorbed to CM-cellulose and eluted as CM1. As salmon gonadotrophin has been shown to be acidic in nature, it is not surprising that the vast majority of the biological activity recovered would not adsorb to CM-cellulose. Fraction CM1 was then chromatographed on DEAE-cellulose as described by Cornell and Pierce (1973) in their

separation of human LH and TSH. Under these conditions DEAE-1, the unadsorbed fraction, contained all the gonadotrophic activity recovered from the column, although this was only 12% of that applied. Human LH was eluted in the corresponding peak by Cornell and Pierce (1973) although the TSH activity remained bound to the ion-exchanger and was eluted later. Although the chromatographic and electrophoretic properties were distinct, the aminoacid composition of the two related salmon fractions SG-CM2 and SG-DEAE-1 were very similar. The final evidence for a marked similarity, though not identity, in sequence between SG-CM2 and SG-DEAE-1, were the peptide maps which indicated both fractions to be nearly homogeneous, the maps being no more complex than that of bovine LH. In summary, the bulk of the biochemical data favours only a single gonadotrophin being present in Teleostean pituitaries. Although the present study on the Elasmobranchs is not as comprehensive as could be wished, it too points to only a single gonadotrophin.

The bioassays employed in the purification of fish gonadotrophins do not allow any speculation as to whether the purified gonadotrophins are biologically 'LH' or 'FSH' like or possess both activities. Very few bioassays, especially those employing non-mammalian materials, have been shown to be specific for only one of the gonadotrophins. The two most widely used bioassays known to be specific for only one of the gonadotrophins, the ovarian ascorbic acid depletion test (Parlow, 1961) for LH, and the method of Steelman and Pohley (1953) for FSH, both proved unresponsive to o-GTH (Burzawa-Gérard and Fontaine, 1965; reviewed in Burzawa-Gérard, 1974). That employed frequently in the purification of non-mammalian gonadotrophins has been the induction of spermiation in anurans, and in spite of some discrepancies, there has been a tendency to

consider this response as being primarily dependent on an 'LH' like factor (van Oordt and de Kort, 1969). Licht (1973a) re-examined this assay and concluded that the amuran spermiation response is not specific for either mammalian pituitary gonadotrophin. He also found that the ratio between the potencies of both highly purified and NIH preparations of LH revealed large discrepancies between the amuran response and other assays of these same gonadotrophin preparations. The unusually high apparent potency of the purified LH fractions in the amphibian led him to question the use of the amuran spermiation test for monitoring the purification of gonadotrophins. Another widely used assay has been the maturation of oocytes in vitro, especially from amphibia. In the case of oocytes from Xenopus laevis, their relatively high specificity for LH from a wide variety of tetrapod species has been demonstrated (Thornton, 1971; reviewed by Licht and Papkoff, 1976), and this has made the in vitro ovulation response particularly valuable for comparative studies on pituitary gonadotrophins. Although the oocytes from other other species may prove to be less specific for LH among the gonadotrophins, it would seem likely that trout-GTH, tested on trout oocytes, and Acipenser-GTH, tested on Rana, Bufo and Acipenser oocytes, are both displaying an 'LH' like activity.

Assays based on the stimulation of cAMP production in either testicular or ovarian homogenates (Fontaine, Burzawa-Gérard and Delerue-Lebelle, 1970; Idler, Bazar and Hwang, 1975a) may well be measuring more of an 'FSH'-like factor in the gonadotrophin because of the much higher incidence of seminiferous tubules as opposed to interstitial cells. The quail interstitial cell assay employed in this study appears specific for LH among the avian and mammalian hormones tested, and because of its



great sensitivity with respect to most bioassays this type of assay may prove extremely useful in further purification studies. Possibly it could be used with cells isolated from the homologous species. Both c-GTH and onc-GTH can stimulate all the various functional aspects of gonadal activity, thus favouring again the hypothesis that only one gonadotrophin exists in the pituitary (reviewed by Fontaine, 1975).

The difficulty in choosing appropriate assays is compounded by the fact that although some lower vertebrates contain two distinct molecules resembling the FSH and LH of mammals, the two types of gonadotrophin may not show the same specificity in lower vertebrates that they do in mammals. For example, studies with highly purified mammalian hormones in reptiles have demonstrated that both FSH and LH may stimulate gonadal growth and gametogenesis, as well as ovulation and steroid production by testes and ovaries (reviewed by Licht and Midgley, 1976b). Furthermore, with regard to progesterone production by turtle ovaries (Crews and Licht, 1975) and androgen production by lizard testes (Tsui, 1976) there is evidence that both mammalian gonadotrophins stimulate the same type of tissues in the gonad. Similar results have been obtained with purified FSH and LH from non-mammalian sources (specific references quoted in Licht and Midgley, 1976b). This overlap in both the type and site of action of FSH and LH in reptiles suggests that the location and/or the specificity of gonadotrophin binding sites in reptilian and mammalian glands may differ markedly. Thus the interpretation of bioassay results on an 'LH' or 'FSH' like activity basis remains difficult to resolve, and the only way to demonstrate convincingly the existence of two gonadotrophins with separate biological activities in fish will be by using a number of different bioassays in one study, pre-

ferably employing tissue from more than one class of vertebrates. In this respect the dogfish may be an excellent experimental animal, as each testis contains ampullae at various stages of development arranged transversely through the testis (Dobson, 1974), and thus by longitudinal sectioning it should be possible to isolate strips containing ampullae with the majority of germ cells at a specific stage of spermatogenesis. If two distinct gonadotrophins can be isolated from the dogfish pituitary, ampullae containing germ cells at different stages may respond to a varying degree to the two hormones, this being determined either by incorporation of ^{32}P -phosphate into the ampullae, or by cAMP/steroid production.

There are a number of approaches that support the argument that fish possess two gonadotrophins. The first comes from the use of purified mammalian gonadotrophins, where many workers have obtained positive results with LH but failed to elicit reactions with FSH (Pickford and Atz, 1957; Ball, 1960; Dodd, 1960). Mammalian LH, when injected into Teleosts has frequently been found to cause reactions usually associated with FSH in the mammal, as well as an LH-type of reaction. As only LH of the two mammalian hormones was active in fish, Hoar (1966) postulated a second FSH-type hormone controls those actions normally associated with FSH in the mammals. Part of the reason for the differential effects of the two mammalian hormones may have been that the LH, which gave positive results in many cases, was much purer than the FSH. While the most potent LH produced, a human preparation, is between 3.0 and 10.7 x NIH-LH-S1, measured in the OAAD assay, FSH preparations as high as 400 x NIH-FSH-S1 have been reported (summarised in Sairam and Papkoff, 1974). It is generally considered that NIH-LH is around 50% pure, while the NIH-FSH is about 2%, so equal doses of both contain vastly

different amounts of the biologically active hormone. Secondly, the dualistic concept is suggested by histological evidence. As indicated in the recent reviews of Donaldson (1973) and Reinboth (1972), two different types of gonadotrophic cells have been observed in the pituitary glands of eel, pacific and sockeye salmon, goldfish, mullet and sunfish. As these authors point out, however, the results are not conclusive since only one gonadotrophin was found in the pituitaries of Poecilia latipinna, catfish, the blind Mexican cavefish and gonadectomized sockeye salmon. The immunological evidence, reviewed by Doerr-Schott, 1976, is also confusing. In teleosts the cells labelled by ovine LH antiserum correspond to gonadotrophic cells or classical basophils (van Oordt, 1968; Ball and Baker, 1969). The cytoimmunochemical reactions are positive in the salmon (McKeown and van Overbeke, 1971), carp (Billard et al., 1971), goldfish and stickleback (Follenius and Dubois, quoted in Doerr-Schott, 1976). No fluorescence was detected after applying an anti-ovine FSH to the hypophysis of O. nerka (McKeown and van Overbeke, 1971), although the absence may be due to a lack of specificity of mammalian FSH antiserum for the presumptive 'FSH' hormone of fishes (Doerr-Schott, 1976). Even in mammals the LH and FSH antisera used do not always enable a clear distinction to be made between FSH and LH producing cells.

The work of Hyder (1970) has shown that in Tilapia there is a differential development of the gametogenic and steroidogenic tissues of the testes. Two extremes of interstitial tissue development can be seen, the lowest at a time when active spermatogenesis is in progress and spermatozoa are present in the sperm ducts, and the highest when practically all the spermatogenic products are drained away. He suggests that two pituitary hormones (an FSH and an LH) would best explain this situation,

the FSH-like principle probably being active largely in the early phases of testicular growth, while the LH-like principle probably reaches its maximal effect at about the time of spermiation.

As Burzawa-Gérard (1974) has shown, it is possible to reconcile this differential stimulation of the gametogenic and steroidogenic tissues in a number of ways. One is that the different tissues are sensitive to different concentrations of a single hormone. Sundararaj et al. (1972) showed that the catfish required different concentrations of gonadotrophin to maintain vitellogenesis or to cause ovulation. Another is that stimulation of the steroidogenic tissue results in secretions that in turn stimulate the gametogenic tissue. As Hyder (1970) points out, this may be the case in the early phases of the secondary spermatogenic wave, but does not appear to be the case in the early phases of the primary wave. Another possibility suggested by Burzawa-Gérard (1974) is that an inhibitory factor may prevent the stimulation of one type of tissue while the other is stimulated.

3) The stability of fish gonadotrophins

Although mammalian and avian gonadotrophins appear to be fairly stable around neutral pH, and many do not lose activity after storage as dry powders for two years at room temperature, this stability does not seem to apply to fish gonadotrophins. However, even mammalian FSH has proved difficult to isolate and purify, which Liu and Ward (1975) suggest may be due to its instability during the course of isolation. The purified salmon gonadotrophin of Idler et al. (1975b,c) was stable in Tris buffers containing EDTA and/or dithiothreitol, and samples did not lose significant activity when stored in these buffers for several months at -73°C. The results of repeated freezing and thawing of the G-75

fractions, however, were attributed to two (or more) gonadotrophins, one of which showed greater lability to phase change. Using their procedure A, a loss of 75% of the original biological activity was reported after dialysis and ultrafiltration, which they speculate may be due to the use of harsher conditions in this procedure. Also the specific activity of SG-fractions decreased between 10 and 25% after lyophilization. A single test of the effect of lyophilization on dogfish gonadotrophin showed that over 50% of the biological activity was lost following this treatment, so the latter was discontinued as a drying step. Idler *et al.* (1975b) also showed that heating homogenates of pituitaries extracted with buffer B at 60°C for one minute, in an effort to remove extraneous protein and denature proteolytic enzymes, resulted in a loss of 60% of the total activity. Salmon gonadotrophin thus appears to be more heat labile than mammalian glycoprotein hormones, where Papkoff *et al.* (1965) have even introduced a heating step at one stage to inactivate proteolytic activity in the crude extracts! Pierce, Faith and Donaldson (1976) also noted major losses in biological activity upon further purification of salmon gonadotrophin SG-G-100. Although the cause was unknown, they put forward the explanation of increasing ease of dissociation or denaturation upon purification. These authors also consider that the success of Idler *et al.* (1975b,c) in preventing loss of activity, though not completely, was due to avoidance of freeze drying at intermediate steps and possibly by preventing oxidation during purification by the addition of low concentrations of dithiothreitol.

In the purification of trout-GTH (Breton, Jalabert and Reinaud, 1976), the physical characteristics of the first trout gonadotrophin obtained led them to suppose that this preparation had been obtained in a

partially dissociated state, the dissociation being completed during ultracentrifugation. They again considered fish gonadotrophic hormones to be more fragile than mammalian gonadotrophins, as it seems that dissociation can be spontaneous for both t-GTH and c-GTH.

The problem encountered with the dogfish gonadotrophin preparation was not dissociation, but rather an aggregation of the hormone. There was also a loss of biological activity of the fractions upon storage as dry powders at -20°C , with the most potent fraction showing the greatest loss of activity. These fractions were not repeatedly thawed and refrozen during storage, so it appears as though the dogfish gonadotrophin, at least prepared in the way described, aggregates spontaneously as a dry powder at -20°C . The ConA-2 fraction from purification 3 has been stored in solution at -70°C for over ten weeks, and when an aliquot was iodinated and filtered on G-100 it was retarded to the same extent as rat FSH. There appears to have been no aggregation of this fraction - so far! As fresh dogfish gonadotrophin is retarded on G-100, it is most likely that aggregation occurred subsequent to the initial extraction of GTN. Unfortunately, at no stage in the early purifications was it possible to pinpoint the position of aggregation because only later was aggregation suspected. However, Figure 28 indicates that the biologically active gonadotrophin emerged at two positions, one retarded and one not. Although the column dimensions were such that too much weight ought not to be put on this result, it is possible that this was the only demonstration of the gonadotrophin in a partially aggregated state.

Other factors which point to the fact that the gonadotrophin aggregated subsequent to ConA-Sepharose chromatography are that the initial biological potency estimate in the ^{32}P -assay and that obtained two

weeks later in vivo in quail of fraction ConA-2 (purification 1) are not significantly different, and also that ConA-Sephadex chromatography during purification 3, under the same conditions as applied to the two previous purifications, did not cause aggregation. It appears therefore that the fractions aggregated subsequent to ConA-Sephadex chromatography, and as the procedures employed after this step in purifications 1 and 2 were different, it looks as though no particular chemical treatment caused the aggregation.

There are a few mentions concerning aggregation of gonadotrophins in the literature, although all refer to mammalian hormones. Gray (1967) demonstrated that human FSH seemed to be mixture of three components of molecular weights of approximately 68,000; 34,000 and 17,000. The original preparations of FSH, which contained all three forms, had been obtained by chromatography using elution with salt gradients. After prolonged storage following dialysis, these were converted principally into the high molecular weight form. No evidence of the existence of a trimer (ca. 100,000 Daltons) was observed. Gray (1967) also states that the association-dissociation process seems to have no effect on the biological activity of the hormone, peaks corresponding to all three molecular weight forms being active in the ovarian augmentation assay. Butt, Crooke and Cunningham (1961) also appeared to obtain aggregation in human FSH during purification, while Sairam and Papkoff (1974) consider it likely that Roos and Gemzell (1965) were dealing with aggregated preparations. Stockell Hartree (personal communication) also has seen aggregation in some mammalian gonadotrophins. However, although aggregation has been reported in a number of cases previously, only dimers, with molecular weights 60,000-70,000, have been confirmed,

whereas dogfish gonadotrophin appears to have aggregated to a molecule with a molecular weight equal to or greater than 100,000 (judging by its behaviour on G-100), and at no time has the presence of smaller aggregates been seen. As to the question of whether the loss of biological activity is related to the aggregation of the dogfish gonadotrophin, this is unknown, although it is probably more likely that the two phenomena are linked. Stockell Hartree (personal communication) has no data on the biological activity of aggregates, and although Gray (1967) reports that all three of his human FSH preparations of different molecular weight possess biological activity, no data are provided. His results also disagree with most studies of dissociation in gonadotrophins which indicate that the dissociated subunits possess little, if any, biological activity (reviewed by Sairam and Papkoff, 1974).

4.) The location of gonadotrophin in the elasmobranch pituitary

As discussed in Chapter 1, there is evidence for gonadotrophic activity in the other lobes of the dogfish pituitary besides the ventral lobe. To recapitulate briefly, the ultrastructural study by Knowles et al. (1975) and the biological assay of various lobes by Firth and Vollrath (1973) have indicated the presence of a gonadotrophin in the median lobe. Goddard and Dodd (quoted in Dodd, 1955) reported that the injection of extracts of skate (Raja batis) neurointermediate lobe into immature mice produced a three-fold increase in uterine weight, which would appear to indicate the presence of gonadotrophic activity in this lobe. Scanes et al. (1972) confirmed earlier reports by showing that the ³²P-chick bioassay responded only to the ventral lobe at the doses used. Thus all lobes of the elasmobranch pituitary have at some time been suggested to contain gonadotrophin. The studies reported here have

used "classical" purification procedures in an attempt to locate gonadotrophic activity in the other lobes of the dogfish pituitary besides the ventral lobe. Results obtained using the ConA-2 glycoprotein fractions from either rostral plus median, or neurointermediate lobes confirm and extend the results of Scanes et al. (1972). Injection of a dose of the respective ConA-2 fraction equivalent to either 14.6 R + M lobes or 10.8 NI lobes failed to elicit a response, whereas a dose equivalent to 0.125 ventral lobes of the ConA-2 fraction from the corresponding ventral lobe purification would have given a significant response. When assayed using the much more sensitive interstitial cell assay, both R + ML and NIL ConA-2 fractions gave dose-response curves parallel to the standard, estimates of the potency of the two fractions showing that the total activity present in the NIL ConA-2 fraction was 8 times that present in the R + ML ConA-2 fraction. Unfortunately, as the potency of the corresponding VL extract was not estimated in the interstitial cell assay, it is impossible to make direct comparisons with extracts of the other lobes. However, as the chick assay and the interstitial cell assay gave the same potency estimates of both fractions CM1 and CM2, it seems reasonable to conclude that these two assays would give approximately the same potency estimate for fraction ConA-2 from ventral lobes. Bearing this assumption in mind, the R + ML ConA-2 fraction contains 0.18% and the NIL ConA-2 fraction 1.46% of the activity present in the ventral lobe ConA-2 fraction. This is not in agreement with the results of Firth and Vollrath (1973), who using the Xenopus oocyte maturation assay concluded that the mean ventral lobe potency was eight times that of the mean median lobe potency. Even allowing for the fact that the ratio of biological potencies between two fractions may differ depending on the bioassay employed (Licht and Midgley, 1976b), it

is difficult to reconcile the two sets of data. It is also possible that the Xenopus assay is measuring a gonadotrophin not detected by either the ^{32}P -chick assay or by the interstitial cell assay, but this again seems unlikely.

The results presented in this study are more in accord with the likelihood that the gonadotrophin extracted during the purification of other lobes of the pituitary besides the ventral is an artifact produced by the method of separating the various lobes. It is difficult to believe that no contamination could take place, especially as the ventral lobe is connected to the median by a hollow stalk. During the dissection of the pituitary from the whole animal pressure is applied to the top of the brain that could easily force the colloid present in the ventral lobe up the stalk into the other lobes. Also, the instruments used for dissecting the various lobes must become contaminated when a large batch of glands is dissected.

5) The immunoassay of fish gonadotrophin

Although the use of radioimmunoassays in studies relating to fish endocrinology are still in their infancy, they have already made a major contribution to the study of reproduction in fish, and have answered many of the questions that had proved very difficult before their introduction. Only two immunoassays have been fully validated, one for salmon gonadotrophin (Crim, Meyer and Donaldson, 1973; Tan, 1976) and the other for carp gonadotrophin (Breton, Karm, Buzawa-Gérard and Billard, 1971). Tan (1976) has also shown that a heterologous assay based upon a salmon antiserum and a carp antigen may prove useful in studies on other teleost species. The use of these assays has allowed a gonadotrophin releasing hormone to be demonstrated in the hypothalamus

from the carp (Breton, Jalabert and Weil, 1975) and the goldfish (Crim, Peter and Billard, 1976), a situation analogous to that found in mammals and birds, and probably also the brown trout, Salmo trutta, which is capable of responding to synthetic mammalian gonadotrophin releasing hormone (Crim and Cluett, 1974). Variations in the plasma gonadotrophin profile have been demonstrated, both during sexual maturation in a variety of salmonids (Crim, Watts and Evans, 1975) and during ovulation in the carp (Breton, Billard, Jalabert and Kann, 1972). In a preliminary study Crim and Evans (1976) have shown that radioimmunoassay estimates of circulating gonadotrophin may be a valuable tool for assessing the usefulness of the treatment of fish with pituitary hormones in the manipulation of their reproductive cycles in aquaculture.

As yet, it has not been possible to develop a radioimmunoassay for dogfish gonadotrophin, although the two major ingredients, a gonadotrophin of sufficiently high purity and a reasonable antiserum, have both been obtained. Antisera raised against the GTN and ConA-2 fractions from purification 1 are probably poor antisera as they possessed no antigonadotrophic activity when tested in vivo in chicks. They were raised soon after the purification began in an effort to develop an immunoassay for dogfish gonadotrophin as quickly as possible. In retrospect they have probably only confused the problems relating to the immunoassay. The initial assay developed, using iodinated CM2 and either anti-ConA-2 or anti-CM2, appears to measure a high molecular weight contaminant present in both the iodinated gonadotrophin and also in very large amounts in the plasma. Due to the aggregation of the CM2 fraction it has been impossible to separate the gonadotrophin from the high molecular weight non-gonadotrophin suspected of being present, and thus

the assay presumably measures both of these materials. It is more difficult to explain why similar results with dogfish plasma have been obtained in the assay using iodinated ConA-2 from purification 3 and anti-CM2. Here any high molecular weight material had been separated from the gonadotrophin during the purification by gel filtration in G-100, and the subsequent ConA-2 glycoprotein fraction produced contains biologically active gonadotrophin in a non-aggregated state, even after iodination. Use of this iodinated preparation in the assay should mean that no high molecular weight material is being measured, although again dogfish plasmas, even at high dilutions, inhibit completely the binding of the label to the antiserum, indicating very high amounts of some material causing this effect. A possible answer to this problem is that the dogfish plasma contains something causing a non-specific inhibition of binding between the label and the antiserum. However, dogfish plasmas included in other immunoassays run routinely in this department, those for chicken LH, salmon GTH and a heterologous avian FSH assay, do not inhibit the binding of the label to its respective antiserum. As the methods employed are basically the same in all the assays, if dogfish plasma did contain a non-specific factor inhibiting binding in the dogfish immunoassay, this factor would be expected to do the same in any immunoassay. A way around the problem may be to raise antisera against ConA-2 (purification 3), which should contain no antibodies against the high molecular weight material presumed to be present in the fractions from the earlier purifications. This would also be an antiserum raised against a non-aggregated gonadotrophin, as the anti-ConA-2 and anti-CM2 may well contain antibodies against the aggregated gonadotrophin, although anti-CM2 has been shown to be capable of neutralizing the effect of a fresh ventral



lobe extract injected into chicks, where the gonadotrophin is non-aggregated. The first anti-ConA-2 (purification 3) bleeds are now available although they have not yet been tested.



SUMMARY

The mature female dogfish, Scoyliorhinus canicula, has been shown to be cyclical in its reproductive physiology. Gravimetric data, supplemented by observation, have shown that the dogfish has an extended, if not continuous, egg-laying season. The gonadosomatic index (G.S.I) shows marked annual variation, however, being highest during late winter and lowest in autumn. Both plasma testosterone and oestradiol concentrations showed marked annual variation in mature female dogfish, the rise and fall preceding similar changes in the G.S.I. by about three months. Oestradiol could not be detected in the plasma of either immature females or males at any stage of maturity.

Bioassay estimates of the gonadotrophic content of the ventral lobe of the pituitary from mature females demonstrated that this too showed a high degree of annual variation, although the cycle was more difficult to correlate with either changes in the G.S.I. or plasma steroid concentration. Whereas the gonadotrophin content of the female ventral lobe varies by a factor of at least 80 over the year, that of the male varies much less, and never approaches the value obtained in the female throughout much of the year.

Intravenous injection of ventral lobe extract into intact, mature males caused elevation of plasma testosterone concentration after 90 minutes. Ventral lobe extract also caused a four-fold increase in plasma testosterone concentration in hypophysectomized males after 4 hours. An extract of neurointermediate lobe caused a significant elevation of plasma testosterone in hypophysectomized males as well, although the elevation was not as great nor as long lasting as that caused by ventral lobe extract. An extract of rostral plus median lobes had no effect on

plasma testosterone concentration.

Mammalian gonadotrophin, injected intravenously, had little effect on plasma testosterone or oestradiol concentrations in hypophysectomized females. A small rise was observed in both steroid concentrations between 4 and 6 hours post-injection following FSH administration. There was no effect with LH.

Plasma steroid concentrations, both testosterone and oestradiol, fell rapidly when the fish were maintained in tanks. Various pituitary operations did not affect the character of this fall. Ventral lobectomy caused a complete cessation of egg-laying. Ablation of the other pituitary lobes had no effect on the rate of egg-laying.

Gonadotrophin(s) were extracted and partially purified using biochemical methods similar to those employed in mammalian studies. Two batches, each of approximately 1000 ventral lobes, were homogenized separately in a mixture of ammonium acetate/ethanol and the glycoprotein fraction (GTN) precipitated by the addition of three volumes of ethanol. This was followed by affinity chromatography of the GTN fractions on ConA-Sepharose which proved most effective. Most of the protein emerged unadsorbed from the column, but this fraction (ConA-1) contained only a small amount of the total gonadotrophic activity (7%), although a slightly larger amount of the TSH activity (31%). The majority of both the gonadotrophic and TSH activities were eluted subsequently in a ConA-2 fraction by using a buffer containing glucoside. This step gave 9 and 25-fold increases in gonadotrophic potency respectively in two purifications. The first ConA-2 fraction was passed through a column of CM-cellulose. About 90% of the protein was unadsorbed and was eluted in the first fraction (CM1). Increasing the buffer molarity eluted a small amount of

a second fraction (CM2). The gonadotrophic activity was split equally between the CM1 and CM2 fractions, with the result that CM2 has a specific activity much greater than CM1. At first glance, these results suggest that the dogfish produces two gonadotrophins, but subsequent bioassay data suggest that this is not the case. Potency estimates of CM1 and CM2 were 0.08 and 1.04, x NIH-LH-S19 respectively in the chick assay, and 0.06 and 1.00 x NIH-LH respectively in the interstitial cell assay. This suggests that the activity in CM1 represents "LH" that did not bind to the CM-cellulose column, rather than a separate 'FSH', although this requires further study using a range of bioassays.

Gel-filtration on Sephadex G-100 of the CM2 fraction or the ConA-2 fraction from the second purification showed that virtually none of the protein was retarded. The unretarded protein peak contained all the detectable gonadotrophic activity, although the yield was much lower than expected. Subsequent gel-filtration of fresh ventral lobe extract suggests that the partially purified gonadotrophic fractions had aggregated and lost some biological activity. A third purification on a smaller number of lobes produced a reasonably potent gonadotrophic fraction that behaved similarly to mammalian gonadotrophins upon gel-filtration. These studies indicated that dogfish gonadotrophin was similar to other fish gonadotrophins in being more labile than mammalian gonadotrophins.

The same biochemical techniques, when applied to either neuro-intermediate or rostral plus median lobes, yielded very small amounts of material, having very low gonadotrophic activities. These were 1.10 and 0.15%, respectively, of the total gonadotrophic activity recovered from the same number of ventral lobes collected from the same fish. It appears,



therefore, as though the ventral lobe is the main, and probably the only, source of gonadotrophin in the dogfish pituitary.

Both GTN and ConA-2 fractions, when injected into rabbits, produced poor antisera. An antiserum prepared against CM2, however, contained anti-gonadotrophic activity, demonstrated by its ability to inhibit the increase in ^{32}P -uptake into chick testes caused by ventral lobe extract. Using this antiserum, and various iodinated dogfish gonadotrophin fractions, it has as yet proved impossible to set up a radioimmunoassay to measure circulating gonadotrophin concentrations. Some of the possible reasons for this are discussed.

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